Membrane-Associated Purine Metabolizing Enzyme Activities of Human Peripheral Blood Cells†

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ABSTRACT: Sealed and unsealed plasma membrane vesicles were prepared from human erythrocytes and lymphocytes. Phosphoribosylpyrophosphate synthetase (PRibPP synthetase), hypoxanthine phosphoribosyltransferase (HPRTase), and adenine phosphoribosyltransferase (APRTase) activities are detectable on both inside-out and right-side-out sealed vesicles. Ghost preparations were about 0.2%, 1%, and 1.2% of the total erythrocyte and 0.5%, 5.3%, and 9.7% of the lymphocyte APRTase, HPRTase, and PRibPP synthetase activities. The rapid decrease in these enzyme activities, upon further purification of the membranes, seemed to suggest that they might be loosely bound extrinsic proteins. Evidence confirming the localization of these enzymes on the cell surface was obtained by measuring production of [^{14}C]AMP by intact cells in medium containing [^{14}C]adenine, ribose 5-phosphate, and Mg^{2+}ATP. The formation of AMP was linear with time and number of cells present. Magnesium and phosphate exerted different effects on the production of extracellular AMP than on intracellular, which involves transport as well as phosphoribosylation. Cytosoluble and membrane-bound APRTase and PRibPP synthetase exhibited different catalytic properties and sensitivities to effectors. Membranes of erythrocytes of HPRTase-deficient patients contain little or no HPRTase activity when assayed in the absence of Triton. Reisolation of these membranes from admixture with normal hemolysates did not result in any bound activity; thus, the membrane-bound activity is not an artifact of the isolation procedure. Lysis with Triton released activity equal to about half that of control membranes. This is further evidence that the enzyme is firmly bound to the membrane.

We have previously reported that 5-phosphoribosyl-1-pyrophosphate synthetase (PRibPP synthetase, EC 2.7.6.1) and a small portion of the hypoxanthine phosphoribosyltransferase (HPRTase, EC 2.4.2.8) isolated from rat intestinal mucosa are membrane associated. The coexistence of these two activities in the fraction of bowel involved in absorption has led to the suggestion that the synthetase and phosphoribosyltransferases are part of a coupled transport system (Yip et al., 1980a).

Adenine phosphoribosyltransferase (APRTase, EC 2.4.2.7) and HPRTase have been found to be partially localized in the membrane and involved in active transport in bacteria (Hochstadt & Quinlan, 1976). Further evidence for the involvement of HPRTase in purine transport in mammalian cell systems has also been reported from the same laboratory (Prasad et al., 1981). Membrane-bound HPRTase has been reported in human fibroblasts (Willers et al., 1977), and both APRTase and HPRTase activities were found to associate with human erythrocyte ghosts (DeBruyn & Oei, 1977).

We previously reported that there are distinct differences between the PRibPP synthetase from nucleated and nonnucleated cells in molecular weight, aggregative nature, and response to the in vivo inhibitory effect of 2′-deoxycoformycin (Yip et al., 1978, 1980b). The present investigation is designed to explore differences in distribution of membrane-associated PRibPP synthetase and phosphoribosyltransferase activities, and to evaluate the possible physiological significance of such findings. Peripheral blood cells are used in these studies. Leukocytes are nucleated but are normally low in de novo biosynthetic activity compared to other types of nucleated cells. Erythrocytes are nonnucleated, void of de novo purine biosynthetic activity, and contain no demonstrable subcellular organelles, thus providing a unique supply of pure plasma membrane for our studies. In this study, we have shown that the membane-bound enzymes are different in regulatory responses, and this may explain physiologic roles and pharmacologic interactions. Most strikingly, we have found one of the HPRTase-deficient mutants that possesses no detectable intracellular enzyme activity but has half of the normal membrane-associated HPRTase activity after Triton lysis.

Materials and Methods

Most of the nonradioactive chemicals were obtained from Sigma Chemical Co., St. Louis, MO, and radioactive substrates were purchased from New England Nuclear, Boston, MA, unless otherwise stated.

Isolation of Intact Cells. Erythrocytes and lymphocytes were separated from fresh heparinized blood by Ficoll Paque (Pharmacia) gradient centrifugation. Lymphocytes were further purified by passage through a cotton wool column (removal of platelets) and by hypotonic lysis [removal of red blood cells (RBC)] (Boyum, 1976; Scholar & Calabresi, 1973). The isolated cells were washed once with phosphate-buffered saline (PBS) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and twice with PBS. Smears of the final preparations were examined microscopically for contamination. The number of cells in the preparations were determined microscopically by using a Thomas-diluting pipet with PBS as the diluting fluid.

Subcellular Fractionation of Lymphocyte Membranes. The procedures described in our previous report for rat intestinal mucosa cells were followed (Yip et al., 1980a). Isolated lymphocytes were lysed with 40 volumes of 0.5 mM sodium phosphate, pH 8.0, buffer by gentle stirring for 1 h at 4 °C or by using a Potter–Elvejem homogenizer. This was followed by differential centrifugation at 2000*g*, 20000*g*, and 100000*g* for 10, 15, and 60 min, respectively. The pellets collected after each step of centrifugation were resuspended in buffer with or without 2% Triton X-100 added for enzyme assays.

Preparation of Blood Cell Membrane Vesicles. The method described by Steck & Kant (1974) was followed for the

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0006-2960/82/0421-6972$01.25/0

preparation of erythrocyte ghosts. Hemolysis was initiated by rapidly mixing 1 mL of packed cells with approximately 40–60 mL of 5 mM sodium phosphate buffer, pH 8.0, with or without the presence of 1 mM $MgSO_4$. The absence of Mg^{2+} leads to the preparation of sealed inside-out vesicles. The sealed right-side-out ghosts were formed by spontaneously resealing shortly after lysis in the presence of magnesium ions in dilute buffer. They were also formed by resuspending thoroughly washed unsealed membrane pellets in 40 volumes of PBS and were then incubated at 37 °C for 40 min to induce resealing. The ghosts were then pelleted and washed twice more in PBS. For the preparation of inside-out vesicles, each milliliter of pelleted unsealed ghosts was diluted to about 40 mL with 0.5 mM phosphate, pH 8.0, buffer. After 18 h on ice, the membranes were pelleted at 28000*g* for 30 min. The membranes were resuspended to 1 mL in the low ionic strength buffer by vortex mixing and passed 3–5 times through a 27-gauge needle on a 1-mL syringe to complete vesiculation. The preparation was further purified by ultracentrifugation on a 1.03 g/mL dextran barrier solution. All the preparations were tested for their sidedness by measuring their sialic acid accessibility to sialidase, their acetylcholinesterase accessibility, and their NAD–cytochrome *c* oxidoreductase accessibility. Detailed assay procedures have been described (Steck & Kant, 1974). For preparation of membrane vesicles from lymphocytes, about 25 mL of fresh lymphocyte concentrates (from the New York Blood Bank, residues of cytoplasmophoresis) was used. Cells were resuspended in 40 volumes of 5 mM KPB, pH 8.0, disrupted by using a Potter–Elvejem homogenizer (Walsh & Crumpton, 1977). Gradient centrifugation was used to differentially separate various subcellular fractions. The plasma membrane fraction was used to prepare sealed vesicles.

Enzyme Assays. For erythrocyte enzymes, previously described methods were used (Yip et al., 1978; Yip & Balis, 1975a,b). A slight modification of our reported assays for PRibPP synthetase was used for the lymphocyte preparations. The reaction mixture for PRibPP synthetase contained 70 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.4), 0.7 mM ETDA, 1.7 mM ETSH, 7 mM Mg^{2+}, 33 mM PO_4^{3-}, 0.67 mM ribose 5′-phosphate (R-5-P), 0.67 mM ATP, 20 mM fluoride ion, 0.2 mM inosine 5′-phosphate (IMP), 0.125 mM [^{14}C]adenine (sp act. 4.5 Ci/mol), and 5 μL of partially purified erythrocyte APRTase (sp act. ≃ 2 IU/mg of protein). Assays for APRTase and HPRTase from lymphocyte membrane preparations were essentially the same as those described for hemolysates (Yip & Balis, 1975a,b), except that 20 mM F^- was added to the reaction mixture, which increased the net activity of membrane preparations significantly. No effect of fluoride was observed with erythrocyte preparations.

Intact Cell Experiment. About 10^7 freshly prepared lymphocytes and 10^9 erythrocytes were incubated in 0.1 M Tris-HCl, pH 7.4, 0.05 M NaCl, and 2.5 mM dextrose buffer solution with radioacive purine and various effectors and substrates. The total volume of the reaction mixture was 200 μL. The cells were incubated at 37 °C for 1 h. The reaction was terminated by immersing the tubes in an ice water bath followed by centrifugation at 4 °C. The nucleotides formed in the medium were chromatographed on DEAE-cellulose paper (Whatman DE-81), and the radioactivity was assayed. The reacted cells were washed 3× with ice-cold PBS and lysed in 100 μL of water. The lysate tubes were placed in a boiling water bath for 2 min. The clarified supernatant of the cell lysates was chromatographed on DEAE-cellulose paper. The radioactive purine transported as well as the amount converted into nucleotides was measured.

Table I: Purine Metabolizing Enzyme Activities of Human Erythrocyte Ghosts[a]

	enzyme activity [pmol (mg of protein)$^{-1}$ min^{-1}]					
	APRTase		HPRTase		PRibPP synthetase	
Triton addition	−	+	−	+	−	+
right-side-out ghosts	10	21	315	591	111	259
sealed inside-out vesicles	2	5	6	12	11	19

[a] From 1 mL of well-washed erythrocytes, 0.7–1 mL of slightly pinkish loosely packed right-side-out ghosts (about 3 mg of protein) or 0.2–0.3 mL of creamy-white inside-out vesicles (about 1 mg of protein) were obtained. The starting cells contained 0.152 unit/mL APRTase, 0.608 unit/mL HPRTase, and 0.211 unit/mL PRibPP synthetase activities. The Triton-solubilized right-side-out ghosts preparation contained 0.29×10^{-3} unit/mL APRTase, 6.1×10^{-3} unit/mL HPRTase, and 2.6×10^{-3} unit/mL PRibPP synthetase. The inside-out vesicle preparation contained 0.02×10^{-3} unit/mL APRTase, 0.05×10^{-3} unit/mL HPRTase, and 0.092 0.092×10^{-3} unit/mL PRibPP synthetase. One unit of activity is the activity of enzyme needed to form 1 μmol of product in 1 min.

Protein Determination. The method of Lowry et al. (1951) was followed.

Results

Purine Metabolizing Enzyme Activity of Human Erythrocyte Ghosts. Activities of APRTase, HPRTase, and PRibPP synthetase were shown in both the right-side-out and inside-out sealed erythrocyte membrane vesicles (Table I). Enzyme activities of vesicles were doubled when 2% Triton X-100 was present in the assay mixture. The amount of APRTase, HPRTase, and PRibPP synthetase from the right-side-out ghost preparation was equivalent to 0.2%, 1%, and 1.2%, respectively, of the total erythrocyte enzyme activity. Furthermore, the inside-out vesicles expressed less than 0.1% of total activity. The right-side-out ghosts were prepared by spontaneous resealing of membrane vesicles in the presence of Mg^{2+}, while the resealing process for inside-out ghosts was time consuming and laborious. The latter was further purified by dextran gradient ultracentrifugation. The comparatively much higher enzyme activities seen with the right-side-out ghosts might be due to the gradual loss of the loosely bound enzymes during the process of obtaining the inside-out vesicles. The sidedness of the preparations was tested by the accessibility of four markers. Right-side-out ghosts have 93% sialic acid accessibility to sialidase and 100% acetylcholinesterase accessibility, while the inside-out vesicles have shown no accessibility to either. On the other hand, the inside-out vesicles thus prepared have 81% and 120% accessibility to glyceraldehyde-3-phosphate dehydrogenase and NADH–cytochrome *c* oxidoreductase, respectively, while the right-side-out ghost preparations have 25% and 0% accessibility, respectively, to these enzymes.

Purine Metabolizing Enzyme Activities of Human Lymphocyte Membrane Preparations. Following procedures described for the subcellular fractionation of rat intestinal mucosa cells (Yip et al., 1980a), we observed that about 9.7% of the PRibPP synthetase, 5.3% of the HPRTase, and 0.5% of the APRTase activities of the lymphocytes were cytoplasmic membrane bound (Table II). Triton solubilization of the pellets increased the enzyme activities in the P_2 fraction 3–4-fold, but no increase was seen in the highly fragmented P_3 fraction. Resealing P_2 vesicles in the presence of Mg^{2+} retained all the enzyme activities (Table III). In the absence of Mg^{2+},

Table II: Purine Metabolizing Enzyme Activities Associated with Various Subcellular Fractions of Normal Human Leukocyte Preparations

fractions	volume (mL)	[protein] (mg/mL)	enzyme activity [pmol (mg of protein)$^{-1}$ min^{-1}] APRTase −[c]	APRTase +[d]	HPRTase −	HPRTase +	PRibPP synthetase −	PRibPP synthetase +
100000g supernatant	190[a]	3.14	278	306	217	220	70	74
P_1, 2000g pellet	20	1.55	15	62	123	296	162	268
P_2, 2000g pellet[b]	5	3.12	26	95	134	461	114	305
P_3, 100000g pellet	1	1.84	384	372	2480	2370	3367	2788

[a] Washed lymphocytes were lysed in 40 volumes of 5 mM phosphate buffer, pH 8.0, at 4 °C overnight with gentle stirring. [b] Percent enzyme activities associated with plasma membrane (P_2) fraction are calculated as APRTase 0.5%, HPRTase 5.3%, and PRibPP synthetase 9.7%. These values were derived from the data obtained with the Triton-solubilized fraction. [c] Minus indicates no Triton added. [d] Plus indicates addition of Triton.

Table III: Purine Metabolizing Enzyme Activities of Sealed Vesicle Preparations of Normal Lymphocytes

Triton addition	enzyme activities [pmol (mg of protein)$^{-1}$ min^{-1}] APRTase −	APRTase +	HPRTase −	HPRTase +	PRibPP synthetase −	PRibPP synthetase +
right-side-out vesicles[a]	6	20	72	394	111	788
inside-out vesicles[b]	4	23	45	92	68	208

[a] The unsealed plasma membrane fraction (P_2) was incubated in 40 volumes of PBS at 37 °C for 40 min to induce resealing in a right-side-out manner. [b] Resealing was done in 40 volumes of 0.5 mM phosphate, pH 8.0, as indicated in the text.

dextran gradient ultracentrifugation gave inside-out vesicles that retained only part of the enzyme activities. Sidedness assays were performed on these lymphocyte preparations. Sialic acid and cholinesterase accessibilities were 56% and 80%, respectively, in the right-side-out vesicles and none in the inside-out vesicles, while the latter preparation had 60% and 52% accessibility to NADH–cytochrome c oxidoreductase and acetylcholinesterase, respectively.

Measurement of Intact Cell-Surface Enzyme Activities. Results obtained above indicate the localization of PRibPP synthetase, HPRTase, and APRTase activities on both the erythrocyte and the lymphocyte membrane surfaces. For further confirmation of this observation, intact erythrocytes and lymphocytes were incubated in medium containing [^{14}C]adenine and various substrates and effectors of PRibPP synthetase. If indeed these are enzyme activities on the cell membrane surface, than after 1 h of incubation at 37 °C one should be able to measure the nucleotide formation in the incubation mixture after removal of the cells. Results of such studies are presented in Table IV. Radioactive nucleotide formation was measurable outside of both lymphocytes and erythrocytes. This demonstrated that PRibPP synthetase activity was present on the outer membrane surface. The average size of lymphocytes is 10 μm, and that of erythrocytes is between 7.2 and 7.0 μm. The 100 times more nucleotide formed outside of the former cells cannot be explained on the basis of their differences in cell surface area alone but can be accounted for on the basis of their proliferative capacity. This difference cannot be accounted for on the basis of cell leakage. Erythrocytes are far more fragile than lymphocytes under all conditions. If leakage has contributed any significant amount of extracellular nucleotides, then the amount produced by equal number of erythrocytes and lymphocytes in the medium should be much higher for the former; exactly the opposite is shown in Table IV. Phosphate and Mg^{2+} affected both the PRibPP synthetase activity on the cell surface and the formation of nucleotides within the cells. Comparing the results of experiments 1 and 3 in Table IV, one observed that the addition of ATP and R-5-P stimulates purine uptake. Experiment 4 indicates the existence of membrane APRTase since some labeled AMP is still formed in the media in the absence of exogenously added APRTase. It was further determined that under our experimental conditions, the extracellular formation of nucleotides was linear with time up to 90 min of incubation at 37 °C. Linearity was also observed when the amount of erythrocytes changed from 1.6×10^8 to 13×10^8 cells in each incubation medium. When lymphocytes from a leukemic patient were incubated in medium similar to the control in Table IV, 437.4 pmol of nucleotides $(10^6 \text{ cells})^{-1}$ h^{-1} was formed extracellularly, which is about 5 times more than that produced by normal lymphocytes.

Effects of Magnesium and Phosphate on Cell-Surface Enzyme Activity and Purine Incorporation. Fresh erythrocytes were incubated with [^{14}C]adenine in isotonic dextrose buffer, and in the presence and absence of partially purified APRTase and substrates for PRibPP synthetase (Figures 1 and 2). After 1 h of incubation at 37 °C, the formation of [^{14}C]AMP was measured in the medium as well as within the cells. The experiments were done under (a) varying Mg^{2+} concentrations

Table IV: Formation of Radioactive Nucleotides with Intact Cells Incubated in Medium Containing [^{14}C]Adenine

expt	incubn medium	radioactive nucleotides formed in media [pmol $(10^6 \text{ cells})^{-1}$ h^{-1}] lymphocytes	erythrocytes	radioactive nucleotides formed within cells [pmol $(10^6 \text{ cells})^{-1}$ h^{-1}] lymphocytes	erythrocytes
1	complete reaction[a]	88.98 ± 19.91	0.95 ± 0.09	112.87 ± 3.82	2.88 ± 0.87
2	no PO_4^{3-}	22.38	0.08	46.14	0.03
3	no ATP, no R-5-P	1.02	<0.01	87.16	1.15
4	no partially purified APRTase	17.80	0.07	148.83	3.48
5	no Mg^{2+}[b]	<0.01	<0.01	75.24	2.05

[a] About 10^7 lymphocytes or 10^9 erythrocytes were incubated in isotonic Tris-HCl-buffered dextrose (2.5 mM) that contained 0.125 mM [^{14}C]Ade, 5 mM Mg^{2+}, 10 mM PO_4^{3-}, 1 mM ATP, 1 mM R-5-P, and 5 μL of partially purified APRTase. Blanks for measuring the nucleotide formation in medium were the medium that contained only cells and [^{14}C]Ade. Cells that were destroyed by boiling were used for blanks of transport measurements. Cells were counted before and after the incubation; no loss was observed. [b] Cells were thoroughly washed with EDTA buffers prior to assay.

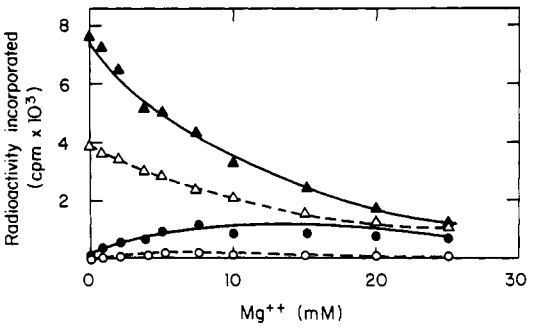

FIGURE 1: Effects of magnesium on cell-surface enzyme activity and purine transport. About 10^9 normal erythrocytes were incubated in isotonic dextrose buffer with or without added PRibPP synthetase substrates and partially purified APRTase and various amounts of Mg^{2+}. The concentrations of the substrates and effectors are as follows: ATP, 1 mM; R-5-P, 1 mM; [^{14}C]Ade, 125 mM; PO_4^{3-}, 5 mM; partially purified APRTase, 0.5 μL. Incubation was at 37 °C for 1 h. (▲) Nucleotide formed within the cells in the presence of PRibPP synthetase substrates and effectors in the medium; (△) Nucleotides formed within the cells in the absence of ATP, R-5-P, and partially purified APRTase in the medium; (●) nucleotides found in medium with enzyme substrates and effectors; (○) nucleotides found in medium without ATP, R-5-P, and partially purified APRTase.

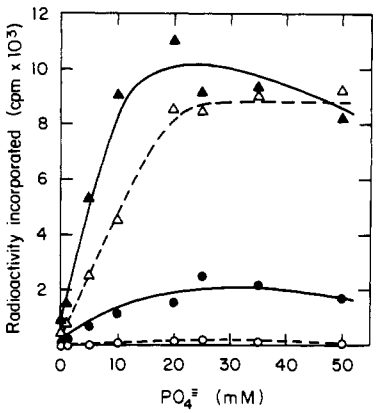

FIGURE 2: Effects of phosphate on cell-surface enzyme activity and purine transport. Assay conditions were the same as those described in Figure 1, except that all the incubation media contained 5 mM Mg^{2+} and phosphate concentration varied. (▲) Nucleotides formed within the cells in the presence of PRibPP synthetase substrates and effectors in the medium; (△) nucleotides formed within cells incubated in medium without added ATP, R-5-P, and partially purified APRTase; (●) nucleotides found in the medium with enzyme substrates and effectors added; (○) nucleotides found in the medium with no enzyme substrates or APRTase added.

and (b) varying PO_4^{3-} concentrations. The [^{14}C]AMP formed in the medium is a measure of the PRibPP synthetase activity on the cell surface. The fact that cell counts before and after the incubation were unchanged assured that the integrity of the cells was maintained. No formation of nucleotides was measurable in the medium when either ATP, R-5-P, or both were absent. These observations ruled out the possibility that the extracellular nucleotides were due to cell leakage. Adenine incorporation was stimulated by the presence of PRibPP synthethase substrates (Figures 1 and 2). The Mg^{2+} and PO_4^{3-} stimulation profiles of the cell-surface PRibPP synthetase were identical with those reported on its cytosol counterpart (Yip et al., 1980c; Yip & Balis, 1980). It must be borne in mind that the same enzyme can behave differently on and off the membrane (Hochstadt & Quinlan, 1976). The effects of these two ions on the simultaneous transport and phosphoribosylation of adenine (Ade) intracellularly were different. Mg^{2+} inhibited and PO_4^{3-} stimulated the process. Since the reaction Ade + PRibPP → AMP + PP_i catalyzed by APRTase has been

Table V: Combined Effects of Phosphate and Magnesium Ions on Erythrocyte [^{14}C]Adenine Incorporation[a]

[phosphate] (mM)	rate of incorporation [pmol (10^6 cells)$^{-1}$ h^{-1}]		
	0 mM Mg^{2+}	5 mM Mg^{2+}	10 mM Mg^{2+}
1	1.17	0.45	0.29
5	2.06	1.38	1.10
10	2.05	2.25	2.14
20	2.12	2.51	2.70

[a] The experimental conditions are similar to those described in Figures 1 and 2.

reported to be strongly stimulated by Mg^{2+} and inhibited by the presence of PO_4^{3-} (Srivastava & Bentler, 1971; Hori & Henderson, 1966a,b), the above observations support the argument that translocation of adenine with concomitant conversion to AMP within the membrane matrix occurs, and diffusion followed by intracellular phosphoribosylation might not be the only mechanism of adenine transport.

Interrelated Effects of Mg^{2+} and PO_4^{3-} on Purine Incorporation. The effects of Mg^{2+} and PO_4^{3-} on purine incorporation are interrelated. The inhibition observed with Mg^{2+} diminishes with increasing concentrations of PO_4^{3-}. This is illustrated in Table V. At 1 mM PO_4^{3-}, addition of 10 mM Mg^{2+} to the incubation medium produced 75% inhibition of [^{14}C]Ade incorporation into cells compared to that seen when Mg^{2+} was absent. The inhibitory effect of Mg^{2+} was abolished when PO_4^{3-} was 10 mM or higher; in fact, slight stimulation was observed at such high phosphate concentrations.

Differential Effects of Mg^{2+} and PO_4^{3-} on Purine Metabolizing Enzymes on and off the Membrane. Unsealed erythrocyte membrane vesicles were prepared. Part of these vesicles were solubilized by the addition of 0.2% Triton. The PRibPP synthetase, and APRTase from the original hemolysate, membrane vesicle suspension and the Triton-solubilized membrane preparation were assayed under various conditions. The results are presented in Table VI. It is shown here that both APRTase and PRibPP synthetase on the membrane have lesser requirements for Mg^{2+} for activation than do the hemolysate enzymes. When Mg^{2+} was omitted in the assays, only trace amounts of hemolysate APRTase and PRibPP synthetase activities were measurable. However, with membrane preparations, close to 10% of the control activities still remained. The Triton-solubilizing fraction behaves more like the membrane-bound enzymes in their responses to PO_4^{3-} and Mg^{2+}. The result seemed to suggest an intrinsic difference between membrane and cytoplasmic origin rather than the effect of localization.

Purine Metabolizing Enzyme Activity of HPRTase-Deficient Erythrocyte Ghosts. About 2 mL of fresh erythrocytes was obtained from a HPRTase-deficient patient and a normal donor. The hemoglobin-free unsealed erythrocyte ghosts were prepared in the absence of Mg^{2+} to avoid spontaneous resealing. After hemolysis and centrifugation, part of the membrane pellets from the patient were resuspended in normal hemolysate and vice versa for 1 h at 4 °C before they were further processed with seven hypotonic buffer washings. Enzyme assays were done on the treated and untreated unsealed ghosts and ghosts that had been washed less than 7 times. It is shown in Table VII that the preparations have obtained a constant specific activity of all the enzymes tested after being washed 5 times. Magnesium seems to affect the HPRTase binding to the membrane more strongly than it does the other two enzymes. Right-side-out ghosts prepared in the presence of Mg^{2+} (as shown in Table I) contained more than 10 times

Table VI: Differential Effects of Mg^{2+} and PO_4^{3-} on Enzymes on and off the Erythrocyte Membrane[a]

	enzyme activity [pmol (mg of protein)$^{-1}$ min^{-1}]					
	APRTase from			PRibPP synthetase from		
assay conditions	hemolysate	membrane suspension	solubilized membrane	hemolysate	membrane suspension	solubilized membrane
complete reaction	432	13.5	17.7	306	335	229
$-Mg^{2+}$	1.5	1.41	1.24	0.2	19	26
$+PO_4^{3-}$ [b]	339	11.2	15.0			
$-PO_4^{3-}$ [c]				3	89	94
$-Mg^{2+}, -PO_4^{3-}$				0	29	0

[a] The unsealed erythrocyte membranes were prepared by hemolysis of erythrocytes in 60 volumes of 5 mM phosphate buffer, pH 8.0 in the absence of Mg^{2+}. The hemolysate and the unsealed ghost suspension in the absence and presence of 0.2% Triton X-100 were assayed for enzyme activities. The APRTase control assay contained 2.5 mM PRibPP, 4.2 mM Mg^{2+}, and 0.125 mM [^{14}C]Ade, and all in 0.05 M Tris-HCl buffer at pH 8.0. The PRibPP synthetase control assay contained 70 mM Tris buffer (pH 7.4), 0.7 mM EDTA, 1.7 mM ETSH, 7 mM Mg^{2+}, 33 mM PO_4^{3-}, 0.67 mM R-5-P, 0.67 mM ATP, 0.125 mM [^{14}C]Ade, and 5 μL of partially purified APRTase in a total volume of 150 μL. [b] Besides the added 5 mM PO_4^{3-} to the APRTase assay, the enzyme preparations (prepared and suspended in 5 mM phosphate buffer) had contributed 1 mM PO_4^{3-} to the final assay mixture. [c] The enzyme preparation had contributed 1 mM PO_4^{3-} to the final assay mixture in the absence of added PO_4^{3-}.

Table VII: Purine Metabolizing Enzyme Activities of HPRTase-Deficient Erythrocyte Membrane Preparations

	enzyme activity [pmol (mg of protein)$^{-1}$ min^{-1}]					
	APRTase		HPRTase		PRibPP synthetase	
hemolysate						
normal control	333		1335		831	
HPRTase-deficient patient	640		0		739	
membrane prepn[a]						
Triton addition	−	+	−	+	−	+
normal control after 5 washes	9.4	16	23	45	109	145
normal control after 6 washes	13	18	21	46	153	215
normal control after 7 washes	15	21	23	42	173	222
normal membrane suspended in patient's hemolysate for 1 h before succeeding 7 washes	7.2	14	23	42	170	191
membrane prepn from patient after 7 washes	9.6	13	0	23	439	745
membrane prepn from patient suspended in normal hemolysate for 1 h before 7 washes	7.6	15	0	21	402	722

[a] Unsealed erythrocyte ghosts, prepared in lysing fresh erythrocytes in 40 volumes of 5 mM pH 8.0 phosphate buffer in the absence of Mg^{2+}. The subsequent washings were done with the same buffer. About 0.3–0.5 mL of creamy-white loosely suspended membrane suspension (2–4 mg of protein) was obtained from 1 mL of erythrocytes.

as much HPRTase activity as either the unsealed ghosts or the inside-out ghosts which were both prepared in the absence of Mg^{2+}. The most interesting observation presented in Table VII is that the unsealed ghosts prepared from HPRTase-deficient erythrocytes had half of the normal membrane-associated enzyme activity in the presence of membrane solubilizer. Incubation of the patient's membrane preparation with normal hemolysate, or vice versa, did not change the enzyme content of the membranes. These observations assured the authenticity of the membrane origin of these enzymes. Furthermore, the hemolysate from the HPRTase-deficient patient had a normal amount of PRibPP synthetase activity, but the unsealed ghosts of the patient were associated with 3.5 times more of the enzyme activity than the normal preparation, which might be the underlying cause of the observed concomitant elevation of the PPibPP level in HPRTase-deficient erythrocytes.

Discussion

The importance of the purine recycling process is 2-fold: (a) defects are related to many diseases, e.g., Lesch-Nyhan syndrome, severe combined immunodeficiency disease, glycogen storage disease II, and gout (Balis, 1976); (b) many analogues of purine nucleosides and their bases have been used in chemotherapy of cancer (Langen, 1975).

It is generally assumed that the mammalian purine metabolizing enzymes HPRTase, APRTase, and PRibPP synthetase are soluble, cytoplasmic enzymes. The studies here with intact human erythrocytes and leukocytes and with their unsealed and sealed membrane vesicles demonstrate that part of these enzyme activities are associated with cell membranes.

Many years have passed since the discovery of the X-linked metabolic disorder the Lesch-Nyhan (L-N) syndrome, in which the most obvious biochemical lesion is the absence of HPRTase activity in tissue extracts (Seegmiller et al., 1967) and the elevated level of PRibPP (Green et al., 1970) and APRTase activity (Rubin et al., 1969). Yet, the biological significance of purine salvage has still remained obscure. The assumption has been made by many that the blockade of purine salvage leads to the neurologic symptoms. The primary flaw in this reasoning is that another group of patients have reduced levels of HPRTase but are free of the same neurologic aberrations (Yu et al., 1971). Of particular interest to our present studies is the fact that we have observed individuals without the stigmata of L-N disease who have essentially zero HPRTase in their red cell lysate but have normal capacity to convert [^{14}C]hypoxanthine ([^{14}C]Hyp) into radioactive IMP with their intact leukocytes (Dancis et al., 1973). Similar observations have also been reported more recently on an intelligent, nonmutilating patient with features of the L-N syndrome, whose fibroblasts are able to convert Hyp into nucleotides (Bakay et al., 1979). These observations seem to suggest that there might be membrane-associated variants of HPRTase, and the different distribution of such variants among patients may contribute to the severity of the syndrome. Willers et al. (1977) have studied the hypoxanthine incorporation and resistance to 8-azaguanine in cultured human fibroblasts. They observed that cells from patients with L-N syndrome showed almost no Hyp incorporation and resistance to concentrations of 8-azaguanine up to 1 mM whereas cells of patients with partial HPRTase deficiency demonstrated various patterns of hypoxanthine uptake and partial resistance to 8-azaguanine. They have also obtained some evidence to suggest the existence of a membrane-associated HPRTase

variant in human fibroblasts. Our results presented in Table VII confirmed the existence of a membrane-associated HPRTase variant. DeBruyn & Oei (1977) have demonstrated both HPRTase and APRTase activities on practically hemoglobulin-free erythrocyte ghosts, further supporting the existence of membrane-associated phosphoribosyltransferases. That PRibPP synthetase from mammalian tissue may be membrane associated was first indicated when Roth et al. (1974) purified this enzyme from rat liver by using a procedure that could not distinguish between particulate and soluble enzymes. At the same time, Lebo & Martin (1977) presented some evidence indicating that the enzyme from rat hepatoma cells might be particulate. None of the above observations have been confirmed. Our recent report on the rat intestinal mucosa membrane-associated PRibPP synthetase and HPRTase activities seems confirmatory (Yip et al., 1980a).

There are two types of membrane proteins: intrinsic, which are inserted into the bilayer to varying degrees, and extrinsic, which are bound to the charged surface of the bilayer. In view of the rapid decrease of PRibPP synthetase, HPRTase, and APRTase activities upon purification of the membranes, one would assume these enzymes may be the loosely bound extrinsic proteins. Similar conclusions were also drawn by DeBruyn & Oei (1977) when they observed that the pH affected the binding of erythrocyte ghost phosphoribosyltransferases. The membrane-associated purine phosphoribosyltransferases reported from bacteria also seem to be loosely bound extrinsic proteins, since they can be released by osmotic shock (Hochstadt-Ozer & Stadtman, 1971).

Interestingly, when DeBruyn & Oei (1974) incubated intact normal human erythrocytes with radioactive purine bases in the presence of exogenous phosphoribosyl pyrophosphate (PRibPP), labeled AMP was found in the medium. This is what we observed when ATP and R-5-P replaced PRibPP. We have detected these enzyme activities on both sides of the membranes (Tables I and III) and on the outer surface of the intact cells (Table IV, Figures 1 and 2). Therefore, if blood cell membrane-associated PRibPP synthetase, APRTase, and HPRTase are indeed extrinsic proteins, then carrier systems must exist to transport them between the inner and outer membrane surface.

Many studies have indicated the importance of the transport of purine and pyrimidine bases and nucleosides across the cell membrane and that changes in this capacity of a cell are closely related to alterations in its growth properties (Berlin & Oliver, 1975). The exact physiological role and mechanisms are not clearly defined. Emerging evidence suggests that the relative responsiveness and acquired resistance of tumor cells to some anticancer agents are related to the extent of their transport, intracellular accumulation, and retention. There are two predominant schools of thought as to the mechanisms of purine base transport by mammalian cells. The first adheres to the concept that purine base uptake occurs in a variety of cell systems by a group translocation mechanism catalyzed by phosphoribosyltransferases (Hochstadt, 1974; Hochstadt & Quinlan, 1976; Jackman & Hochstadt, 1976). The alternate school believes that purine base transport, in some cell lines, is distinct from metabolism (Berlin & Hawkins, 1968; Berlin, 1970). Results from the studies of Zylka & Plagemann (1975) on Hyp/Gua phosphoribosyltransferase-deficient Novikoff cells supported the conclusion of the latter. Later, by using a rapid kinetic technique, Marz et al. (1979) were able to measure the purine/pyrimidine influx into cells in which substrate conversion to nucleotides was negligible due either to lack of the appropriate enzymes or to depletion of cells of ATP (PRibPP). With their transport kinetic data, they concluded that facilitated diffusion is the mechanism for purine and pyrimidine transport in mammalian cells and that group translocations do not occur in such cell systems. On the other hand, many enzymes which were considered to be cytosol are emerging as partially membrane associcated, e.g., HPRTase (Prasad et al., 1981; Yip et al., 1980a–c), PRibPP synthetase (Yip et al., 1980a–c), adenosine deaminase, and inosine phosphorylase (Li & Hochstadt, 1976). These observations seem to suggest that if purine/pyrimidine transport is involved and regulated by a membrane-associated multienzyme complex, then the product of this transport machinery can be anything from base to nucleotide. Our results further confirmed the membrane localization of some of these purine metabolizing enzymes and support the enzyme-mediated group translocation mechanism in purine uptake by mammalian cells.

The studies of Li et al. (1978) on the role of metal ions in the mechanism of PRibPP synthetase from *S. typhimurium* have indicated that this enzyme requires two divalent cations per catalytic site. The role of Mg^{2+} is not only to supply Mg^{2+}ATP as substrate for the enzymatic reaction but also to support the enzyme in a stereoconfiguration that is more accessible to substrate. Our results on the studies of polyamine inhibition on purine metabolizing enzymes (Yip & Balis, 1980) had not only supported their proposed mechanism for PRibPP synthetase but also further extended the dual Mg^{2+} role in the APRTase and HPRTase reaction. If these enzymes are indeed involved in membrane purine transport function, then the Mg^{2+} inhibition on purine transport, as shown in Figure 1 and Table V, and the difference in the Mg^{2+} requirement for membrane and cytosol enzyme, as shown in Table IV, seem to suggest that the enzymes of (or on) the membrane are in the stereoconfigurations which no longer require Mg^{2+} for activation, and on the contrary are inhibited by the excess of its presence.

PRibPP synthethase from human hemolysate has an absolute requirement of PO_4^{3-} for activation (Fox & Kelley, 1971). The results presented in Table VI indicate that the PO_4^{3-} requirement for membrane PRibPP synthetase was lower than the cytosol counterpart. We have shown previously that PO_4^{3-} stabilized PRibPP synthetase upon thermal inactivation; addition of Mg^{2+} at low phosphate concentration as shown in Table V is consistent with the previous observation that the magnesium phosphate complex is not an effector for PRibPP synthetase.

We have observed that stimulation of surface PRibPP synthetase by the presence of its substrates, ATP and R-5-P, and its effector, PO_4^{3-}, also stimulated purine transport (Table IV and Figure 1). This seems to indicate that membrane-bound PRibPP (produced by the membrane-bound PRibPP synthetase) is able to stimulate purine transport. PRibPP is a very labile compound (Yip & Balis, 1980) subject to various phosphatase reactions (Fox & Marchant, 1977). Membrane-bound PRibPP may be protected from such hydrolysis by its location and can be transmitted immediately to the purine phosphoribosyltransferases in the membrane vicinity; thus, phosphoribosylation and transport of a purine moiety occur simultaneously through these membrane-bound enzymes. Many observations reported in the literature are supportive of this proposed scheme: (1) The membrane-bound HPRTase and APRTase of *Escherichia coli* are capable of nucleotide formation without added PRibPP (Hochstadt & Quinlan, 1976). (2) Both APRTase and HPRTase can bind PRibPP and be stabilized in the absence of other substrates (Yip & Balis, 1975a,b; Krenitsky & Papaioannou, 1969). (3) The initial burst of AMP in the absence of added effector observed

by Groth & Young (1971) was interpreted as evidence that APRTase from rat liver normally binds a phosphorylated ribose group which can initiate the enzyme reaction in the absence of Mg^{2+}. (4) Kinetic studies of HPRTase and APRTase indicate that the enzymes bind with PRibPP first before reacting with their prospective purine (Hori & Henderson, 1966a,b; Henderson et al., 1968). ATP is detectable in serum and various body fluids as are various sugar compounds. Therefore, the proposed purine membrane transport mechanism is consistent with the existing data.

Acknowledgments

We thank Dr. Francis Collins of Yale University School of Medicine for his cooperation in supplying the HPRTase-deficient cells and for his informative discussions.

References

Bakay, B., Nissinen, E., Sweetman, L., Francke, U., & Nyhan, W. L. (1979) *Pediatr. Res. 13*, 1365–1370.

Balis, M. E. (1976) *Adv. Clin. Chem. 18*, 213–246.

Berlin, R. D. (1970) *Science (Washington, D.C.) 168*, 1539–1545.

Berlin, R. D., & Hawkins, R. A. (1968) *Am. J. Physiol. 215*, 932.

Berlin, R. D., & Oliver, J. F. (1975) *Int. Rev. Cytol. 42*, 287–336.

Boyum, A. (1976) *Scand. J. Immunol., Suppl. No. 5*, 9–15.

Dancis, J., Yip, L. C., Cox, R. P., Piomelli, S. J., & Balis, M. E. (1973) *J. Clin. Invest. 52*, 2068–2074.

DeBruyn, C. H., & Oei, T. L. (1974) *Purine Metabolism in Man* (Sperling et al., Eds.) pp 223–227, Plenum Press, New York.

DeBruyn, C. H., & Oei, T. L. (1977) *Adv. Exp. Med. Biol. 76*, 139–152.

Fox, I. H., & Kelley, W. N. (1971) *J. Biol. Chem. 246*, 5739–5748.

Fox, I. H., & Marchant, P. J. (1977) *Adv. Exp. Med. Biol. 76*, 85–91.

Green, M. L., Bayless, J. R., & Seegmiller, J. E. (1970) *Science (Washington, D.C.) 167*, 887–889.

Groth, D. P., & Young, L. G. (1971) *Biochem. Biophys. Res. Commun. 43*, 82–87.

Henderson, J. F., Brox, L. W., Kelley, W. N., Rosenbloom, F. M., & Seegmiller, J. E. (1968) *J. Biol. Chem. 243*, 2514–2522.

Hochstadt, J. (1974) *CRC Crit. Rev. Biochem. 2*, 259–310.

Hochstadt, J., & Quinlan, D. (1976) *J. Cell. Physiol. 89*, 839–852.

Hochstadt-Ozer, J., & Stadtman, E. R. (1971) *J. Biol. Chem. 246*, 5304–5311.

Hori, M., & Henderson, J. F. (1966a) *J. Biol. Chem. 241*, 1406–1411.

Hori, M., & Henderson, J. F. (1966b) *J. Biol. Chem. 241*, 3404–3408.

Jackman, L. E., & Hochstadt, J. (1976) *J. Bacteriol. 126*, 312–326.

Krenitsky, T. A., & Papaioannou, R. (1969) *J. Biol. Chem. 244*, 1271–1277.

Langen, P. (1975) *Antimetabolites of Nucleic Acid Metabolism.*

Lebo, R. V., & Martin, D. W. (1977) *Purine Metabolism in Man* (Sperling, O., & Wyngaarden, J. B., Eds.) pp 570–574, Plenum Press, New York.

Li, C., & Hochstadt, J. (1976) *J. Biol. Chem. 251*, 1181–1187.

Li, T. M., Mildvan, A. S., & Witzer, R. L. (1978) *J. Biol. Chem. 253*, 3918–3923.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Marz, R., Wohlhueter, R. M., & Plagemann, P. G. W. (1979) *J. Biol. Chem. 254*, 2329–2338.

Prasad, R., Shopsis, C., & Hochstadt, J. (1981) *Biochim. Biophys. Acta 643*, 306–318.

Roth, D. G., Sheton, E., & Deuel, T. F. (1974) *J. Biol. Chem. 249*, 291–296.

Rubin, C. S., Balis, M. E., Poumelli, S., Berman, P. H., & Dancis, J. (1969) *J. Lab. Clin. Med. 74*, 732–736.

Scholar, E. M., & Calabresi, P. (1973) *Cancer Res. 33*, 94–103.

Seegmiller, J. E., Rosenbloom, F. M., & Kelley, W. N. (1967) *Science (Washington, D.C.) 155*, 1682–1684.

Srivastava, S. K., & Bentler, E. (1971) *Arch. Biochem. Biophys. 142*, 426–434.

Steck, T. L., & Kant, J. A. (1974) *Methods Enzymol. 41*, 172.

Walsh, F. S., & Crumpton, M. J. (1977) *Nature (London) 269*, 307–311.

Willers, I., Held, K. R., Singh, S., & Goedde, H. W. (1977) *Clin. Genet. 11*, 193–200.

Yip, L. C., & Balis, M. E. (1975a) *Biochem. Biophys. Res. Commun. 63*, 722–729.

Yip, L. C., & Balis, M. E. (1975b) *Biochemistry 14*, 3204–3208.

Yip, L. C., & Balis, M. E. (1980) *Biochemistry 19*, 1849–1856.

Yip, L. C., Roome, S., & Balis, M. E. (1978) *Biochemistry 17*, 3286–3291.

Yip, L. C., Yeh, A. K., & Balis, M. E. (1980a) *Am. J. Physiol. 239*, 266–271.

Yip, L. C., Tedde, A., & Balis, M. E. (1980b) *Biochem. Pharmacol. 29*, 2888–2890.

Yip, L. C., Yu, T. F., & Balis, M. E. (1980c) *Purine Metabolism in Man, IIIa* (Rapado, A., Watts, R. W. E., & DeBruyn, C. H., Eds.) pp 307–312, Plenum Press, New York.

Yu, T. F., Balis, M. E., Krenitsky, T. A., Dancis, J., Silvers, D. N., Elion, G. B., & Gutman, A. B. (1971) *Ann. Intern. Med. 74*, 255–264.

Zylka, J. J., & Plagemann, P. G. W. (1975) *J. Biol. Chem. 250*, 5756–5767.