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SURFACE PROPERTIES OF ERYTHROCYTES: NORMAL, PAROXYSMAL NOCTURNAL HEMOGLOBINURIA AND GLUTATHIONE-TREATED CELLS

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SUMMARY

We have examined surface parameters of erythrocytes and found differences that distinguish among normal, paroxysmal nocturnal hemoglobinuria (PNH) and glutathione-treated cells.

1. The acetylcholinesterase activity of PNH cells is reduced compared to normal cells, and the reduction in activity parallels the severity of the disease, but the acetylcholinesterase activity of GSH-treated normal cells is normal.

2. Lactoperoxidase-catalyzed labeling of intact erythrocytes demonstrates that PNH erythrocytes bind much more ^{125}I than normal cells while GSH-treated cells bind less.

3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of isolated erythrocyte membranes reveals anomalies in the distribution of Coomassie blue-stained bands which are not seen in GSH-treated erythrocytes.

4. The number of acetylcholinesterase activity sites revealed by binding of [^3H]diisopropylfluorophosphate to isolated erythrocyte membranes was increased in PNH cells and decreased in GSH-treated cells compared to normal cells.

5. The number of *p*-chloromercuribenzene sulfonate molecules bound to intact erythrocytes is decreased in PNH erythrocytes, but not in GSH-treated erythrocytes, compared to normal cells.

6. Na^+ uptake at 4 °C is increased to a greater extent in GSH-treated erythrocytes than in PNH erythrocytes compared to normal values.

7. These results suggest that the PNH characteristic involves a constellation of differences in surface properties and that these properties are not shared by GSH-treated cells. GSH-treated cells are, therefore, not an adequate model for PNH cells.

INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare, acquired blood disease which is usually fatal. Nocturnal episodes of hemolysis give rise to "coca-cola

Abbreviations: BMHP, 1-bromomercuri-2-hydroxypropane; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PCMBS, *p*-chloromercuribenzene sulfonate; PNH, paroxysmal noctural hemoglobinuria; DFP, diisopropylfluorophosphate.

colored" morning urine, the classical presenting symptom. The incidence of PNH is approximately two patients per million (population) and it appears in all races and both genders with equal frequency. PNH appears to be acquired rather than inherited, as there has never been more than one case reported in any family[1]. The disease has been clinically well described for the past century but it is still unclear why the hemolytic episodes occur particularly at night[2]. The triggering mechanism for cell lysis appears to be binding of activated complement from the plasma[3].

One of the characteristics of PNH erythrocytes is their low acetylcholinesterase activity. Those cells which are most sensitive to complement lysis have no acetylcholinesterase activity and those relatively insensitive to complement lysis have somewhat reduced activity[4]. The decrease in acetylcholinesterase activity of PNH compared to normal erythrocytes is a convenient measure of the severity of the disease, since it parallels the proportion of complement-sensitive cells. It has been demonstrated that lysis of normal cells by complement does not affect erythrocyte acetylcholinesterase activity[4]. The involvement of acetylcholinesterase in the PNH defect has been debated. It is extremely unlikely that the loss of enzyme activity is responsible for the PNH defect. Acetylcholinesterase is located on the outer surface of the erythrocyte membrane[5]. It is quite possible that the basic PNH defect changes the surface of the erythrocyte so that the cell binds activated complement in an abnormal manner and the surface-located enzyme activity is altered. The accumulated evidence suggests that the PNH anomaly is confined to the membrane surface. This project was conceived of as an investigation of the surface properties of normal and PNH erythrocytes.

Study of PNH has been hampered by the difficulty of obtaining blood samples from patients who are already moderately to severely anemic. Attempts have been made to produce PNH cells by treating normal cells *in vitro* with various agents. To date the most promising method for producing PNH-like cells is treatment with reduced glutathione[6]. It was decided to include a group of GSH-treated erythrocytes in the study.

Several parameters of surface properties were chosen for investigation. The activity of acetylcholinesterase in the various experimental groups seemed interesting in view of its correlation with the severity of the disease so this measure was included. We also included a clinical diagnostic measure of the disease, the sucrose hemolysis test[7]. This test gave us a measure of the proportion of the red cell population that was complement sensitive.

Lactoperoxidase can be used to catalyse the iodination of protein tyrosines. When this reaction is performed on intact erythrocytes, only one molecular weight class of membrane proteins and one glycoprotein are iodinated[8,9]. If ghosts are used for the same procedure, most of the proteins are iodinated. It was concluded that in the intact red cell, the iodinated protein and glycoprotein are uniquely available at the surface of the cell. We compared the lactoperoxidase-catalyzed iodination of intact normal, PNH and GSH-treated erythrocytes.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of erythrocyte membranes dissolved in sodium dodecyl sulfate was used to compare the molecular weight distribution of both membrane proteins and glycoproteins in the experimental groups.

Acetylcholinesterase, like most esterases has a serine active site, which can

be covalently labeled with diisopropylfluorophosphate (DFP). Using radioactive DFP, it is possible to measure the number of acetylcholinesterase active sites per cell[10]. This technique was used on normal, PNH and GSH-treated erythrocytes in an attempt to determine whether the low acetylcholinesterase activity characteristic of PNH erythrocytes was due to loss or inactivation of the enzyme.

There have been several suggestions that stromal sulfhydryl groups are involved in the PNH defect. Vaccari and Baldini[11] found fewer non-reactive or hidden sulfhydryl groups in the membranes of PNH red cells compared to normal red cells. The ability of various sulfhydryl agents such as GSH, 2-aminoethylisothiouronium bromide and cysteine to produce "PNH-like cells" supports the central role of membrane sulfhydryls in the defect of the PNH cell[6,12]. We measured the availability of membrane sulfhydryls to titration with two sulfhydryl agents, 1-bromomercuri-2-hydroxypropane (BMHP) and *p*-chloromercuribenzene sulfonate (PCMBS).

One of the last parameters included was the measurement of Na^+ uptake at 4 °C in normal, PNH and GSH-treated erythrocytes.

METHODS

Fresh blood was collected into Vacutainers containing acid–citrate–dextrose solution. The blood was centrifuged at $2000 \times g$ for 10 min and the plasma and buffy coat removed. The cells were washed three times with an equal volume of isotonic sodium phosphate buffer (310 imosM, pH 7.5). The cells were centrifuged after each wash and the supernatant and remaining white cells removed by aspiration. The cells were suspended in an equal volume of the wash medium and stored at 4 °C. Ghosts were prepared by osmotic shock by addition of 8 vol. of 60 imosM phosphate-buffered saline (pH 7.2) containing 1 mM EDTA. The mixture was allowed to stand at room temperature for 15 min and was then centrifuged at $10\,000 \times g$ for 10 min. The supernatant was removed and an equal volume of 60 imosM Tris-buffered saline (pH 7.2) containing 1 mM EDTA was added, and the procedure was repeated. The final wash was done with 8 vol. of 30 imosM Tris-buffered saline (pH 7.2) containing 1 mM EDTA. The ghosts obtained were creamy white. Both red cell and ghost counts were performed as red cell counts with a Coulter Counter.

Solutions of 0.325 M GSH were prepared immediately before use by dissolving GSH obtained from Calbiochem in distilled water and adjusting the pH of the solution to 8.0 with 10 M NaOH. The osmolarity of the resultant solution was measured with an Advanced Osmometer and an equiosmolar solution of phosphate-buffered saline was prepared from 1 M NaCl and isotonic sodium phosphate buffer. The saline solution was adjusted to pH 8.0. 1 vol. of packed red cells was incubated with 3 vol. of GSH or saline for 1 h at 37 °C. Cells were then centrifuged at $2000 \times g$ for 10 min, the supernatant removed, and the packed cells washed with 4 vol. of isotonic sodium phosphate buffer (pH 7.5). The centrifugation and wash were repeated for a total of three washes. The cells were resuspended in 1 vol. of isotonic buffer and stored at 4 °C.

Hemolysis of red cells in low ionic strength media (sucrose hemolysis test) was done by the method of Hartmann *et al.*[7] using "sugar–water" and plasma

from the same sample. The difference in hemolysis in the presence and absence of plasma was termed "complement mediated hemolysis". 100% hemolysis was determined by incubating red cells with 0.5% Triton X-100. This was found to be more reproducible than either hemolysis in distilled water or hemolysis of frozen and thawed cells.

Our results are expressed per cell rather than per ml of packed cells because erythrocytes treated with high concentrations of GSH packed differently than normal cells. Counts were performed with a Coulter Counter.

Acetylcholinesterase determinations were done by a modification of the method of Ruch (unpublished). A 2-ml system containing 5 mM acetylcholine iodide and 0.01 ml packed red cells in barbital–KCl buffer (pH 8.0) was used. Cells were incubated for 30 min at 37 °C and the reaction was stopped by the addition of 2 ml of freshly prepared Triton–alkaline hydroxylamine reagent (2% Triton X-100–14% NaOH –14% NH_2OH, (2:1:1, by vol.)). The tubes were mixed well, 3 ml of 1.75% $FeCl_3 \cdot 6H_2O$ in 1 M HCl was added, and absorbance at 560 nm against a blank containing cells and reagent but no acetylcholine iodide was measured. One set of triplicates containing no cells was included in the incubation to control for nonenzymatic hydrolysis of acetylcholine iodide. The difference in absorbance was related to a standard curve to determine the number of μmoles acetylcholine iodide utilized.

Lactoperoxidase-catalyzed iodination of intact erythrocytes was done by the method of Phillips and Morrison[8] using $Na^{125}I$ from New England Nuclear and lactoperoxidase from Calbiochem. The reaction was initiated by addition of 5 μl of 2.2 mM H_2O_2 to the 2-ml system containing individual magnetic fleas to insure thorough mixing. An additional 5 μl of 2.2 mM H_2O_2 was added every 5 min to maintain the concentration in the presence of erythrocyte catalase and glutathione peroxidase. The reaction was carried out for 40 min at 37 °C and was stopped by washing the cells three times with 1 vol. of cold isotonic phosphate buffer (pH 7.5). Radioactivity of the cells was determined with a Baird Atomic spectrometer.

Disc gel electrophoresis was done in 0.1 and 1.0% sodium dodecyl sulfate containing polyacrylamide gels using Calanco disc gel apparatus according to the procedures of Maizel[13] or Fairbanks *et al.* [14]. E-C Apparatus slab gel electrophoresis equipment was used for 6-mm thick slab gels which were made according to the method of Fairbanks *et al.*[14]. Electrophoresis was carried out for 6–7 h at approx. 200 mA and 100 V. Gels were stained by the method of Fairbanks *et al.*[14] using Coomassie blue or Amido Black for protein and periodic acid–Schiff for glycoprotein. Stained gels were scanned in a Gilford spectrophotometer equipped with a linear transport accessory (Fig. 1). After scanning, the gels were sliced and the radioactivity of the individual slices was measured in a Nuclear Chicago well-counter. Counts *minus* background (which was less than 300 counts/slice per 10 min) were corrected to the number of cells and reported as counts per 10 min per slice per 10^8 cells.

Specific labeling of erythrocyte membrane acetylcholinesterase was done by the method of Bellhorn *et al.*[15]. Sears and Weed[16] have recently demonstrated that when radioactive DFP is incubated with red cells, the major portion of the label is found intracellularly rather than on the membrane, so we used ghost

preparations for our experiments. [^{3}H]DFP was obtained from New England Nuclear, nonradioactive DFP from Calbiochem and butyrylcholine iodide from Eastman Kodak Company. Radioactivity of the ghost preparations was determined

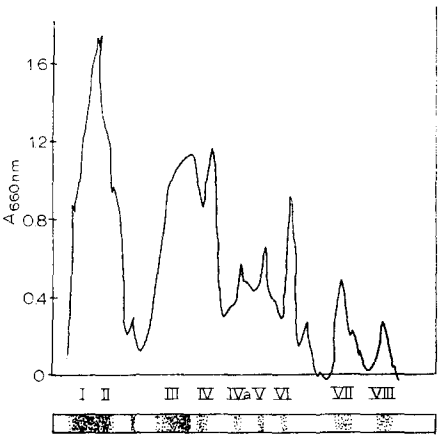

Fig. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of erythrocyte membranes. Maizel gel containing 5% acrylamide, 1% sodium dodecyl sulfate, stained with Coomassie blue. The bottom portion of the figure is a diagram of the stained gel. The top portion of the figure is a reproduction of the Gilford scan of the gel done at 660 nm. The top of the gel is to the left.

by scintillation counting in an aqueous cocktail containing Instagel from Packard Instrument. Radioactivity was determined after sodium dodecyl sulfate–polyacrylamide gel electrophoresis in unstained gels by the method of Leon and Bohrer[17].

Binding of sulfhydryl agents was determined by incubating a 5% suspension of washed erythrocytes in isotonic phosphate buffer (pH 7.5) containing either $5 \cdot 10^{-4}$ M ^{203}Hg-labeled PCMBS (0.1 μCi/ml) or $1 \cdot 10^{-4}$ M ^{197}Hg-labeled BMHP (1.0 μCi/ml), both obtained from Amersham, Searle. The cells were incubated for 30 or 45 min at room temperature and were then centrifuged at $2000 \times g$ for 5 min, the supernatant fluid was removed and the cells washed with an equal volume of isotonic phosphate buffer (pH 7.5) three times. Cells were suspended in an equal volume of the wash medium and the radioactivity measured in a Baird Atomic spectrometer.

Na^+ uptake was determined by incubating a 5% suspension of washed erythrocytes in isotonic NaCl containing 0.1 μCi ^{22}Na per ml for 20 h at 4 °C. The cells were then washed three times with an equal volume of isotonic choline chloride and the radioactivity of the cells measured in the (Baird Atomic) spectrometer.

RESULTS AND DISCUSSION

Many differences were found in the parameters of surface properties that were investigated. These differences clearly distinguish among PNH, normal and GSH-treated cells.

One of the first differences that became apparent was that GSH-treated normal

TABLE I

PRODUCTION OF GSH-TREATED CELLS

Source	*Date*	*Treatment*	*% Hemolysis*	*μmoles acetylcholine hydrolyzed per h per*		
				ml packed cells	*mg hemoglobin*	*10^8 cells*
Outdated	11/19/70	0.635 M GSH	29.3	334	—	—
Banked		0.635 iosM NaCl	0	354	—	—
Outdated	11/23/70	0.635 M GSH	34.5	245	19.2	7.42
		0.635 iosM NaCl	2.3	337	19.1	6.57
	12/4/70	0.635 M GSH	34.2	335	14.2	6.28
		0.635 iosM NaCl	0	346	18.0	5.54
		0.3175 M GSH	17.4	321	15.6	6.68
		0.3175 iosM NaCl	0	327	17.5	6.18
Fresh defibrinated	12/10/70	0.325 M GSH	17.7	349	13.3	10.80
		0.325 iosM NaCl	0	371	14.8	5.70
		Control	0	304	15.1	5.79

cells had normal acetylcholinesterase activity, while PNH cells had lowered acetylcholinesterase activity. We found that the acetylcholinesterase activity of GSH-treated erythrocytes remained normal whether the blood was outdated or fresh, defibrinated or collected into acid–citrate–dextrose solution (Table I). Treatment with twice the GSH concentration did not change the activity nor did varying the length of treatment. There was only one variable that seemed to alter these findings — the particular source of the blood. Blood from one normal subject, D.M., consistently showed a reduction in acetylcholinesterase activity when incubated with 0.325 M GSH (Table II). But we emphasize — this was not found with any other source of blood.

Our finding that GSH treatment fails to lower the acetylcholinesterase activity of normal erythrocytes has recently been confirmed by Stead and Rosse[18]. We feel that the decrease in acetylcholinesterase activity found both in our anomalous normal subject D.M. and by Kann *et al.*[6] may be explained by the recent findings that there are several different forms of acetylcholinesterase and that these forms are genetically determined[19]. One variant may be susceptible to alteration by GSH while the others are not.

In other respects the acetylcholinesterase results were consistent with the literature. PNH erythrocytes had reduced acetylcholinesterase activity compared to normal erythrocytes and this reduction paralelled the severity of the disease. The most severely affected patients had acetylcholinesterase activities as low as 50% of normal values, while one patient, who had only minor hemolytic episodes and could be identified at present as having PNH only through laboratory tests, had acetylcholinesterase levels that were 92% of normal values (Table III).

Results from the sucrose hemolysis test confirmed that both PNH and GSH-treated erythrocytes undergo complement-mediated hemolysis, while normal erythrocytes do not. Complement-mediated hemolysis was two to five times higher in the

TABLE II

ACETYLCHOLINESTERASE ACTIVITY OF CONTROL, GSH- AND SALINE-TREATED ERYTHROCYTES

Values are given in μmoles acetylcholine hydrolyzed/10^8 cells per h.

Date 1970	*Source*	*Control*	*GSH (%)*	*Saline (%)*
3/10	OO	5.54	5.95 (107)	6.38 (115)
7/19	BS	5.30	6.07 (115)	6.83 (120)
10/11	DM	7.91	4.19 (53)	7.22 (91)
11/11	GK	6.56	6.50 (99)	7.22 (110)
12/13	DM	5.20	4.25 (82)	6.94 (133)

TABLE III

ACETYLCHOLINESTERASE ACTIVITY OF PNH PATIENTS

Source	*Date*	*Acetylcholine-esterase activity***	*% of normal*	*Severity of disease*
Normal	—	6.21 ± 0.42*	100.0	—
OA	2/9	2.46	39.6	Severe
	2/17	3.36	54.1	
	3/10	2.80	45.0	
	7/19	2.54	40.8	
DF	2/17	4.58	73.8	Moderate/
	3/10	3.28	52.8	Severe
LB	10/11	7.26	117.0	Minimal
	11/11	6.09	98.0	
PC	10/11	3.96	63.7	Severe
ML	12/13	4.45	71.7	Moderate
RO	7/13	5.60	90.0	***
FI	7/13	6.55	105.0	***

* Average ± standard error for eight normals.
** μMoles acetylcholine hydrolyzed/10^8 cells per h.
*** Hemolytic disease other than PNH.

GSH-treated cells than in the PNH cells. Saline-treated normal cells undergo an increased nonspecific hemolysis compared to untreated normal cells, but show absolutely no complement-mediated hemolysis. Complement-mediated hemolysis was the one parameter studied that PNH and GSH-treated cells shared. We concluded that the treatment with GSH was able to change the surface of the erythrocyte so that binding with activated complement increased.

Lactoperoxidase-catalyzed labeling of the outermost components of the intact red blood cell with ^{125}I was markedly different among the experimental groups. The PNH patients tested bound up to five times more ^{125}I per cell than

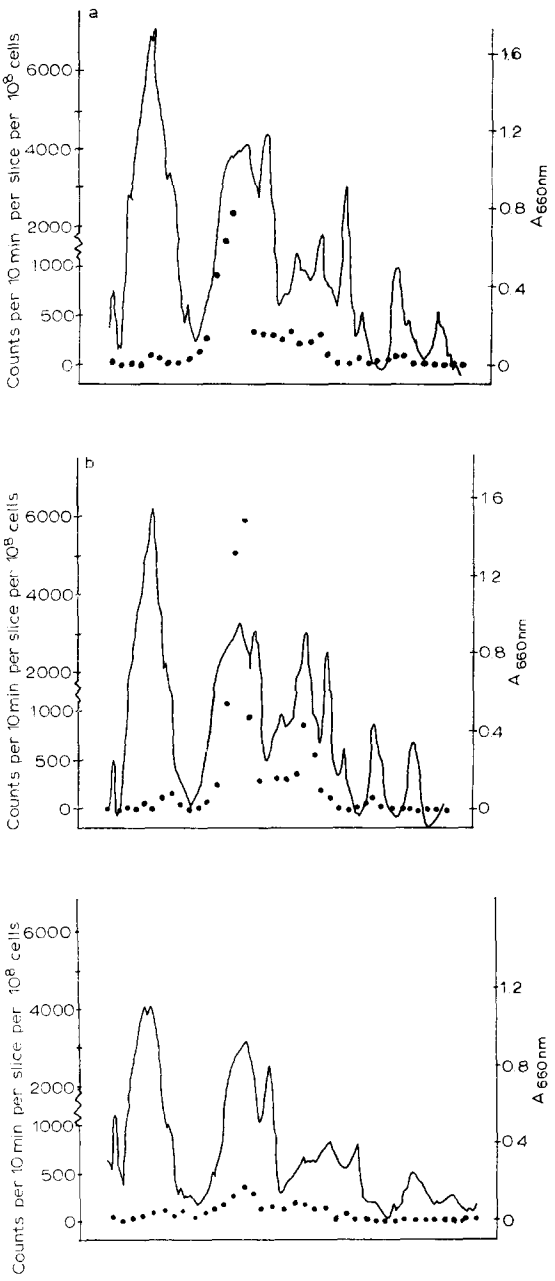

Fig. 2. Lactoperoxidase-catalyzed iodination of intact erythrocytes. Iodination was performed as in Methods. Isolated membranes were dissolved in 2% sodium dodecyl sulfate and electrophoresed in Maizel gel containing 5% polyacrylamide and 1% sodium dodecyl sulfate. Gels were stained with Coomassie blue scanned at 660 nm, then sliced in consecutive sections and the ^{125}I-radioactivity per slice determined. Reproduction of the Gilford scan with the radioactivity per slice per 10^8 cells represented by the superimposed dots. (a) Normal cells. (b) Cells from PNH patient OA. (c) GSH-treated normal cells.

TABLE IV

COMPARISON OF THE ACETYLCHOLINESTERASE ACTIVITY, AND NUMBER OF MOLECULES OF ^{125}I AND [^{3}H]DFP BOUND PER CELL EXPRESSED AS RATIO TO THE NORMAL CONTROL

	Acetylcholinesterase	^{125}I	[^{3}H]*DFP*
Normal	1.00	1.00	1.00
GSH-treated	1.07	0.28	0.42
GSH-treated DM	0.67	2.94	0.68
Saline-treated	1.15	0.17	0.85
PNH			
PC	0.64	5.48	2.92
OA	0.45	1.57	1.52
LB	0.98	5.00	1.42

the normal controls, while the GSH-treated cells bound much less ^{125}I per cell than the normal (Table IV). When iodinated intact cells were ghosted and the ghost proteins examined electrophoretically on sodium dodecyl sulfate–polyacrylamide gels, the distribution of label could be observed (Fig. 2). One particular Coomassie blue-stained band is always observed to contain the most label. This band has a molecular weight of approx. 90 000. The results shown in Fig. 2 suggest that in PNH erythrocytes there is more lactoperoxidase-catalyzed iodination of both Band III, the protein–glycoprotein usually labeled, and Band V compared to the normal. GSH-treated erythrocytes appear to have less label at Bands III and V than normal erythrocytes. There are several possible explanations for this finding. Since the ability of the enzyme to catalyze iodination depends upon it binding directly to the tyrosine molecule acted upon[20], it is very possible that the arrangement of the iodinated protein and/or glycoprotein in the PNH cell differs enough from the normal cell to make it more accessible to the enzyme, or merely to change the kinetics of the reaction. It is also possible that the total number of exposed tyrosines is increased in the PNH cell compared to the normal cell. In either case, the increased binding of ^{125}I to the PNH cell suggests to us that the surface components of the PNH cell are arranged differently from those in the normal cell.

At the gel concentration we used, the iodinated glycoprotein and protein components of the membrane migrate together. It was therefore impossible to distinguish whether the difference in iodination observed for PNH, compared to normal cells, was due to a difference in the iodination of the protein, the glycoprotein, or both.

The number of Coomassie blue-stained electrophoretic bands seen on sodium dodecyl sulfate–polyacrylamide gels appeared the same for all the groups examined. The molecular weights of the Coomassie blue-stained bands also appeared to be unaltered. However, the distribution of stain among the various molecular weight classes did differ significantly for the PNH compared to normal cells. The PNH patients examined have slightly altered proportions, although the differences are not the same for all. Table V gives a comparison of the percentage distribution of Coomassie blue stain on the gels. Patient O.A. has approximately three times the normal content of Band V (approx. 90 000 daltons) which has been located at

TABLE V

DISTRIBUTION OF COOMASSIE BLUE ON SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHOROGRAMS

Area of each peak as per cent of the total area from Gilford scans measured at 660 nm; average of duplicates. Peaks numbered as in Fig. 1.

	Group	*I and II*	*III*	*IV*	*IVa*	*V*	*VI*	*VIa*	*VII*	*VIII*
Normal	BS	47.4	28.3	9.5	0.9	2.5	4.2	0.9	4.2	2.2
	DM	42.2	28.7	9.6	2.8	2.3	4.3	0.6	5.6	4.1
PNH	OA	43.8	27.8	8.6	0.6	*6.8*	3.1	2.0	4.4	3.5
	PC	43.9	25.8	8.1	2.3	1.5	2.9	1.1	6.3	*9.3*
	LB	43.3	27.8	8.8	1.6	1.6	3.7	0.8	4.9	*6.9*

the outer surface of the erythrocyte membrane[21]. Both patients, P.C. and L.B., have an increased amount of Band VIII (approx. 20000 daltons) and the more severely affected patient, P. C., has a greater proportion of this band. It is possible that the differences in the electrophoresis patterns seen in the PNH cells are due to proteolytic breakdown of selected higher molecular weight components of the membrane. Fairbanks *et al.*[14] have emphasized the problems of proteolytic attack and we have followed all their suggestions to minimize this problem. Even if the differences are due to proteolytic attack, there appears to be something different about the PNH membrane structure that makes proteolysis more likely in PNH than normal cells, which suggests that there are differences in the arrangement of proteins within the membrane. If, on the other hand, these differences in the Coomassie blue-stained patterns are due to real differences in the protein content of the membrane, then we still may conclude that the architecture of the PNH erythrocyte membrane differs from that of a normal cell.

Acetylcholinesterase is a superficial enzyme which may be removed from human ghosts, but not intact erythrocytes, with relatively mild procedures[22]. Several possible explanations have been offered for the reduced acetylcholinesterase activity of PNH erythrocytes, including loss of the enzyme from the cell surface, and the presence of an inhibitor[23]. It is also possible that the enzyme is present in an altered form or that it is inaccessible to substrate in the diseased cells. Our data suggests that the most probable explanation is that it is present in an altered form, for specific labeling of the active site serine of acetylcholinesterase with radioactive DFP indicates that there are a greater number of acetylcholinesterase active sites per cell in PNH than in normal cells (Fig. 3). Acetylcholinesterase has two sites to which substrate must bind to be acted upon, an anionic binding site and an esteratic active site[24]. DFP binds only to the esteratic site. Our evidence suggests that in the PNH membrane, the conformation of acetylcholinesterase is disturbed just enough to disrupt the anionic binding site without affecting the esteratic site. The increased binding of DFP in PNH compared to normal cells may be due to either of two possibilities. There may actually be a larger number of acetylcholinesterase molecules, albeit defective, on the PNH cell than on the normal cell, or there may be exposure of normally masked molecules. There is not enough evidence at this time to dis-

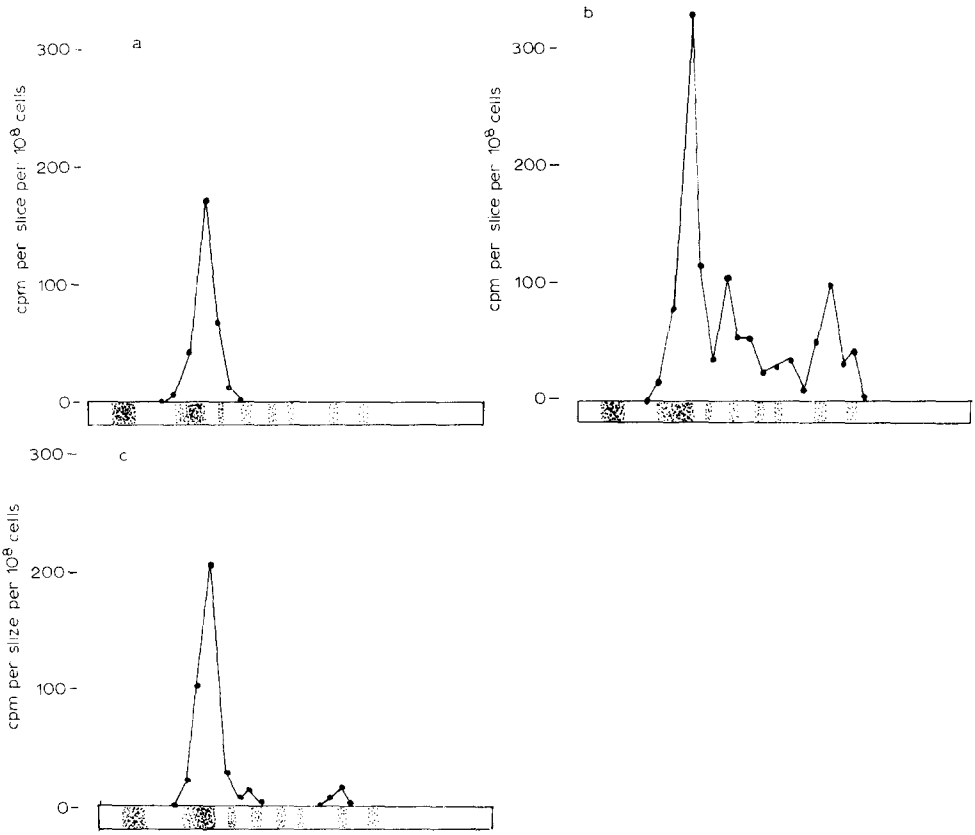

Fig. 3. [^{3}H]DFP distribution in erythrocyte membrane proteins. Isolated membranes were reacted with [^{3}H]DFP according to Bellhorn, *et al.*[15], dissolved in 2% sodium dodecyl sulfate and electrophoresed in 6-mm thick Fairbanks gels containing 5.6% acrylamide and 1% sodium dodecyl sulfate. The gels were sliced in consecutive sections and the radioactivity per slice per 10^8 cells determined. Diagram of a parallel gel stained with Amido Black is shown at the bottom of the figure. (a) Normal cells. (b) Cells from PNH patient OA. (c) Cells from PHN patient LB.

tinguish which of these two is the more likely, but there has been some suggestion that detergent solubilization of erythrocyte membranes releases a number of acetylcholinesterase molecules which are normally masked[25].

Shapiro *et al.*[26] have shown that the sulfhydryl agents PCMBS and BMHP react with different classes of sulfhydryl groups in the intact erythrocyte membrane. PCMBS reacts with sulfhydryl groups involved in the maintenance of the Na^+–K^+ barrier; these groups are affected by ionizing radiation. BMHP reacts with sulfhydryls that are more superficial, involved in a permeability function other than Na^+ and K^+ and are not radiosensitive. Binding of PCMBS was reduced 20–30% in two patients who were severely affected. It was normal for both the minimally affected patient and the GSH-treated cells. There was no significant difference in the binding of BMHP among the experimental groups (Table VI).

Sulfhydryl groups are intimately involved in the maintenance of the proper structure and functioning of the red blood cell membrane[26,27]. Several authors have

TABLE VI

COMPARISON OF THE ACETYLCHOLINESTERASE ACTIVITY, NUMBER OF MOLECULES OF [^{197}Hg]BMHP AND [^{203}Hg]PCMBS BOUND AND ^{22}Na FLUX PER CELL EXPRESSED AS RATIO TO THE NORMAL CONTROL

	Acetyl-cholinesterase	*[^{197}Hg]BMHP*	*[^{203}Hg]PCMBS*	*^{22}Na*
Normal	1.00	1.00	1.00	1.00
GSH-treated	1.07	1.04	0.98	3.30
Saline-treated	1.15	1.07	1.27	2.00
PNH				
OA	0.45	1.05	0.68	—
DF	0.63	1.01	0.79	—
LB	0.98	0.93	0.99	2.60
ML	0.72	—	1.03	1.65

found a reduction in the sulfhydryl content of the membranes from PNH compared to normal erythrocytes[11,28]. These differences were confined to the reactive oxidized and non-reactive or masked sulfhydryl groups. When measurement of the sulfhydryl groups in PNH and normal cells was done with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), there were no significant differences found[29]. However, DTNB measures only the reactive reduced sulfhydryl groups unless used in conjunction with detergent and reducing conditions, so the data from DTNB titration does not conflict with the earlier data. Furthermore, there was some suggestion that a small proportion of the sulfhydryl groups located on the outside of the membrane might be abnormal. That suggestion was borne out by our findings that there is a decrease in the number of PCMBS molecules bound per cell in PNH cells compared to normal cells.

Uptake of $^{22}Na^+$ in cells incubated at 4 °C was twice normal in the PNH cells and three times normal in the GSH-treated cells (Table VI). This suggests to us that there is at least a partial breakdown in the Na^+–K^+ permeability barrier of the PNH cell membrane which may involve the PCMBS-titrable sulfhydryl groups. The raised ($Na^+ + K^+$)-activated ATPase activity characteristic of PNH erythrocytes may be a necessary adaptation to maintain the proper cation makeup within the cell in the face of an increased cation leak[30].

We have found a group of surface properties which seem to distinguish clearly between PNH and normal cells. These properties include an increased lactoperoxidase-catalyzed iodination of surface components, a difference in the distribution of membrane proteins, an increase in the number of acetylcholinesterase-active sites per cell available to DFP, a decrease in the sulfhydryl groups titrable with PCMBS and an increase in the Na^+ uptake at 4 °C.

GSH-treated cells do not appear to be a good model for PNH cells because they do not share these properties with PNH cells. GSH-treated cells bound fewer molecules of ^{125}I per cell during lactoperoxidase-catalyzed iodination, fewer acetylcholinesterase-active sites per cell were available to DFP, and there was a much larger increase in the Na^+ uptake at 4 °C. Furthermore, GSH-treated cells had normal acetylcholinesterase activity, while the activity in PNH cells is reduced.

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