

Membrane Lipid Depletion in Hyperpermeable Red Blood Cells: Its Role in the Genesis of Spherocytes in Hereditary Spherocytosis *

HARRY S. JACOB WITH THE TECHNICAL ASSISTANCE OF STEPHANIE LOWRY

(From *St. Elizabeth's Hospital and Tufts University School of Medicine, Boston, Massachusetts*)

Abstract. Hereditary spherocytosis (HS) red cells lose membrane lipids excessively during incubation *in vitro*. Individual phosphatides as well as cholesterol are lost in proportion to their content in membranes, suggesting that fragments of membrane are removed. Supplementation of HS red cells with glucose during incubation has no consistent protective effect, whereas diminishing the excessive sodium flux through these cells by suspending them in either sodium-free or hypertonic media prevents membrane fragmentation. The characteristic excessive increase in osmotic fragility which occurs in incubated HS red cells results both from inordinate accumulation of intracellular sodium ions which produces osmotic swelling, and from depletion of surface material which generates microspherocytosis. Inhibiting both of these processes by incubating HS red cells in sodium-free media completely prevents increases in osmotic fragility despite prolonged incubation.

Normal red cells rendered hyperpermeable to cations by exposure either to *n*-butanol or to inhibitors of membrane sulfhydryl groups, lose membrane lipid upon incubation in a similar fashion to untreated HS red cells; perfectly smooth microspherocytes, akin to those seen in HS, are thereby generated.

I conclude that depletion of membrane lipids in HS which leads to microspherocytosis is correlatable with the excessive cation flux and possibly to the stimulated metabolism of acidic phosphatides in these red cells. It is suggested that this relation is derived from the fact that these phosphatides are in some way involved in maintaining the proper alignment of repeating membrane lipoprotein units, and that this function is adversely affected when these molecules are turning over more rapidly in response to increased cation flux.

Introduction

Recent evidence has demonstrated that the hemolytic process in hereditary spherocytosis (HS) involves disordered physiology of the red cell membrane (1). Two processes have received attention: (a) altered flux of sodium ions through

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Address requests for reprints to Dr. Harry S. Jacob, St. Elizabeth's Hospital, 736 Cambridge Street, Brighton, Mass. 02135.

the HS red cell membrane, and (b) altered metabolism and stability of the lipids in these red cell membranes.

Investigations into the former have demonstrated that the permeability of HS membranes to extracellular sodium ions is excessive (2, 3), and in response the rate of active pumping of this cation outward from the cell is increased to twice normal (3, 4). When generation of ATP, which is required for cation pumping (5-8) is optimal, compensation is adequate and cation homeostasis is preserved. Under suboptimal conditions, as with entrapment of red cells in the splenic micro-

vasculature, or during their prolonged incubation *in vitro*, intracellular sodium and water accumulate, leading to osmotic swelling, increased osmotic fragility, and ultimately to osmotic hemolysis (3). It has been suggested that this process underlies the destruction of HS red cells entrapped in the spleen (1).

The second process, that of disordered behavior of membrane lipids, may relate to the former. As in other tissues (9), the accelerated pumping of cations in HS red cells results in a concomitant stimulation of anabolism of certain membrane phospholipids (10). Mainly those "acidic" phosphatides with the greatest binding avidity for cations (11, 12) are involved. Furthermore, membrane phospholipid metabolism has been demonstrated to be altered concomitantly with cation flux alterations that are provoked in HS and normal red cells (10). Thus, concomitant with the increased flux of sodium across HS red cell membranes, their incorporation of inorganic phosphate-³²P into acidic phosphatides occurs at roughly twice normal rates, and this is reduced toward normal when sodium flux is artificially reduced (10). Similar considerations in a variety of tissues have led to the suggestion that phospholipids are generally crucial in the transport of cations through membranes (9).

In addition, an earlier observation that HS red cells have a propensity to lose surface lipids upon prolonged incubation (13) has been more fully documented recently (14). It has been emphasized (15) that the resulting loss in surface material would generate a more spheroidal and osmotically fragile red cell, one characteristic of that found in this disease in the circulation or especially after incubation *in vitro*.

The process by which HS red cells become depleted of surface lipids was the subject of the present investigation. It has previously been suggested (16) that phospholipids, whose molecular structures comprise both hydrophobic and hydrophilic components, have structural importance in all membranes, interacting both with other lipids and with the protein components thereof. It was hypothesized in this investigation that the increased metabolism of phospholipid molecules in HS red cell membranes, which in turn relates to the increased flux of sodium ions across these mem-

branes (10), might render the cell vulnerable to fragmentation. To examine this hypothesis, the stability of red cell membranes as measured by their lipid content has been investigated under conditions in which sodium flux across red cells has been varied by the following experimental maneuvers: sodium flux was (a) diminished in HS red cells and (b) increased in normal red cells; further (c) intracellular levels of sodium were raised acutely in normal red cells and (d) the permeability of red cells to cations was increased by chemical modification of the membrane structure. Results have been partially presented in preliminary form elsewhere (17).

Methods

Incubation procedures. Five patients, all of whom had been splenectomized in the past, served as donors of HS red cells. The patients, from four families, had normal blood element counts (18) including reticulocyte percentages at the time of the studies. The following characteristics, considered typical of the disease (19), were present in every donor: (a) a congenital hemolytic anemia affected at least two family members; (b) complete clinical remission occurred after splenectomy; (c) spherocytes were obvious on examination of the peripheral smear; and (d) associated with this, typically increased osmotic fragilities of fresh and particularly of incubated blood were noted. These five donors have been utilized in previous studies of the pathogenesis of HS (1, 3, 10, 20). Normal red cells were obtained from healthy male volunteers.

Fresh blood, drawn aseptically into heparin was centrifuged at 3000 rpm, the buffy coat removed, and the cells resuspended in the sterile buffer incubation medium. After recentrifugation and a second removal of any remaining buffy coat, all under aseptic conditions, the red cells were resuspended in buffered incubation medium to a hematocrit of 50. Leukocytes in these cell suspensions numbered less than 1000 and platelets less than 20,000/mm³. The incubation medium described previously (3) was, unless otherwise stated, a buffered 5% dialyzed human serum albumin¹ solution containing the following

¹ Normal human serum albumin was obtained from the Massachusetts Public Health Biologic Laboratories as a 25% solution and contained 10⁻⁴ M protein-bound fatty acid. After overnight dialysis against several changes of appropriate buffer, the solution was diluted to a final concentration of 5% in buffer. This medium was used rather than plasma because previous studies indicated that plasma when incubated undergoes alterations in its lipid moieties which affect the content and turnover of lipids of the membranes of red cells incubated therein (21, 22). Nonetheless it is acknowledged that the medium used in the studies might itself have unsuspected effects on red cell membrane lipids which have not been analyzed. If so,

constituents: 3.5 mM K⁺; 160 mM Na⁺; 30 mM PO₄²⁻; 25 mM HCO₃⁻; and Cl⁻, balance of anions. Glucose was added after dialysis and when appropriate to a final concentration of 22 mmoles/liter. The medium was isosmolar with normal plasma as determined by freezing point depression. Media in which the tonicity or cation content was altered were prepared by appropriate manipulation of the bathing fluid during the dialysis of the albumin. The media were rendered sterile by Seitz filtration, and 500 U of penicillin/ml and 250 µg of streptomycin/ml were added to the media. The cell suspension was equilibrated to a pH of 7.4 with a 95% oxygen and 5% carbon dioxide mixture before incubation. After removal of a sample of cells for counting of blood elements and hematocrit determination, the remaining cell suspension was divided into equal aliquots of usually 1 ml and incubated with shaking for various periods as 37°C in separate sterile stoppered tubes after addition of a volume of buffered incubation medium sufficient to produce a final hematocrit of the incubated cell suspensions of 5–10%; this prevented a significant decrease in pH of the system during prolonged incubation. Under these conditions pH diminished by no more than 0.07 U in 24 hr and autohemolysis did not exceed 4%. The effects of incubation on HS red cells were compared to those of red cells from normal subjects incubated at the same time under identical conditions. In all experiments depicted alterations in lipid content are presented per original ml of red cells.

Extraction and analysis of red cell lipids. Before and after the various periods of incubation, the total aliquot of the red cell suspension in each tube was washed three times with 30 volumes of ice-cold isotonic saline and finally made to a hematocrit of roughly 50%. Lipids were extracted from the cell suspension by procedure III of Ways and Hanahan (23). This procedure has been shown to yield lipid virtually free of nonlipid contaminants. Aliquots of the lipid extract were analyzed for phosphorus by the methods of Lowry and coworkers (24) scaled up to a convenient level. Triplicate determinations agreed within ±2%. Values of lipid phosphorus were multiplied by 25 to yield total phospholipid. Triplicate cholesterol measurements by the method of Brown and coworkers (25) agreed within ±3% but only if the period for color development was kept constant at 30 min for standard and experimental samples. Lipid extract in small volumes of chloroform-methanol (2:1 v/v) was spotted on glass plates coated with silica gel G as described by Stahl (26), and ascending thin-layer chromatographic separation of the individual phosphatides was performed with the solvent system, chloroform-methanol-acetic acid-water (500:300:80:40 v/v) as described by Skipski, Peterson, and Barclay (27). The separated phosphatides were localized with iodine or sulfuric acid spray and were identified by comparison with reference compounds, by group-specific sprays, and by deacylation and chromatography of water-soluble products on paper (28) as previously described (10). After development and identifica-

tion, the area of silica gel containing each phosphatide was removed from the plate, eluted with recoveries of 70–80%, as described by Skipski and coworkers (27), or with recoveries of 90–100%, as previously described (10), and analyzed for phosphorus in triplicate by the method of Lowry and coworkers (24). Intracellular contents of red cell sodium were measured as previously described (3).

Results

Depletion of membrane lipids during incubation of HS red cells. The loss of lipids from normal and HS red cells during incubation for 24 hr at 37°C is shown in Table I. In the absence of glucose significant amounts of phospholipids and cholesterol, which comprise virtually all of the lipid of red cells (23), were lost from HS red cells. Depletion of both types of lipids was of the same degree; the mean loss of 11.8% in total phospholipids was not significantly different from the 13.1% loss in cholesterol. By contrast, loss of lipids from normal red cells during parallel incubations averaged between 1 and 2%; the difference between HS and normal red cells was highly significant ($P < 0.001$). These results confirm earlier studies of Reed and Swisher (14). As also seen in Table I, the addition of glucose to the incubated red cells only minimally diminished the mean phospholipid and cholesterol loss from HS red cells to a degree that was not statistically significant. In fact, in experiments with red cells from patients 4 and 5, glucose may have increased lipid loss during the 24 hr of incubation. After

TABLE I
Loss of membrane lipids during incubation of red cells*

	Pa- tient	Phospholipid loss		Cholesterol loss	
		No glucose	Glucose	No glucose	Glucose
		%		%	
Hereditary spherocytosis	1	14.2	11.7	16.7	10.4
	2	21.2	—	17.6	—
	3	11.0	2.0	11.0	4.0
	4	7.8	12.7	13.0	20.0
	4	3.0	5.0	3.6	5.6
	5	13.6	—	13.3	—
	5	12.0	15.0	16.3	19.0
	Mean ±SE	11.8 ± 2.1	9.3 ± 2.5	13.1 ± 1.8	11.8 ± 3.3
Normal (9)					
	Mean ±SE	1.8 ± 0.6		1.1 ± 0.7	

* Washed red cell suspensions from normal and HS patients were incubated at 37°C in buffered albumin media for 24 hr with or without added glucose (0.022 moles/liter). Mean values for normal cells with or without glucose supplementation are pooled. For P values see text.

it is presumed that normal and HS red cells would be similarly affected.

incubation and centrifugation of the red cell suspension, 40–70% of the phospholipid and cholesterol lost from the red cells could be identified in lipid extracts prepared from the supernatant incubation medium. As also reported by Reed and Swisher (14) chromatographic separation of phosphatides from the extracted lipids of incubated HS red cells revealed no disproportionate loss of any single phosphatide species.

In several experiments, one of which is shown in Fig. 1, loss of phospholipid (top) and cholesterol (bottom) from HS red cells (white circles) could be appreciated after 4 hr of incubation and continued unabated thereafter. In five experi-

ments HS red cells lost from 0–11% of their phospholipids and cholesterol in a 4–6 hr period. Normal cells (black circles) lost little or no lipid even during the more prolonged 24-hr period of incubation.

Effect of diminished sodium flux on lipid depletion in HS red cells. In previous studies (3) it was demonstrated that HS red cells underwent less autohemolysis in vitro and survived in the circulation for longer periods after incubation in media in which sodium flux was curtailed. Thus HS red cells suspended in media rendered hypertonic by the addition of sucrose, an impermeable osmotically-active molecule, or in media in which

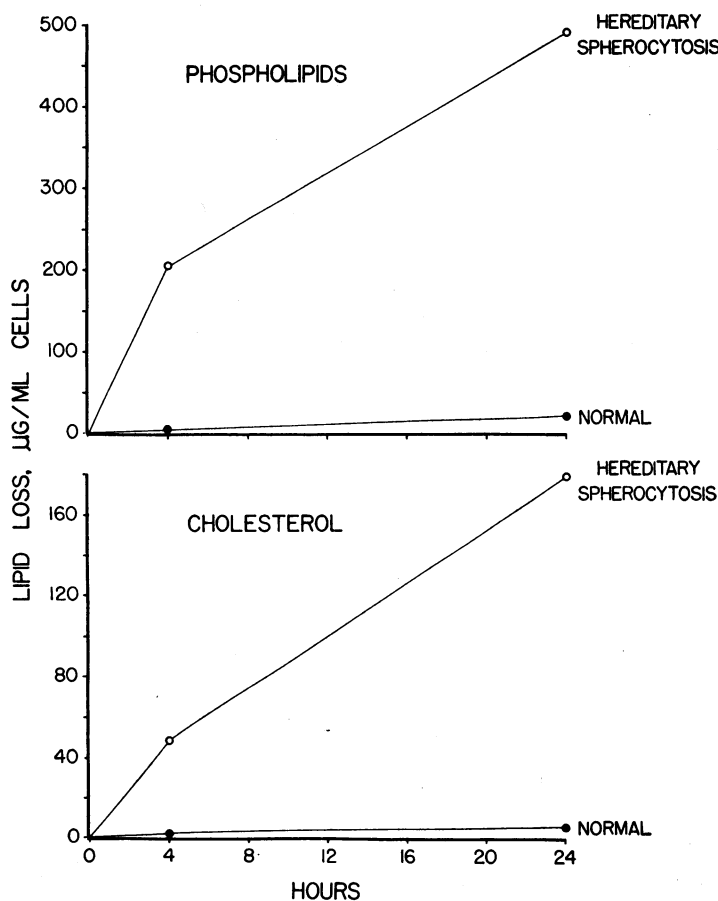


FIG. 1. MEMBRANE LIPID DEPLETION DURING RED CELL INCUBATION. Washed hereditary spherocytosis (HS) red cells (white circles) lose both phospholipid (upper) and cholesterol (lower) after brief periods of incubation at 37°C, whereas normal red cells (black circles) lose little or none. The experiment depicted is representative of six experiments performed in media with or without glucose supplementation and utilizing five HS and four normal donors.

sodium ions were replaced by choline, had diminished sodium flux and manifested greater viability relative to HS cells suspended in control media, both in vitro and in vivo (3). In addition, incorporation of inorganic phosphate- ^{32}P ($^{32}\text{P}_i$) into membrane phospholipids was diminished under these circumstances (10). As shown in Table II, the depression of sodium flux with either of these two media inhibited the loss of lipids from HS cells during prolonged incubation.

The increase in osmotic fragility after incubation of HS red cells was correlated with their loss of surface lipids as shown in Fig. 2. When HS red cells were incubated in a medium in which choline replaced sodium (white circles), depletion of membrane lipids was curtailed relative to that of an aliquot of the same cells incubated in the usual sodium-containing medium (black circles) as shown in the left portion of Fig. 2. The osmotic fragility of the choline-incubated HS red cells reflected this protection as shown in the right portion of Fig. 2. That is, the excessive fragility of HS red cells which characteristically accompanies incubation for 24 hr in sodium-containing media

TABLE II
Inhibition of lipid loss from HS red cells by diminishing cation flux*

Experiment	Cation flux		% Inhibition, $\frac{a-b}{a} \times 100$
	Control (a)	Diminished (b)	
	$\mu\text{g lipid lost/ml RBC}\dagger$		
S1	473	95	80
S2	581	30	95
C1	137	0	100
C2	698	15	98
C3	455	20	96

* Two aliquots of washed HS red cells were incubated at 37°C for 24 hr in the usual control buffered albumin medium (see Methods) or in either albumin media rendered hypertonic by addition of 80 mM sucrose ("S" experiments) or in media in which Na^+ was replaced by choline ("C" experiments). The two noncontrol media reduce cation flux through red cells incubated therein (see text). All media contained 0.022 M glucose and HS red cells from three donors are represented.

† The loss of phospholipid plus cholesterol is presented.

was not apparent in red cells incubated in the sodium-free medium. The fragility of these incubated cells approximated that of fresh, unincubated cells depicted by the dashed line. No acute alterations in osmotic fragility occurred in

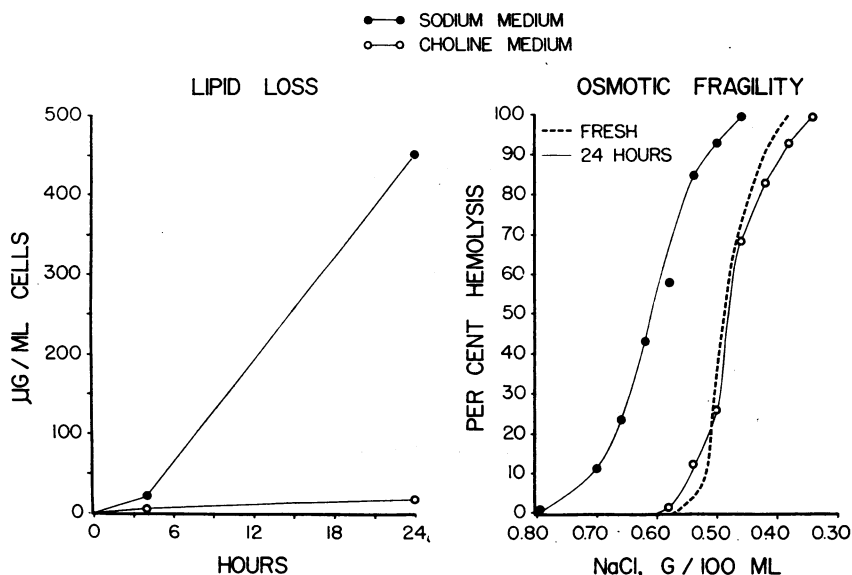


FIG. 2. CONCOMITANT PREVENTION OF LIPID DEPLETION (LEFT) AND INCREASE IN OSMOTIC FRAGILITY (RIGHT) IN HS RED CELLS INCUBATED IN Na -FREE MEDIA. The characteristic depletion of total membrane lipids and gain in osmotic fragility observed in HS red cells incubated at 37°C in the usual Na -containing suspension media (black circles) was curtailed in another aliquot of the same cells incubated in media in which choline replaced Na ions (white circles). All media contained 0.022 M glucose and the experiment depicted is representative of three experiments performed with red cells from two HS donors.

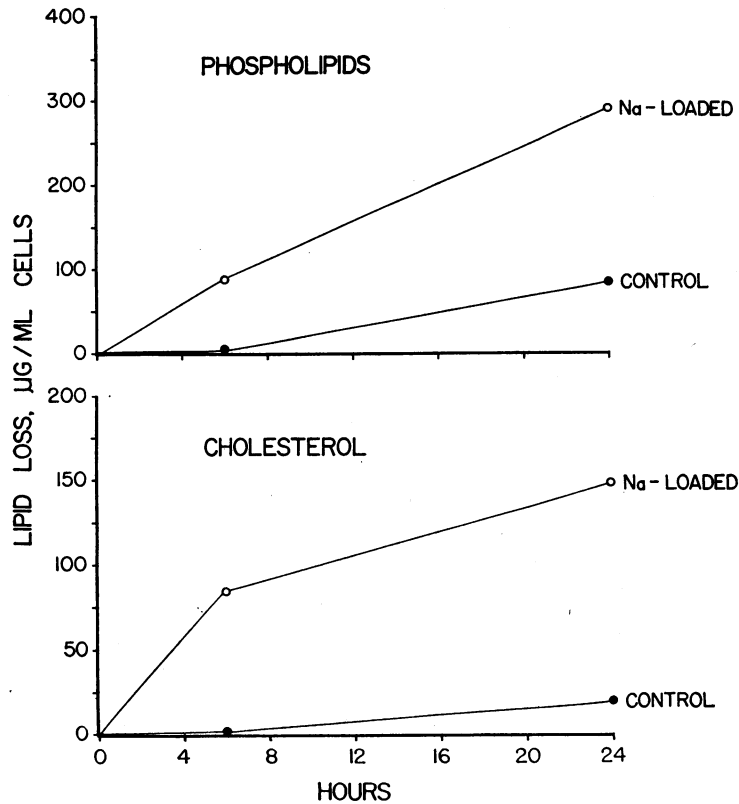


FIG. 3. MEMBRANE LIPID DEPLETION DURING INCUBATION OF NA-LOADED RED CELLS. Butanol-treated red cells of high Na content (19.5 meq/liter cells) (white circles), lose phospholipid (upper) and cholesterol (lower) upon incubation at 37°C in glucose-containing media to a greater degree than similarly treated control cells of normal Na content (8.7 meq/liter cells) (black circles). Na-loaded and control cells were prepared by suspending at 10°C two aliquots of packed cells in equal volumes of *n*-butanol diluted to 0.5 moles/liter in either 0.15 M NaCl or KCl, respectively. The cells were incubated for 20 min, and thereafter Na-loaded cells were washed and incubated in the usual albumin medium of high Na concentration. Control cells were washed and incubated similarly in medium of identical composition except for replacements of all but 20 meq/liter Na⁺ by K⁺. The experiment depicted is representative of two experiments performed.

fresh HS red cells suspended in choline-containing media. In two other experiments, the mean cell fragility of HS red cells was lessened by 0.16 and 0.14 g/100 ml NaCl with incubation in sodium-poor, relative to sodium-rich media. The possibility that choline might have other unsuspected effects on red cell stability and fragility not related to lessened sodium flux was also excluded. Thus, excessive lipid losses and osmotic fragilities of HS red cells continued unabated in cells exposed for 30 min to choline and then incubated in either the usual high-sodium medium

or in a medium in which half the usual content of sodium was replaced by choline.

Effect of increased sodium flux on lipid depletion in incubated red cells. A reversible increase in membrane permeability can be produced in normal red cells by exposing them for brief periods to cold 0.5 M butanol (29, 30). During the period of butanol exposure red cells rapidly incorporate extracellular cations into their intracellular space. After removal of butanol and washing of the cells, membrane permeability normalizes and cells loaded with extracellular cations are thereby ob-

tained (31). Cells loaded with sodium by this procedure actively pump this cation more rapidly (30), have increased ATPase activity (31), and in our hands incorporate $^{32}\text{P}_i$ into membrane acidic phosphatides to an increased degree (10). As shown in Fig. 3 such sodium-loaded red cells (white circles) also lose surface lipids in excess of similarly treated control cells (black circles) whose sodium content was normal after exposure to butanol in media of low sodium (high potassium) concentration. At zero time the sodium content of the sodium-loaded cells in Fig. 3 was 19.5 meq/liter cells as compared to 8.6 meq/liter cells for the control cells, and there was virtually no difference between lipid contents of the two cell types; e.g., 109 and 111 μg of phospholipid P/ml cells and 1.14 and 1.14 mg cholesterol/ml cells in sodium-loaded and control cells, respectively. In a similar experiment (not shown) red cells with sodium concentrations of 14 and 8.7 meq/liter lost 400 and 50 μg of lipid/ml red cells, respectively, after 24 hr of incubation. Autohemolysis was less than 2% in 24 hr in these experiments.

Cation flux is also increased in red cells that are exposed to inhibitors of membrane sulfhydryl activity such as paramercuribenzoate (PMB) or *N*-ethylmaleimide (NEM) (32). Incubation with these compounds leads to cellular sodium gain and potassium loss mainly through an increased leak rate of these ions through the cell membrane (32).

TABLE III
Effect of sulfhydryl inhibition on lipid loss from incubated red cells*

Experiment	Lipid loss	
	Control RBC	SH-inhibited RBC
	$\mu\text{g/ml RBC}$	
1	84	355
2	212	419
3	11	328
4	15	433
5	170	466
6	170	551
Mean \pm SE	110 \pm 35	425 \pm 33†

* Two aliquots of washed normal red cells were incubated for 24 hr at 37°C with or without *p*-mercuribenzoate, 1.25 $\mu\text{moles/ml}$ RBC (Experiments 1-5), or with or without *N*-ethylmaleimide, 5.0 $\mu\text{moles/ml}$ RBC (Experiment 6). The sum loss of phospholipid plus cholesterol is presented. Autohemolysis was less than 2% in all cell suspensions depicted.

† *P* value for difference between control and SH-inhibited red cells < 0.001.

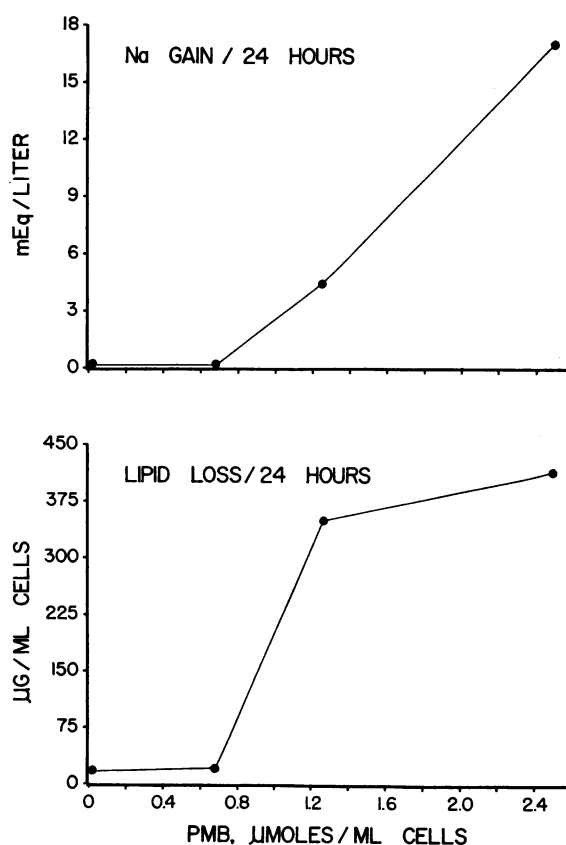


FIG. 4. CONCOMITANT ACCUMULATION OF INTRACELLULAR NA (UPPER) AND DEPLETION OF MEMBRANE LIPIDS (LOWER) IN RED CELLS INCUBATED WITH PARAMERCURIBENZOATE (PMB). At doses of PMB above 0.7 $\mu\text{mole/ml}$ cells accumulation of Na occurs in red cells incubated at 37°C for 24 hr. Concomitantly, but not before, abnormal depletion of membrane total lipids is noted. Autohemolysis was less than 2% in all cell suspensions depicted.

As shown in Table III normal red cells rendered hyperpermeable to cations by exposure to non-hemolytic doses of PMB (Experiments 1-5) and NEM (Experiment 6) lost significantly greater amounts of lipids than did untreated red cells which were incubated in parallel. This excessive loss of lipids was inhibited by roughly 75% in three experiments when sulfhydryl-inhibited red cells were incubated in media of low sodium (sucrose or choline) content (not shown). As with HS red cells no loss of any one lipid exceeded that of any other. Thus in three experiments utilizing different quantities of PMB the per cent losses of phospholipids from red cells incubated for 24 hr were 6.9, 9.6, and 32.1; the corresponding per cent losses of cholesterol were 11.2, 9.7, and

31.3, respectively. In addition no individual phosphatide was depleted in excess of any other.²

That the induction of hyperpermeability in red cells and their loss of membrane lipids are closely related phenomena is shown in Fig. 4. With increasing concentrations of PMB a threshold dose is reached at which sodium ions begin to accumulate in incubated red cells (upper Fig. 4). At this dose, but not before, significant loss of membrane lipid during incubation was noted (lower Fig. 4).

The loss of surface material which resulted from depletion of membrane lipid generates typical microspherocytes similar to those observed in HS; this is seen in Fig. 5. Reflecting their loss of surface material, red cells exposed to nonhemolytic doses of PMB first became smaller and developed surface projections (upper right, Fig. 5); with

² In an experiment utilizing 2.5 μ moles PMB/ml cells, in which roughly 10% of the total phospholipids and cholesterol had been lost during incubation for 24 hr, the major phosphatides retained the same percentage distributions as found before incubation, as follows: sphingomyelin, 25.7% (initial), 25.0% (final); lecithin, 29.6%, 29.1%; and phosphatidyl ethanolamine 19.8%, 24.3%.

further incubation these projections were lost and perfectly smooth microspherocytes were produced (lower right, Fig. 5). Untreated red cells retained their biconcavity throughout the incubation period (left, Fig. 5).

In two experiments normal red cells which were rendered hyperpermeable by suspension in hypotonic media, a procedure previously shown to increase membrane phospholipid metabolism (10), lost lipid in excess of control cells. The effect was not as great as that observed with butanol or sulfhydryl inhibitors.

The effect on the lipid stability of red cells of partially inhibiting active cation transport was studied in incubation mixtures containing the cardiac glycoside ouabain. The results were inconsistent. Ouabain diminished slightly the lipid loss from HS red cells in two experiments and increased it slightly in two others.

Discussion

The present results indicate that in a variety of situations, all of which have in common excessive cation flux through red cells, membrane lipids

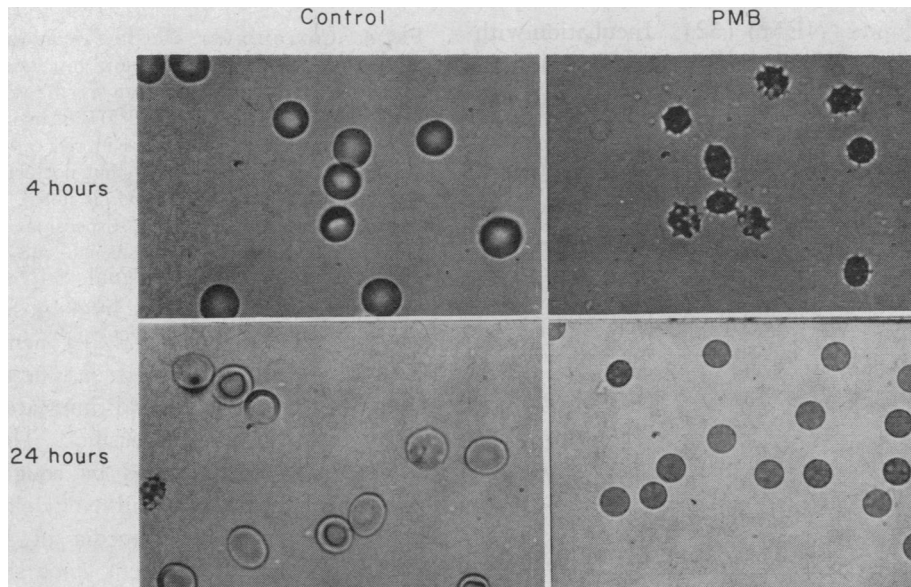


FIG. 5. EFFECT OF PARAMERCURIBENZOATE (RIGHT) ON RED CELL MORPHOLOGY. Red cells exposed for brief periods to nonhemolytic doses of PMB (1.75 μ moles/ml cells) develop surface irregularities and become smaller (upper right). With more prolonged incubation, measurable depletion of membrane lipid occurs (see Fig. 4) which is associated with loss of surface excrescences and generation of smooth microspherocytes (lower right). Untreated red cells (left) retain their biconcavity.

tend to be lost. Thus, HS red cells whose sodium flux is generally about twice normal (2-4), lose roughly 15% of their membrane lipids during 24 hr of incubation, whereas normal cells lose little or none. Furthermore the loss of lipids from HS red cells can be curtailed if sodium flux is diminished. As also found by others (14), the loss of individual lipids is exactly proportionate to their content in the cell membrane, no individual phosphatide being either disproportionately depleted or spared. This implies that pieces of membrane are separated from the cell during incubation, and that this process underlies the observed depletion of lipid. The sizes of the eluted pieces of membrane and their degree of homogeneity remain obscure. Green and his coworkers (33) have provided evidence that various membranes, including those of red cells, are formed from stable, repeating lipoprotein units. Thus membranes can be made to disaggregate into these units and reaggregate into unimolecular layers, ultimately to become vesicular membranes again by perturbation of the hydrophobic bonds (34) which bind one repeating unit to another. From such evidence it is reasonable to suspect that the loss of lipid from incubated red cells which was observed in the present studies, in fact might represent elution of lipoprotein macromolecules of variable sizes. Examples of such large pieces have been observed with electron microscopy by Weed and Bowdler (15) to be "fragments" or "buds" about 1/10 the size of the parent red cells from which they are derived. The sequence in the right portion of Fig. 5 which shows hyperpermeable (PMB treated) red cells forming multiple surface excrescences and later becoming perfectly smooth microspherocytes, supports the notion that relatively large surface buds can be removed in toto during the process. The possibility that smaller units are also eluted has not been investigated, although inhomogeneity in the size of released membrane units is suggested by the inconstant recoveries of eluted lipid in the supernatants of centrifuged, incubated red cells.

The manner by which excessive cation flux and membrane disorganization are linked remains obscure. It seems pertinent, however, that the metabolism of certain acidic phosphatides is increased in all situations studied in which excessive

cation flux and lipid depletion coexist; i.e., in (a) HS red cells; (b) butanol-treated, sodium-loaded red cells; (c) in osmotically swollen red cells (10); and in (d) spherocytes produced by heating red cells at 50°C.³ Conversely, when metabolism of these acidic phosphatides is normalized as when HS red cells are suspended in choline media to suppress sodium flux (10), excessive lipid loss ceases. Although no mechanistic interpretation of these correlations is as yet available at least two possibilities might be examined in the future. Firstly, these results are consistent with the suggestion that fragmentation occurs when certain phosphatides, which may act by electrostatic bonds to cement together the lipid and protein components of the membrane (16), have their properties altered; this may occur when turnover of their phosphate groups is stimulated during excessive cation flux through red cell membranes (10). Alternatively, abundant data have been collected and reviewed recently by Korn (35), which suggest that membrane lipid-protein bonds are, in fact, hydrophobic (34), and that the resulting lipoprotein units are repeating and are bound together by these same forces. In one current model of membrane structure offered by Green and his coworkers (33), this unit is coated only on its upper and lower surfaces with lipid. Neighboring units, which are similarly coated, are prevented from attachment except at mutually uncovered (protein) side surfaces. A single layered sheet of lipoprotein is generated which curves and finally fuses at its open ends into a vesicular membrane. Removal of lipid from such a membrane produces disaggregation and bunching of the altered repeating units into chaotic, nonmembranous structures (33). A similar sequence of lipid depletion followed by membrane disorganization may underlie the fragmentation of incubated red cells observed in the present studies.

Reed and Swisher (14) have postulated another mechanism for excessive cell fragmentation in HS. These authors conceive of cellular ATP as crucial to membrane stability; with its loss, fragmentation would occur. This postulate was supported by their findings that glucose supplementation (and therefore presumably ATP preservation) diminished the depletion of lipids from incubated HS

³ Jacob, H. S. Unpublished observations.

red cells. In the present studies we were not able to confirm these data. In our hands glucose inconsistently affected lipid loss in HS red cells; in fact, in some experiments glucose may have increased lipid depletion slightly (patients 4 and 5, Table I).⁴ The reasons for the discrepant results are unknown although they may relate to the differing incubation procedures in the two studies. Plasma was utilized as the suspending media by the former authors whereas lipid-poor, buffered albumin was utilized in the present study. It has been recently reported both from our laboratory (10) and from another (37) that phospholipid metabolism as measured by ³²P_i incorporation is markedly diminished in plasma relative to buffered nonplasma systems; this observation may have possible significance in explaining the differing results. In addition the higher hematocrit values of the suspensions utilized by the previous authors resulted in larger pH decrements due to lactate accumulation during incubation than in the dilute suspensions utilized in the present studies. This difference would be most apparent with glucose supplementation. As previously reported (38), incubation of HS red cells in media of diminished pH decreases autohemolysis. It seems possible, therefore, that the decrement in lipid loss with glucose supplementation as noted by others might reflect an effect of pH per se. However, preliminary experiments have failed to validate this possibility. In red cells from two HS patients, loss of lipids was not affected by glucose in any consistent way; regardless of whether pH was allowed to fall during incubation. The results utilizing ouabain in the incubation mixtures also make it questionable that a diminished ATP content is principally related to red cell fragmentation. This glycoside

⁴ This finding is of interest since both in our laboratory³ and that of Dacie (36) autohemolysis of red cells from a small proportion of otherwise typical HS patients has been observed to paradoxically increase with glucose supplementation. The relationship of this observation to that of increased lipid loss with glucose supplementation as reported in the present studies is under investigation. To date, red cells from case 5 (Table I) and one other patient, not utilized in the present studies, have had levels of autohemolysis, after 48 hr of incubation, which were not improved by glucose supplementation. In both cases loss of lipids/24 hr was either not reduced or, in fact, was increased by glucose. Red cells from case 5 were used in Experiments S2 and C2 (Table II).

partially inhibits the ATPase reaction required for active cation pumping, but does not affect other ATP-dependent reactions. It might be expected thereby that more ATP would be made available by ouabain to function in its postulated role of preserving membrane integrity. In fact, ouabain does tend to increase ATP levels in incubated red cells (39), yet as shown by both Reed and Swisher (14) and by ourselves it has no particular effect on lipid stability in HS red cells.

Since red cell lipid is localized completely in membranes, its loss from the cell diminishes surface material. The least surface area for a given cellular volume being a sphere, it follows that such surface diminution produces a more spheroidal cell. The diminished distensibility of such a surface-depleted cell results in an increased propensity to hemolyze with the osmotic swelling which occurs in hypotonic solutions; that is, results in increased osmotic fragility. HS red cells, therefore, are victims of two additive processes both culminating in the excessive osmotic fragility which occurs with incubation of these cells; i.e., (a) the tendency to accumulate osmotically-active sodium ions (followed by water) due to increased permeability to this cation (2, 3); and (b) the tendency to lose surface material and hence distensibility. The latter process has recently been emphasized more fully (15). Inhibiting both processes by incubating HS red cells in media in which choline replaces sodium, completely prevents any increase in osmotic fragility for as long as 24 hr (Fig. 2).

It seems likely that these two processes are also involved in the hemolytic process which occurs in HS in vivo. The continuously accelerated sodium flux across the HS membrane results in the continuing tendency of the circulating HS red cell to lose surface material. As a result the cell becomes more spheroidal with time, and thereby its likelihood of being retarded in its passage through the fenestrated, basement membrane filter of the splenic microvasculature (40) is enhanced, perhaps due to its diminishing plasticity (41, 42). Once temporarily sequestered, sodium and water are measurably accumulated (13), osmotic swelling occurs, and passage through the basement membrane apertures is further discouraged. With persistent entrapment osmotic fragility increases (43, 44), ultimately leading to osmotic lysis.

It is stressed that the spleen is not required for the production of microspherocytes in hyperpermeable red cells. Such surface-depleted cells are produced readily *in vitro* (Fig. 5) and are found in abundance in the HS patient after remote splenectomy. In fact as shown by Emerson and coworkers (43, 44), with more prolonged red cell survival which occurs in HS after splenectomy, the bulk of red cells actually are of increased spheroidicity relative to the presplenectomy state. This, with the presumptive evidence that normal red cells are more spheroidal and of decreased cellular lipid content with maturation (45), suggest that the process of membrane loss may occur continuously in red cells and is simply accelerated in the excessively permeable HS red cell. A small fraction of red cells whose osmotic fragility is markedly increased does seem to be produced by exposure to the microvasculature of the spleen, disappearing after splenectomy (43, 44). These cells may owe their excessive fragility to accumulation of sodium and water, reflecting their temporary stasis in the spleen (13) or to a depletion of surface material if fragments of membrane are pinched off when these cells traverse the apertures in the splenic vasculature (46), or most likely to a combination of both.

I conclude that the primary defect in HS red cells is their increased permeability to sodium ions. This, in turn, doubly and additively jeopardizes their viability; firstly, by directly increasing the tendency for the cell to accumulate sodium and water which ultimately must produce osmotic hemolysis; this process is of serious consequence especially when conditions for active pumping of sodium back out of the cell are suboptimal as when the cells are entrapped in the splenic microvasculature. Secondly, increased cation permeability, by stimulating the metabolism of certain membrane lipids renders the HS cell liable to fragmentation; this generates a more spheroidal cell which in turn presages its splenic entrapment, at the same time diminishing its ability to resist the osmotic swelling which accompanies such entrapment.

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