JCI The Journal of Clinical Investigation

Erythrocyte lipid loss in hereditary spherocytosis.

C F Reed, S N Swisher

J Clin Invest. 1966;45(5):777-781. https://doi.org/10.1172/JCI105392.

Research Article

Journal of Clinical Investigation
Vol. 45, No. 5, 1966

Erythrocyte Lipid Loss in Hereditary Spherocytosis *

CLAUDE F. REED † AND SCOTT N. SWISHER

(From the Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.)

Probable manifestations of the intrinsic corpuscular defect leading to shortened red blood cell (RBC) survival in hereditary spherocytosis (HS) have been induced by *in vitro* incubation of these erythrocytes (2–4). The classical *in vitro* changes that have been described are a marked increase in autohemolysis in the absence of metabolized substrate and an increase in osmotic fragility.

Young, Izzo, Altman, and Swisher (4) showed that a number of substrates utilized by RBC to produce ATP could substitute for glucose in preventing the exaggerated *in vitro* hemolysis in HS erythrocytes. The exact role of cellular ATP in preventing these changes has, however, been debated. Selwyn and Dacie (3) suggested that, particularly in the absence of metabolized substrate, a premature degeneration of the membrane occurred in HS erythrocytes during *in vitro* incubations. In a preliminary report (1), we described an *in vitro* loss of lipid in HS erythrocytes not found in normal cells that was minimized, but not prevented completely, by the presence of glucuose. These findings appeared to be a specific manifestation of the membrane degeneration postulated by Selwyn and Dacie (3) and to represent an example of the mechanism, originally proposed by London and Schwartz (5), whereby RBC cellular energy is utilized in preventing changes in membrane lipids; in HS erythrocytes, it appeared that utilization of energy through this pathway was inefficient.

More recently, Jacob and Jandl (6) have emphasized the importance of colloid osmotic lysis, rather than some energy dependent membrane degeneration, as the primary event responsible for the *in vitro* changes observed in HS erythrocytes in the absence of metabolized substrate. This view depends on the observation of Bertles (7) that at the usual sodium gradient between plasma and cell water found in humans, the influx of sodium ions into HS erythrocytes is greater than into normal cells, and also on the well-known energy requirement for maintenance of normal cationic gradients across the cell membrane (8). A detailed consideration of these two proposals may be found (9).

The present report describes the lipid composition of HS erythrocytes. It also describes in detail the loss of lipid observed in these erythrocytes during *in vitro* incubations and the effect of glucose in minimizing this loss.

Methods

A total of seven patients with HS who had undergone splenectomy were studied. The diagnosis was established in each case by the usual criteria, which included characteristic findings in the peripheral blood and bone marrow, the characteristic patterns of autohemolysis and osmotic fragility, the expected genetic pattern of transmission, the presence of an enlarged and congested spleen at operation, and the correction of the hemolytic anemia after splenectomy. All of the subjects with HS had had their spleens removed at least 1 year before the study, had hematocrits ranging between 40 and 46%, and had reticulocyte counts of 2% or less. Seven hematologically normal donors were used as controls.

By a sterile technic, blood was collected from the subjects into sufficient Na_2-EDTA to achieve a final plasma concentration of 5×10^{-3} mole per L. Incubation of the blood was in siliconized or polyethylene flasks at 37° C in a Dubnoff shaker adjusted to 90 oscillations per minute for 24 hours. For each subject, three types of incubation were used: *1*) whole blood with sufficient glucose added to achieve a final glucose concentration of 30 mmoles per L, *2*) whole blood with sufficient glucose

* Submitted for publication September 9, 1965; accepted February 4, 1966.

This investigation was partially supported by the U. S. Army Medical Research and Development Command, Department of the Army, under research contract DA-49-193-MD-2656.

A preliminary report of this work has previously been presented (1).

† U. S. Public Health Service, National Heart Institute, research career program award HE-6234.

Address requests for reprints to Dr. Claude F. Reed, Dept. of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, N. Y. 14620.

added to achieve a final concentration of 30 mmoles per L and ouabain to produce a final concentration of 1×10^{-4} mole per L, and 3) the subject's RBC resuspended at a hematocrit of 35 to 40% in serologically compatible plasma from which glucose had been removed by prior dialysis against a 1 mM phosphate buffer. At the end of the 24-hour period of incubation, the mean pH and range were, for the normal RBC with glucose added, 6.98 (6.88 to 7.05); for normal RBC without glucose, 7.21 (7.12 to 7.30); for HS erythrocytes with added glucose, 6.94 (6.85 to 7.0); and for HS erythrocytes without glucose, 7.25 (7.15 to 7.28). All incubations were cultured at the end of the 24-hour experimental period and found sterile. Penicillin, 100 U per ml, and streptomycin, 0.1 mg per ml, were added to some of the incubations and had no effect on the results.

Samples of RBC were taken for lipid analysis before and after each incubation. The cells were separated from the plasma and washed three times in 0.15 M NaCl at 4° C, with careful removal of the buffy coat. Red blood cell counts were determined on the washed cell suspension with a model B Coulter counter.

The extraction, separation, and quantitation of the erythrocyte lipids were carried out as previously described (10). Lipid nitrogen was determined on some of the samples by using a modification of the method described by Minari and Zilversmit (11). The digestion mixture of Lang (12) was used and a longer digestion period, 10 hours at 280° C, was found necessary for complete digestion of lipid samples, particularly those containing sphingomyelin. Glucose was determined by a modification of the anthrone method (13), autohemolysis by the method of Young and associates (4). Probabilities were calculated with Student's *t* test (14).

Measurements of cellular ATP, sodium and potassium content, and critical hemolytic volumes were also made on the erythrocytes used in these studies; these results are reported separately (9).

Results

Lipid composition of HS erythrocytes. The lipid composition of the erythrocytes from the seven patients with HS in this study is shown in Tables I, II, and V. The amount of lipid per cell and the distribution of the lipid are virtually identical to that found in the normal RBC.

TABLE I

*Distribution of total lipid in normal and hereditary spherocytosis erythrocytes**

RBC	Phospholipid	Cholesterol†	Other neutral lipid†	Other
Normal				
Mean	65	23	2	10
Range	62–69	22–24	0–3	8–12
HS				
Mean	65	24	1	10
Range	60–69	20–30	0–3	9–12

* Analysis of erythrocytes from seven patients with hereditary spherocytosis compared with the results obtained from 20 normal subjects previously reported from this laboratory (10). Abbreviations: HS = hereditary spherocytosis, and RBC = red blood cells.
† Per cent by weight.

TABLE II

*Lipid composition per cell in normal and HS erythrocytes**

RBC	Total lipid	Lipid phosphorus	Cholesterol
	$g \times 10^{-12}$	$\mu g \times 10^{-9}$	$g \times 10^{-12}$
Normal			
Mean	4.95	11.5	1.13
1 SD	0.14	0.45	0.13
HS			
Mean	5.10	12.2	1.27
1 SD	0.33	0.65	0.19

* Analysis of RBC from seven patients with HS compared with results obtained from 20 normal subjects previously reported from this laboratory (10).

Loss of RBC lipid during the in vitro incubations. The per cent of total lipid lost by normal and HS erythrocytes in the three types of experiments used is shown in Table III. The normal RBC lost relatively little lipid during the 24-hour period, and the HS erythrocytes lost more lipid in each case. The addition of glucose reduced the mean total lipid loss in HS erythrocytes from 20 to 11%. This difference is significant ($p < 0.05$). The difference between the mean total lipid lost by normal (7%) and HS erythrocytes (20%) in the experiments where no glucose was present is also significant ($p < 0.01$). In the experiments with added glucose, the mean lipid loss was 3% in the normal and 11% in the HS erythrocytes ($p < 0.10$). The addition of ouabain did not affect the lipid loss significantly in either RBC.

In both the normal and HS erythrocytes such loss of lipid as did occur involved all lipids present in the cell membrane in proportion to their

TABLE III

Per cent total lipid lost per cell in normal and HS erythrocytes after in vitro incubation for 24 hours at 37° C

Additive	RBC*	
	Normal	HS
Glucose	3 ± 2.7	11 ± 2.5
Glucose and ouabain	6 ± 2.5	8 ± 5.2
None	7 ± 2.5	20 ± 2.0

* Mean ± 1 SE of RBC from seven normal subjects and seven patients with HS.

TABLE IV

*Analysis of lipid lost by HS erythrocytes during in vitro incubation for 24 hours without glucose**

	Total lipid	Lipid phosphorus	Cholesterol	Lipid nitrogen†
		% lost	*% lost*	*% lost*
Mean	20	20	24	23
1 SE	2	1.7	2.6	4.6

* Results from RBC of seven patients with HS.
† Lipid nitrogen was determined on three subjects only.

concentrations. The loss of RBC lipid was greatest in those incubations in which HS erythrocytes were used in the absence of glucose. Table IV shows that the losses of lipid phosphorus, lipid nitrogen, and total cholesterol were proportionately equal to each other and to the measured loss of total lipid in these experiments. Table V compares the distribution of the four quantitatively major phospholipids in normal and HS erythrocytes before and after *in vitro* incubation for 24 hours in the absence of glucose. The phospholipid composition of HS erythrocytes, in terms of major lipid classes, was identical to that found in normal RBC, and the loss of 20% of lipid phosphorus by HS erythrocytes during incubation for 24 hours without glucose was proportionately distributed among the individual phospholipids.

In the glucose-supplemented experiments, the residual concentration of glucose was, in each case, more than 5 μmoles per ml of blood at 24 hours; thus, a concentration of glucose exceeding that normally found *in vivo* was present throughout. Over the first 12 hours, glucose utilization averaged 2.3 μmoles per ml RBC per hour for the HS erythrocytes and 1.85 μmoles per ml RBC for the normal cells. During the second 12 hours, the respective average rates of glucose utilization were 1.2 and 0.9 μmoles per ml RBC per hour. This decrease in glucose utilization during the second 12 hours may have been due (15) to a fall in pH, which was not prevented in these experiments, but as shown above, the pH of the blood at the end of the incubation period was virtually identical in the HS and normal groups.

TABLE V

*Per cent distribution of lipid phosphorus in normal and HS erythrocytes before and after in vitro incubation for 24 hours without glucose**

Phospholipid	Normal Erythrocytes	HS erythrocytes	
		Before incubation	After incubation
Sphingomyelin	22±1.9	22±1.5	23±1.4
Lecithin	30±1.5	27±1.9	27±2.9
Phosphatidyl serine	15±1.3	15±2.1	13±1.9
Phosphatidyl ethanolamine	25±1.3	24±1.1	24±1.8
Other	8	12	13

* Mean ± 1 SD of RBC from seven patients with HS compared with results obtained from 20 normal subjects previously reported from this laboratory (10).

In both normal and HS erythrocytes at 24 hours, cellular ATP was absent in the experiments without added glucose but present in the incubations to which glucose had been added. The expected inhibition of active cation transport by ouabain was observed. These results are presented in detail elsewhere (9).

Autohemolysis was 1% or less in all the incubations of normal RBC. The mean and range in the HS incubations were *a*) with added glucose, 2% (0.5 to 3.2); *b*) with added glucose and ouabain, 3% (1.7 to 5.2); and *c*) without added glucose, 6% (0.8 to 19.5). If the one value of 19.5% is excluded, the mean and range for the HS incubations without glucose become 3% (0.8 to 5.0). Thus, it appears that a mean loss of as much as 20% of erythrocyte lipid may precede gross hemolysis, and that the addition of ouabain does not materially increase the hemolysis of HS or normal RBC during *in vitro* incubations for 24 hours if glucose is present.

Discussion

Both the amount and distribution of lipid per RBC were normal in the seven patients with HS studied. De Gier and associates (16), as well as Phillips and Roome (17), have previously reported that the distribution of erythrocyte phospholipids is normal in these patients, whether or not splenectomy has been performed. Studies in our laboratory on three patients with HS who have not had splenectomy indicate that the amount of total lipid per cell may be somewhat lower in these patients, but that the distribution of the lipid is normal. Thus, the suggestion that the proportion of phosphatidyl ethanolamine is reduced in HS erythrocytes as a basic feature of this disease (18) has not been confirmed in three independent laboratories.

The period of 24 hours for incubation of the RBC was chosen for detailed analysis of the lipid

loss and the effect of glucose in minimizing this change because pilot experiments (1) indicated that hemolysis was minimal in HS erythrocytes incubated for this period without glucose, as found by Selwyn and Dacie (3), whereas loss of erythrocyte lipid was nearly maximal. In our hands, prolongation of incubation of HS RBC without glucose beyond 24 hours increases the degree of hemolysis but does not increase the loss of any lipid in the unhemolyzed cells beyond about 25%. Prankerd (19), on the other hand, has reported observing a loss of about 50% of erythrocyte cholesterol and total lipid phosphorus in HS erythrocytes incubated for 48 hours without glucose, but little or no loss of cholesterol (the only lipid measured) after 24 hours of incubation. He did not investigate the effect of glucose on lipid loss. The reasons for the discrepancy between Prankerd's findings and ours may be due in part to methodological differences (10).

The central features of the present study are the observed uniform loss of all erythrocyte lipids and the effect of glucose in minimizing this loss. The effect of glucose in decreasing the loss of lipid in HS erythrocytes may well be related to the maintenance of cellular ATP concentrations. This proposed effect of ATP does not seem related to its role in actively maintaining normal cationic gradients across the cell membrane, since inhibition of this function with ouabain did not cause an increased loss of cellular lipid. Similarly, the observed effect of glucose is not due to alterations in the rate of ATP-dependent *de novo* lipid synthesis, since this does not occur in the mature erythrocyte (20). Some shift in the equilibrium point of exchange between plasma and erythrocyte lipids, a process that might require ATP, would not account for the uniform loss of lipid observed. The exchange of lipids is not symmetrical, as cholesterol exchanges rapidly (5, 21) and the phospholipids much more slowly and at rates that vary for the individual compounds (22, 23). The reversible acylation of membrane lysophosphatides has also been suggested as a mechanism accounting for some turnover of membrane lipid constituents (24–26), and Oliveira and Vaughan (24) have postulated a requirement for ATP by this process. The actual amount of phospholipid turnover involved is probably quite small, however, and the process has not been found to differ from normal in HS erythrocytes (26). Furthermore, alterations in the rate of acylation of erythrocyte lysophosphatides would not affect the amount of cellular cholesterol or the amount of total lipid. None of the above mechanisms, which may play a role in the turnover of erythrocyte lipid, would account for the observed equal loss of cholesterol, lipid nitrogen, lipid phosphorus, and of the major individual phospholipids in HS erythrocytes. This finding suggests that the observed changes may be due to loss of whole fragments of the membrane containing all of the component lipids. The actual demonstration of the formation of such fragments from HS erythrocytes during the course of the present experiments and the possible role of reduced concentrations of cellular ATP in their genesis are discussed elsewhere (9).

Summary

Erythrocyte lipid composition has been found to be normal in seven patients with hereditary spherocytosis (HS). During *in vitro* incubation for 24 hours, erythrocytes from patients with HS lose substantially more lipid than do normal erythrocytes. The loss involves all cellular lipids equally and is reduced by the presence of glucose, but not affected by ouabain.

The present results suggest that some mechanism exists in erythrocytes through which energy is utilized in preventing loss of membrane substance and that this mechanism is altered in HS.

Acknowledgments

We wish to thank Mrs. Marion Murphy and Mrs. Geraldine Roberts for their technical assistance.

References

1. Reed, C. F., and S. N. Swisher. Abnormalities of *in vitro* behavior of structural lipids of red blood cells from patients with hereditary spherocytosis (abstract). J. clin. Invest. 1960, **39**, 1019.
2. Ham, T. H., and W. B. Castle. Relation of increased hypotonic fragility and of erythrostasis to the mechanism of hemolysis in certain anemias. Trans. Ass. Amer. Phycns 1940, **55**, 127.
3. Selwyn, J. G., and J. V. Dacie. Autohemolysis and other changes resulting from the incubation *in vitro* of red cells from patients with congenital hemolytic anemia. Blood 1954, **9**, 414.
4. Young, L. E., M. J. Izzo, K. I. Altman, and S. N. Swisher. Studies on spontaneous *in vitro* autohe-

molysis in hemolytic disorders. Blood 1956, **11**, 977.

5. London, I. M., and H. Schwarz. Erythrocyte metabolism. The metabolic behavior of cholesterol of human erythrocytes. J. clin. Invest. 1953, **32**, 1248.
6. Jacob, H. S., and J. H. Jandl. Cellular permeability in the pathogenesis of hereditary spherocytosis. J. clin. Invest. 1963, **42**, 944.
7. Bertles, J. F. Sodium transport across the surface membrane of red blood cells in hereditary spherocytosis. J. clin. Invest. 1957, **36**, 816.
8. Harris, J. E. The influence of the metabolism of human erythrocytes on their potassium content. J. biol. Chem 1941, **141**, 579.
9. Weed, R. I., and A. J. Bowdler. Metabolic dependence of the critical hemolytic volume of human erythrocytes: relationship to osmotic fragility and autohemolysis in hereditary spherocytosis and normal red cells. J. clin. Invest. 1966, **45**, in press.
10. Reed, C. F., S. N. Swisher, G. V. Marinetti, and E. G. Eden. Studies of the lipids of the erythrocyte. I. Quantitative analysis of the lipids of normal human red blood cells. J. Lab. clin. Med. 1960, **56**, 281.
11. Minari, O., and D. B. Zilversmit. Use of KCN for stabilization of color in direct nesslerization of Kjeldahl digests. Analyt. Biochem. 1963, **6**, 320.
12. Lang, C. A. Simple microdetermination of Kjeldahl nitrogen in biological materials. Analyt. Chem. 1958, **30**, 1692.
13. Bartlett, G. R. Methods for the isolation of glycolytic intermediates by column chromatography with ion exchange resins. J. biol. Chem. 1959, **234**, 459.
14. Dixon, W. J., and F. J. Massey. Introduction to Statistical Analysis, 2nd ed. New York, McGraw-Hill, 1957, p. 121.
15. Murphy, J. R. Erythrocyte metabolism. II. Glucose metabolism and pathways. J. Lab. clin. Med. 1960, **55**, 286.
16. De Gier, J., L. L. M. van Deenen, R. A. Geerdink, K. Punt, and M. C. Verloop. Phosphatide patterns of normal, spherocytic and elliptocytic red blood cells. Biochim. biophys. Acta (Amst.) 1961, **50**, 383.
17. Phillips, G. B., and N. S. Roome. Quantitative chromatographic analysis of the phospholipids of abnormal human red blood cells. Proc. Soc. exp. Biol. (N. Y.) 1962, **109**, 360.
18. Kates, M., A. C. Allison, and A. T. James. Phosphatides of human blood cells and their role in spherocytosis. Biochim. biophys. Acta (Amst.) 1961, **48**, 571.
19. Prankerd, T. A. J. Studies on the pathogenesis of haemolysis in hereditary spherocytosis. Quart. J. Med. 1960, **29**, 199.
20. Van Deenen, L. L. M., and J. de Gier. Chemical composition and metabolism of lipids in red cells *in* The Red Blood Cell, 1st ed., C. Bishop and D. M. Surgenor, Eds. New York, Academic Press, 1964, pp. 282–285.
21. Hagerman, J. S., and R. G. Gould. The *in vitro* interchange of cholesterol between plasma and red cells. Proc. Soc. exp. Biol. (N. Y.) 1951, **78**, 329.
22. Hevesey, G., and L. Hahn. Turnover of lecithin, cephalin and sphingomyelin. Det Kongelige Danske Videnskabernes Selskab, Biologiske Meddelelser 1940, **15**, 1.
23. Reed, C. F. Studies of *in vivo* and *in vitro* exchange of erythrocyte and plasma phospholipids. J. clin. Invest. 1959, **38**, 1032.
24. Oliveira, M. M., and M. Vaughan. Incorporation of fatty acids into phospholipids of erythrocyte membranes. J. Lipid Res. 1964, **5**, 156.
25. Mulder, E., J. de Gier, and L. L. M. van Deenen. Selective incorporation of fatty acids into phospholipids of mature red cells. Biochim. biophys. Acta (Amst.) 1963, **70**, 94.
26. Robertson, A. F., and W. E. M. Lands. Metabolism of phospholipids in normal and spherocytic human erythrocytes. J. Lipid Res. 1964, **5**, 88.