The Selective and Conjoint Loss of Red Cell Lipids

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ABSTRACT The pattern of lipid loss from the membrane of red cells incubated in serum is influenced by the availability of glucose. Under homeostatic conditions with respect to glucose, cholesterol alone is lost. This results from esterification of free cholesterol in serum by the serum enzyme, lecithin: cholesterol acyltransferase, and is associated with a proportional decrease in membrane surface area, reflected by an increased osmotic fragility. This selective loss of membrane cholesterol also occurs in hereditary spherocytosis (HS) red cells, even after incubation for 65 hr in the presence of glucose. The loss of free cholesterol from red cells relative to its loss from serum, under these conditions, is greatest at higher hematocrits, similar to those found in the spleen.

Although the selective loss of membrane cholesterol increases the spherodicity of normal red cells, it does not lead to a change in their rate of glucose consumption, and both the loss of cholesterol and the increase in osmotic fragility are reversible in vitro. Moreover, normal red cells made osmotically fragile by cholesterol depletion in vitro rapidly become osmotically normal and survive normally after their reinfusion in vivo.

In contrast to this selective loss of membrane cholesterol, red cells incubated in the absence of glucose lose both cholesterol and phospholipid. This occurs more rapidly in HS than normal red cells and is followed by a disruption of cation gradients and then by hemolysis. Cholesterol and phospholipid lost under these conditions is not restored during subsequent incubations in vitro.

Selective loss of membrane cholesterol is a physiologic event secondary to an altered state of serum lipids. It is reversible both in vitro and in vivo and neither influences cellular metabolism nor impairs viability. Conjoint loss of phospholipid and cholesterol, however, results from intrinsic injury to the red cell membrane which results from prolonged metabolic depletion.

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INTRODUCTION

In 1939 Ham and Castle proposed that erythrostasis was an important feature of red cell destruction in diseases of man (3). Increased osmotic fragility, the hallmark of erythrostasis, was seen when normal red cells were pooled in the spleen (3, 4), as well as elsewhere (5), or were incubated in vitro (3, 6). The effects of erythrostasis in vitro could be largely prevented, however, by frequent resuspension of red cells in fresh serum during incubation (3). Two components responsible for this change in osmotic fragility have been described: an increase in cell volume due to cation accumulation (7, 8), and a decrease in cell surface area due to loss of membrane lipids (8–13).

Murphy has reported that, although a normal cation content could be maintained by red cells when incubated in serum under conditions of low hematocrit and adequate glucose, the cells nonetheless became osmotically fragile (8). This was due to a loss of membrane cholesterol, but not phospholipid (8, 11), and resulted from esterification of free cholesterol in serum by the serum enzyme, lecithin: cholesterol acyltransferase (14, 15), with subsequent equilibration between the free cholesterol compartments of serum and red cells (8, 11, 16). The cholesterol of mature red cells is confined to the membrane where it exists entirely in the free form.

In contrast to this selective loss of red cell cholesterol, two recent studies have shown that red cells incubated in vitro lose both cholesterol and phospholipid, that they are lost in equivalent amounts, and that more is lost from hereditary spherocytosis (HS) than from normal red cells (9, 10, 17). HS cells are of particular interest because of their inordinate susceptibility to splenic pool-

ing exposing them to the conditions of erythrostasis (18–20).

The meaning of lipid loss as observed in vitro is unclear, and a deficiency of lipid in red cells of patients with hemolytic anemia has not been well documented. However, the acquisition of lipid by HS red cells in vivo was found to reduce spherocytosis and permit a prolonged lifespan (1).

These studies were undertaken to delineate the conditions under which membrane lipids are lost individually or conjointly in vitro from HS and normal red cells and to assess the influence of lipid loss on cellular metabolism and viability.

METHODS

Studies in vitro. Red cells obtained from defibrinated blood were incubated at cell concentrations ranging from 6 to 60%. For incubations at hematocrits of 10% or less, the red cells were freed of white cells and platelets by washing with sterile Hanks' balanced salt solution, brought to a hematocrit of 30–40% in Hanks', and adjusted to the appropriate hematocrit by the addition of serum. For incubations at higher hematocrits, red cells were washed in serum rather than buffer and resuspended in serum. Glucose was added as 5% dextrose in water. When comparisons between normal and HS cells were made, a common serum was used for both. Incubations of quadruplicate 0.5–2.0-ml aliquots of red cell suspensions containing penicillin, 500 units/ml, were performed in sterile, stoppered, 16 × 150 mm culture tubes or 28 × 58 mm vials in a Dubnoff metabolic shaker placed in a 37°C atmosphere shaking at 130 oscillations/min. Free cholesterol–depleted serum was obtained by incubating fresh, cell-free serum at 37°C for 36–48 hr. Extraction (21) and measurement of red cell cholesterol (22), phospholipid (23), and serum free cholesterol (24) were performed as described previously (11). Red cell extracts were washed three times with 1/5 volume of 0.05 M KCl. This procedure removed greater than 99% of phosphate added as Na_2HPO_4, glucose-6-PO_4, and adenosine 5'-triphosphate. Standard errors (95% confidence limits) of extraction and measurement were: red cell cholesterol ±1.8%, red cell phospholipid ±1.3%, and serum free cholesterol ±1.4%. Values for normal red cell lipids at zero time were: cholesterol 12.6–13.8 $\mu g/10^8$ cells, and lipid phosphorus 10.8–11.6 $\mu g/10^9$ cells. In patients with HS these values were: cholesterol 12.9–13.6, and lipid phosphorus 10.3–11.7. For the quantitative recovery of serum free cholesterol after incubation, the supernatant serum was pooled with the supernatant of cell washes, lyophilized, and extracted with 30 ml of acetone: ethanol (1: 1, v/v) at 50°C for 10 min. The glucose content of whole cell suspensions was determined using the $Ba(OH)_2$-$ZnSO_4$ precipitation, glucose oxidase method (25). Cell Na and K were calculated from measurements of their concentrations in whole blood and serum and of hematocrit and are expressed as content per 10^{13} cells rather than concentration. Osmotic fragility was performed as modified (11) from Emerson et al. (19).

Studies in vivo. For measurement of their osmotic fragility and survival after transfusion, red cells which had been incubated at a hematocrit of 10% in vitro were centrifuged and resuspended in sterile saline to yield a hematocrit of 30%, to which was added 1/5 volume of sterile acid-citrate-dextrose and 100 μc $Na_2{}^{51}CrO_4$. After 20 min at room temperature the cells were washed once with saline and infused into the cell donor. Osmotic fragilities of the transfused cells and of the recipient's cells were determined as previously described (11, 26) using 0.5 ml of whole blood and 2.5 ml of test solution. Because no additional correction was made for either the volume of unhemolyzed cells or the per cent cell water, these fragilities are greater than measurements made using the same red cells but at lower cell concentrations. For the "preinjection" osmotic fragility, 0.1 ml of the cell suspension transfused was added to 10 ml of blood drawn just before the transfusion. Survival curves were calculated by the method of least squares using counts per minute per gram of hemoglobin in the 5 min sample as 100%. This value agreed within ±10% with the theoretical 100% calculated from the counts per minute injected assuming a blood volume of 7% of body weight. Surface counting was performed over the liver, spleen, and precordium at 1 min intervals beginning at the time of injection and continuing for at least 45 min thereafter.

Selection of subjects. Three patients with hereditary spherocytosis, as determined by family history, red cell morphology, osmotic fragility, and autohemolysis, served as cell donors for studies in vitro. Two had undergone splenectomy 7 and 11 yr previously. Normal subjects were free of hematologic disease. All subjects who served as recipients of autologous ^{51}Cr-labeled red cells were apprised of the experimental details and gave their free consent. The protocol of this study was approved by the Review Committee, Thorndike Memorial Laboratory.

RESULTS

Selective loss of cholesterol in vitro. When HS or normal red cells were incubated at a hematocrit of 6% for 65 hr in a common serum containing glucose at a concentration of 300 mg/100 ml, there was a progressive fall in the red cell content of cholesterol, while no significant change occurred in the red cell content of phospholipid (Fig. 1). This decline in red cell cholesterol reflects the new equilibrium partition established between cell and serum free cholesterol in the face of partial depletion of the combined pool.

To test the effect of cell concentration on this partition, normal red cells were washed in serum and incubated at three hematocrits ranging from 20 to 60% in fresh serum containing glucose, 500 mg/100 ml (Table I). At zero time and after incubation for 24 hr, red cells and serum were quantitatively recovered and their free cholesterol content measured. A preferential loss of cholesterol from red cells occurred at higher hematocrits, the level of free cholesterol in serum being maintained, while at lower hematocrits a proportionately greater fall in serum free cholesterol was observed. No change occurred in the red cell content of phospholipid. Thus, although cell and serum free cholesterol equilibrate with each other and can be considered as a single pool, in the face of partial depletion of this pool the partition of free cholesterol within it is not constant but rather is related to the cell concentration.

To assess the effect which variations in the serum: cell ratio have on the acyltransferase reaction, the amount of

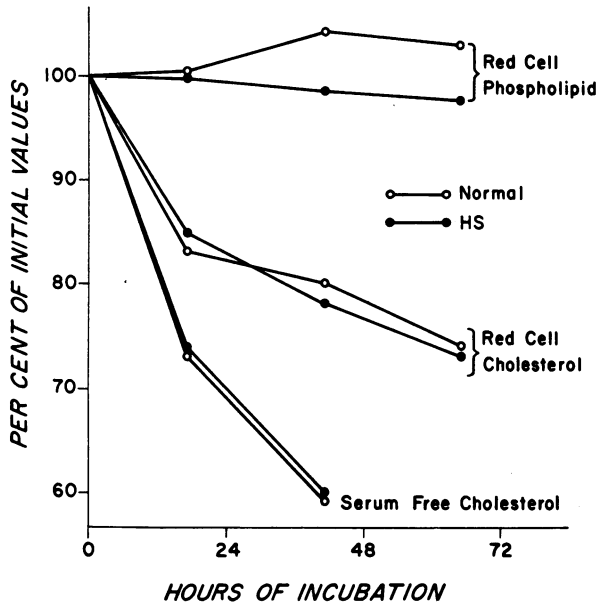

FIGURE 1 Effect of incubation on red cell and serum lipids. Red cells from a normal subject and a patient with hereditary spherocytosis (HS), who had undergone splenectomy, were incubated at a hematocrit of 6% at 37°C in serum pooled from both and containing added glucose. There was a fall in both the serum and red cell content of free cholesterol but no change in the red cell content of phospholipid.

cholesterol esterified during these incubations was related to the serum volume as follows: (*μg cholesterol lost from cells* + *μg free cholesterol lost from serum*)/*volume of serum*. The total cholesterol esterified per milliliter of serum was very similar at the three hematocrits (215, 222, and 238 μg/ml serum).

Since both the spherocytes in HS (7) and those induced by sulfhydral blocking agents (27) manifest an increased glycolytic rate, the effect of membrane cholesterol depletion on the rate of glucose utilization was examined. On two separate occasions, normal red cells were incubated at a hematocrit of 7% for 72 hr in either fresh normal serum or serum which had been heated to 56°C to prevent cholesterol esterification, each serum containing glucose at a concentration of 280 mg/100 ml. The average rate of glucose utilization in the heated serum was 2.2 μmoles/ml of packed cells per hr during the 1st 24 hr, 1.5 μmoles during the 2nd 24 hr, and 1.2 μmoles during the 3rd 24 hr. In spite of a decrease in cell cholesterol in fresh serum which averaged 37% at 72 hr, the rate of glucose utilization remained the same as in red cells which were incubated in heated serum, in which cholesterol loss was prevented. There was no change in the red cell content of phospholipid under either condition of incubation.

To test the ability of red cells to replenish cholesterol lost under the conditions described above, red cells were incubated for 12 hr in serum which had previously been allowed to undergo cholesterol esterification (Fig. 2). Cholesterol was lost from these red cells and there was a proportional increase in osmotic fragility. The medium was then replaced by serum which had been heated to

TABLE I

*Effect of Hematocrit on the Loss of Free Cholesterol from Serum and Red Cells**

Hematocrit	Loss of free cholesterol, per cent of initial values + SE	
	Serum	Cells
	%	%
20%	36.8 ±9.8	20.6 ±5.2
40%	12.5 ±2.0	16.3 ±5.8
60%	2.3 ±2.2	9.0 ±2.6

* Normal red cells were incubated at 37°C for 24 hr in fresh serum with added glucose. Free cholesterol was quantitatively recovered from cells and serum. The data are averaged from two similar experiments. No change occurred in the red cell content of phospholipid.

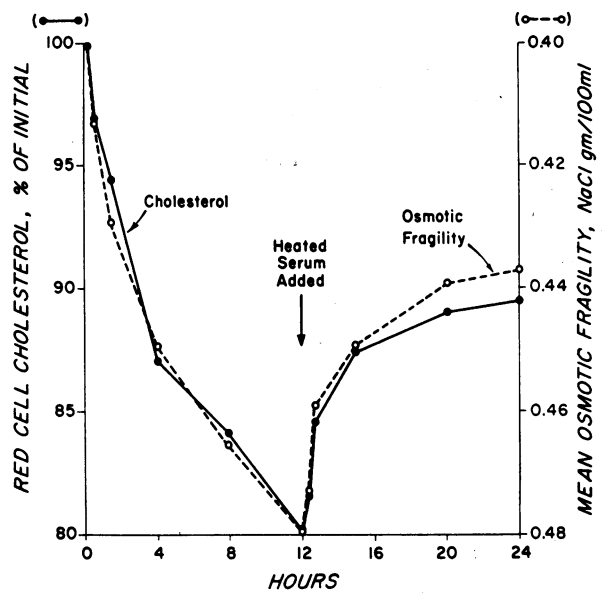

FIGURE 2 Depletion and repletion of cholesterol in normal red cells in vitro. Normal red cells were incubated at a hematocrit of 10% at 37°C in autologous serum depleted of free cholesterol by prior incubation. After 12 hr the cells were sedimented and resuspended in autologous serum which had been heated to 56°C, and the incubation was continued for another 12 hr. Data shown are the averages from three separate experiments. During the 1st 12 hr, while in cholesterol depleted serum, there was a loss of red cell cholesterol and an increase in osmotic fragility. Both returned toward normal during incubation for a 2nd 12 hr in normal, heated serum.

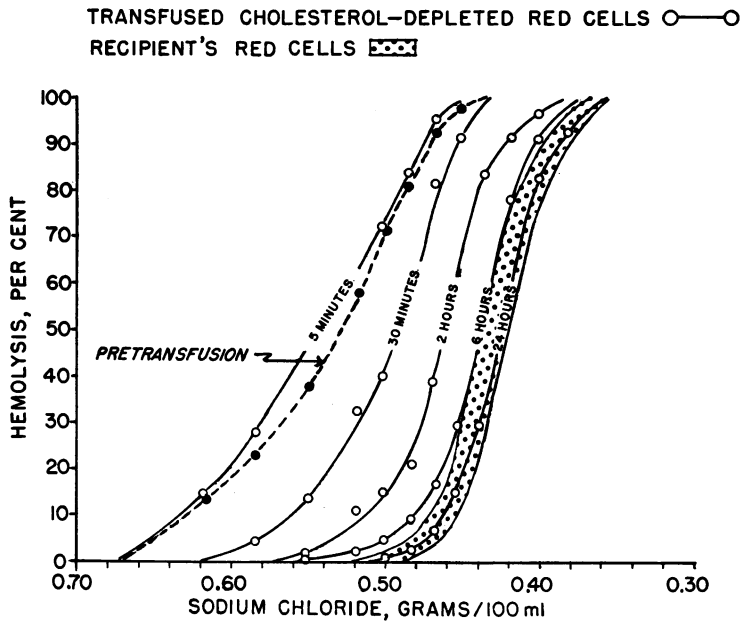

FIGURE 3 Recovery of cholesterol depleted red cells in vivo. After their incubation for 12 hr in free cholesterol-depleted, autologous serum, as in Fig. 2, normal red cells were labeled with ^{51}Cr and infused into the cell donor. The transfused, osmotically fragile cells rapidly regained a normal osmotic fragility.

56°C to prevent esterification and which therefore had a full complement of free cholesterol. During this second incubation there was a partial restoration of both the cholesterol content and the osmotic resistance of these normal red cells. There was no change in the phospholipid content of these red cells. Under the conditions of low hematocrit used, as also shown previously (8, 11), there were no changes in cell volume or cation content,

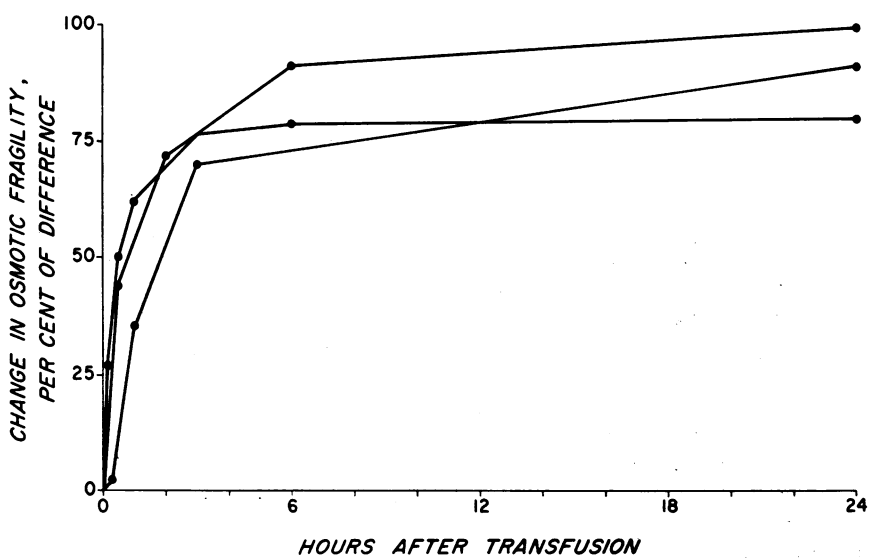

FIGURE 4 Rates of recovery of cholesterol-depleted red cells in vivo (see Fig. 3). Data are presented as the change in mean osmotic fragility after transfusion expressed as a per cent of the increase in mean osmotic fragility which had occurred during incubation before transfusion. The return toward normal occurred rapidly, being half complete in approximately 1 hr.

TABLE II

*Influence of Glucose on the Loss of Lipid by HS Red Cells**

	Change in lipid, per cent of initial values	
	Cholesterol	Phospholipid
	%	%
Heated serum, (+) glucose	−0.8	+1.0
Fresh serum, (+) glucose	−11.8	+1.3
Heated serum, (−) glucose	−14.0	−13.7
Fresh serum, (−) glucose	−15.0	−10.2

* Red cells from a patient withHS who had undergone splenectomy were incubated for 36 hr at a hematocrit of 6% at 37°C in serum which had been dialyzed against glucose-free Hanks' balanced salt solution. A portion of the serum had been heated to 56°C for 30 min. Glucose, 300 mg/100 ml, was added to a portion of both fresh and heated serum.

which might themselves influence osmotic fragility. That such changes were avoided is also indicated by the lack of change in the osmotic fragility of red cells incubated for the entire 24 hr in serum which had been heated to 56°C. Such red cells preserved both their cholesterol and phospholipid.

Effect of cholesterol depletion on red cells in vivo. To study the fate in vivo of red cells depleted of cholesterol in vitro, red cells from three normal subjects were incubated in autologous, free cholesterol-depleted serum for 12 hr, after which they were labeled with ^{51}Cr and reinfused into the cell donor (Fig. 3). The loss of cell cholesterol in vitro ranged from 10 to 15% and there was a proportional increase in osmotic fragility. After reinfusion into the cell donor there was a rapid and progressive return of osmotic fragility to normal. By analogy with the studies in vitro it is inferred that the transfused red cells rapidly regained cholesterol. Repletion of these red cells in vivo was complete, in contrast to their partial repletion in vitro, and occurred rapidly, the repair process being half complete within approximately 1 hr (Fig. 4). Survival of the transfused cells was normal (^{51}Cr half survival = 22.5, 26.0, and 26.7 days), and no sequestration was observed, even transiently, over the liver or spleen.

Loss of cholesterol and phospholipid in vitro. The selective loss of membrane cholesterol in vitro in these and previous (8, 11) studies contrasts with reports of a loss of membrane phospholipid as well as cholesterol during incubation in vitro, particularly in HS (10, 13, 17). Because glucose exhaustion appeared to play a role in two of these studies (10, 13), the role of cellular metabolism in the loss of membrane lipid was assessed (Table II). Red cells from a patient with HS who had undergone splenectomy were washed in glucose-free Hanks' solution and resuspended in compatible normal serum which had previously been dialyzed free of glucose. A portion of the serum had been heated to 56°C to destroy acyltransferase activity. During incubation in fresh dialyzed serum to which glucose had been added, HS red cells selectively lost cholesterol as described above. This loss was prevented in heated glucose-containing serum. In the absenec of glucose, however, there was a loss of cholesterol and phospholipid in both fresh and heated sera. Thus the loss of phospholipid occurs only in the face of glucose exhaustion.

The events of lipid loss as related to glucose exhaustion are depicted in Fig. 5, which compares observations in two patients with HS who had undergone splenectomy and three normal subjects. Similar results, except for a more rapid glucose consumption, were obtained in a third patient with HS who had an intact spleen. Over the 1st 12 hr of incubation, during most of which time glucose was present, lipid loss involved cholesterol alone, cation gradients were maintained, and no hemolysis occurred. After the exhaustion of glucose, and occurring more rapidly in HS than in normal red cells, phospholipid plus equivalent, additional amounts of cholesterol were lost, Na and K gradients were disrupted, and finally hemolysis ensued. Neither cholesterol nor phospholipid could be added to normal red cells incubated under these conditions for 36 hr and then incubated an additional 12 hr in fresh or heated serum containing glucose, 400 mg/100 ml.

DISCUSSION

Selective loss of cholesterol. The equilibrium exchange of free cholesterol between serum (or plasma) and red cells causes quantitative changes in the latter paralleling changes in the former (8, 11). As the esterification of cholesterol in vitro reduces the total quantity of free cholesterol in the red cell–serum pool, a new equilibrium is established between cells and serum resulting in a reduction in the amount of cholesterol in both compartments. The partition of free cholesterol between the cell and serum compartments under these conditions is a function of the cell volume relative to the serum volume. At low cell concentrations relatively more cholesterol is lost from serum than from red cells, while at high cell concentrations little cholesterol is lost from serum in comparison to the greater per cent lost from red cells. Thus, in areas of erythroconcentration, as in the spleen, cholesterol esterification might proceed to the selective disadvantage of red cells, present in high concentration, and shape change resulting from cholesterol loss might impede red cell release from the spleen. However, having gained access to the general circulation the red cell can rapidly replete the cholesterol which has been lost. This was seen when red cells made sphero-

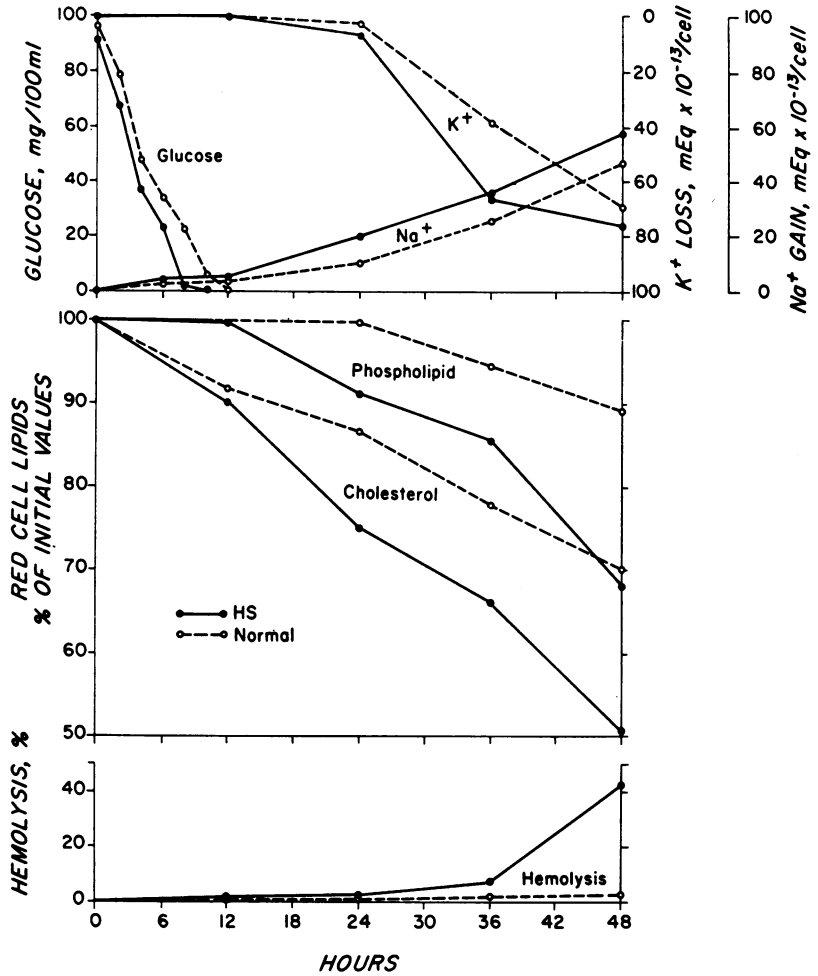

FIGURE 5 Effect of incubation on red cells. Red cells from normal subjects and patients with HS who had undergone splenectomy were incubated at a hematocrit of 25% at 37°C in fresh serum. Data shown are averaged from two HS patients and three normal subjects. While glucose was present, only cholesterol was lost from both HS and normal red cells. After glucose exhaustion, and occurring more rapidly in HS than normal, phospholipid was lost as well, Na and K gradients became disrupted, and finally hemolysis ensued.

cytic by cholesterol depletion in vitro were reinfused in vivo. The speed with which the process of repletion occurs probably accounts for the lack of splenic sequestration and the normal survival of these transfused red cells. Cholesterol repletion is more rapid than the acquisition of excess cholesterol by normal red cells as occurs after their transfusion into patients with obstructive jaundice, a process which is also reversible but which occurs over the course of 48 hr (11). The demonstration of cholesterol repletion in vitro and a parallel return of osmotic fragility toward normal in this study contrasts in part with Murphy's finding of cholesterol repletion occurring without an accompanying change in osmotic fragility (8). Possibly the shorter duration of incubation in our study accounts for this discrepancy.

Although the exact role in vivo of cholesterol esterification as studied in serum in vitro has been debated (28), the weight of recent evidence would ascribe a major role to acyltransferase in comparison with a lesser role played by cholesterol esterification in the liver (28–31). Norum and Gjone (32) have recently described a family which lacks serum acyltransferase, although their ability to esterify cholesterol in the intestine is present. Affected individuals have very low concentrations of cholesteryl esters in plasma. That the serum acyltransferase reaction may serve as a regulator of red cell cholesterol is indicated by the high content of cholesterol in red cells of the patients with acyltransferase deficiency (33), as well as in patients with obstructive jaundice (11) in whom acyltransferase activity is also decreased

(11, 34). However, factors other than acyltransferase have also been shown to influence red cell cholesterol, e.g., elevations of serum bile salts, as occur in obstructive jaundice, cause a shift in the serum: cell free cholesterol partition favoring an accumulation of cholesterol by the red cell (11).

Lecithin is limiting in the acyltransferase reaction in serum (8). When the cell: serum ratio was varied, however, the amount of cholesterol which became esterified was related only to the volume of serum present in spite of a large reservoir of lecithin in red cells. Thus, it appears that the lecithin of red cells does not readily enter into the acyltransferase reaction.

Conjoint loss of cholesterol and phospholipid. When incubations in vitro were performed at a low hematocrit and in the presence of adequate glucose, HS red cells behaved the same as normal red cells in that they selectively and reversibly lost cholesterol without any change in phospholipid content. When glucose was absent, however, there was a loss of phospholipid plus equivalent amounts of cholesterol, more rapidly from HS than normal red cells, and this loss was not reversible in vitro. Red cells incubated under these conditions have been shown to have a markedly shortened survival when injected in vivo (7). Such incubations may well reflect changes which occur during red cell pooling in the spleen where the tendency to deplete available sources of glucose is far greater than in the peripheral blood (35). A loss of both cholesterol and phospholipid which was particularly marked in HS red cells and which was partially preventable by adding glucose has been reported by Reed and Swisher (10) using an incubation system similar to that of the present study except for a higher hematocrit and the presence of ethylenediaminetetraacetic acid (EDTA). Similarly, Langley and Axel (13) observed that the lipid loss from normal red cells, particularly the phospholipid loss, was diminished by adding glucose. Lipid loss related to metabolic depletion would be expected to occur more rapidly in HS red cells since these cells are metabolically hyperactive (7, 36). On the other hand, Jacob (17) has recently reported the loss of cholesterol and phospholipid by HS red cells incubated in buffer, even in the presence of adequate glucose, and has ascribed this to the increased cation flux which HS red cells maintain (7). The difference between these results and our own may be due to the absence of serum lipoproteins in the previously reported study. However the data herein reported demonstrate that, in the presence of serum lipoproteins, increased cation pumping does not lead to membrane lipid loss in HS. The loss of phospholipid from the red cell membrane was consistently seen only after several hours of metabolic exhaustion. The loss of cholesterol and phospholipid under these conditions has been attributed to membrane fragmentation by Weed and Reed (37). However, Langley and Axell (13) have recently shown that a loss of membrane protein does not accompany this lipid loss, thus indicating that pieces of membrane are not lost but rather that the membrane affinity for lipids is altered.

Function of membrane lipids. A specific role for cholesterol in the red cell membrane has not been defined, although the membrane of red cells as compared with other membranes is particularly rich in cholesterol (38). Although red cells were made spheroidal by the loss of 37% of their membrane cholesterol, they did not alter their rate of glycolysis. This contrasts with the increased glycolytic rate both in red cells made spherocytic with sulfhydral inhibitors (27) and in the spherocytes of HS (7, 35), conditions associated with an increased membrane permeability to cations (7, 27).

Cholesterol appears to play a major role in determining cell shape. Murphy has recently provided data demonstrating a high concentration of cholesterol along the convexity of normal red cells (39). Its loss from the membrane causes a closely correlated loss of surface area as manifested by increased osmotic fragility (8, 11), while its acquisition leads to a gain in surface area (11). An extreme distortion of red cell membrane configuration in "spur cell" anemia has been associated, in several cases, with a massive increase in the cholesterol: phospholipid ratio (40–42). These quantitative alterations in cholesterol of the red cell membrane are all reversible and probably do not influence cell viability except insofar as they cause changes in cell shape (1, 11, 42).

A more specific role for phospholipids has been suggested by studies of monovalent (43, 44) and divalent (45) cation flux in red cells and nervous tissue and of coordinated enzymatic activity in mitochondria (46). In the present study, phospholipid loss occurred only under conditions of metabolic failure, and was indicative of profound cellular injury.

The HS red cell manifests most dramatically the effects of erythrostasis. Spheroidal at birth (47), it is predisposed to splenic pooling where spheroidicity is enhanced by both low pH (48) and cholesterol loss. After glucose exhaustion, cell sphering is further compounded by a gain of sodium and finally a loss of membrane cholesterol *and* phospholipid. All but the latter are reversible if the red cell, with so much tending to keep it in the spleen, can find its way into the homeostatic milieu of the general circulation.

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