# Modification of Red Cell Membrane Structure by Cholesterol-Rich Lipid Dispersions

## A MODEL FOR THE PRIMARY SPUR CELL DEFECT

RICHARD A. COOPER, ELIZABETH C. ARNER, JAMES S. WILEY, and
SANFORD J. SHATTIL

From the Hematology–Oncology Section, Department of Medicine, University
of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

A B S T R A C T   Cholesterol-rich membranes are the hall-mark of "spur" red cells. Spur cells accumulate cholesterol from cholesterol-rich serum lipoproteins. Previous studies suggested that this added cholesterol is responsible for both the altered morphology and the destruction of spur cells. To examine this process in the absence of other serum factors, cholesterol-lecithin dispersions with varying amounts of unesterified cholesterol (C) relative to phospholipid (P) were prepared, and their influence on normal human red cells was studied.

Cholesterol-rich lipid dispersions (C/P mole ratio > 1.0) transferred cholesterol to both red cell membranes and serum lipoproteins, and cholesterol-poor dispersions (C/P mole ratio < 1.0) depleted red cells of cholesterol. Changes in membrane cholesterol paralleled changes in membrane surface area, as calculated from osmotic fragility, with a 0.22% variation in surface area per 1.0% variation in cholesterol content. Cold-induced compression of membrane surface area was increased in cholesterol-poor red cells (C/P = 0.4), whereas the surface area of cholesterol-rich membranes (C/P = 1.80) underwent no compression. Although the Na and K permeability of red cells severely depleted of cholesterol was increased, lesser degrees of depletion had no effect, and the permeability of cholesterol-rich cells was normal. However, increasing membrane cholesterol caused a progressive decrease in red cell deformability, as measured by filtration.

Cholesterol-poor red cells were spherocytic in appearance and cholesterol-rich cells were broad and flat, indicative of their surface areas. In addition, cholesterol-rich cells had an irregular contour due to folding of the periphery of the cell. This shape abnormality was identical to that of both spur cells after splenectomy and normal red cells incubated in spur serum. Normalization of the C/P of spur serum by added phospholipid prevented the increase in membrane cholesterol and surface area and the transformation of cell shape.

These studies establish that the cholesterol content of red cells is dependent on the C/P of their milieu, either lipoproteins or cholesterol-lecithin dispersions. Moreover, the surface area, deformability, and contour of cholesterol-rich red cells are a direct function of their increased membrane C/P. Although cholesterol-rich spur cells are further modified in the circulation of patients with spleens, this abnormality of the membrane lipid bilayer, induced by cholesterol-rich cholesterol-lecithin dispersions, represents the primary spur cell defect.

## INTRODUCTION

Spur cell anemia is an acquired hemolytic process occurring in patients with severe liver disease, usually alcoholic cirrhosis (1–3). Recent studies have shown that in patients with spur cells there is an increase in the ratio of free (or unesterified) cholesterol relative to phospholipid both in serum and in red cells; moreover a strong correlation exists between this cholesterol-to-phospholipid disproportion in serum low-density lipoproteins (LDL)[1] and the same disproportion in red cell

---

[1] *Abbreviations used in this paper:* C/P, cholesterol-to-phospholipid mole ratio; HDL, high-density lipoprotein; LCAT, lecithin-cholesterol acyl transferase; LDL, low-density lipoprotein.

membranes (4). Because the spur cell phenomenon is acquired by normal red cells incubated in serum from patients with spur cells or by normal red cells infused into patients with spur cells, we have suggested that the serum lipoprotein abnormality is etiologic in the genesis of spur cells. However, other factors in patients' serum that might be active in vitro or in vivo could not be excluded. Therefore, we undertook to establish an artificial system that reproduced the cholesterol disproportion in the serum of patients with spur cells to study this factor in the absence of other influences.

The sonication of phospholipids, such as lecithin, alone or with varying amounts of cholesterol, disperses the lipid in aqueous buffers (5). In 1968 Bruckdorfer, Edwards, Graham, and Green demonstrated that the cholesterol in sonicated mixtures of cholesterol and lecithin exchanges with the cholesterol in both LDL and red cell membranes (6, 7). When red cells were incubated in sonicated mixtures that contained a cholesterol-to-phospholipid mole ratio (C/P) of 1.0, they underwent no change in cholesterol content; however red cell membranes lost cholesterol when incubated with cholesterol-phospholipid mixtures having a C/P of < 1.0. We have used cholesterol-rich sonicated mixtures (C/P > 1.0) similarly prepared to reproduce the serum lipoprotein abnormality of patients with spur cells. The finding that these cholesterol-rich dispersions were capable of inducing the spur cell defect in vitro afforded us an opportunity to assess certain physical characteristics of cholesterol-rich red cells and to apply this to a further understanding of the pathophysiology of spur cell anemia.

## METHODS

*Preparation and analysis of sonicated lipids and serum lipoproteins.* L-α-dipalmitoyl lecithin (General Biochemicals Div., Mogul Corp., Chagrin Falls, Ohio), 40 mg, and cholesterol (Sigma Chemical Co., Inc., St. Louis, Mo.) in amounts up to 80 mg were added to 10 ml of 0.155 M NaCl in a fluted metal container surrounded by an ice slurry and subjected to 70 W for 60 min with a Branson sonifier (Branson Instruments Co., Stamford, Conn.) with a standard tip. After sonication, 4.0 ml of normal human serum previously heated to 56°C for 30 min or 4.0 ml of human serum albumin (Sigma Chemical Co.) in 0.155 M NaCl at a concentration of 2.0 g/100 ml was added, and the serum-sonicate or albumin-sonicate mixture was centrifuged at 21,800 *g* for 30 min to sediment undispersed lipid. Except in dispersions containing more than 40 mg of cholesterol, only trace amounts of lipid were sedimented.

Serum and sonicate lipids were extracted with acetone: ethanol (1:1) for measurement of cholesterol (8), free cholesterol (9), and lipid phosphorus (10). Phospholipid values were calculated by multiplying lipid phosphorus values times 25. Individual phospholipids were quantitated by thin layer chromatography with chloroform: methanol: glacial acetic acid: water (25:15:4:2) (11).

Individual serum lipoproteins were separated by density centrifugation with KBr (12). The density of sonicated lipids was determined by centrifugation at 108,000 *g* for 16 h at various KBr concentrations.

Serum and sonicate free cholesterol were labeled with [³H]cholesterol by the addition of 50 μCi in 10 μl of ethanol to either 10 ml of serum or albumin-sonicate and subsequent incubation for 6 h at 37°C.

*Incubation of red cells and analysis of red cell membranes.* For incubation, freshly obtained normal human red cells were washed three times with Hanks' balanced salt solution and resuspended at a hematocrit of 10% in Hanks' containing penicillin (100 U/ml). Red cell suspensions were mixed with equal volumes of serum-sonicate or albumin-sonicate mixtures and incubated in stoppered 16 × 150-mm test tubes or stoppered 250-ml Ehrlenmeyer flasks in a shaking water bath for varying times up to 24 h.

Cell morphology was assessed in wet preparations and dried, Wright's-stained smears. For scanning electron microscopy, cell suspensions were fixed with 2% glutaraldehyde (Polysciences, Inc., Warrington, Pa.) in 0.1 M phosphate buffer, and electron micrographs were kindly prepared by Dr. Ronald Weinstein, Tufts University School of Medicine.

The osmotic fragility of red cells after incubation was determined after further dilution with 1.5 vol of Hanks', as described previously (3). Except where noted, all determinations were made at room temperature. For variations in temperature, red cells and lysing solutions were brought to a constant temperature and held there during lysis. Red cells were sedimented within 5 min of addition to lysing solutions by centrifugation for 2 min in a Serofuge (Clay-Adams, Div. of Becton, Dickinson & Co., Parsippany, N. J.). For measurement of critical hemolytic volume, 0.2 ml of cell suspension was added to 0.6 ml of lysing solution. Microhematocrits were determined in triplicate after centrifugation for 5 min in a microhematocrit centrifuge (International Equipment Company, Needham Heights, Mass.). Surface area was calculated from osmotic fragility according to Castle and Daland (13) as described previously (14).

For measurement of red cell membrane lipids, red cells were washed three times with 0.155 M NaCl and extracted with isopropanol and chloroform (15) for measurement of cholesterol (8), lipid phosphorus (10) and individual phospholipids (11) as described previously (3). Red cell ghosts were prepared according to Dodge, Mitchell, and Hanahan (16).

For measurement of Na and K fluxes after incubation, inosine (10 mM) and adenine (2 mM) were added to cell suspensions 1 h before the end of incubation to assure that adenine nucleotides were normal during these measurements (17). Cell cation concentration, sodium influx, sodium efflux, and potassium influx were measured as described previously (18).

For measurement of cell filtration, red cells after incubation were adjusted to an hematocrit of 0.1% by the addition of Hanks' balanced salt solution, and triplicate 10-ml aliquots were filtered through 2.5 cm, 8.0 μm Millipore filters (Millipore Corp., Bedford, Mass.) at 10 mm Hg negative pressure. The time for evacuation of fluid from the chamber above the filter was taken as filtration time.

## RESULTS

*Characteristics of lipid dispersions.* The C/P of dispersions prepared by sonication was similar to the C/P of lipids added before sonication for all mixtures except that obtained by the addition of more than 40 mg of cholesterol to 40 mg of lecithin, and only in the latter case was there a significant amount of lipid in the pellet

after centrifugation at 21,800 $g$. Thus, for example, the C/P of dispersions prepared from a mixture of 23 mg cholesterol plus 40 mg lecithin was 1.0; 40 mg + 40 mg = 1.9; 80 mg + 40 mg = 2.2. 84% of both the cholesterol and phospholipid present in dispersions with a C/P of 1.0 passed through Millipore filters of 0.45 μm pore size.

To assess the chemical stability of dispersions, the cholesterol and phospholipid content of sonicated lipids, with and without added serum, was measured at various times during storage at 0°C and 37°C. Samples were centrifuged immediately before lipid determinations. There was no change in the total lipid content or the C/P ratio over the course of 30 days at either temperature. When analyzed by thin layer chromatography, more than 95% of the phospholipid both before and after sonication was lecithin, 1–2% was recovered in the area of phosphatidyl-serine, and the remainder was recovered in the region of lysolecithin. Thus, it appears that neither sonication itself nor storage after sonication had a discernible effect on the synthetic lipids studied.

The buoyant density of sonicated lipids was determined by measuring their flotation in varying concentrations of KBr at 108,000 $g$ for 16 h. At the density of 0.155 M NaCl, all sonicated lipids were sedimented by their centrifugation. Pure phospholipid dispersions floated incompletely at $d = 1.063$ and completely at $d = 1.21$. Cholesterol-containing dispersions with a C/P of 1.0 and 2.2 floated partially at $d = 1.022$ and completely at $d = 1.035$. It therefore was possible to separate cholesterol-containing dispersions from high-density lipoprotein (HDL), but it was not possible to separate these dispersions from LDL.

To examine the interaction of lipid dispersions and serum lipoproteins, dispersions with a C/P of 2.2 were incubated with normal HDL for 20 h at 37°C, and thereafter HDL and the dispersions were separated by centrifugation at $d = 1.063$. The C/P of HDL increased from 0.22 before incubation to 0.41 when HDL was separated immediately after mixing. The C/P of HDL increased thereafter to 1.04 at 2 h, 1.23 at 8 h, and 1.44 at 20 h. Thus, although cholesterol-rich lecithin dispersions did not fuse with HDL, their excess cholesterol transferred to HDL.

*Effect of cholesterol-lecithin dispersions on red cell membrane lipids.* Previous investigators have demonstrated that an equilibrium exchange exists between the cholesterol in red cells and that in whole serum (19), isolated serum lipoproteins (20, 21) or cholesterol-lecithin dispersions (7). In each case, 50% equilibrium is reached in approximately 2 h. Red cells at a hematocrit of 10% were incubated with equal volumes of either serum or dispersions with a C/P of 1.0, each containing [³H]cholesterol. The incorporation of radioactivity into red cells was measured over the course

of 24 h. Under both conditions the time to 50% equilibration was 2.3 h. Thus, the exchange equilibrium of free cholesterol between red cells and cholesterol-lecithin dispersions follows the same time course as the exchange rate with human serum lipoproteins.

Although the movement of cholesterol between sonicated dispersions and red cells did not require the presence of serum proteins, to carry out studies of red cell morphology, sonicated lipids were mixed with either heated normal serum or human serum albumin. Because the C/P of normal serum is approximately 0.5, the addition of serum decreased the total C/P of the serum-sonicate mixture. No changes were observed in red cells incubated in a serum-sonicate mixture consisting of dispersed lipid with a C/P of 1.0 plus normal serum, the C/P of this mixture being approximately 0.9. The effect of serum-sonicate mixtures with a normal (0.9) or increased (1.85) C/P on the cholesterol content of red cells is depicted in Fig. 1. During incubation for 24 h the cholesterol content of red cells incubated in the presence of cholesterol-rich dispersion increased by 150%, whereas there was no change in the cholesterol content of red cells incubated with cholesterol-normal dispersions. In neither case was there any change in the red cell content of phospholipid nor in the percent composition of the various phospholipids. Moreover, the C/P of ghosts prepared at the end of 24 h was identical to that of intact cells. Thus, red cells incubated with cholesterol-rich lecithin dispersions acquired membrane cholesterol.
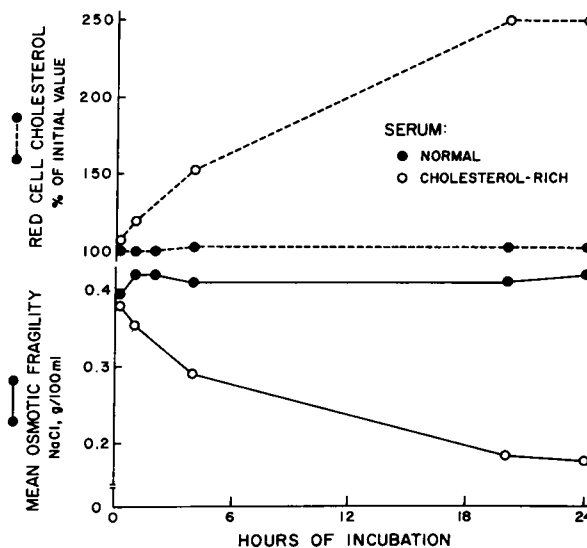


FIGURE 1 Osmotic fragility and cholesterol content of red cells incubated in normal and cholesterol-rich serum. Heated serum was enriched with free (unesterified) cholesterol by the addition of cholesterol-lecithin dispersions with a C/P of 2.2.
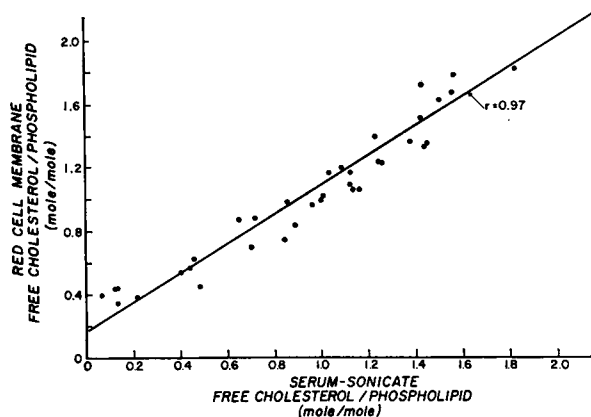
FIGURE 2  Relationship between the C/P of the serum-sonicate medium and red cells incubated in it. The C/P of serum was varied by the addition of cholesterol-dipalmitoyl lecithin dispersions.

The effect of dispersions of varying cholesterol-phospholipid composition on the C/P of red cell membranes is shown in Fig. 2. The C/P of cholesterol-lecithin dispersions correlated closely with that of red cell membranes over the entire range studied. These results are very similar to observations previously made with lipoproteins obtained from patients with liver disease (3). In none of these incubations, over the complete range of C/P mole ratios studied, was there any change in the red cell content of phospholipid. Thus, the lipid changes observed resulted from the selective acquisition or loss of cholesterol by red cells.

*Effect of red cell C/P on membrane surface area.* Red cells that had lost cholesterol were osmotically fragile, whereas red cells that had gained cholesterol were resistant to osmotic lysis. The osmotic fragility curves were symmetrical and upright, and therefore only the 50% hemolysis point is depicted in Figs. 1 and 3. The time course of the acquisition of osmotic resistance coincided with the acquisition of membrane cholesterol (Fig. 1), and the time course of cholesterol loss coincided with an increasing osmotic fragility. This close relationship between osmotic fragility and membrane C/P existed over a broad range of values in red cells incubated with either serum-sonicate (Fig. 3) or albumin-sonicate mixtures of varied C/P ratios.

Because osmotic fragility is a measure of the surface area-to-volume ratio of cells, it can serve as a measure of surface area only if there has been no change in cell volume. In the studies reported herein, red cells maintained a normal volume and a normal total cation content (Table I) during incubation. From the formula of Castle and Daland (13), it was calculated that the osmotic fragility data depicted in Fig. 3 represents a range

of surface area from a loss of 13% at a C/P of 0.3 (67% loss of cholesterol) to a gain of 24% at a C/P of 1.8 (100% gain of cholesterol). Thus, over the range of 167% in terms of membrane cholesterol ($-67 - +100\%$) membrane surface area changed 37% ($-13 - +24\%$). This is equivalent to a change in membrane surface area of 0.22% for each 1.0% change in red cell membrane cholesterol content, a value identical to that observed previously when red cells were incubated in serum depleted of free cholesterol by the enzyme lecithin-cholesterol acyl transferase (LCAT) or serum enriched in free cholesterol as obtained from patients with liver disease (14, 22).

That the changes seen in osmotic fragility were a result of changes in surface area was further confirmed by measurements of the critical hemolytic volume of red cells exposed to hypotonic solution. Red cells which had lost cholesterol and had a C/P of 0.41 had a volume increase of 30% at the point of 50% lysis, as compared with 76% for red cells that had undergone no change in cell lipids after incubation. In contrast, cholesterol-rich red cells with a C/P of 1.62 underwent a volume increase of 122%, and those with a C/P of 1.80 increased 172%.

Jacobs and Parpart observed in 1931 that the osmotic fragility of red cells was influenced by the temperature at which the osmotic fragility was measured (23). Careful studies carried out more recently by Murphy established that this difference in osmotic fragility is due to a decrease in cell surface area under conditions of decreased temperature (24). The effect of membrane cholesterol on the degree to which this change in cell surface area occurs is depicted in Fig. 4. The mean osmotic fragility of normal red cells increased 0.06 g/100 ml NaCl as the temperature was lowered from 37° to 0°C. This is equivalent to a decrease in surface area of 6.2%. Red cells depleted of cholesterol by incubation with cholesterol-poor serum-sonicate mixtures, which had a C/P = 0.40 − 0.42, underwent a more striking change in osmotic fragility when temperature was lowered, the change between 37° and 0°C being equivalent to a change in surface area of 8.6%. In contrast, cholesterol-rich red cells (C/P = 1.75), which had an increased surface area and therefore a decreased osmotic fragility, underwent no significant change in osmotic fragility as the temperature was lowered from 37° to 0°C. Thus, a decreased amount of membrane cholesterol permits a greater than normal degree of compression of cell surface area, whereas an increase in membrane cholesterol fixes the membrane in an expanded form.

*Effect of red cell C/P on membrane deformability and permeability.* For red cells to traverse pores of small diameter, they must undergo a change in shape. Therefore, spherocytic red cells, which lack the excess mem-

TABLE I

Cell Na and K Content and Flux under Conditions of Varying Membrane Cholesterol

| Red cell C/P | Total Na influx | Na efflux | | K influx | | Cell cations | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | | Total | Ouabain-sensitive | Total | Ouabain-sensitive | Na | K | Total |
| | *meq/liter/h* | *meq/liter/h* | | *meq/liter/h* | | *meq/liter* | | |
| 0.42 | 4.13 (2.90–5.11)* 5‡ | 4.15 (4.0–4.3) 2 | 2.20 (2.10–2.30) 2 | 2.37 (2.10–2.64) 2 | 1.83 (1.56–2.09) 2 | 16.0 (15.1–16.8) 4 | 87.3 (86.6–88.0) 2 | 103.3 |
| 0.54 | 2.19 (2.17–2.20) 2 | — | — | — | — | — | — | |
| 0.90 | 2.07 (1.84–2.30) 6 | 2.86 (2.55–3.20) 3 | 1.73 (1.60–1.90) 3 | 1.81 (1.68–2.01) 3 | 1.33 (1.25–1.46) 3 | 7.8 (6.3–9.3) 6 | 95.9 (94.3–97.9) 4 | 103.5 |
| 1.34 | 1.83 (1.77–1.89) 2 | — | — | — | — | — | — | |
| 1.62 | 1.90 (1.66–2.15) 5 | 2.75 (2.70–2.80) 2 | 1.72 (1.65–1.80) 2 | 1.47 (1.44–1.50) 3 | 1.13 (1.06–1.20) 3 | 8.3 (8.0–8.5) 4 | 93.1 (92.4–93.9) 2 | 101.4 |
| 1.85 | 2.07 (2.04–2.10) 2 | — | — | — | — | — | — | |

\* Range.
‡ Number of observations.

brane to accommodate a shape change, do not readily pass through filters with 3–8 μm pores (25). This characteristic of spherocytic cells was manifested by cholesterol-poor red cells (C/P = 0.40), which had a filtration time through 8 μm Millipore filters more than 20 times normal. The effect of added membrane cholesterol on filtration time is shown in Fig. 5. Little change in filterability was noted when the C/P of red cells was increased to 1.28. However, with further increases in membrane cholesterol relative to phospholipid, filtration time progressively lengthened to 80% more than normal at a membrane C/P of 2.00.

The content of sodium and potassium and the fluxes of these cations in red cells whose membranes had a range of C/P ratios is shown in Table I. Red cells which had become markedly cholesterol-depleted during 20 h of incubation had an increase in cell sodium concentration and a reciprocal decrease in cell potassium concentration, resulting in a total cation concentration that was normal. All ion fluxes measured were increased in these cells, the magnitude of this increase varying from approximately 30% to 100%. Red cells somewhat less severely depleted of cholesterol had a normal sodium influx. The Na and K content and fluxes in cholesterol-

rich red cells were all within or very near the normal range.

*Effect of red cell C/P on cell morphology.* When viewed in wet preparation by means of phase micros-
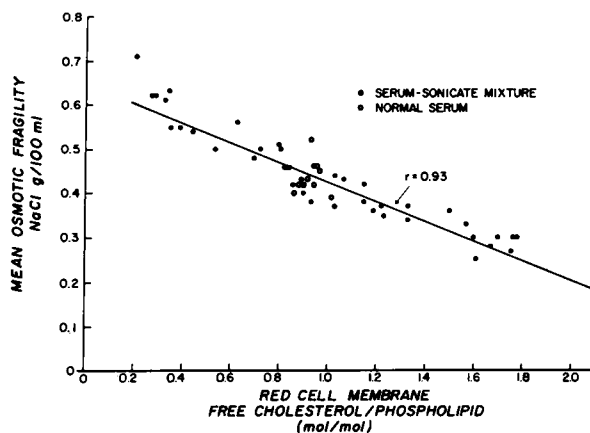


FIGURE 3 Relationship between the C/P of red cell membranes and the osmotic fragility of red cells. Red cells were incubated with normal serum or with mixtures of serum and cholesterol-lecithin dispersions, as in Fig. 2.
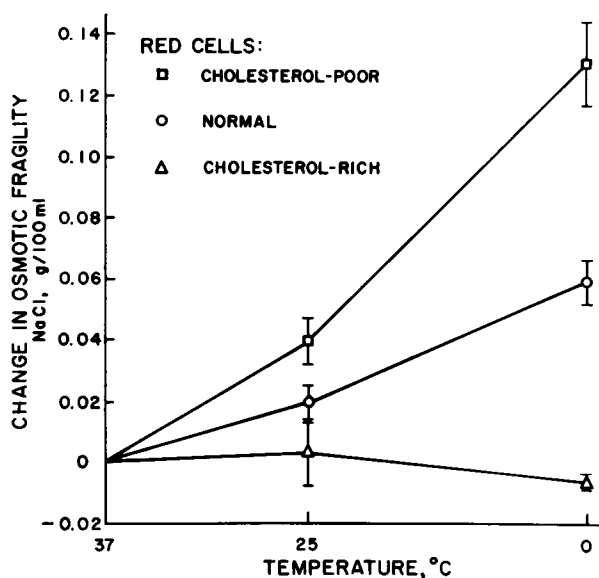
FIGURE 4 Effect of temperature on red cell osmotic fragility. Values for mean osmotic fragility±1 SE are the averages of four experiments. The average C/P of cholesterol-poor red cells was 0.41 and of cholesterol-rich red cells, 1.75.

the accumulation of cholesterol and the change in cell morphology associated with cholesterol accumulation (Fig. 8). In addition, these red cells underwent no change in the osmotic fragility. This "normalization" of spur serum occurred in patient A (serum-free cholesterol = 3.05 mg/ml; serum phospholipid = 4.13 mg/ml) upon the addition of 3.20 mg lecithin/ml of serum; in patient B (serum-free cholesterol = 0.90 mg/ml; serum phospholipid = 1.39 mg/ml) this occurred upon the addition of 1.60 mg lecithin/ml of serum. Thus, it appears that the changes in cholesterol content, morphology, and surface area that normal red cells undergo when incubated in either artificial serum-sonicate mixtures or serum from patients with spur cells are closely interrelated and directly dependent upon the amount of free cholesterol relative to phospholipid in the serum environment.

## DISCUSSION

These studies demonstrate that the C/P of the medium in which red cells exist directly influences the C/P within the red cell membrane. No change in either the phospholipid content or the phospholipid composition of red cells was observed in these studies, and changes

copy, cholesterol-depleted red cells were spheroidal in appearance, indicative of their decreased surface area (Fig. 6). Red cells incubated with serum-sonicate mixtures that caused no change in membrane C/P or osmotic fragility were normal in appearance. In contrast, cholesterol-rich cells were broad and flat; some were targeted in appearance but most had an irregular contour due to folding of the periphery of the cell. This abnormal morphology of cholesterol-rich red cells was not apparent after 90 min of incubation; however it was approximately half maximal at 6 h and maximal by 24 h, a time course similar to that of the changes in membrane cholesterol and osmotic fragility shown in Fig. 1. The targeting of some cells and the undulating margin of others can be best appreciated in the scanning electron micrographs of these red cells in Fig. 7. In dried, Wright's-stained smears, these cholesterol-rich red cells had a regular pattern of spiculation around the periphery, similar to that of red cells incubated in vitro in serum from patients with spur cells reported previously (3).

To gain further insight into the relationship between the change in cell cholesterol and the change in cell contour that normal red cells undergo in serum from patients with spur cells, the effect which normalizing C/P ratio of spur cell serum would have on the morphology of red cells subsequently incubated in that serum was studied in serum freshly obtained from two patients with spur cells. The addition of sonicated lecithin in saline to spur serum prevented both
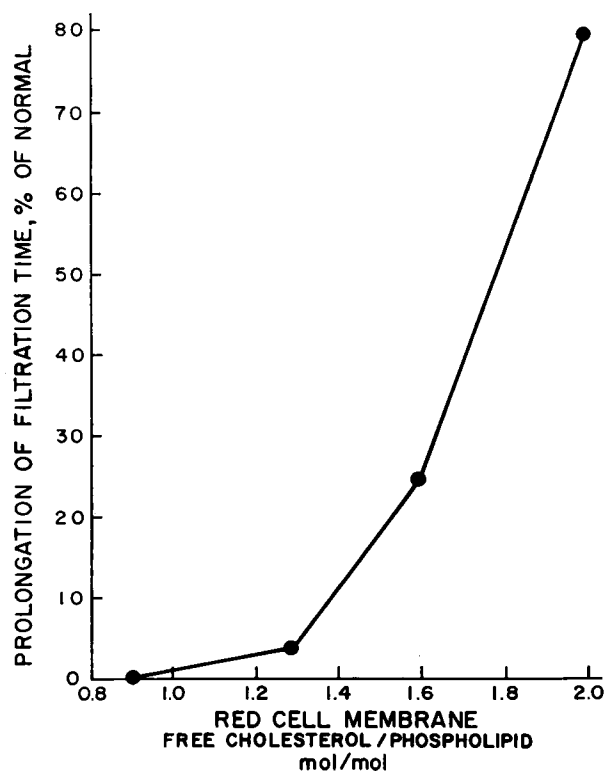


FIGURE 5 Effect of increasing membrane C/P on red cell filtration time. Values shown are the averages of two experiments.

$C/_L =$     0.45                 0.90                1.95

FIGURE 6 Phase microscopy of red cells after incubation with serum-lipid dispersion mixtures. Red cells with a C/P of 0.45 were spherocytic and those with a C/P of 0.90 (normal) were normal in appearance. Cholesterol-rich red cells (C/P = 1.95) were broad and flat and most had irregular margins.

in the C/P of red cell membranes resulted from a selective acquisition or loss of cholesterol. The effects on red cell shape and lipid composition seen here with cholesterol-poor liposomes are similar to those reported previously when red cells were depleted of cholesterol in vitro by incubation in serum in which cholesterol was depleted through the action of the enzyme LCAT (26, 27) or in vivo after the infusion of pure phospholipid dispersions in rodents (28). Studies with cholesterol-rich cholesterol-lecithin dispersions reproduce the spur cell defect, and they provide support for the concept that cholesterol is directly responsible for the abnormality of shape and deformability that characterizes spur cells.

*Morphology of cholesterol-rich red cells.* That it is added cholesterol per se that accounts for the morphologic abnormality of cholesterol-rich red cells appears to be substantiated by several sets of data. First, after incubation with either serum from patients with spur cells (3) or with cholesterol-rich dispersions, the time course of the appearance of abnormal cell shapes corresponds to the rate of acquisition of membrane cho-

lesterol. Second, the fact that cholesterol-rich red cells with folded margins can be produced in a totally artificial system excludes other factors present in the serum of patients with spur cells, such as bile acids (29), as important in the genesis of the morphologic abnormality. Third, when phospholipid was added to serum from patients with spur cells so as to render it no longer capable of transferring cholesterol to normal red cells, the red cells incubated in this serum underwent no change in either osmotic fragility or shape.

The reason for the irregular contour of cholesterol-rich red cells remains unclear. It has been suggested that biologic membranes are not uniform but rather that they may have "rigid" as well as "fluid" domains within the bilayer (30). It has also been suggested that with very high cholesterol concentrations within membranes, lecithin-cholesterol complex formation may occur (31). Either of these phenomena would create a nonuniformity within the cholesterol-rich membrane that may account for the irregularities of contour.

The morphology and surface area of red cells made cholesterol-rich in vitro differ in two respects from spur
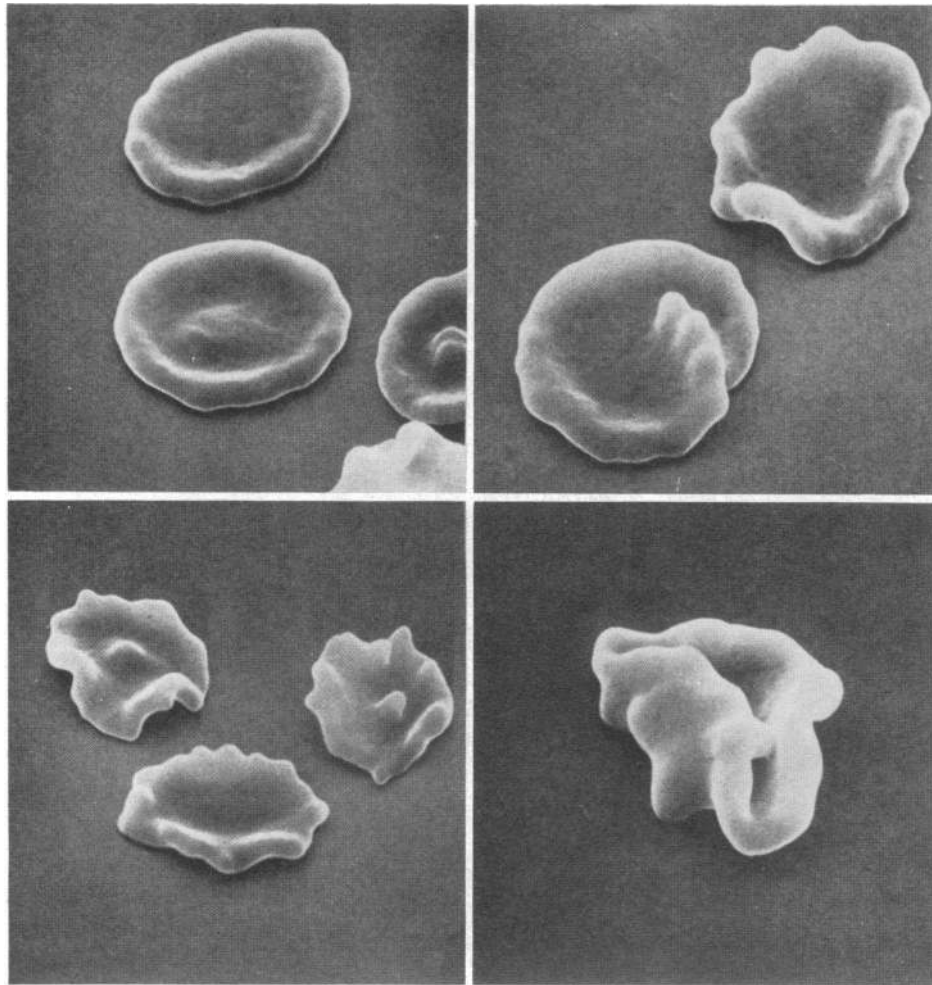
*Cholesterol-Rich Red Cell Membrane*     121

FIGURE 7 Scanning electron micrographs of cholesterol-rich red cells. All cells are from the same preparation. The range of morphologic variation is shown.

cells as obtained from patients. First, the contour of spur cells is not folded with an undulating periphery; instead, spur cells are bizarrely spiculated, resembling hereditary acanthocytes; and, second, spur cells have a normal surface area (3, 32), whereas red cells made cholesterol-rich in vitro have an increased surface area. Both of these differences result from physical changes that occur in the membranes of cholesterol-rich red cells as they circulate in vivo. Thus, $^{51}$Cr-labeled normal red cells acquire surface area when infused into patients with spur cells, but this added surface area is subsequently lost over the course of 5–10 days in vivo (3). In the absence of the spleen, these secondary physical changes no longer occur, and spur cells are not bizarrely spiculated with a normal surface area, but rather their surface area is increased and they are morphologically identical to the cholesterol-rich red cells obtained in vitro (32).

*Relation to other abnormalities of lipoprotein composition.* Abnormal serum lipoproteins have been described in a number of human conditions, and some of these are also associated with abnormalities of red cell lipids. However, the abnormality of lipoprotein composition characterized by a disproportionate serum C/P of the magnitude produced in cholesterol-rich cholesterol-lecithin dispersions or observed in patients with spur cells appears to be unique to the spur cell patient. Patients with cirrhosis who do not have spur cells also have cholesterol-rich LDL; however the C/P of their lipoproteins ranges from 0.8 to 1.1 (normal = 0.72±0.08); whereas in patients with spur cells it ranges from 1.1 to 1.6 (4). Red cells in these latter patients are targeted in appearance and rich in both cholesterol and lecithin (4, 33); however, the C/P of these target cells is less than 1.1, too low to cause

Red
Cell
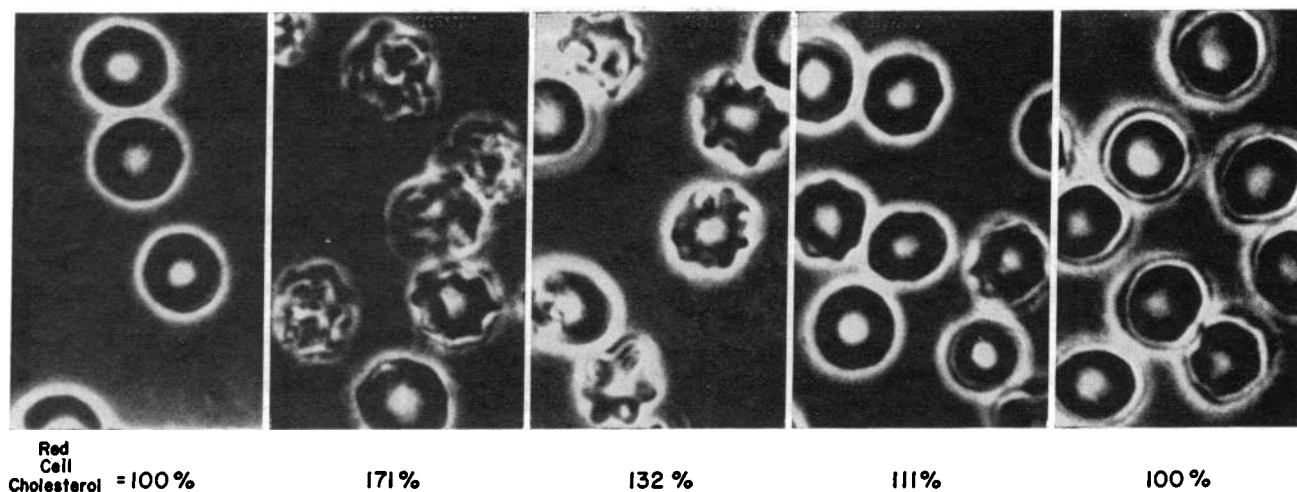Cholesterol = 100%     171%          132%          111%          100%

FIGURE 8  Cholesterol content and morphology of normal red cells incubated in normal serum
(left panel) or serum from a patient with spur cells to which had been added dipalmitoyl
lecithin dispersions increasing (left to right) from 0 to 3.2 mg/ml of serum.

a prolongation of filtration time in vitro or premature cell destruction in vivo.

In obstructive jaundice of long duration and in familial LCAT deficiency, abnormal lipoprotein particles accumulate in plasma, consisting primarily, if not exclusively, of phospholipid (mainly lecithin) and cholesterol in a C/P close to 1.0 (34, 35), and, like patients with cirrhosis, these patients have targeted red cells rich in both cholesterol and lecithin (4, 33, 36). While the current studies explain the accumulation of cholesterol by red cells incubated with liposomes or lipoproteins disproportionately rich in free cholesterol, they do not shed light on the mechanism of accumulation of lecithin in cirrhosis, obstructive jaundice, and familial LCAT deficiency.

Although apparently unique among human conditions, the spur cell abnormality is very similar to the abnormality acquired by guinea pig red cells under conditions of prolonged cholesterol feeding. In these animals, LDL is rich in free cholesterol, and, in addition, a new species of LDL appears containing free cholesterol and phospholipid in a C/P of approximately 2.0 (37). Studies using guinea pig serum in vitro demonstrate the same phenomena seen in human red cells incubated with serum from patients with spur cells or with cholesterol-rich liposomes in vitro (38).

*Effect of cholesterol on Na and K permeability.* The permeability of red cells to Na and K was increased only in those red cells depleted of 67% of their cholesterol. These red cells also had an increase in Na concentration and a reciprocal decrease in K concentration. Na influx was within the normal range in red cells that had lost only 40% of their cholesterol. In

contrast, Poznansky, Kirkwood, and Solomon have reported a 15–45% decrease in K influx in red cells that had lost only 30% of their cholesterol (39). Red cells that had gained cholesterol had normal Na and K concentrations and normal permeabilities to Na and K. This differs from observations made in cholesterol-rich guinea pig red cells, in which a 60% decrease in ouabain-sensitive Na efflux and a 30% decrease in the ouabain-insensitive Na efflux have been demonstrated (40). It should be noted that the previously reported decreased erythritol permeability of cholesterol-rich guinea pig red cells (40) and the increased glycerol permeability of cholesterol-depleted human red cells (41) are probably in error, since, as Gottfried and Robertson have recently shown (42), the methods used to study the permeability of these nonelectrolytes are strongly influenced by the osmotic fragility of the red cell.

*Contribution of cholesterol to red cell surface area.* It appears from the present study, as well as from our previous studies with serum from patients with liver disease and serum with an active LCAT (22), that there is a variation in surface area of approximately 0.22% for every 1% variation in red cell cholesterol content, suggesting that cholesterol accounts for 22% of red cell surface area. This value is consistent with current views of membrane structure that indicate that the red cell membrane is a lipid bilayer with associated and intercalated protein (43). Cholesterol contributes approximately 38 $Å^2$/molecule to the surface area of the cell (44), and, at low surface tension, phospholipid molecules each contribute approximately 85 $Å^2$ (45). If glycolipids and free fatty acids contribute 3–5% to

*Cholesterol-Rich Red Cell Membrane*     123

cell surface area and that, in addition, protein contributes 15–20%, then the cholesterol of a normal human red cell would account for 20–25% of membrane surface area, a value close to that observed in these studies.

*Immobilization of the lipid bilayer in cholesterol-rich red cells.* Although the lipid composition of red cells varies among mammals, cholesterol accounts for a relatively constant percent (46). The interaction between cholesterol and the hydrocarbon chains of phospholipid within the membrane appears to restrict the motion of the first six (47) to eight (48) phospholipid carbon atoms from the bilayer surface, permitting increased motion of the remainder of the hydrocarbon chain in the hydrophobic region of the membrane bilayer. In this way cholesterol maintains the membrane bilayer in an "intermediate fluid condition," permitting greater fluidity of hydrocarbon chains in the gel phase while restricting molecular motion in the liquid crystal phase (49). This concept is supported by a variety of physical measurements, including differential scanning calorimetry (50), laser-Raman spectroscopy (51), proton nuclear magnetic resonance (52), electron paramagnetic resonance (48), and X-ray diffraction (53). The intermediate fluid condition is apparent at a C/P of approximately 1.0, i.e. at a ratio of one cholesterol to two hydrocarbon chains. In the current studies, the C/P approached 2.0, i.e one cholesterol for every one hydrocarbon chain. The effect of this added cholesterol on the molecular motion, or fluidity, of the membrane was seen in three ways: First, as we have reported separately, motion of the fluorescent probe, 12-(9-anthroyl) stearic acid, is decreased in the region of the hydrocarbon chains nearest the surface of both spur cells obtained from patients and cholesterol-rich cells prepared in vitro (54). Similar observations have been made in cholesterol-rich guinea pig red cells (55). Second, the ability of the lipid bilayer to undergo compression as lateral motion of the hydrocarbon chains was decreased in the cold (56) was not observed in cholesterol-rich red cells. This is consistent with the concept that lateral motion was already restricted by excess membrane cholesterol, which fixed the membrane in an expanded form. Third, immobilization of the lipid bilayer in cholesterol-rich red cells was seen grossly as an impaired ability of these cells to change shape and pass through filters of small pore size. Similar results have been obtained with normal red cells made cholesterol-rich by incubation in spur serum (3).

Inhibition of molecular motion by excess membrane cholesterol is not unique to red cells, but appears to be a general property of mammalian cell membranes. In platelets it causes an increased sensitivity to epinephrine

(57), and in murine lymphoma cells it results in a decreased malignant potential (58). In red cells, restricted motion at the molecular level is translated to decreased deformability at the macroscopic level, and this appears to underlie the premature destruction of spur cells in vivo.

## ACKNOWLEDGMENTS

## REFERENCES

1. Smith, J. A., E. T. Lonergan, and K. Sterling. 1964. Spur-cell anemia. Hemolytic anemia with red cells resembling acanthocytes in alcoholic cirrhosis. *N. Engl. J. Med.* 271: 396–398.
2. Silber, R., E. Amorosi, J. Lhowe, and H. J. Kayden. 1966. Spur-shaped erythrocytes in Laennec's cirrhosis. *N. Engl. J. Med.* 275: 639–643.
3. Cooper, R. A. 1969. Anemia with spur cells: a red cell defect acquired in serum and modified in the circulation. *J. Clin. Invest.* 48: 1820–1831.
4. Cooper, R. A., M. Diloy-Puray, P. Lando, and M. S. Greenberg. 1972. An analysis of lipoproteins, bile acids, and red cell membranes associated with target cells and spur cells in patients with liver disease. *J. Clin. Invest.* 51: 3182–3192.
5. Sessa, G., and G. Weissmann. 1968. Phospholipid spherules (liposomes) as a model for biological membranes. *J. Lipid Res.* 9: 310–318.
6. Bruckdorfer, K. R., P. A. Edwards, and C. Green. 1968. Properties of aqueous dispersions of phospholipid and cholesterol. *Eur. J. Biochem.* 4: 506–511.
7. Bruckdorfer, K. R., J. M. Graham, and C. Green. 1968. The incorporation of steroid molecules into lecithin sols, β-lipoproteins and cellular membranes. *Eur. J. Biochem.* 4: 512–518.
8. Zlatkis, A., B. Zak, and A. J. Boyle. 1953. A new method for the direct determination of serum cholesterol. *J. Lab. Clin. Med.* 41: 486–492.
9. Brown, H. H., A. Zlatkis, B. Zak, and A. J. Boyle. 1954. Rapid procedure for determination of free serum cholesterol. *Anal. Chem.* 26: 397–399.
10. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* 234: 466–468.
11. Skipski, V. P., R. F. Peterson, and M. Barclay. 1964. Quantitative analysis of phospholipids by thin-layer chromatography. *Biochem. J.* 90: 374–378.
12. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* 34: 1345–1353.
13. Castle, W. B., and G. A. Daland. 1937. Susceptibility of erythrocytes to hypotonic lysis as a function of discoidal form. *Am. J. Physiol.* 120: 371–383.
14. Cooper, R. A., and J. H. Jandl. 1968. Bile salts and cholesterol in the pathogenesis of target cells in obstructive jaundice. *J. Clin. Invest.* 47: 809–822.
15. Rose, H. G., and M. Oklander. 1965. Improved proce-

dure for the extraction of lipids from human erythrocytes. *J. Lipid Res.* **6**: 428-431.

16. Dodge, J. T., C. Mitchell, and D. J. Hanahan. 1963. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* **100**: 119-130.

17. Whittam, R., and J. S. Wiley. 1968. Some aspects of adenosine triphosphate synthesis from adenine and adenosine in human red blood cells. *J. Physiol. (Lond.).* **199**: 485-494.

18. Wiley, J. S., and R. A. Cooper. 1974. A furosemide-sensitive cotransport of sodium plus potassium in human red cells. *J. Clin. Invest.* **53**: 745-755.

19. Hagerman, J. S., and R. G. Gould. 1951. The *in vitro* interchange of cholesterol between plasma and red cells. *Proc. Soc. Exp. Biol. Med.* **78**: 329-332.

20. Ashworth, L. A. E., and C. Green. 1964. The transfer of lipids between human $\alpha$-lipoprotein and erythrocytes. *Biochim. Biophys. Acta.* **84**: 182-187.

21. Basford, J. M., J. Glover, and C. Green. 1964. Exchange of cholesterol between human $\beta$-lipoproteins and erythrocytes. *Biochim. Biophys. Acta.* **84**: 764-766.

22. Cooper, R. A., and J. H. Jandl. 1969. Red cell cholesterol content: a manifestation of the serum affinity for free cholesterol. *Trans. Assoc. Am. Physicians Phila.* **82**: 324-330.

23. Jacobs, M. H., and A. K. Parpart. 1931. Osmotic properties of the erythrocyte. II. The influence of pH, temperature and oxygen tension on hemolysis by hypotonic solutions. *Biol. Bull. (Woods Hole).* **60**: 95-119.

24. Murphy, J. R. 1967. The influence of pH and temperature on some physical properties of normal erythrocytes and erythrocytes from patients with hereditary spherocytosis. *J. Lab. Clin. Med.* **69**: 758-775.

25. Jandl, J. H., R. L. Simmons, and W. B. Castle. 1961. Red cell filtration and the pathogenesis of certain hemolytic anemias. *Blood J. Hematol.* **18**: 133-148.

26. Murphy, J. R. 1962. Erythrocyte metabolism. III. Relationship of energy metabolism and serum factors to the osmotic fragility following incubation. *J. Lab. Clin. Med.* **60**: 86-109.

27. Cooper, R. A., and J. H. Jandl. 1969. The selective and conjoint loss of red cell lipids. *J. Clin. Invest.* **48**: 906-914.

28. Robins, S. J., and A. Miller. 1974. Red cell cholesterol depletion and the formation of spiculated cells in vivo. *J. Lab. Clin. Med.* **83**: 436-443.

29. Cooper, R. A., F. A. Garcia, and C. Trey. 1972. The effect of lithocholic acid on red cell membranes in vivo. *J. Lab. Clin. Med.* **79**: 7-18.

30. Oldfield, E., and D. Chapman. 1972. Dynamics of lipids in membranes: Heterogeneity and the role of cholesterol. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **23**: 285-297.

31. Darke, A., E. G. Finer, A. G. Flook, and M. C. Phillips. 1971. Complex and cluster formation in mixed lecithin/cholesterol bilayers. Cooperativity of motion in lipid systems. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **18**: 326-330.

32. Cooper, R. A., D. B. Kimball, and J. R. Durocher. 1974. The role of the spleen in membrane conditioning and hemolysis of spur cells in liver disease. *N. Engl. J. Med.* **290**: 1279-1284.

33. Neerhout, R. C. 1968. Abnormalities of erythrocyte stromal lipids in hepatic disease. *J. Lab. Clin. Med.* **71**: 438-447.

34. Hamilton, R. L., R. J. Havel, J. P. Kane, A. E. Blaurock, and T. Sata. 1971. Cholestasis: lamellar structure of the abnormal human serum lipoprotein. *Science (Wash. D. C.).* **172**: 475-478.

35. Glomset, J. A., K. R. Norum, and W. King. 1970. Plasma lipoproteins in familial lecithin:cholesterol acyltransferase deficiency: lipid composition and reactivity in vitro. *J. Clin. Invest.* **49**: 1827-1837.

36. Gjone, E., H. Torsvik, and K. R. Norum. 1968. Familial plasma cholesterol ester deficiency. A study of the erythrocytes. *Scand. J. Clin. Lab. Invest.* **21**: 327-332.

37. Sardet, C., H. Hansma, and R. Ostwald. 1972. Characterization of guinea pig plasma lipoproteins: the appearance of new lipoproteins in response to dietary cholesterol. *J. Lipid Res.* **13**: 624-639.

38. Sardet, C., H. Hansma, and R. Ostwald. 1972. Effects of plasma lipoproteins from control and cholesterol-fed guinea pigs on red cell morphology and cholesterol content: an in vitro study. *J. Lipid Res.* **13**: 705-715.

39. Poznansky, M., D. Kirkwood, and A. K. Solomon. 1973. Modulation of red cell $K^+$ transport by membrane lipids. *Biochim. Biophys. Acta.* **330**: 351-355.

40. Kroes, J., and R. Ostwald. 1971. Erythrocyte membranes —effect of increased cholesterol content on permeability. *Biochim. Biophys. Acta.* **249**: 647-650.

41. Bruckdorfer, K. R., R. A. Demel, J. De Gier, and L. L. M. Van Deenan. 1969. The effect of partial replacements of membrane cholesterol by other steroids on the osmotic fragility and glycerol permeability of erythrocytes. *Biochim. Biophys. Acta.* **183**: 334-345.

42. Gottfried, E. L., and N. A. Robertson. 1974. Glycerol lysis time as a screening test for erythrocyte disorders. *J. Lab. Clin. Med.* **83**: 323-333.

43. Singer, S. J., and G. L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. *Science (Wash. D. C.).* **175**: 720-731.

44. Vandenheuvel, F. A. 1963. Study of biological structure at the molecular level with stereomodel projections. I. The lipids in the myelin sheath of nerve. *J. Am. Oil Chem. Soc.* **40**: 455-471.

45. Bar, R. S., D. W. Deamer, and D. G. Cornwell. 1966. Surface area of human erythrocyte lipids: reinvestigation of experiments on plasma membrane. *Science (Wash. D. C.).* **153**: 1010-1012.

46. Rouser, G., G. J. Nelson, S. Fleischer, and G. Simon. 1968. Lipid composition of animal cell membranes, organelles and organs. *In* Biological Membranes: Physical Fact and Function. D. Chapman, editor. Academic Press. London. 5-70.

47. Verma, S. P., and D. F. H. Wallach. 1973. Effects of cholesterol on the infrared dichroism of phosphotide multibilayers. *Biochim. Biophys. Acta.* **330**: 122-131.

48. Hubbell, W. L., and H. M. McConnell. 1971. Molecular motion in spin-labeled phospholipids and membranes. *J. Am. Chem. Soc.* **93**: 314-326.

49. Chapman, D. 1968. Recent physical studies of phospholipids and natural membranes. *In* Biological Membranes: Physical Fact and Function. D. Chapman, editor. Academic Press, Inc., New York. 125-202.

50. Ladbrooke, B. D., R. H. Williams, and D. Chapman. 1968. Studies on lecithin-cholesterol-water interactions by differential scanning calorimetry and X-ray diffraction. *Biochim. Biophys. Acta.* **150**: 333-340.

51. Lippert, J. L., and W. L. Peticolas. 1971. Laser Raman investigation of the effect of cholesterol on conforma-

tional changes in dipalmitoyl lecithin multilayers. *Proc. Natl. Acad. Sci. U. S. A.* **68**: 1572–1576.

52. Penkett, S. A., A. G. Flook, and D. Chapman. 1968. Physical studies of phospholipids. IX. Nuclear resonance studies of lipid-water systems. *Chem. Phys. Lipids.* **2**: 273–290.

53. Levine, Y. K., and M. H. F. Wilkins. 1971. Structure of oriented lipid bilayers. *Nat. (New Biol.).* **230**: 69–72.

54. Vanderkooi, J., S. Fischkoff, B. Chance, and R. A. Cooper. 1974. Fluorescent probe analysis of the lipid architecture of natural and experimental cholesterol-rich membranes. *Biochemistry.* **13**: 1589–1595.

55. Kroes, J., R. Ostwald, and A. Keith. 1972. Erythrocyte membranes—compression of lipid phases by increased cholesterol content. *Biochim. Biophys. Acta.* **274**: 71–74.

56. Luzzati, V. 1968. X-ray diffraction studies of lipid-water systems. *In* Biological Membranes: Physical Fact and Function. D. Chapman, editor. Academic Press, Inc., New York. 71–123.

57. Shattil, S. J., R. Anaya-Galindo, R. A. Cooper, and R. W. Colman. 1974. Platelet hypersensitivity induced by cholesterol incorporation. *Clin. Res.* **22**: 405a.

58. Shinitsky, M., and M. Inbar. 1974. Difference in microviscosity induced by different cholesterol levels in surface membrane lipid layer of normal lymphocytes and malignant lymphoma cells. *J. Mol. Biol.* **85**: 603–615.