INFLUENCE OF CHOLESTEROL CONTENT ON RED CELL MEMBRANE VISCOELASTICITY AND FLUIDITY

A. CHABANEL, M. FLAMM, K. L. P. SUNG, M. M. LEE, D. SCHACHTER, AND S. CHIEN
Department of Physiology, Columbia University College of Physicians and Surgeons, New York, New York 10032

ABSTRACT The purpose of this investigation was to correlate the viscoelastic properties and lipid fluidity of the red blood cell membrane to its lipid composition. The viscoelastic properties of human red cells that had been enriched or depleted in cholesterol were determined by the micropipette technique. The lipid fluidity of the outer and inner leaflets of the erythrocyte membrane was concurrently assessed by steady state fluorescence depolarization. The elastic modulus and the viscosity moduli of the erythrocyte membrane showed no significant differences between the cholesterol-modified and the control cells. Cholesterol enrichment decreased the lipid fluidity of the outer membrane leaflet alone, and cholesterol depletion increased the fluidity mainly of the inner leaflet.

INTRODUCTION

The ability of red cells to undergo marked deformation is a necessary condition for their flow through the microcirculation, because the diameter of human red cells is 8 μm, while that of the capillaries through which these cells must pass is on the order of 4–5 μm. The deformability of red cells is also an important factor in reducing the bulk viscosity of blood flowing through large vessels.

The deformability of red cells is determined by three major factors: the viscoelastic properties of the membrane, the geometry of the cell, especially the ratio of cell surface area to cell volume, and the viscosity of the intracellular medium. Many methods to measure red-cell deformability have recently emerged (Chien, 1977; Mohandas et al., 1979). The relative importance of the three determining factors may vary according to the method used to measure red-cell deformability.

Previous investigators have studied the deformability of red blood cells after modification of their cholesterol-to-phospholipid ratio (C/PL). Using the technique of filtration through 8.0 μm Millipore filters at 10 mm Hg negative pressure, Cooper et al. (1975) found a decrease in red cell filterability as C/PL increased from 1.28 to 2.00. Determining the velocity of complete entry of single red cells into a micropipette with an internal diameter of 3 μm, Suda et al. (1978) also found a decreased deformability as the C/PL ratio increased from 0.6 to 1.8. Performing capillary viscometry on suspensions of red blood cells from cholesterol-fed guinea pigs with the red cell C/PL ranging from 1.1 to 1.6, Rogaush (1978) found no alteration of the suspension viscosity. Note that the different techniques used may have different sensitivities to the various factors involved in the deformability of red blood cells.

To examine possible changes in the intrinsic viscoelastic properties of the membrane owing to modifications in cholesterol content, we used the micropipette technique in the present investigation (Evans and Hochmuth, 1976). In this method the application of a negative aspiration pressure causes a segment of an intact red cell to enter a micropipette with an internal diameter of 0.8 to 1.5 μm. The use of such small micropipettes allows the determination of the intrinsic membrane material properties without a significant contribution by cell geometry. From the time course of cell entry and the equilibrium length of the aspirated segment, the elastic modulus and the viscosity of the membrane were calculated using a mathematical model described by Chien et al. (1978). On the same red blood cells' preparation these results were compared with estimates of the membrane lipid fluidity of cholesterol enriched and depleted red blood cells. The lipid fluidity of the whole membrane was monitored by the fluoresence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) (Shinitzky and Inbar, 1976), and the lipid fluidity of each hemileaflet of the membrane was assessed by using an impermeant pyrene fluorophore, the stachyose derivative of pyrene butyryl hydrazide (SPBH), as described by Cogan and Schachter (1981).

MATERIALS AND METHODS

Cholesterol, egg lecithin, and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). DPH was obtained from Aldrich Chemicals Co., Inc. (Milwaukee, WI). SPBH was prepared in our laboratory as described by Cogan and Schachter (1981).

Preparation of Cholesterol-Modified Red Blood Cells

Normal human erythrocytes were isolated by centrifugation from the freshly drawn blood of healthy donors. The buffy coat was discarded, and

the cells were washed three times with a buffer composed of 5 mM sodium phosphate, pH. 7.4, 145 mM NaCl, and 5 mM KCl. In four experiments the cholesterol content of human erythrocyte membranes was altered by incubation at 37°C of intact erythrocytes with sonicated dispersions of cholesterol and dipalmitoylphosphatidylcholine, as described by Cooper et al. (1975); molar ratios of cholesterol/dipalmitoylphosphatidylcholine in the dispersions were either 0 (cholesterol depletion), 1 (control), or 2–3 (cholesterol enrichment). In four other experiments the cholesterol content was modified by incubating the cells in heat-inactivated plasma enriched with either cholesterol (enrichment), lecithin (depletion) or no lipid (control) as described by Shinitzky (1978). In one additional experiment an isotonic vehicle containing polyvinylpyrrolidone (PVP) was substituted for the plasma as described by Shinitzky et al. (1979). Changes in membrane lipids were monitored directly by chemical estimation of cholesterol (Zlatkis et al., 1953) and total phospholipid (Fiske and Subbarow, 1925) or indirectly by determination of the fluorescence anisotropy of DPH (Flamm and Schachter, 1982).

Deformability Measurements

Micropipettes with a radius (R_p) of 0.4 to 0.8 μm were used to study the viscoelastic properties of the erythrocyte membranes. The same micropipette was used throughout a given experiment. The methodology is described in detail by Chien et al. (1978), who showed that the deformational entry of the erythrocyte into the micropipette in response to a step aspiration pressure (ΔP) exhibits a two-phase behavior. After an initial rapid phase (phase I) of deformation, there is a continued, slower phase (phase II) with the final maximum steady state deformation (D_{pm}) attained within a 20-s period of observation. The membrane elastic modulus is calculated from the stress-strain relationship between $(\Delta P)R_p$ and D_{pm}/R_p. When the aspiration pressure is removed, the deformed erythrocyte segment in the micropipette decreases in length with time, and there is a single phase of relaxation leading to the complete recovery of cell shape. The membrane viscosity of the various phases is calculated as the product of the time constant of the response and the membrane elastic modulus. Chien et al. (1978) observed that the membrane viscosity of phase I varies inversely with the level of deforming stress, $(\Delta P)R_p$, or the degree of deformation, D_{pm}/R_p. This dependence of phase I viscosity on shear rate or stress was confirmed in the present study. Therefore, the phase I viscosity cannot be compared between different cells unless the stress or strain is specified. To assess the effects of cholesterol enrichment or depletion on phase I viscosity, we divide the experimental value by the control value determined at the same D_{pm}/R_p, and this dimensionless ratio is termed the viscosity index. This index equals one when the experimental value equals the control value obtained at the same D_{pm}/R_p. Normalization of the experimental data on phase I viscosity by dividing it by the control data at the same $(\Delta P)R_p$ yields similar results as normalization at the same D_{pm}/R_p. Phase II viscosity and the viscosity of the relaxation phase do not vary with different degrees of deformation. All measurements were made at room temperature (21°–24°C).

Scanning Electron Microscopy

Red blood cell specimens were fixed in 2.5% glutaraldehyde in phosphate buffer. After two phosphate buffer rinses, the specimens were processed through an ethanol dehydration series from 30 to 100% ethanol. The cells were resuspended in 100% ethanol and gently dropped on to a glass coverslip that had been cleaned with ethanol. After air drying, the coverslip with cells was glued on to aluminium stubs (JEOL USA, Electron Optics Div., Peabody, MA) with silver conductive paint. The stubs with samples were coated with gold-palladium in a sputterer (Hummer I; Technics, Alexandria, VA), and the specimens were viewed and photographed in a scanning electron microscope (JEOL-25; JEOL USA, Electron Optics Div.) at 30° tilt and 25 kV.

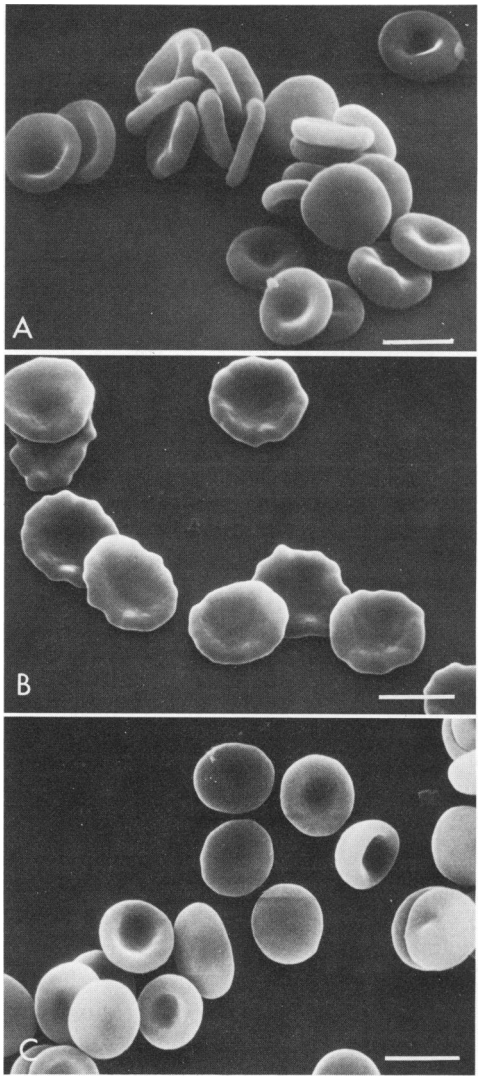

FIGURE 1 Scanning electron migrographs of (*A*) control erythrocytes, (*B*) cholesterol-enriched erythrocytes, and (*C*) cholesterol-depleted erythrocytes. Magnification of 3000 (bar = 5 μm).

Lipid Fluidity Measurements

Lipid fluidity[1] of the whole red cell membrane was assessed by fluorescence depolarization measurements using DPH as a probe (Shinitzky and Inbar, 1976). SPBH was used to assess outer and inner leaflet fluidity as decribed by Cogan and Schachter (1981). The impermeant probe SPBH was inserted selectively into the outer hemileaflet by treatment of intact cells and primarily into the inner hemileaflet by treatment of leaky erythrocyte membranes due to the greater uptake at the endofacial surface. Intact human erythrocytes (20% suspension) or leaky erythrocyte ghost membrane suspensions (1.25–1.50 mg protein ml^{-1}) were incubated with various concentrations of SPBH (intact cells, 75–225 μM; leaky ghosts 5–20 μM) for 30 min at 37°C. The cells and ghost

[1]The term "lipid fluidity" as applied to lipid fluorophores in membrane bilayers is used to denote the relative motional freedom of the lipid fluorophore and incorporates the concepts of rate of rotation and extent of rotation, as previously discussed (Cogan and Schachter, 1981).

TABLE I

ELASTIC MODULUS OF CHOLESTEROL-MODIFIED RED BLOOD CELLS

Experiment No.	Cholesterol/phospholipid molar ratio			Elastic modulus		
	Enriched	Control	Depleted	Enriched	Control	Depleted
				10^{-3} dyn/cm		
1	1.23	0.80	0.54	3.82 ± 0.16	3.54 ± 0.13	3.61 ± 0.14
2	1.35	0.87	0.56	3.00 ± 0.05	2.24 ± 0.05	2.94 ± 0.07
3	1.35	0.80	0.48	4.77 ± 0.09	6.45 ± 0.25	6.26 ± 0.13
4	1.20	0.87	0.55	10.50 ± 0.70	8.50 ± 0.70	7.70 ± 0.35
5	1.56	0.76	—	4.47 ± 0.12	3.85 ± 0.10	—
6	1.69	0.90	—	3.13 ± 0.06	3.05 ± 0.10	—
7	1.25	0.71	—	4.68 ± 0.16	4.44 ± 0.30	—
8	1.33	0.84	—	7.61 ± 0.18	8.25 ± 0.17	—
9	1.20	0.69	0.42	4.04 ± 0.14	4.00 ± 0.11	3.94 ± 0.10

Cholesterol/phospholipid molar ratios were determined from the DPH anisotropy values. Some of the cell preparations studied in the micropipette tests described here were the same as those used by Flamm and Schachter (1982), i.e., the values for cholesterol/phospholipid ratio in experiments 4–8. The data experiments 1–3 and 9 were obtained from additional cell preparations studied. Cholesterol modification was induced by incubation with sonicated dispersions of lipid in experiments 5–8 (Cooper et al., 1978) by treatment with inactivated plasma in experiments 1–3, 9 (Shinitzky, 1978) and by treatment with an albumin-containing vehicle (Shinitzky et al., 1979) in experiment 4, as described in Methods. Values of elastic moduli (mean ± SEM) were the average of ~30 measurements.

membranes were washed to remove unincorporated probes; the cells where then lysed osmotically to obtain the membrane preparations (Cogan and Schachter, 1981). All membrane suspensions were adjusted to a protein concentration of 0.15–0.20 mg · ml^{-1} and steady state fluorescence depolarization was measured in a polarization spectrofluorimeter (SLM Instruments, Inc., American Instrument Co., Urbana, IL), with corrections for light scattering as previously described (Cogan and Schachter, 1981). The results are expressed in terms of the fluorescence anisotropy parameter, $[(r_0/r) - 1]^{-1}$ (where r is the observed fluorescence anisotropy and r_0 the maximal limiting anisotropy of the probe), which varies inversely with the lipid fluidity, i.e., the motional freedom of the fluorophore.

RESULTS

Fig. 1 shows the scanning electron micrographs of the red blood cells after enrichment or depletion of their cholesterol content. Control cells were discocytes after 23 h of incubation with a mean diameter of 7.4 ± 0.1 μm. Cholesterol-enriched cells after 23 h of incubation displayed the characteristic flat pancake shape (Cooper et al., 1975) with an irregular contour due to the folding of the periphery of the cells; the cell diameter (8.4 ± 0.1 μm) was significantly greater than that of the control cells ($P < 0.005$). Cholesterol-depleted cells, after 5 h of incubation, had a smaller diameter (6.7 ± 0.1 μm) than control cells ($P < 0.005$), some of which were stomatocytes (Chailley et al., 1981). We determined the surface area of the erythrocytes by allowing them to swell in hypotonic medium with graded reduction in osmolality. Serial microphotographs were taken under the light microscope and the diameter of each cell was measured in several directions. The diameter of the cell when it became spherical (equal diameter in all

TABLE II

VISCOSITY MODULI OF CHOLESTEROL-MODIFIED RED BLOOD CELLS

Experiment No.	Phase I viscosity index			Phase II viscosity			Viscosity in recovery		
	Enriched	Control	Depleted	Enriched	Control	Depleted	Enriched	Control	Depleted
					10^{-4} dyn-s/cm			*10^{-4} dyn-s/cm*	
1	0.93 ± 0.13	1	1.10 ± 0.14	16.74 ± 1.05	14.69 ± 1.09	14.03 ± 1.14	0.81 ± 0.13	0.73 ± 0.07	0.84 ± 0.11
2	1.16 ± 0.13	1	1.84 ± 0.15	9.90 ± 0.66	6.58 ± 0.42	9.08 ± 0.67	1.72 ± 0.23	1.96 ± 0.23	2.25 ± 0.25
3	0.58 ± 0.09	1	0.76 ± 0.05	17.52 ± 1.03	19.10 ± 1.24	19.37 ± 1.53	1.22 ± 0.14	2.58 ± 0.21	1.27 ± 0.11
4	1.05 ± 0.13	1	0.63 ± 0.05	74.06 ± 8.51	47.36 ± 5.86	31.87 ± 2.90	—	—	—
5	0.96 ± 0.08	1	—	17.68 ± 1.29	13.70 ± 0.98	—	1.35 ± 0.26	0.86 ± 0.14	—
6	0.82 ± 0.15	1	—	14.99 ± 0.95	13.84 ± 1.03	—	0.87 ± 0.16	0.67 ± 0.08	—
7	1.74 ± 0.37	1	—	24.90 ± 1.41	21.99 ± 2.60	—	2.08 ± 0.46	1.57 ± 0.21	—
8	0.74 ± 0.05	1	—	32.27 ± 1.86	38.47 ± 1.36	—	0.47 ± 0.03	0.46 ± 0.02	—
9	0.87 ± 0.07	1	1.02 ± 0.06	18.14 ± 1.43	13.32 ± 0.83	16.16 ± 1.09	0.41 ± 0.03	0.42 ± 0.05	0.57 ± 0.04

Viscosity data correspond to the same experiments as in Table I. Cholesterol/phospholipid molar ratios are the same as in Table I. Values (mean ± SEM) are the average of ~30 measurements.

directions, as confirmed by moving the cell under the microscope) was used to calculate the surface area. The surface area was 116 ± 3 μm^2 for the control cells (C/PL = 0.99), 163 ± 3 μm^2 for cholesterol-enriched cells (C/PL = 1.62), and 94 ± 2 μm^2 for cholesterol-depleted cells (C/PL = 0.75). These measurements demonstrate a significant increase in red blood cell surface area with cholesterol loading ($P < 0.005$) and a significant decrease with cholesterol removal ($P < 0.005$).

The results of micropipette tests in nine different experiments are presented in Tables I and II. The C/PL molar ratios of control, enriched and depleted erythrocytes were, respectively, 0.80 ± 0.07, 1.35 ± 0.17, and 0.51 ± 0.06. The viscoelastic parameters (mean ± SEM) given for each type of preparation in each experiment are the average of ~30 measurements. The standard deviations of the mean values for the nine experiments are similar in magnitude to the corresponding standard deviations (= SEM × $\sqrt{N}$) obtained within one experiment; this indicates that cellular heterogeneity within each individual is nearly as much as the variation of viscoelastic properties among the mean values of individuals. In Fig. 2, the ratio of each viscoelastic parameter for a cholesterol-modified sample to the value of the corresponding control is plotted against the C/PL molar ratio. The plots show that the experimental points are distributed around the horizontal line drawn for the ordinate value of 1.0, which represents no change in viscoelastic parameters of cholesterol-modified as compared with control red blood cells. Thus, neither the elastic modulus nor the viscosity moduli for the two loading phases and the recovery phase are significantly altered by a sizable change of the C/PL ratio.

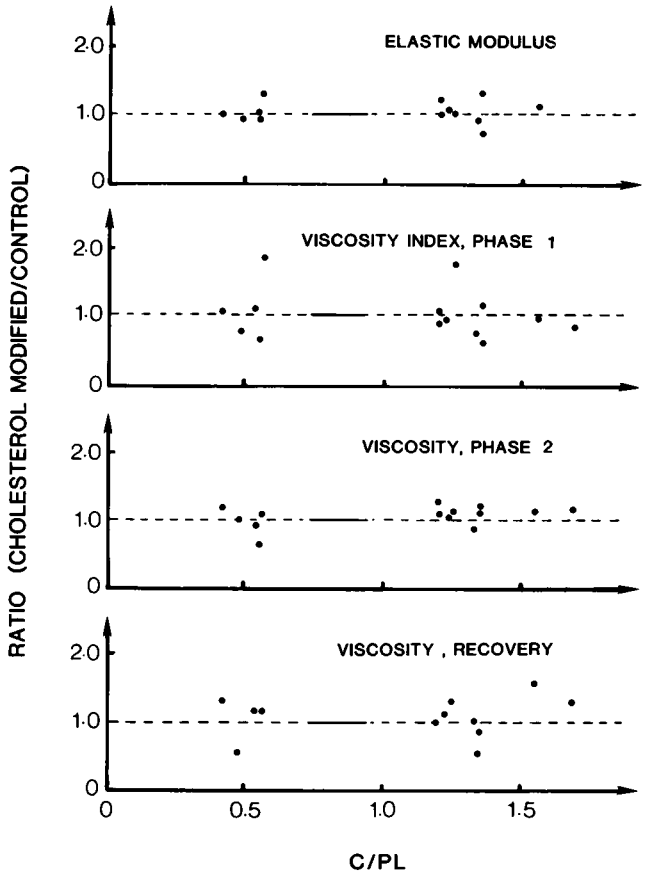

FIGURE 2 Viscoelastic parameters on cholesterol-modified red blood cells. This graph shows the results of nine experiments, each of which consists of ~30 pairs of cells. C/PL were determined from the DPH anisotropy. Each viscoelastic modulus was expressed as the ratio of the value for the cholesterol-modified cells to the value for the control cells. The thick portion of the horizontal line shows the range of C/PL values of control cells. The coefficients of correlation between the viscoelastic parameters and C/PL were not significantly different from 0 in all cases ($P > 0.05$).

Fluorescence polarization studies were performed in five of the preceding experiments and the results illustrated in Figs. 3 and 4. Fig. 3 shows that the DPH anisotropy parameters for control cells (C/PL = 0.82 ± 0.04), enriched cells (C/PL = 1.4 ± 0.09), and depleted cells (C/PL = 0.45 ± 0.03) were, respectively, 2.36 ± 0.45, 3.26 ± 0.15, and 1.70 ± 0.16. This increase of the DPH anisotropy parameter with the C/PL ratio confirms the earlier observations of Cooper et al. (1975). The SPBH anisotropy parameters for the same red blood cell samples (Flamm and Schachter, 1982) are plotted in Fig. 4. The results demonstrate that cholesterol enrichment increased the anisotropy parameter of the impermeant SPBH only in the erythrocyte-loaded (outer leaflet) preparations ($P < 0.005$); no significant effect of enrichment was detected in the ghost loaded preparations (mainly inner leaflet). Fig. 4 also shows that cholesterol depletion decreased the SPBH anisotropy parameter mainly in ghost-loaded preparations ($P < 0.01$) with a less consistent decrease in the erythrocyte-loaded preparations ($P < 0.05$).

DISCUSSION

Although the biophysical properties of cell membranes are generally dependent on their biochemical composition and

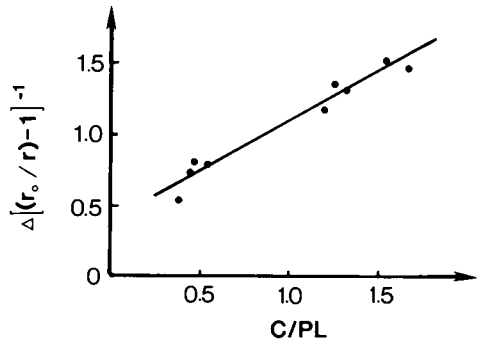

FIGURE 3 DPH fluorescence depolarization studies of cholesterol-modified red blood cell membranes for five out of the nine experiments on viscoelasticity. Variation of the anisotropy parameter, $\Delta[(r_0/r)]^{-1}$, is expressed as the ratio of the value for the cholesterol-modified cells to the value for the control cells. The coefficient of correlation was 0.977.

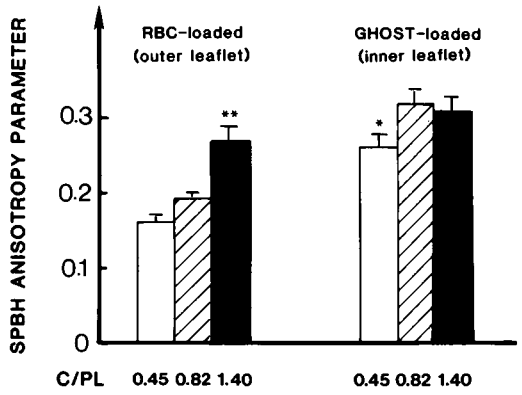

FIGURE 4 SPBH fluorescence depolarization studies of the same samples as in Fig. 3 for cholesterol-depleted (□), cholesterol-enriched (■), and control (▨) red blood cells. ** , $P < 0.005$ when compared with control; *, $P < 0.01$ when compared with control. Adapted from Flamm and Schachter (1982).

molecular organization, the specific factors determining the rheological properties of red blood cells are not yet completely understood. To elucidate the relationship between membrane structure and cell deformability, we studied the biophysical properties of the red cell membrane by varying one of its components, membrane cholesterol. If the lipid fluidity of the membrane is an important determinant of its deformability, variations in lipid fluidity due to alterations in membrane cholesterol should lead to alterations in cell deformability.

The micropipette technique used to assess cell deformability in our studies tests the intrinsic viscoelastic properties of the membrane and is insensitive to the cell geometry and shape. Using this technique, we found that changes in the membrane cholesterol content did not alter significantly the measured viscoelastic properties of the red cell membrane. Because in these same preparations the membrane lipid fluidity did change with altered cholesterol content, it appears that the lipid fluidity is not a significant determinant of the viscoelasticity of the whole membrane. Our results can be interpreted to support the conclusions of prior authors (Evans and Hochmuth, 1976; Chien, 1977; Lux, 1979; Palek and Liu, 1979) that the protein cytoskeletal network is mainly responsible for the rheological behavior of the erythrocyte membrane. In this regard, note that modifications in membrane cholesterol content can influence erythrocyte membrane proteins. Borochov et al. (1979) showed changes in the labeling of endofacial proteins, including spectrin and actin, following cholesterol modification. Similarly Basu et al. (1980) demonstrated enhanced binding of anti-Rh_o(D) antibodies to the exofacial surface of cholesterol-enriched erythrocytes. The present study shows that the cholesterol-induced changes in membrane proteins, notably the alterations in erythrocyte spectrin and actin, do not significantly affect the viscoelastic properties of the intact membrane.

In several previous investigations (Cooper et al., 1975; Suda et al., 1978; Shiga et al., 1979) it was reported that the deformability of red blood cells decreased when the C/PL ratio increased. The method used in these studies were the filtration technique (Cooper et al., 1975) and the total aspiration of single cells into a 3-μm diameter micropipette (Suda et al., 1978; Shiga et al., 1979). In both techniques the penetration of the entire cell through a pore or into a micropipette is involved in the deformability measurement. These measurements are therefore sensitive to cell geometry (cell volume, cell surface area, and their ratio) and cell shape, and the results do not necessarily reflect membrane properties. The increase in membrane surface area resulting from cholesterol loading should lead to an increased deformability of the whole cell. The findings of a decreased whole cell deformability by Cooper et al. (1975) and Shiga et al. (1979) probably result from the altered cell shape. The possibility of an apparent reduction in whole cell deformability due to an increased cell adhesiveness to the filter pores or the micropipette should also be considered, since the incorporation of cholesterol into the membrane has been shown to enhance the binding of certain substances to the outer membrane surface (Shinitzky et al., 1979; Basu et al., 1980).

The selective decrease in fluidity of the outer hemileaflet owing to cholesterol enrichment may signify that the excess cholesterol enters into the outer leaflet preferentially. Such a mechanism would lead to an expansion of this leaflet relative to the inner leaflet and result in the characteristic pancake shape of the cholesterol enriched red blood cells. This result is in accord with the bilayer couple theory of Sheetz and Singer (1974) and Sheetz et al. (1976) that predicts a change in the relative composition of the two hemileaflets will affect the overall shape of the cell membrane. The selective expansion of the outer leaflet relative to the inner is expected to change the curvature of the membrane. The present study indicates that the alterations in red cell surface area, curvature, and lipid fluidity resulting from modifications in membrane cholesterol content are not associated with a change in membrane viscoelasticity as tested by micropipette aspiration.

This investigation was supported by the U.S. Public Health Service research grant HL16851 from the National Heart, Lung, and Blood Institute and the National Institutes of Health grants AM21238 and GM07367. A. Chabanel was the recipient of a fellowship from the Delegation Generale a la Recherche Scientifique et Technique, France.

Received for publication 4 January 1983 and in final form 20 May 1983.

REFERENCES

Basu, M. K., M. Flamm, D. Schachter, J. F. Bertles, and A. Maniatis. 1980. Effects of modulating erythrocyte membrane cholesterol on Rh_o(D) antigen expression. *Biochem. Biophys. Res. Commun.* 95:887–892.

Borochov, H., R. E. Abbott, D. Schachter, and M. Shinitsky. 1979.

Modulation of erythrocyte membrane proteins by membrane cholesterol and lipid fluidity. *Biochemistry*. 18:251–255.

Chailley, B., F. Giraud, and M. Claret. 1981. Alterations in human erythrocyte shape and the state of spectrin and phospholipid phosphorylation induced by cholesterol depletion. *Biochim. Biophys. Acta*. 643:636–641.

Chien, S. 1977. Principles and techniques for assessing erythrocyte deformability. *Blood Cells*. 3:71–99.

Chien, S., K. L. P. Sung, R. Skalak, S. Usami, and A. Tözeren. 1978. Theoretical and experimental studies on viscoelastic properties of erythrocyte membrane. *Biophys. J.* 24:463–487

Cogan, U., and D. Schachter. 1981. Asymmetry of lipid dynamics in human erythrocyte membranes studied with impermeant fluorophores. *Biochemistry*. 20:6396–6403.

Cooper, R. A., E. L. Arner, J. S. Wiley, and S. J. Shattil. 1975. Modification of red cell membrane structure by cholesterol-rich lipid dispersions. *J. Clin. Invest*. 55:115–126.

Cooper, R. A., M. H. Leslie, S. Fishkoff, M. Shinitzky, and S. J. Shattil. 1978. Factors influencing the lipid composition and fluidity of red cell membrane in vitro: production of red cell possessing more than two cholesterol per phospholipid. *Biochemistry*. 17:327–331.

Evans, E. A., and R. M. Hochmuth. 1976. Membrane viscoelasticity. *Biophys. J.* 16:1–11.

Fiske, G. H., and Y. Subbarow. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* 66:375–400.

Flamm, M., and D. Schachter. 1982. Acanthocytosis and cholesterol enrichment decrease the lipid fluidity of the outer but not of the inner leaflet of the human erythrocyte membrane. *Nature (Lond.)*. 298:290–292.

Lux, S. E. 1979. Spectrin-actin membrane skeleton of normal and abnormal red blood cells. *Semin. Hematol*. 16:21–51.

Mohandas, N., W. M. Phillips, and M. Bessis. 1979. Red blood cell deformability and hemolytic anemias. *Semin. Hematol*. No. 2. 16:95–114.

Palek, J., and S. C. Liu. 1979. Dependence of spectrin organization in red blood cell membranes on cell metabolism: implications for control of red cell shape, deformability and surface area. *Semin. Hematol*. 16:75–93.

Rogaush, H. 1978. Red cell deformability and adaptation in cholesterol-fed guinea pigs. *Pfluegers Arch. Eur. J. Physiol*. 373:39–42.

Sheetz, M. P., and S. J. Singer. 1974. Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions. *Proc. Natl. Acad. Sci. USA*. 71:4457–4461.

Sheetz, M. P., R. G. Painter, and S. J. Singer. 1976. Biological membranes as bilayer couples. III. Compensatory shape change induced in membranes. *J. Cell Biol*. 70:193–203.

Shiga, T., N. Maeda, T. Suda, K. Kon, M. Sekiya, and S. Oka. 1979. Rheological and kinetic dysfunctions of the cholesterol-loaded human erythrocytes. *Biorheology*. 16:363–369.

Shinitzky, M., and M. Inbar. 1976. Microviscosity parameters and mobility in biological membranes. *Biochim. Biophys. Acta*. 433:133–149.

Shinitzky, M. 1978. An efficient method for modulation of cholesterol level in cell membranes. *FEBS (Fed. Eur. Biochem. Soc.) Lett*. 85:317–320.

Shinitzky, M., Y. Skornick and N. Haran-Ghera. 1979. Effective tumor immunization induced by cells of elevated membrane-lipid microviscosity. *Proc. Natl. Acad. Sci. USA*. 76:5313–5316.

Suda, T., N. Maeda, M. Sekiya, K. Matsuoka, R. Tokita, and T. Shiga. 1978. Influences of membrane cholesterol on the human red cell properties. *Med. J. Osaka Univ*. 39:21–27.

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.