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CHOLESTEROL OXIDASE SUSCEPTIBILITY OF THE RED CELL MEMBRANE

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We have used the highly variable and conditional susceptibility of cholesterol to cholesterol oxidase to probe molecular rearrangements in the human red cell membrane. Cholesterol in the intact erythrocyte normally is not a substrate for this enzyme. Susceptibility was induced however, by these pretreatments: mild enrichment in membrane cholesterol, exposure to ≥ 0.03% (3 mM) glutaraldehyde and warming in dilute salt solutions (μ approx. 0.001). Cholesterol reactivity in dilute salt solutions emerged only following a lag of 30 min or more. The lag time was shortened by raising the temperature, by reducing the salt concentration or by treating with glutaraldehyde. The induced sensitivity to the enzyme was inhibited by restoring physiologic ionic strength or by introducing 0.1 mol lysophosphatidylcholine per mol cholesterol into the membrane. (In striking contrast, lysophosphatidylethanolamine and lysophosphatidylserine did not inhibit oxidation.) The various effectors of cholesterol oxidase sensitivity strongly influenced the impact of the others, suggesting that each shifted cholesterol toward or away from an enzyme-sensitive disposition. None of these effects was observed in pure cholesterol or red cell membrane lipids dissolved in detergent, which were uniformly highly reactive with the enzyme. We conclude that the observed variation in cholesterol oxidase sensitivity reflects changes in the organization of the bilayer, perhaps a lateral redistribution of lipids which creates cholesterol-rich phases or domains in which cholesterol is more or less accessible to the enzyme. If so, the time-dependent increase in cholesterol susceptibility during warming at low ionic strength might be a novel indicator of the kinetics of phase changes in the bilayer of the red cell.

Introduction

Cholesterol oxidase attacks cholesterol at its 3β-hydroxyl group to form Δ^4-cholestenone and H_2O_2 [1,2]. The rate of the reaction is highly dependent on the environment of the substrate, presumably because highly water-insoluble sterols are typically sequestered within hydrophobic ensembles. Thus, the susceptibility of solubilized cholesterol to the enzyme varies markedly with the type of detergent in which it is dissolved [3]. The oxidation of cholesterol in synthetic lipid vesicles is strongly affected by their phospholipid composition and sterol content [4,5]. Cholesterol at the cytoplasmic surface of the red cell membrane is a far better substrate for the oxidase than cholesterol at the outer surface [6]. We have recently provided preliminary evidence that, while erythrocytes are normally resistant to cholesterol oxidase, the entire cholesterol pool in intact red cells becomes susceptible when the cholesterol level is increased from the normal value of 0.8 to approx. 0.9 mol/mol phospholipid [7] or following exposure of cells to

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Abbreviation: C/P, cholesterol/phospholipid molar ratio.

buffers of very low ionic strength [8].

Sterols are universal components in the plasma membranes of eukaryotic cells; however, their precise disposition and functions in the bilayer are uncertain [9,10]. We have therefore used cholesterol oxidase susceptibility to explore organizational features of the human red blood cell bilayer not otherwise appreciated.

Experimental procedures

Materials. Cholesterol oxidase (EC 1.1.3.6; *Brevibacterium* sp.) was used as obtained from Beckman Instruments, Inc., Carlsbad, CA. The stated activity of the enzyme was 17 IU/mg. Analysis of the preparation by gel electrophoresis [11] showed a single band. The absence of phospholipase C activity was demonstrated as described [12]. Cholest-4-en-3-one (cholestenone) was purchased from Steraloids (Wilton, NH), lyso-[^{14}C]palmitoylphosphatidylcholine was purchased from New England Nuclear (Boston, MA), and the unlabeled lysophosphatides were from Sigma Chemical Co. (St. Louis, MO). Phospholipase A_2 (bee venom) was from Sigma. Spectroscopic grade heptane was obtained from Fisher Scientific. All other chemicals used were of reagent grade.

Membrane preparations. Human blood was obtained fresh from normal donors or from outdated units generously provided by the University of Chicago Blood Bank. Outdated cells were more prone to hemolysis during prolonged incubation and tended to be more rapidly oxidized by the enzyme but otherwise exhibited the same behaviour as fresh cells. Erythrocytes, sealed and unsealed ghosts, and inside-out and right-side out vesicles were prepared as described [13]. Enrichment and depletion of erythrocyte cholesterol were carried out as described [7]. Ghost sealing was assessed by floatation on density barriers of Dextran 70 [14]. In some experiments, isolated membranes were digested at very low ionic strength at 37°C for prolonged periods. Since such incubations induce ghost breakdown to inside-out vesicles, we routinely preincubated unsealed ghosts to complete the vesiculation process prior to the addition of the enzyme (see legends). Such preparations contain predominantly inside-out and unsealed right-side-out vesicles, providing nearly complete accessibility of the cytoplasmic cholesterol [13].

Treatment of cells with phospholipase A_2. The procedure described by Lubin et al. [15] was followed. Briefly, cholesterol-enriched red cells were incubated in 20 vol. of buffer containing 150 mM NaCl, 5 mM KCl, 5.5 mM Na_2HPO_4, 0.8 mM NaH_2PO_4, 0.25 mM $CaCl_2$, 0.25 mM $MgSO_4$, 10 mM dextrose (pH 7.4); 15 IU phospholipase A2 was added and the mixture incubated for 1 h at 37°C. Phospholipid degradation was assessed by thin-layer chromatography as described by Roelofsen and Zwaal [16].

Assays. The cholesterol and phospholipid content of ghosts was determined as previously described [7] and expressed as the molar ratio of cholesterol to phospholipid. Cholestenone was assayed spectrophotometrically [8] on 25–50 μl aliquots of packed red cells or membranes, which normally contain approx. 25–50 μg cholesterol.

Results

The studies described below document a dramatic dependence on experimental conditions of the susceptibility to oxidation by cholesterol oxidase of red cell membrane cholesterol. The effects of ionic milieu, temperature, cholesterol content, covalent modification, and intercalated amphipathic compounds are considered. In order to probe separately the two surfaces of the membrane, we compared intact cells, sealed and unsealed ghosts, and membrane vesicles of right-side-out and inside-out orientation.

Dependence of cholesterol oxidation on membrane preparation

Gottlieb [6] first reported that whereas the cholesterol at the outer surface of erythrocytes is not susceptible to oxidation by cholesterol oxidase in physiological saline, cholesterol in unsealed ghosts and inside-out vesicles is readily oxidized. We have examined this point systematically, since it could signify either of two different processes: (a) the cholesterol of the cytoplasmic surface might be more reactive than at the outer surface, and (b) the membrane might instead be altered by hemolysis and/or isolation.

Fig. 1 summarizes such experiments. While intact erythrocytes were not a substrate for the cholesterol oxidase, sealed ghosts prepared directly from freshly-lysed, unwashed membranes (sealed red ghosts) were distinctly, albeit slowly, oxidized. Ghosts washed free of hemoglobin prior to sealing (sealed white ghosts) were oxidized at three times the rate of the sealed red ghosts. Finally, red ghosts (and white ghosts; not shown) in which the enzyme was present at both surfaces were digested much more rapidly than when only the outer surface was attacked. In other experiments, we found that inside-out and right-side-out vesicles were equally oxidized by cholesterol oxidase at a rate equivalent to that seen with the parent unsealed white ghosts.

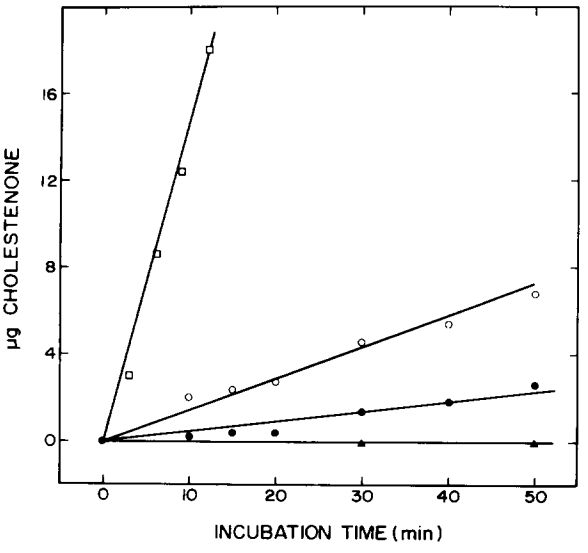

Fig. 1. Reactivity of the two membrane surfaces to cholesterol oxidase in different preparations. ▲———▲, Outer surface of red cells. Red blood cells were washed in 150 mM NaCl/5 mM NaP_i (pH 7.5) and digested in 19 vol. of this buffer containing 1 IU/ml cholesterol oxidase at 37°C. Data are expressed as µg cholestenone per ml suspension (which contained 50 µl packed cells, hence approx. 50 µg cholesterol). ●———●, Outer surface of red ghosts. Cells were lysed in 40 vol. 5 mM NaP_i (pH 8). The ghosts were pelleted and incubated in 19 vol. 150 mM NaCl/5 mM naP_i (pH 7.5) for 30 min at 37°C to induce sealing. Enzyme then was added to 1 IU/ml and the procedure described for red cells repeated. ○——— ○, Outer surface of white ghosts. Hemoglobin-free ghosts were prepared by three washes in 5 mM NaP_i (pH 8) and then sealed and assayed as above. □———□, Both surfaces of red ghosts. Red ghosts were prepared and assayed as above, except that the pellet following hemolysis was incubated at 0°C for 5 min in 19 vol. 150 mM NaCl/5 mM NaP_i (pH 7.5) containing 1 IU/ml cholesterol oxidase prior to sealing to allow enzyme penetration to the cytoplasmic space.

Thus, during the course of preparation of right-side-out vesicles, the susceptibility of the outer membrane surface to oxidation progressed from a negligible value in intact cells to equivalence with the highly-reactive cytoplasmic surface. We conclude that both of the aforementioned hypotheses are correct; i.e., (a) the susceptibility of the outer surface changes during membrane isolation, and (b) the cytoplasmic surface is initially a much better substrate for the enzyme than the outer surface.

That the susceptibility to oxidation of the sealed red and white ghosts preparations was not a reflection of their partial permeability to the enzyme was assured by measuring the mean radius of their hemolytic holes [14]: 6–8 Å for the red ghosts and 10–13 Å for the white ghosts. Cholesterol oxidase has $M_r = 33\,000$ [1], hence a calculated ideal Stokes' radius of 22 Å. In addition, probes such as Dextran 10 do not permeate such ghosts [14]. Furthermore, if some of the ghosts had been unsealed, the time courses in Fig. 1 would have been biphasic. Instead, the oxidation invariably went to completion in a first order fashion.

Bloj and Zilversmit [17] have recently argued that the limited cholesterol oxidase susceptibility of the intestinal brush border reflects extensive cholesterol binding to plasma membrane proteins. This hypothesis is a poor explanation for our results, given that all of the red cell cholesterol exchanges rapidly across the membrane and reacts with the enzyme as a single pool at both surfaces [8]. Rather, we infer that the environment of the cholesterol is different at the two membrane surfaces.

These data lend support to the hypothesis that subtle but substantial alterations in the organization of the bilayer accompany the reduction of the intact cell membranes to ghosts and vesicles [13]. The progress of the outer surface toward a reactivity similar to the cytoplasmic surface is in accord with the suggestion that the holes in unsealed ghosts foster redistribution of lipid between the two leaflets of the bilayer [18].

Induction of cholesterol oxidase susceptibility in intact erythrocytes by incubation at low ionic strength

We have published preliminary evidence that the normal insensitivity of red cells to cholesterol oxidase can be overcome by a small increase in their cholesterol content [7] or by prior incubation at very low ionic strength [8]. These effects are illustrated in Fig. 2. Incubation in a very low ionic strength medium had no immediate effect on the rate of red cell cholesterol oxidation. However, exposure of the cells to 0.5 mM sodium phosphate (pH 8) for approx. 15 min at 37°C either in the presence or the absence of the enzyme caused the cells to become increasingly good substrates, as signified by an accelerating time course of oxidation (Panel A, O——O and △——△). A 1-h preincubation of cells without enzyme in this buffer abolished the lag entirely and promoted a simple first-order pattern of oxidation (●——●). Further preincubation at 37°C up to about 3 h led to increasing acceleration once enzyme was added. These results indicate that the low ionic strength effect was on the cells alone rather than on the enzyme or on an enzyme-cell interaction.

Enrichment of the cholesterol/phospholipid mole ratio (C/P) in the erythrocyte from the normal value of 0.77 to C/P = 1.33 also abolished the lag (Fig. 2B). A 60-min preincubation in 0.5 mM sodium phosphate further accelerated the first-order oxidation of cholesterol in the enriched cells by a factor of 2.8. The duration of the lag at low ionic strength was highly temperature-dependent. For example, in Fig. 3A the lag was much shorter at 37°C than at 30°C. It can be seen in Fig. 3B that activation disappeared entirely below 20°C.

In contrast to intact cells, ghosts and vesicles derived from red cell membranes showed no time and temperature-dependent stimulation by preincubation at very low ionic strength (not shown). Indeed, a contrary effect was observed: preincubation at very low ionic strength caused inhibition of the subsequent oxidation of ghosts or inside-out vesicles in the presence of isotonic saline (Fig. 4). The inhibition took the form of a lag which increased with increasing time of preincubation at very low ionic strength.

Cation and cholesterol dependence of cholesterol oxidation

After activation by preincubation at very low ionic strength, the oxidation of red blood cell

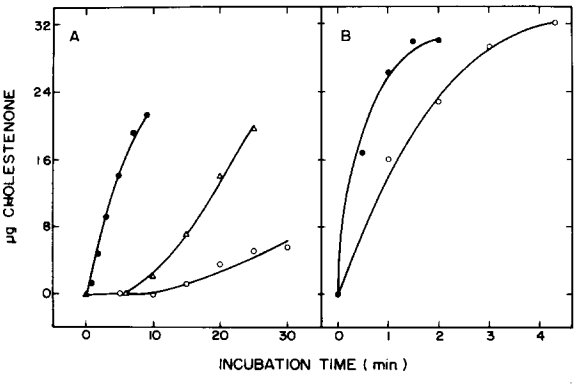

Fig. 2. Effect of preincubation at 37°C at low ionic strength on oxidation of normal and enriched red cells. Control (panel A) and cholesterol-enriched (panel B) red cells were washed twice in 0.5 mM NaP_i (pH 7.5)/310 mM sucrose and resuspended in 19 vol. of the same buffer. After preincubation at 37°C for 0 min (O——O), 10 min (△——△), and 60 min (●——●), cholesterol oxidase was added to 1 IU/ml and the time course of oxidation determined at 37°C on 0.5 ml aliquots of the suspension containing 25 μl red cells. The membrane cholesterol to phospholipid mol ratio was 0.77 and 1.33 in control and enriched preparations, respectively.

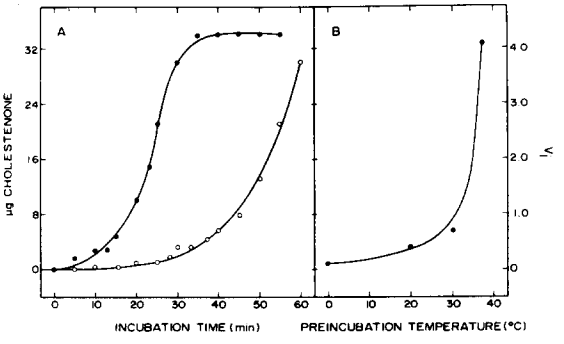

Fig. 3. Preincubation at low ionic strength stimulates oxidation of red cells. (Panel A). Red cells were washed twice in 40 vol. 0.5 mM NaP_i (pH 7.5)/310 mM sucrose and digested in 19 vol. of this buffer containing cholesterol oxidase (1 IU/ml) at 30°C (O——O) and 37°C (●——●). The oxidation of 0.5 ml aliquots of the suspension was determined as described in Methods. (Panel B). Red cells were washed in low ionic strength buffer as in (A). The cells were preincubated for 1 h in 1.5 vol. 0.5 mM NaP_i (pH 7.5)/310 mM sucrose at the indicated temperatures and then suspended in the same buffer containing cholesterol oxidase at 37°C to give final concentrations of 0.05 ml red cells and 1 IU enzyme per ml. The rate of oxidation at 37°C (in μg cholestenone/min per unit enzyme) was determined at 37°C on 0.5 ml aliquots of the suspension.

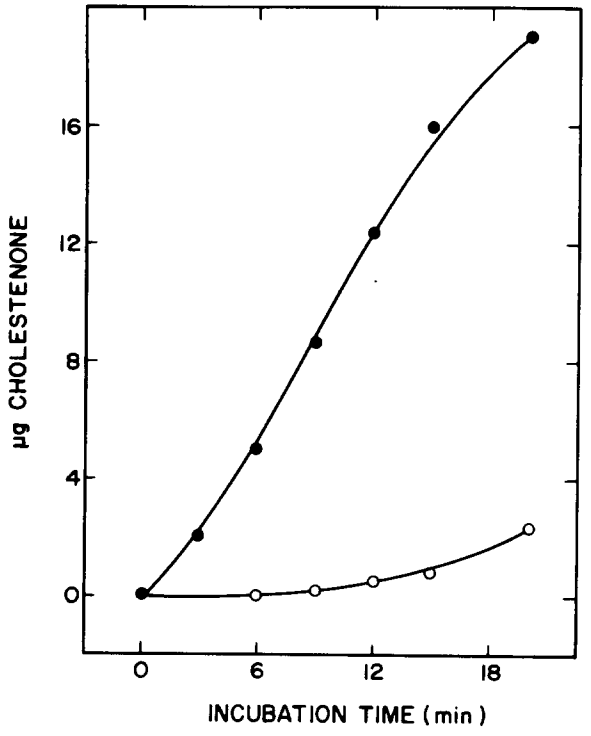

Fig. 4. Preincubation of ghosts at low ionic strength inhibits oxidation at high ionic strength. Unsealed white ghosts were either not preincubated (●———●) or were washed in 40 vol. 0.5 mM NaP_i (pH 8) and preincubated for 1 h at 0°C in 30 vol. of this buffer (○———○) and then pelleted. Suspensions were prepared containing 150 mM NaCl/5 mM NaP_i (pH 7.5), 0.05 ml ghosts/ml and 1 IU/ml cholesterol oxidase. The mixtures were brought rapidly to 37°C and the time-course of oxidation determined on 1.0 ml aliquots of the suspensions.

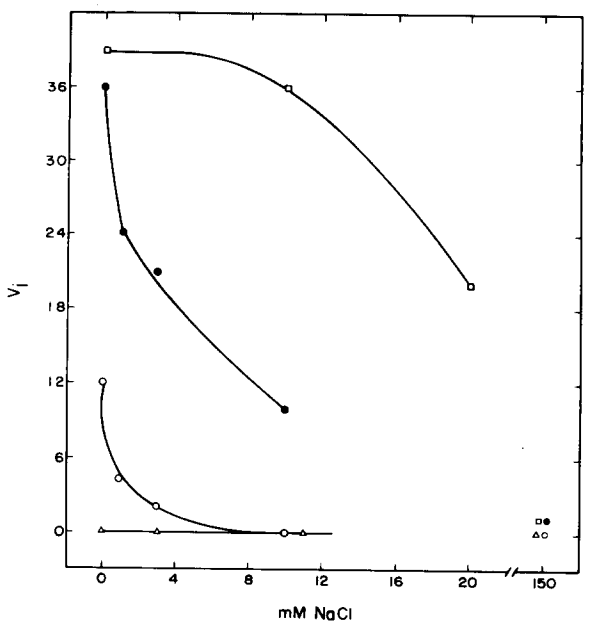

Fig. 5. Effect of ionic strength on the oxidation of red cells and membranes of varied cholesterol content. Red cells, enriched in or depleted of cholesterol as described [7], were washed twice in 0.5 mM NaP_i (pH 7.5) containing the indicated concentrations of NaCl and the amount of sucrose necessary to maintain isotonicity at 310 mosM. The cells were then incubated for 1 h at 37°C in 19 vol. of the same buffers. Cholesterol oxidase (1 IU/ml) was added and the initial rate of oxidation (µg cholestenone/min per unit enzyme) measured on 0.5 ml aliquots of the suspension. The cholesterol contents of the red cells were 0.65 (△———△), 0.81 (○———○) and 1.13 (●———●) mol cholesterol/mol phospholipid. In another experiment (□———□), inside-out vesicles of normal cholesterol content were prepared by warming ghosts for 30 min at 37°C in 0.5 mM NaP_i (pH 7.5) [13], preincubated for 10 min at 37°C at the indicated ionic strength and then digested with 0.5 IU/ml cholesterol oxidase, half of that used in the other curves.

cholesterol was highly dependent on the salt concentration in the medium (Fig. 5). Oxidation was immediately and profoundly inhibited by the addition of as little as 10 mM NaCl to the cells. Isolated membrane vesicles, predominantly inside-out in orientation, were far less sensitive to the inhibitory effect of the electrolyte; their rate of oxidation was reduced only to one-half at 20 mM NaCl. Furthermore, oxidation of isolated vesicles but not normal cells was detectable at physiologic ionic strength. In marked contrast, oxidation of pure cholesterol and ghosts dissolved in taurodeoxycholate was stimulated up to 2-fold when the ionic strength was increased from $\mu = 0.0015$ to $\mu = 0.165$.

Fig. 5 also shows the interplay between ions and membrane cholesterol content as determinants of oxidation rate. As noted previously [7] a small elevation of membrane cholesterol, here from C/P = 0.81 to C/P = 1.3, conferred susceptibility on intact cells in isotonic buffers. Furthermore, the oxidation of cholesterol-enriched cells was more stimulated than the oxidation of normal cells at low ionic strength and showed no lag period such as seen in Figs. 3 and 4. This enhancement is particularly evident at 10 mM NaCl where control cells are unreactive while enriched cells are highly reactive to cholesterol oxidase. Finally, a slight reduction in membrane cholesterol (from C/P = 0.81 to C/P = 0.65) abolished susceptibility to the enzyme at all salt concentrations (Fig. 5).

TABLE I

THE EFFECT OF ION SPECIES ON CHOLESTEROL OXIDATION

Hemoglobin-free unsealed ghosts were washed in 5 mM imidazole acetate (pH 7.5) and incubated in 6 vol. of this buffer for 15 min at 37°C to induce vesiculation. The vesicles were pelleted and resuspended in 19 vol. 5 mM imidazole acetate (pH 7.5; $\mu = 0.004$) containing various electrolytes at the ionic strength indicated. Cholesterol oxidase was added to 0.05 IU/ml and the initial rate of oxidation measured at 37°C on 1.0 ml aliquots of the suspension, as described in Methods.

Increment in ionic strength	Added salt	v_i (μg cholestenone/min per unit enzyme)
0.004	None	33.0
0.009	5 mM sodium acetate	19.4
	1.67 mM sodium sulphate	24.6
	1.67 mM calcium acetate	1.5
0.019	15 mM sodium acetate	7.2
	5 mM sodium sulphate	9.8
	5 mM calcium acetate	0.8

Similar variations in the relative rates of cholesterol oxidation were observed when the cytoplasmic surface of ghosts of varied cholesterol content was oxidized at different ionic strengths; however, as noted above, the cytoplasmic surface was always more reactive than the outer surface under comparable conditions.

It was of interest to establish whether the effect of electrolytes on cholesterol oxidation was specifically attributable to the binding of cations or anions or simply to charge screening; i.e., to a simple ionic strength effect. We therefore reacted membrane vesicles with cholesterol oxidase in the presence of 1:1, 2:1, and 1:2 electrolytes. The results are presented in Table I. The divalent cation (Ca^{2+}) was profoundly more potent an inhibitor of oxidation than the monovalent cation (Na^+). The effect of Mg^{2+} was equivalent to that of Ca^{2+} (not shown). The anion species seemed indifferent. We conclude that electrolytes inhibit oxidation by contributing cations which bind anionic membrane sites.

Kinetic analysis of the salt dependence of cholesterol oxidation

The dependence of cholesterol oxidation on membrane concentration obeyed simple Michaelis-Menten kinetics. The K_m for the oxidation of cholesterol in vesicles decreased and the V_{max} increased as the electrolyte concentration was reduced (Table II). (Since the cholesterol concentration in experiments such as that shown in Fig. 5 was typically 25–50 μg/ml, both the K_m and V_{max} contributed to the increased rates of

TABLE II

KINETIC ANALYSIS OF THE OXIDATION OF MEMBRANE VESICLES AND CHOLESTEROL-ENRICHED ERYTHROCYTES

Ghosts were washed in 10 vol. 0.5 mM NaP_i (pH 7.5), resuspended in 3.8 vol. of this buffer and induced to vesiculate by warming for 0.5 h at 37°C. Aliquots of the mixture were suspended at various concentrations in the same buffer containing or lacking 150 mM NaCl and 5 mM NaP_i, pH 7.5 (final). Cholesterol oxidase was added at 0.5 or 0.05 IU/ml to the high and low salt mixtures, respectively, and the initial rate of oxidation at 37°C or 20°C determined on 1 ml aliquots as described in Methods. Final membrane concentrations varied from 0.01–0.2 ml ghost/ml (approx. 10–200 μg cholesterol/ml). Red cells were enriched as described [7] to 1.21 mol cholesterol/mol phospholipid. The cells were mixed at 37°C with 150 mM NaCl/5 mM NaP_i (pH 7.5) to give 0.05 to 0.20 ml cells/ml. Enzyme was added (1 IU/ml) and the initial rate of oxidation determined on 0.05 ml aliquots as described in Methods. In all cases, double reciprocal plots of initial rates vs. cholesterol concentration were linear.

Preparation	Buffer	Temp. (°C)	K_m (μg cholesterol/ml)	V_{max} (μg cholestenone/min per unit enzyme)
Vesicles	150 mM NaCl/5 mM NaP_i	37	125.0	2.8
	0.5 mM NaP_i	37	10.3	101.2
	0.5 mM NaP_i	20	12.2	38.0
Enriched red cells	150 mM NaCl/5 mM NaP_i	37	345.0	30.2

oxidation with decreasing ionic strength; however, the V_{max} effect predominated.) These data suggest that both the association of the enzyme with the substrate and the catalytic event are enhanced at reduced salt concentration.

The K_m of the enzyme for pure cholesterol in isotonic saline containing taurodeoxycholate was immeasurably low in our experiments; i.e., less than 5 μg cholesterol/ml. (This low value is similar to those found for cholesterol oxidase from *Nocardia erythropolis* and *Nocardia* sp., namely 1.1 and 5.4 μg/ml, respectively [1,2]. It appears that the membrane renders cholesterol a poor substrate in all cases but that the degree of inhibition varies widely with conditions.

Effect of intercalators on cholesterol oxidation

The effects of an alkane (decane) and an alcohol (*n*-octanol) on cholesterol oxidation in vesicles are illustrated in Table III. Both agents caused a modest stimulation of oxidation rate at very low ionic strength. Upon addition of 50 mM NaCl, the control membranes became poor substrates while those treated with the intercalators were more than five times more reactive (Table III).

TABLE III

EFFECTS OF DECANE AND OCTANOL ON THE OXIDATION OF MEMBRANE CHOLESTEROL

Hemoglobin-free unsealed ghosts were vesiculated in 30 vol. of 0.5 mM NaP_i (pH 8) for 15 min at 37°C and pelleted by centrifugation. The membranes were suspended in 19 vol. 0.5 mM NaP_i (pH 7.5) with or without 50 mM NaCl and saturating amounts of decane and octanol. After a 15 min incubation at 37°C, enzyme was added (0.05 and 0.5 IU/ml in mixtures without and with NaCl, respectively) and the initial rate of oxidation (v_i) measured on 1.0 ml aliquots of the suspension as described in Methods.

Buffer	Addition	v_i (μg cholestenone/ min per unit enzyme)
0.5 mM NaP_i	none	50
	decane	92
	octanol	74
0.5 mM NaP_i/ 50 mM NaCl	none	3.0
	decane	17.6
	octanol	17.2

Effect of lysophosphatidylcholine on cholesterol oxidation

The addition of small amounts of lysophosphatidylcholine inhibited the oxidation of membrane cholesterol dramatically (Fig. 6). Cholesterol-enriched red cells and ghosts were only slightly less affected than normal red cells (not shown). This effect was reversed by extracting the lysophosphatidylcholine from the membranes with

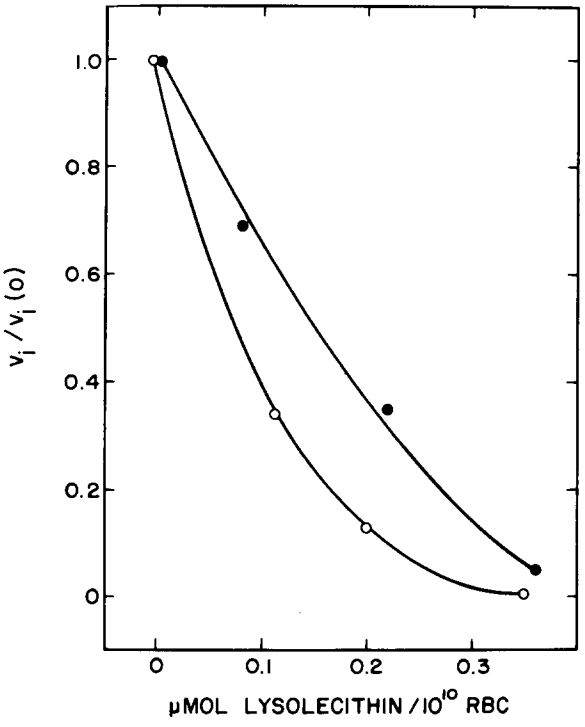

Fig. 6. Effect of lysophosphatidylcholine on the oxidation of normal and cholesterol enriched erythrocytes. Red cells were enriched in cholesterol as described [7], brought to low ionic strength and preincubated for 1 h at 37°C as described in the legend to Fig. 3. Egg lysophosphatidylcholine (containing a trace of synthetic lyso[^{14}C]palmitoylphosphatidylcholine) was added to 0–0.4 μmol per 10^{10} cells and the mixtures incubated for 15 min at 37°C. Lyso[^{14}C]phosphatidylcholine uptake was determined from the radioactivity of aliquots of red cells following washing and expressed per cell number determined by Coulter counting. (One ml packed erythrocytes contains approximately $0.9 \cdot 10^{10}$ cells and 2.6 μmol cholesterol.) Cholesterol oxidase (1 IU/ml) was added to the remainder of the mixtures and the initial rate of oxidation (v_i) determined. Rates are expressed relative to that measured in the absence of added lysophosphatidylcholine, $v_i(0)$. Membrane cholesterol to phospholipid mole ratios were 0.80 and 1.10 for control (○——○) and enriched (●——●) cells, respectively.

bovine serum albumin. The potency of the lysophosphatidylcholine was striking; 50% inhibition of cholesterol oxidase activity occurred when the added lysophosphatidylcholine was 2.5 mol% of the normal red cell cholesterol and 4.2 mol% of the enriched red cell cholesterol. In contrast, the digestion of pure cholesterol and ghosts dissolved in taurodeoxycholate was diminished by only 10–20% in the presence of one mol lysophosphatidylcholine per mol cholesterol.

Egg lysophosphatidylcholine (containing predominantly palmitic and stearic acid esters) and synthetic lysopalmitoylphosphatidylcholine behaved identically. In contrast, lysophosphatidylserine (from bovine brain) and lysophosphatidylethanolamine (from egg yolk) caused only a slight inhibition of red cell and ghost cholesterol oxidation (Table IV). Phospholipase A_2 digestion of intact erythrocytes, which were first enriched in cholesterol in vitro, also inhibited subsequent oxidation of cholesterol. Washing the cells with 1% bovine serum albumin after the phospholipase treatment and prior to exposure to cholesterol oxidase reversed the inhibition, presumably by extracting the lysophosphatides.

TABLE IV

EFFECT OF LYSOPHOSPHATIDES ON THE OXIDATION OF RED CELL AND VESICLE CHOLESTEROL

Red cells were washed twice in 30 vol. 0.5 mM NaP_i (pH 7.5), 310 mM sucrose containing 0.05% bovine serum albumin, resuspended in 10 vol. of this buffer and preincubated for 1 h at 37°C. Lysophosphatides (1 volume) were added to the concentrations indicated and the mixtures incubated for 10 min at 37°C. Cholesterol oxidase (1 IU/ml) was added and the initial rate of oxidation (v_i) measured as described in Methods. Ghosts were washed once in 10 vol. 0.5 mM NaP_i (pH 7.5), resuspended in 4 vol. of this buffer containing 0.05% albumin, and vesiculated by warming for 30 min at 37°C. The initial rate of oxidation of vesicles (0.05 ml membranes/ml) in buffer containing different concentrations of lysophosphatides and 0.05 IU/ml cholesterol oxidase was measured as described in Methods. The uptake of the lysophosphatides was not measured in this experiment (but see Fig. 6). However, from studies of the sensitivity of red cells to crenation and lysis by these compounds, we conclude that the three lysophosphatides were taken up in similar amounts under these conditions. Note that the albumin present binds a significant fraction of the lysophosphatides and is used here to buffer their concentration.

Preparation	Added lysophosphatide		v_i
		mM	(μg cholestenone/ min per unit enzyme)
Red cells	none		3.01
	lysoPC	0.01	1.30
	lysoPC	0.02	0.86
	lysoPE	0.01	2.92
	lysoPE	0.02	2.11
	lysoPS	0.01	2.88
	lysoPS	0.02	2.28
Vesicles	none		41.2
	lysoPC	0.02	6.0
	lysoPC	0.05	1.8
	lysoPE	0.02	46.4
	lysoPE	0.05	25.0
	lysoPS	0.02	49.0
	lysoPS	0.05	43.0

Effect of glutaraldehyde on cholesterol oxidation

While 1% (100 mM) glutaraldehyde is a universally employed fixative for protein systems, it does not significantly interfere with the diffusion of cholesterol either across or out of red cell membranes (Lange, Y., Molinaro, A.M. and Steck, T.L., unpublished observations). Its use to stabilize cells against hemolysis during prolonged incubations led to the incidental observation that the pretreatment of red cells with as little as 0.03% (3 mM) glutaraldehyde stimulated cholesterol oxidation in suitably prepared cells. Half-maximal stimulation occurred following exposure of cells to 10 vol. of approx. 0.3% glutaraldehyde. The highest stimulation we observed was 40-times that of the untreated control.

The stimulatory effect of glutaraldehyde was observed within 5 min of its addition. The effect was not reversed by washing the cells, suggesting covalent binding. (In fact, cholesterol oxidation was increased by washing the glutaraldehyde-treated cells because the aldehyde inactivates cholesterol oxidase.) That the action of glutaraldehyde was specific for the red cell membrane was shown by the absence of stimulation of the oxidation of cholesterol in egg phosphatidylcholine liposomes. Neither 100 mM formaldehyde nor benzaldehyde affected the rate of oxidation of red cell cholesterol.

Relationship among effectors of cholesterol oxidation

The influence of the various pretreatments of red cells was measured as a function of their cholesterol content. The results of one such experi-

ment are shown in Fig. 7. As described previously, enrichment of cells in cholesterol, warming at low ionic strength and treatment with glutaraldehyde all stimulated cholesterol oxidation in red cells, while lysophosphatidylcholine inhibited. Fig. 7 reveals, however, that the action of each effector was through shifting the cholesterol-dependence curve

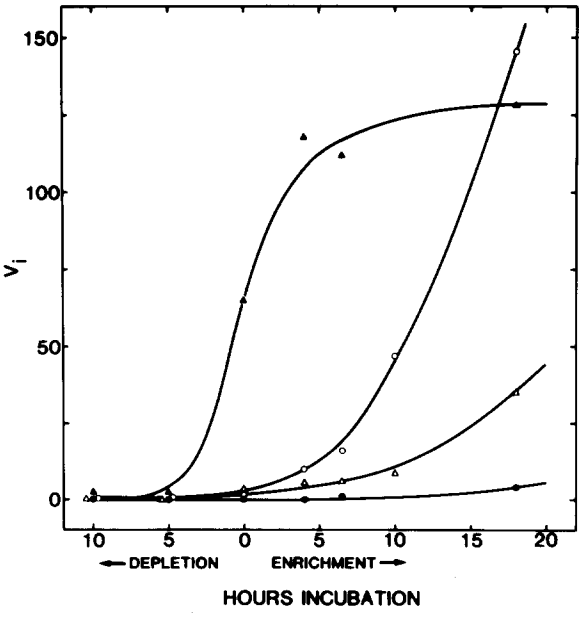

Fig. 7. Relationship of various determinants of cholesterol oxidase sensitivity in red cells. Freshly-drawn red cells were washed in saline and aliquots were incubated for the intervals indicated on the abscissa with phosphatidylcholine vesicles (for cholesterol depletion) or phosphatidylcholine/cholesterol vesicles (for cholesterol enrichment) as described [7]. While not determined here, the cholesterol/phospholipid ratio in many similar experiments ranged from 0.5 to 1.2 mol/mol, the untreated cells containing 0.8 mol cholesterol/mol phospholipid. The aliquots were washed in saline and portions treated as follows: (A) ●———●, approx. 0.2 ml cells of each cholesterol content were suspended to 5.9 ml in 0.15 M NaCl/5 mM NaP_i (pH 7.5) for 10 min at 37°C following which the reaction was started by the addition of 6 IU of cholesterol oxidase. The initial rate of oxidation was determined and is expressed on the ordinate as μg cholestenone/min^{-1} per unit enzyme. (B) ○———○, The cells were treated with 1% glutaraldehyde for 10 min at 0°C, washed twice in saline, and then treated as in part A. (C) ▲———▲, The cells were washed twice with 310 mM sucrose/0.5 mM NaP_i (pH 7.5), resuspended in the same medium to 5.9 ml, preincubated for 1 h at 37°C and then reacted with cholesterol oxidase as in part A. (D) △———△, This experiment was identical to part C, except that the suspensions were made 0.03 mM in lysophosphatidylcholine just before the addition of the enzyme.

to the left (stimulation) or right (inhibition).

The major implication of Fig. 7 is that the various effectors of cholesterol oxidase sensitivity strongly influenced one another. We have obtained other support for this premise. The curve of NaCl inhibition of cholesterol oxidation in isolated membranes (as in Fig. 5) was shifted to the left by the presence of lysophosphatidylcholine. Furthermore, the stimulatory action of glutaraldehyde was potentiated by other pretreatments which rendered membrane cholesterol susceptible to the enzyme. That is, oxidation of red cells in isotonic saline was only slightly stimulated by glutaraldehyde alone, while intact cells activated by enrichment with cholesterol or incubation in low ionic strength buffer were oxidized much more rapidly when also treated with glutaraldehyde (Table V). The stimulatory effect of glutaraldehyde on the oxidation of cells pre-incubated at low ionic strength was to shorten the lag period seen in Fig. 2. Reversal of the low ionic strength activation of cholesterol oxidation by the addition of lysophosphatidylcholine (as in Fig. 6) or high ionic strength

TABLE V

THE EFFECT OF GLUTARALDEHYDE ON THE OXIDATION OF RED CELL CHOLESTEROL

Red cells washed in 150 mM NaCl/5 mM NaP_i (pH 7.5) or 0.5 mM NaP_i (pH 7.5)/310 mM sucrose were suspended in 14 vol. of these buffers. Glutaraldehyde was added to 1% final concentration where indicated and the mixtures incubated for 30 min on ice. The cells were washed and resuspended in the same buffer and incubated for 1 h at 37°C. NaCl (150 mM) and egg lysophosphatidylcholine (0.1 mM) were added as indicated. Cholesterol oxidase was added and the initial rate of oxidation measured on 0.5 ml aliquots of the suspension at 37°C.

Buffer	Addition	v_i (μg cholestenone/min per unit enzyme)
150 mM NaCl/5 mM NaP_i	none	0
	glutaraldehyde	0.6
0.5 mM NaP_i/310 mM sucrose	none	23.9
	glutaraldehyde	147.0
	glutaraldehyde plus NaCl	1.7
	glutaraldehyde plus lysophosphatidylcholine	2.5

buffer (as in Fig. 5) also abolished the stimulatory effect of glutaraldehyde (Table V). Furthermore, the inhibition curve of red cell cholesterol oxidation by NaCl (Fig. 5) was shifted dramatically to the right by pretreating the cells with glutaraldehyde.

Finally, the reason that cytoplasmic surface cholesterol is a substrate for cholesterol oxidase under conditions at which the outer surface is not appears to be simply that the cholesterol-dependence curves for the isolated membrane lie to the left of those for the corresponding intact cells.

Discussion

While the general attributes of lipid bilayers in artificial and biological membranes have been defined with ever-increasing detail over the past 15 years, subtleties of bilayer organization continue to be elucidated. Among these are transverse asymmetry, lateral inhomogeneities, phase behaviour and specificity in lipid-lipid and lipid protein interactions. Here, we have studied the restraints imposed by the human red blood cell membrane on the reaction of cholesterol with cholesterol oxidase.

As in the case in detergent micelles [3] and synthetic liposomes [4,5], the reactivity of cholesterol in the red cell to cholesterol oxidase is strongly influenced by (and hence reports on) the environment of the sterol. Three kinds of specific membrane manipulations which affect neither cholesterol nor cholesterol oxidase directly have a profound impact on cholesterol oxidation in situ; these are exposure to glutaraldehyde, lysophosphatidylcholine, and low salt media. Furthermore, outer-surface cholesterol progressively becomes a better substrate as red cells are converted to sealed ghosts and sealed right-side-out vesicles. A fifth effector is the cholesterol content of the membrane itself, which, above a critical threshold, promotes oxidation dramatically.

The mechanisms of the modulation of cholesterol oxidase susceptibility by these agents are unknown; however, existing data point to specific alterations in the organization of the bilayer. Consider these findings:

(1) The oxidation of the entire pool of red cell membrane cholesterol can proceed, under favorable conditions, with half-times of 3 s or less [8]. These data rule out the hypothesis that cholesterol must leave the membrane and enter the aqueous phase before enzyme attack can occur, since the escape of cholesterol from liposomes [19,20] and intact red cells [21] has a half-time of hours.

(2) None of the effects of salt concentration, lysophosphatidylcholine or cholesterol level on membranes was observed with detergent solutions of red cell membranes or pure cholesterol. Furthermore, the K_m for membrane cholesterol is high and the V_{max} low compared to cholesterol in taurodeoxycholate solution, and these values vary with the state of the membrane. One can imagine that the enzyme is hindered in both the binding and catalytic steps by the environment of the sterol in the bilayer. Cholesterol oxidase does not bind significantly to membranes in which the sterol is not a substrate, in that normal erythrocytes neither compete with ghosts nor exhibit enzyme binding (data not shown).

(3) Anionic groups in the membrane are implicated by the data shown in Table I. Monovalent and, more particularly, divalent cations may screen mutual repulsion among acidic phospholipids, thereby creating an environment in which membrane sterols are more accessible to the enzyme. A similar hypothesis could explain the action of glutaraldehyde, since it presumably lowers the pK of the α-amino groups on aminophospholipids. In this regard, it previously was suggested that cholesterol is a better substrate for the oxidase in liposomes of aminophospholipids than cholinephospholipids because the larger headgroups of the latter reduced access of the enzyme to the substrate [4]. According to this hypothesis, glutaraldehyde modification of aminophospholipids would curtail cholesterol oxidation. That the opposite is the case supports an electrostatic rather than steric explanation of these data.

Since the cytoplasmic surface of the red cell membrane is far richer in anionic phospholipids than the outer surface [22], electrostatic repulsions among the charged polar head groups might increase the availability of the sterol to the enzyme. This would explain why inner-surface cholesterol is more reactive to the oxidase than outer surface cholesterol under identical conditions.

(4)The rate of oxidation of cholesterol increased

sharply at a critical point which was set by the interplay among several variables (Fig. 7). We surmise that there are at least two dispositions of cholesterol in the human erythrocyte membrane: one or more susceptible to cholesterol oxidase and one or more not. The transition between these states presumably reflects a change in the environment of the sterol caused by molecular reorganization of the bilayer, just as the reactivity of red cells and ghosts or inside-out vesicles presumably reflects the differences of the environment of the sterol in the two leaflets of the bilayer. Our data are consistent with the hypothesis that lateral reorganization in bilayers can create sterol-rich domains which are particularly susceptible to cholesterol oxidase, as suggested for artificial liposomes [5]. Alternatively, it could be that rearrangement of the lipids can cause the polar end of the cholesterol molecule to move closer to the aqueous interface by a few angstroms. The striking inhibition by lysophosphatidylcholine at very low levels (< 0.1 mol/mol cholesterol) might be attributable to the suppression of such reorganization.

In this regard, it should be noted that the phospholipase A_2 of cobra venom exhibits "interfacial activation, a preference for substrate in aggregates rather than monomers" [23]. The present system is more complex in that cholesterol is a substrate both at high dilution in detergent and at high concentration in the membrane. The key link seems to be the inhibitory action of neighboring phospholipids.

(5) The most intriguing phenomenon we have observed is the time-dependence of the increase in the enzyme susceptibility of outer membrane surface cholesterol during the warming of red cells at very low ionic strength (Figs. 2 and 3) and the time-dependence of the decline in the susceptibility of cytoplasmic-surface cholesterol in isotonic buffer during preincubation of isolated membranes at very low ionic strength (Fig. 4). Low-salt buffers, in addition to an instantaneous, readily reversible effect on enzymic oxidation rate, have a slow effect on the susceptibility of the membrane to the oxidase. The latter behaviour suggests specific rearrangements at both surfaces of the bilayer, since lateral phase separation in membranes takes many minutes (see, for example, Ref. 24). The inhibition observed after restoration of physiologic salt concentrations may not represent the reversal of such organizational changes, since return to a low salt medium (for a second time) causes activation without a lag even after an hour at high salt. Rather, the lag might represent the slow nucleation of enzyme-susceptible domains; such nuclei could persist after return to high-salt buffers. Our data suggest that cholesterol and cholestenone would equilibrate between laterally contiguous domains far more rapidly than the time required to form them since the cholesterol behaves as a single population with respect to the oxidase in all cases, despite its possible nonuniform distribution. The several effectors of cholesterol oxidation rate could presumably interact with one another (as in Fig. 7) by contributing to or reducing such lipid domains. Physical evidence for variation of lipid phase behaviour with ionic strength has been described recently [25].

Lateral phase separations in membranes containing cholesterol have evoked considerable experimental [26–28] and theoretical attention [29], yet remain poorly understood. The findings presented herein provide a fresh starting point for physical studies on cholesterol dynamics in membranes, the kinetics of phase change in particular.

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