CRITICAL LIPID-PROTEIN STOICHIOMETRIES IN ERYTHROCYTE MEMBRANE REACTIONS GOVERNING PROTECTION AND MORPHOLOGY SWITCHING

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The human erythrocyte may be reversibly switched from its normal discocyte morphology to the echinocyte (Fig. 1). The ligands used to drive the discocyte → echinocyte conversion are lipid anions, which bind in the outer sectors of the cell's membrane. The reverse reaction, echinocyte → discocyte, is driven by exogenous proteins that bind and remove the lipid anions from the cell. Therefore, as Ponder (1) noted, the discocyte ⇌ echinocyte conversion may be used as an indicator for titration of lipid ligands with proteins. Hence the apparent stoichiometry for the following reaction may be determined:

$$\text{ligand (echinocytic concentration)} + \text{protein} \rightleftharpoons \psi\ \text{ligand}\cdot\text{protein} + \text{free ligand (subechinocytic concentration)} \quad (1)$$

The system is illustrated here by determination of the stoichiometry of fatty acid anion binding to serum albumin. The course of the titration depends on (*a*) the amount of ligand needed in the membrane, to drive the discocyte → echinocyte conversion; (*b*) the relationship of the echinocytic concentration of ligand to subechinocytic concentration; (*c*) the relative binding capacity of the erythrocytes for the ligand, with respect to that of the protein (ideally, the ratio is small); (*d*) the dependence of the echinocytogenic reactions on ligand structure, hydrocarbon chain length, unsaturation, and substitution; (*e*) remaining below the ligand critical micelle concentration; (*f*) the sharpness of the titration reactions; (*g*) true reversibility and freedom from rate limitations, not only in the discocyte ⇌ echinocyte equilibria but also in any reactions linked to them such as cell surface modulating reactions (2).

METHODS

The work-up, handling, solvent conditions and adjustment of washed, isotonic erythrocytes to advantageous concentrations was described in two recent papers (2, 3). Only enough cells are needed to fill several microscope fields with ~ 80–150 cells, so that only a few drops of blood are needed. The proteins were crystalline serum albumin and β-lactoglobulin. The fatty acids were prepared by Nu-Chek-Prep Co. (Nu-Chek-Prep Inc., Elysian, MN) and the synthetic phospholipids were products of the Sigma Chemical Co. (St. Louis, MO), examined by chromatography. The titration was carried out as a point-by-point procedure. Each point included a total volume of 0.5 to 1.0 ml, such that the cell concentration was 5×10^7 cells/ml. The required amounts of protein and ligand were added to the cells, in an order corresponding to the desired direction of morphology conversion. After stirring and incubation at 25°C for 300–500 s, the cells were fixed (2), and counted to determine *r*, the fraction of echinocytes.

RESULTS AND DISCUSSION

Fig. 2 plots the data for forward titration (Discocyte → Echinocyte) using laurate anion; y = molecular ligand added/cell. Quantities y_0 and y_p on the abscissa designate the onset of echinocyte formation in the absence and the presence of P molecules added protein/cell. If the protein binds ligand, it becomes titrated or filled with ψ ligands before echinocytes form. Quotient $\psi = (y_p - y_0)/P$ is the apparent stoichiometry, therefore, for reaction 1.

Increasing the total amount or concentration of protein relative to the number of cells used should simplify or optimize use of the method, in analogy to methods of ordinary acid-base titration using colorimetric indicators. Thus, to obtain a sharp end point the buffer capacity of the indicator is kept small relative to the buffer capacity of either excess titrant. Applied here in distribution of a ligand with respect to a protein, and to a cell membrane which is used as a visible indicator, the same considerations apply. With sufficient concentrations of titrants and small numbers of cells, the titration curves are steep and the total ligand binding capacity of the cells is small, yielding a sharp endpoint. Close to $\nu = 2 \pm 1 \times 10^7$ bound organic anionic ligands/cell are generally required to switch the morphology (discocyte → echinocyte (2, 3). Ranging from strongly to comparatively weakly bound fatty acid anions, the total amount of ligand needed to obtain $r = 1.0$ (100% echinocytes) is of the order of 2×10^{-9} to 10×10^{-8} mol of ligand (total) for 5×10^7 cells. The amount of serum albumin needed to titrate this range of lipid (echinocyte → discocyte) is in the range 1–5 nm.

The stoichiometries, ψ, of a number of lipid anions toward serum albumin obtained in this way are listed in

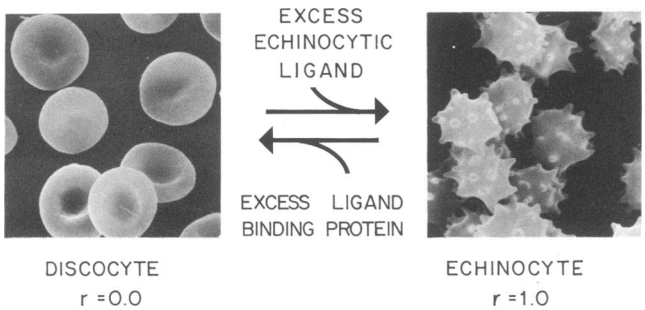

FIGURE 1 Reversible erythrocyte membrane morphology conversion used as the visibly seen (×400 microscopy) indicator reaction for determination of the end point in reaction 1.

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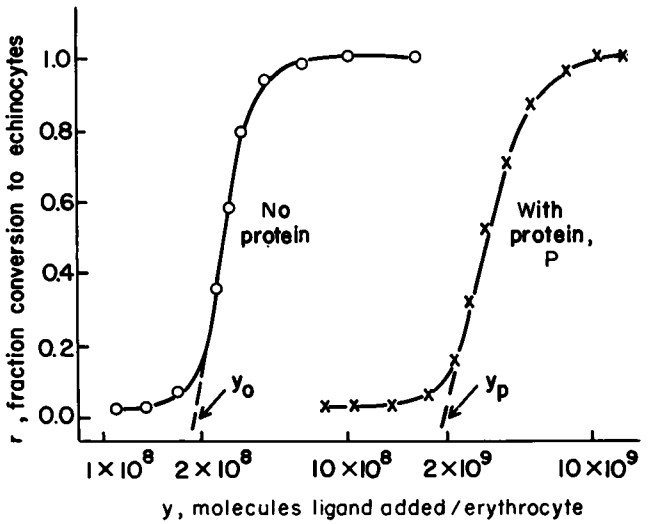

FIGURE 2 Forward titration of the erythrocyte membrane by laurate anion. Displacement of r vs. y plots depends on the amount of protein, P, and on the protein's stoichiometry, ψ, for binding echinocytic ligand.

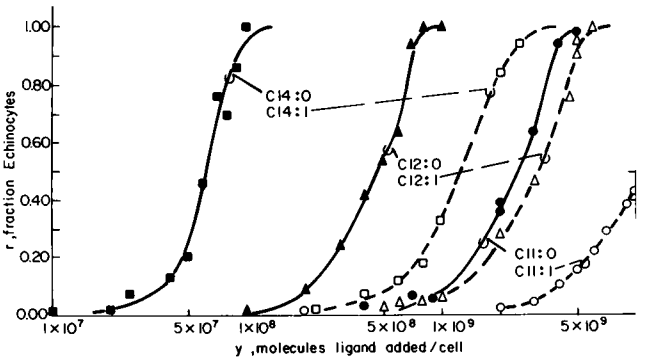

FIGURE 3 Alkane and alkene carboxylate anions: sensitivity to chain length and structure, in titration of erythrocytes. Exogenous protein not involved.

Table I. They may be compared with the apparent number of sites, often designated n, found by more conventional methods. The fatty acid anions, undecanoate (C11:0) to pentadecanoate (C15:0) and the unsaturates C11:1 to C14:1, produce $\psi \sim 5 \pm 2$ to $\psi \sim 12 \pm 2$ according to our values. Steinhardt and Reynold's tabulation (4), for a number of alkane carboxylates and alkane sulfates binding to serum albumin mainly fall in the range of 6 to 11 total sites. The total sites are sums of one or two strong sites ($n_1 = 1$ or 2; $K_1 \sim 10^6 - 10^7$), and sites of intermediate affinity (n_2 5 – 8; $K_2 \sim 10^4 - 10^5$). Spector et al. (5) analyzed the serum albumin-alkane anion interaction in terms of a series of sites, all with differing affinities. They obtained optimum fit with eight to nine principal sites. In our method, and in conventional methods, it is difficult to obtain suitable data using lipids much longer than ~ C16:0. Solubility limitations and critical micelle concentration formation fix the chemical potential for the monodisperse ligand (6), no matter how much total ligand is added.

The number of sites on serum albumin for lysophosphatidyl anions is markedly smaller, $\psi = 1$–2, than for the foregoing alkane carboxylates and for sulfate detergents. Such values are consistent with the scanty literature (7, 8) for serum albumin-lysophosphatidyl interaction, yielding $n = 1$ or 2 sites at most. Alkane chain length and structure govern binding affinities to serum albumin, but the nature of the polar head group governs the number of secondary sites.

Fig. 3 exhibits the sensitivity of the forward (discocyte → echinocyte) titration to relatively small variations in fatty acid anion chain length and chain structure. Extension of the hydrocarbon chain by one carbon group, —CH_2—, displaces the titration plot by nearly an order of magnitude to the left, on the abscissa. Comparing chains of equal length, one unsaturated bond at the penultimate end of the chain displaces the corresponding plot far to the right of its saturated congener. The echinocyte formation plots of closely related fatty acids (homologues) in the C10 to C15 series not shown in Fig. 3, fall in place as expected: that for C13:0 lies between the C12:0 and C14:0 plots, etc. Three lysolipids of the phosphatidyl cholines C16, C14 and C12, have abscissal values, denoted y, at $r = 0.5$, of 10.5×10^7, 1.6×10^7, and 0.8×10^7, respectively, behaving similarly to the alkane carboxylates in their progression. All such results depend on two main contributions: the binding affinities of the lipids with respect to the membrane, and their tendency to break forces normally stabilizing the discocyte and thus to allow lapse to the echinocyte, all in the region of the end-point of the ligand-exogenous protein titration.

TABLE I
NUMBER OF SITES, ψ, OF PLASMA ALBUMIN FOR ANIONIC ECHINOCYTIC LIGANDS VIA MEMBRANE MORPHOLOGY SWITCHING REACTIONS USED AS THE ENDPOINT INDICATOR

Ligand	ψ	Ligand	ψ	Ligand	ψ
C11:0, Undecanoate	10.2	C11:1 10-Undecenoate	9.7	L.C.* Lauroyl	1.7
C12:0, Laurate	7.2	C12:1 11-Dodecenoate	10.5	L.C. Myristoyl	0.9
C13:0, Tridecanoate	5.3	C14:1 9-Myristoleic	10.7	L.C. Palmitoyl	0.5
C14:0, Myristate	7.0			L.C. Oleoyl	0.9

*L.C., lysophosphatidylcholine anion.

Existence of either the discocyte or the echinocyte requires accommodation of the cell's cytoskeleton to these two morphologies. In turn, part of the control over the cytoskeleton is mediated by transmembrane proteins, particularly through glycophorin and possibly through a glycophorin-band 3 complex (3). The intruding lipid anions likely provide more than simply the driving force for echinocyte formation. They may interact directly with the transmembrane proteins to relax controls over the cytoskeleton. There are close to 3×10^5 glycophorin dimers and 6×10^5 such monomers. Insertion of 2×10^7 lipids in the human erythrocyte membrane, triggering echinocyte formation, is equivalent to ~ 70 ligands/glycophorin dimer. If the dimer is compact, all ligands are unlikely to be bound to glycophorin. However, they may form a domain in glycophorin's vicinity of the kind Van Zoelen et al (9) describe.

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THE MOLECULAR BASIS OF ALCOHOL TOLERANCE AND DRUG CROSS-TOLERANCE IN CHRONIC ALCOHOLISM

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Tolerance to ethanol in chronic alcoholics probably arises from alterations in the membrane properties of the nervous system. (Curran and Seeman, 1977; Chin and Goldstein, 1977). We recently reported that liver mitochondrial membranes from rats chronically fed ethanol are resistant to the uncoupling effect of ethanol, which is associated with a resistance to the disordering of the membrane lipid structure by ethanol. (Rottenberg et al., 1980; Waring et al., 1981). This resistance is caused by altered lipid composition of the mitochondrial membranes. We report now that membrane binding of ethanol, anesthetics, and hydrophobic molecules in brain synaptosomes and liver mitochondria from rats is conspicuously reduced after chronic ethanol consumption. These membranes are resistant to structural disordering by both ethanol and halothane. Tolerance, cross-tolerance, and dependence in chronic alcoholics therefore could result, in part, from membrane alterations which inhibit the binding of ethanol and other drugs.

RESULTS AND DISCUSSION

To study the effect of ethanol and halothane on membrane structure we employed two spin-labeled membrane probes. We calculate the order parameter, S, from the hyperfine splitting of the EPR spectra of 5-doxyl stearate (Gaffney, 1975). The partition coefficient of the nitroxide-labeled decane, 5N10, between the hydrophobic membrane and the medium is calculated from the contribution of the bound and free species to the composite EPR spectrum. (Linden et al., 1973.)

Fig. 1 shows the results of these measurements in liver mitochondria and brain synaptosomes from ethanol-fed rats and their controls. It can be seen that membranes from ethanol-fed rats are more rigid than those from the controls. When titrated with ethanol from 25 mM to 1.0 M, control membranes become much more fluid, as indicated by a decrease in the order parameter and an increase in the partition coefficient. Membranes from ethanol-fed rats are much less affected by the addition of ethanol and maintain relatively rigid membranes even at high concentrations of ethanol. Comparing the order parameters and the probe partition coefficients in membranes from ethanol-fed rats and controls, it can be seen that membranes from ethanol-fed animals in the presence of moderate ethanol concentrations are as fluid as membranes from the control in the absence of ethanol; the latter presumably are

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