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12. Liver homogenates were prepared from male Swiss-Webster mice (12 weeks old, 35 to 45 g), Sprague-Dawley rats (250 to 500 g), and Hartley guinea pigs (500 to 600 g). The livers were freed of adhering tissues, perfused with sodium phosphate buffer (*p*H 7.4, ionic strength, 0.2), and homogenized (10 percent, weight to volume) in the same buffer. Differential centrifugation (*2, 3*) produced subcellular fractions. Washed microsomes were resuspended in tris-HCl (*p*H 9.0, ionic strength, 0.1) and assayed within 48 hours of preparation. Cytosolic fractions were diluted with tris-HCl (*p*H 7.4, ionic strength, 0.2). The repelleting procedure often results in a substantial decrease in *trans*-β-methylstyrene oxide hydrolase activity in the unwashed microsomal fraction; the > 96 percent recovery of styrene oxide epoxide hydrolase activity after repelleting indicates that the loss in microsomal activity is due to the removal of the trapped cytosolic fraction.
13. Epoxide hydrolase activity was monitored by gas-liquid chromatography of the *n*-butylboronate diesters of the diols. Homologous diols were used as internal standards. Rates were determined from three replicates of at least four points on a linear time course, and rates of hydration were proportional to the time of incubation and to the protein concentration. The microsomal enzyme activity for which styrene oxide was used as a substrate is similar to that demonstrated elsewhere (*9, 10*).
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X-ray and Neutron Scattering Density Profiles of the Intact Human Red Blood Cell Membrane

Abstract. *Hemoglobin-free human red blood cell membranes have been prepared with glutaraldehyde to maintain an intact structure on partial dehydration. Treatment of resealed ghosts with poly(L-lysine) produced an essentially constant structural unit and permitted correlation of electron microscopy results with x-ray and neutron diffraction profiles. These profiles provide detailed information, for the intact membrane, on the location and relative distribution of lipids and proteins.*

The red cell membrane has been the object of structural investigations for many years. Although its major components have been characterized, and to some extent biochemically localized, their physical distribution is still largely unknown. Until now, no one membrane specimen has been prepared for x-ray diffraction which is sufficiently well organized for reasonable resolution and produces an electron density profile (*1-3*) that is compatible with biochemical results (*4*). We report here the determination of an electron density profile from intact hemoglobin-free erythrocyte membranes that is consistent with biochemical results and compatible with electron micrographs of such membranes. We also present neutron scattering profiles for this system.

In this work we used standard hemoglobin-free Dodge membranes (*5*) that were subsequently resealed (*6*). Removal of water from a suspension of ghosts by equilibrating the pellet over a saturated salt solution has been used to organize membranes on a one-dimensional lattice (*2, 7*). We found that the red cell membrane does not remain intact under these conditions (*8*); at least one major protein component, spectrin (which resides at the cytoplasmic surface), is displaced during the partial dehydration, giving a residual membrane specimen that produces an x-ray periodicity of 55 to 70 Å. Treatment of the membrane suspension with 1 percent glutaraldehyde before partial dehydration eliminated this repeat and produced three to four orders of a 200- to 300-Å periodicity (*9*). As estimated by dry weight, the glutaraldehyde produced less than a 5 percent increase in the membrane mass. Electron micrographs of partially dehydrated membranes fixed in glutaraldehyde and OsO_4 and examined edge-on showed a highly asymmetric pair of collapsed membranes (each membrane about 120 to 150 Å thick) with variable spacing between adjacent membrane pairs (Fig. 1a). Resolution was sufficiently good that we could identify an unstained ribbon ~25 Å thick (presumably the hydrophobic core of a lipid bilayer) bordered by a thin (~25 Å) band of stained material at the extracellular face and a thick stained region (~70 to 100 Å) on the cytoplasmic side.

In order to establish, for the purpose of phasing, a structural unit that remains constant at different lattice spacings and is readily identifiable in electron micrographs, resealed ghosts were cross-bridged at their extracellular surfaces with poly(L-lysine) (producing a mass increase of no more than 1 percent). Poly(L-lysine) is known to aggregate whole cells and ghosts by interacting with the net negative charge on the exte-

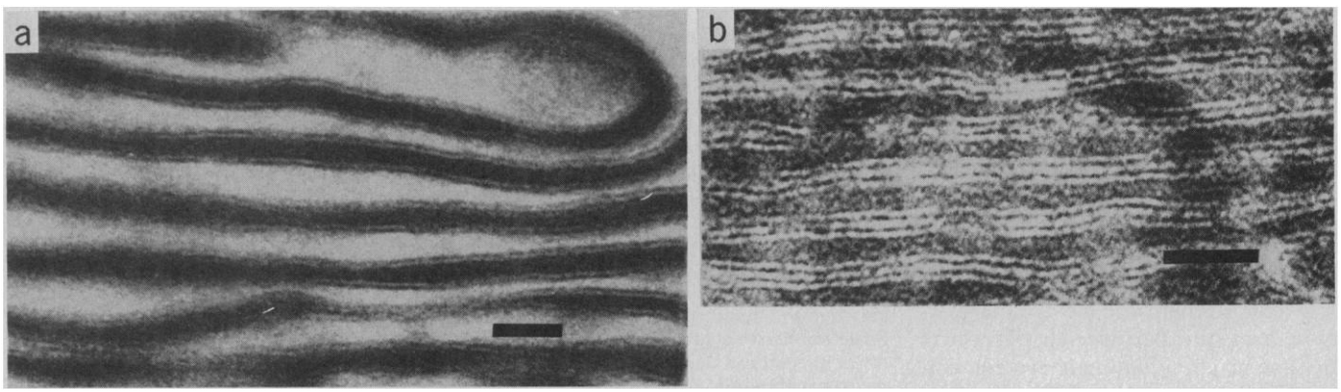

Fig. 1. Electron micrographs of resealed, glutaraldehyde-fixed, and partially dehydrated human red cell ghosts (a) without poly(L-lysine) and (b) treated with poly(L-lysine). Scale bars, 50 nm. In (b) the unit consisting of three thin unstained ribbons corresponds to the extracellular space between membrane pairs and the hydrocarbon cores of the membrane lipid bilayers on either side.

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rior surface of the membrane (*10*). The resealed ghosts are capable of excluding the polycation (80,000 daltons) from their interiors (*11, 12*). As shown in Fig. 1b, after addition of the polycation, the extracellular separation became approximately constant; the unstained hydrocarbon cores were now separated by ~90 to 100 Å as measured from the micrographs.

Diffraction patterns from resealed and poly(L-lysine)-treated membranes were recorded with a position-sensitive detector after partial dehydration for 24 to 48 hours (86 percent relative humidity). After the background profile (collected at 90° to the lamellar scattering) was scaled and subtracted from the lamellar diffraction and a Lorentz correction (*13*) was made to the data, five orders of diffraction were observed—for example, with a 195-Å periodicity at 86 percent relative humidity. An adaptation of the technique of Schwartz *et al.* (*14*) was used to correct for the presence of both lattice and substitutional disorder, as well as to determine the phases of the unit cell transform. This method reduced the choice of phases to two. One set of phases produced an electron density profile that bore no relationship to the electron micrographs (Fig. 1b) of the specimen, and it was therefore eliminated. Model calculations as well as the independent neutron scattering density profile supported the alternate phase assignment.

The electron density profile calculated from the intensity data is shown in Fig. 2b. The unit cell is composed of a pair of asymmetric membranes with their (presumably lipid) bilayer cores separated (center to center) by 72 Å, a result consistent with electron micrographs of this system (Fig. 2a). The two head groups at the extracellular surface ($z = 0$) are only partially resolved in the x-ray profile. The lipid bilayers have a head group separation of 48 Å, some 12 Å larger than that found by Rand and Luzzati (*15*) for extracted lipids alone. The symmetric profile calculated by Stamatoff *et al.* (*2*) for partially dehydrated red cell ghosts is similar to the bilayer part of our profile, and this suggests, contrary to some assertions (*16*), that they were not observing extracted lipids. We suggest, however, that the conditions that Stamatoff *et al.* employed for partial dehydration (low ionic strength and elevated temperature) led to the displacement of spectrin (*17*), the major cytoplasmic protein. Our profile is also strikingly different from that derived by Pape *et al.* (*3*), which gave a head group separation of ~70 Å; the low resolution of their data may explain the difference.

Neutron diffraction data were collected with a two-dimensional position-sensitive detector (*18*) from the same system, with a poly(L-lysine) concentration of 5 mg per 100 ml and partial dehydration at 86 percent relative humidity, at three D_2O:H_2O ratios—1:0 (100 percent D_2O), 3:1 (75 percent), and 2:3 (40 percent). After subtraction of the camera profile, four orders of 192 Å, also broadened by lattice disorder, were observed. The intensity distribution was corrected and unambiguously phased by the same method used for the x-ray data (*14*).

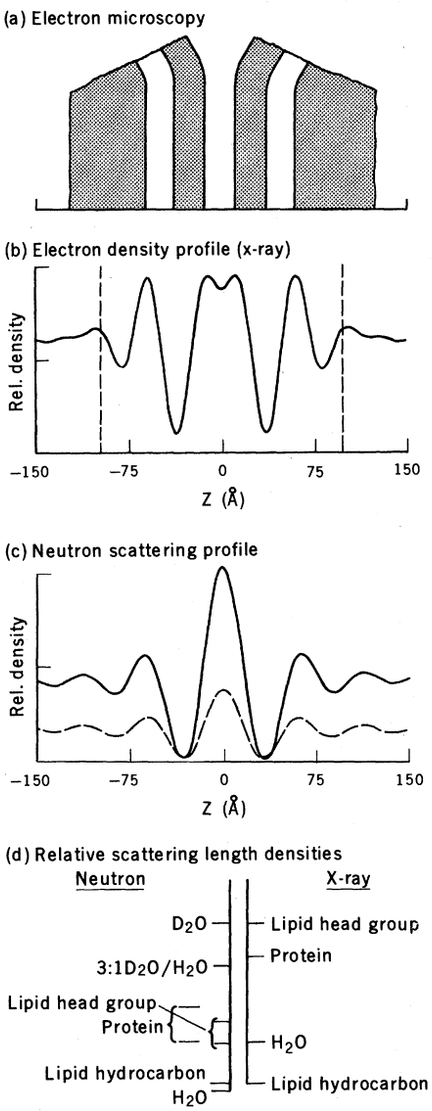

Fig. 2. (a) Scaled schematic representation of membrane pair from electron micrograph (Fig. 1b) of resealed, glutaraldehyde-fixed, and poly(L-lysine)-treated ghosts after partial dehydration. (b) Electron density profile from x-ray diffraction; 195-Å periodicity. (c) Neutron scattering profiles for (—) 100 percent D_2O and (---) 75 percent D_2O; 192-Å periodicity. Calculations require that the unit cell density profiles be zero for $|z| > d_2$, where d is membrane thickness. The abrupt rise in the x-ray scattering profile at $|z| > 80$ Å (b) is probably due to limited resolution in the data. (d) Scale of relative scattering length densities of major membrane components for x-ray and neutron diffraction (D_2O, 6.3×10^{-14} cm/Å^3, and H_2O, -0.6×10^{-14} cm/Å^3 on the neutron scale; lipid head group, 0.465 e/Å^3 and lipid hydrocarbon, 0.288 e/Å^3 on the x-ray scale).

Neutron scattering profiles of the partially dehydrated membranes in 100 percent and 75 percent D_2O are shown in Fig. 2c. Since the zero-order structure factors are unknown, the profiles can be scaled only to within an additive constant. For comparison, the two profiles have been arbitrarily set equal at their hydrocarbon core (this assumes that exchangeable hydrogens are excluded from the center of the lipid bilayers and represents a minimum separation between the two profiles). Since the separation between profiles is larger in the extracellular than in the cytoplasmic region, it can be concluded that more protein is present at the cytoplasmic than at the extracellular surface (see Fig. 2d). The large x-ray scattering density (Fig. 2b) observed at the center of the extracellular region ($z = 0$) is probably a result of the limited resolution of the data (~1/25 Å^{-1}), which is comparable to the distance between the two strongly scattering head groups (~22 Å).

These profiles are consistent with biochemical results, which have shown (*4*) that while all of the major classes of proteins in the red cell can be labeled from the cytoplasmic surface, only 30 percent of the protein can also be tagged at the extracellular surface. The equal heights of the phospholipid head groups in the x-ray profile reflect the nearly equimolar distribution of total phospholipids across the red cell membrane (*4*). More important, a knowledge of the chemical constituents of the membrane, their scattering cross sections, and their positions (identified from scattering profiles and magnitudes) can provide sufficient information to determine quantitatively the distribution of lipid, protein, and water across the membrane (*19*). These profiles also provide a basis for determining the location of cholesterol in the membrane which we are obtaining from a neutron diffraction analysis of membranes substituted with deuterated cholesterol.

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Esterase 6 and Reproduction in *Drosophila melanogaster*

Abstract. *A nonspecific carboxylesterase (esterase 6) of* Drosophila melanogaster *shows greater activity in adult males than in females and is highly concentrated in the anterior ejaculatory duct of the reproductive tract of the male. Esterase 6 is depleted in males by copulation and is transferred to females early during copulation as a component of the seminal fluid. That esterase 6 may be involved in a system controlling the timing of remating is suggested by differences in the activity of this enzyme in a strain of* Drosophila *selected for a decrease in time to remating and by differences in the timing of remating in females initially inseminated by males lacking or having active esterase 6.*

Esterases are among the most genetically variable enzymes known in both plants and animals (*1*). Carboxylesterases (E.C. 3.1.1) have been extensively studied in *Drosophila* and are highly polymorphic (*2*). The first biochemical polymorphism discovered in *Drosophila* was a carboxylesterase, esterase 6 (EST 6), found in *D. melanogaster* (*3*), and genetic variation at the autosomal *esterase 6 (Est 6)* locus has been found in every natural population studied (*1*). With few exceptions (*4*), however, the functions of nonspecific esterases and their in vivo substrates are unknown. Studies (*5*, *6*) on the developmental pattern and tissue distribution of EST 6 have suggested that this enzyme might have a function in some aspect of reproduction. We present results here which show that EST 6 is a component of the seminal fluid of *D. melanogaster*, is active in the reproductive tract of females, and may act to influence the timing of female remating.

Natural populations of *D. melanogaster* are invariably polymorphic for two electrophoretic variants of EST 6 termed EST 6^F and EST 6^S. Two strains of flies derived from a natural population in Bloomington, Indiana, were individually made homozygous for the third chromosome, which carries the structural locus for EST 6. This procedure ensures that only one form of EST 6 is present in flies from either of these two strains. Flies heterozygous for the *Est* 6^F and *Est* 6^S alleles were obtained from a balanced lethal strain carrying the dominant morphological markers, *Stubble* and *Serrate* (*7*). A null stock (*Est* 6^o) lacking esterase activity and producing no detectable EST 6 protein has been described (*6*).

Several investigators have shown that the specific activity of EST 6 may be fivefold greater in 3- to 5-day-old virgin males than in virgin females of the same age (*5*, *6*). In males, the enzyme is highly concentrated in the anterior ejaculatory duct, a reproductive tract, secretory organ connecting the accessory glands and testes to the ejaculatory bulb and penis (*5*, *6*). The possibility that EST 6 is involved in reproduction was tested by determining whether the enzyme was depleted in males by copulation and transferred to females. Virgin males were collected from a stock homozygous for the *Est* 6^S allele, and virgin females were collected from the *Est* 6^o stock. All flies were aged at 25°C for 4 days. The males were divided into six groups of ten flies each. Two groups of males (treatment A) and two groups of females (treatment D) were immediately frozen at −50°C. Two groups of males (treatment B) were indi-

Table 1. Esterase 6 activity (± the standard error of the mean) in virgin and mated *Drosophila melanogaster*. The number of flies in each group was ten.

Treatment	Groups (No.)	Specific activity of EST 6*
A. Virgin *Est* $6^{S/S}$ ♂♂	2	3.289 ± 0.317
B. Once mated *Est* $6^{S/S}$ ♂♂	2	1.046 ± 0.125
C. Twice mated *Est* $6^{S/S}$ ♂♂	2	0.778 ± 0.173
D. Virgin *Est* $6^{o/o}$ ♀♀	2	0.042 ± 0.004
E. *Est* $6^{o/o}$ ♀♀ mated to *Est* $6^{S/S}$ ♂♂	3	0.185 ± 0.035

*Micromoles of β-naphthol per milligram of protein per 30 minutes at 27°C.

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