REFERENCES

1. Stoeckenius, W., R. H. Lozier, and R. Bogomolni. 1979. Bacteriorhodopsin and the purple membrane of Halobacteria. *Biochim. Biophys. Acta.* 505:215–278.
2. Garty, H. D. Cahen, and S. R. Caplan. 1980. Photoacoustic calorimetry of *Halobacterium halobium* photocycle. *Biochem. Biophys. Res. Commun.* 97:200–206.
3. Oesterhelt, D., and W. Stoeckenius. 1971. Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*. *Nature New Biol.* 233:249–152.
4. Ort, D. R., and W. W. Parsons. 1979. Enthalpy changes during the photochemical cycle of bacteriorhodopsin. *Biophys. J.* 25:355–364.

THE RELATIVE LOCATIONS OF INTRAMEMBRANE FLUORESCENT PROBES AND OF THE CYTOSOL HEMOGLOBIN IN ERYTHROCYTES, STUDIED BY TRANSVERSE RESONANCE ENERGY TRANSFER

J. EISINGER AND J. FLORES

Bell Laboratories, Murray Hill, New Jersey 07974 U.S.A.

We have investigated the use of transverse resonance energy transfer (RET) to determine the level of fluorescent probes within the phospholipid regions of the erythrocyte membrane relative to the plane of closest approach of the cytosol hemoglobin. The *n*-(9-anthroyloxy)-stearic and palmitic acids (*n*-AS and *n*-AP) and 9-vinyl anthracene (9-VA) embedded in the membrane were used as donors, and the hemes of the cytosol hemoglobin were the acceptors.

The experiments were analyzed by a model in which the acceptor hemes are constrained to remain in the half space defined by a plane whose normal distance from a donor (D) is d. The hemes are assumed to be uniformly and continuously distributed and their orientations to be dynamically and isotropically averaged. It can be shown that at the high heme concentration inside the erythrocyte (20 mM) and the modest efficiencies of RET encountered here, this model is adequate for determining at least an approximate value of d, but in any case a d_{max}.

RESULTS

Following Wolber and Hudson (1), it is convenient to express the distances and the acceptor density (ρ) as dimensionless parameters, so that $x = d/R_0$ and ρ is the number of acceptors per volume R_0^3, where R_0 is the usual Förster distance. The desired equation for x may then be obtained analytically in terms of the measured average RET efficiency, $<T>$

$$d = xR_0 = [(\pi\rho/6)(<T>^{-1} - 1)]^{1/3}. \qquad (1)$$

This relationship is illustrated graphically in Fig. 1.

$<T>$ was determined by measuring the fluorescence decay of the membrane-bound probes in intact erythrocytes and ghosts made from labeled cells. The decays were measured by means of a monophoton lifetime instrument using a high-pressure hydrogen spark lamp of 2.5-ns half-width. The experimental time profiles were fitted by multi-exponential decay functions of the type

$$I(t) = \sum \alpha_i \exp(-t/\tau_i) \qquad (2)$$

after convolution by the excitation pulse profile. One may then define the mean lifetime of the donor by

$$<\tau> = \sum \alpha_i \tau_i^2, \qquad (3)$$

since $\alpha_i\tau_i$ is the fraction of light with life time τ_i. By comparing $<\tau>$ for intact cells and ghosts (subscripts c and g), i.e., in the presence and absence of the heme acceptors,

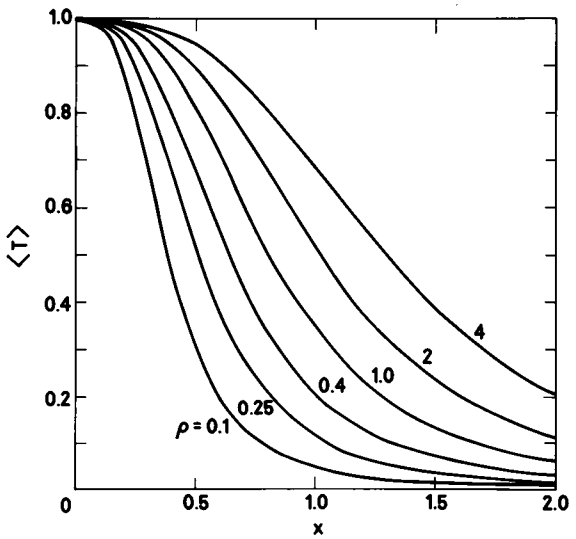

FIGURE 1 The dependence of $<T>$, the dynamically averaged RET efficiency from a donor, D, to a continuum of acceptors beyond a plane whose normal distance from D is x. x is a dimensionless quantity measured as a multiple of the Förster distance R_0. ρ is the density of acceptors per volume R_0^3.

BIOPHYS. J. © Biophysical Society · 0006–3495/82/01/6/02 $1.00
Volume 37 January 1982 6–7

$$<T> = 1-(<\tau>_c/<\tau_g>), \quad (4)$$

and values for d were obtained by substitution of Eq. 4 in Eq. 1.

The results of these experiments are summarized in Table I, which lists for the various donor probes values of the steady-state emission anisotrophy, $<r>$, $<\tau>$, and R_0, in addition to d.

As can be seen from Eq. 1, d depends on the density of hemoglobin molecules near the inner membrane surface. The values of d given in Table I were calculated assuming a uniform distribution of hemoglobin within the cell, which corresponds to a hemoglobin packing fraction of 0.27. If hemoglobin is condensed near the membrane (we assumed face-centered close packing) the packing fraction becomes 0.52 and the d values would be greater, as indicated for n-AS probes in Fig. 2. ρ_{AV} and ρ_{CP} refer to average and closely packed hemoglobin concentrations near the inner membrane surface.

It can be seen that $<r>$ decreases monotonically as n increases for the n-AS and n-AP probes. This is thought to reflect the increased fluidity at the center of the phospholipid bilayer, although a dependence of $<r>$ on n for these probes in paraffin oil has been reported (2). Certainly, the hydrophobic 9-VA has the lowest emission anisotrophy, which suggests that it is free to tumble.

The low $<r>$ values ensure that the uncertainty in d, introduced by orientational effects in the calculation of R_0, does not exceed 20% (3).

The range of d values for the n-AS probes is 12 Å or ~ 2/3 of the distance between the number 2 and 16 carbon atoms for a fully extended hydrocarbon chain. Their level in the membrane is progressively deeper with increasing n and this is consistent with the result of a qualitative study of the location of these probes in dimiristoyl phosphatidylcholine liposomes (4).

The distance of additional membrane probes from the cytosol hemoglobin will be measured in a manner similar to the one discussed here. From such studies, a firmer picture of the probes' levels relative to the phosphate headgroups should emerge.

TABLE I
COMPUTED DISTANCES, d, FROM DONOR

Probe	$<r>$	$<T>$	R_0*	d
			(Å)	(Å)
2-AS	0.19	0.07	33.4	44
6-AS	0.18	0.14	34.6	36
9-AS	0.17	0.21	35.4	32
12-AS	0.13	0.25	36.0	31
16-AS	0.08	0.305	34.8	30
2-AP	0.11	0.10	32.9	38
16-AP	0.05	0.21	34.5	30
9-VA	0.03	0.385	37.9	28

*The average orientation factor was assumed to be 2/3.

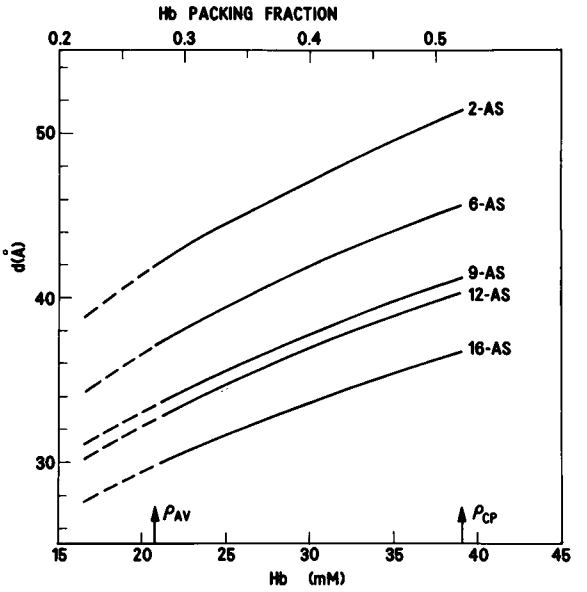

FIGURE 2 The dependence of d on the concentration of hemoglobin near the inner membrane surface of the erythrocyte. The average heme concentration in the red cell is 20.7 mM (ρ_{AV}) and the density for close-packed hemoglobin (39 mM) is indicated by ρ_{CP}. The hemoglobin molecule was assumed to have the shape of a sphere with a diameter of 57Å.

In addition to providing detailed information on membrane structure, transverse and two-dimensional RET experiments may be useful in clinical studies of pathological erythocytes.

NOTE ADDED IN PROOF

We have measured $<T>$ of several membrane probes in erythrocytes as a function of ionic strength (200–800 mosmol). From these data and the asymptotic limit of $<T>$ at ~ 800 mosmol we estimate the heme concentration at the cytosol membrane interface to be 14.3 mM under physiological conditions (295 mosmol). The distances (d) in Table I were obtained with this value, rather than the average cytosol heme concentration.

We wish to thank A. A. Lamola, W. E. Blumberg, and D. M. Zuckerman for helpful discussions and assistance in the instrumentation.

Received for publication 5 May 1981.

REFERENCES

1. Wolber, P. K., and B. S. Hudson. 1979. An analytical solution to the Förster energy transfer problem in two dimensions. *Biophys. J.* 28:197–210.
2. Matayoshi, E. D., and A. M. Kleinfeld. 1981. Emission wavelength dependent decay of the 9-anthroyloxy-fatty acid membrane probes. *Biophys. J.* In Press.
3. Dale, R. E., J. Eisinger, and W. E. Blumberg. 1979. The orientational freedom of molecular probes. The orientation factor in intramolecular energy transfer. *Biophys. J.* 26:161–194.
4. Thulborn, K. R., and W. H. Sawyer. 1978. Properties and the location of a set of fluorescent probes sensitive to the fluidity gradient of the lipid bilayer. *Biochim. Biophys. Acta.* 511:125–140.