fact that r_∞ is seldom <0.1 [cf. Eisinger et al. (1981)]. And finally, the marked differences in fluorescence properties of the probe in solution and bilayers point to some interesting and unusual photophysical properties of this molecule that bear further investigation.

Acknowledgments

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References

Andrich, M. P., & Vanderkooi, J. M. (1976) *Biochemistry 15*, 1257.
Cundall, R. B., Johnson, I., Jones, M. W., Thomas, E. W., & Munro, I. H. (1979) *Chem. Phys. Lett. 64*, 39.
Dale, R. E., Chen, L. A., & Brand, L. (1977) *J. Biol. Chem. 252*, 7500.
Eisinger, J., Blumberg, W. E., & Dale, R. E. (1981) *Ann. N.Y. Acad. Sci.* (in press).
Engel, L. W., & Prendergast, F. G. (1981) *Biochemistry* (following paper in this issue).
Heyn, M. P. (1979) *FEBS Lett. 108*, 359.
Israelachvili, J., Mitchell, D. J., & Ninham, B. (1977) *Biochim. Biophys. Acta 470*, 185.
Kinosita, K., Jr., Kawato, S., & Ikegami, A. (1979) *Biophys. J. 20*, 289.
Lakowicz, J. R., & Prendergast, F. G. (1978) *Science (Washington, D.C.) 200*, 1399.
Lakowicz, J. R., Prendergast, F. G., & Hogen, D. (1979) *Biochemistry 18*, 520.
Lawaczeck, R., Kainosho, M., & Chan, S. I. (1976) *Biochim. Biophys. Acta 443*, 313.
Mantulin, W. W., & Weber, G. (1977) *J. Chem. Phys. 66*, 4092.
Nikitina, A. N., Ter-Sarkisyan, G. S., Milchailov, B. M., & Minchenkova, L. E. (1963) *Opt. Spectrosc. (Engl. Transl.) 14*, 347.
Podo, F., & Blaisie, J. K. (1972) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1032.
Spencer, R. D., & Weber, G. (1969) *Ann. N.Y. Acad. Sci. 158*, 361.
Weber, G., & Bablouzian, B. (1966) *J. Biol. Chem. 241*, 2558.

Values for and Significance of Order Parameters and "Cone Angles" of Fluorophore Rotation in Lipid Bilayers†

L. W. Engel and F. G. Prendergast*

ABSTRACT: A rigid formalism has been developed for the calculation of the order parameter S for fluorescence probes embedded in environments that hinder the motions of the probes and for calculation of a "cone angle" of fluorophore rotation from the order parameters. The motions of the fluorescence probes 1,6-diphenylhexa-1,3,5-triene (DPH) and 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH) embedded in lipid bilayers were then analyzed in terms of the order parameter and the cone angle. Order parameters for such fluorescence can only be compared to the average order parameter over a segment of a fatty acyl chain or of membrane thickness. Also, because the bilayer may be perturbed by the fluorophore at regions distant from the immediate location of the probe, these "averaged" order parameters cannot be easily compared to those calculated from nuclear magnetic resonance data but are more readily compared to order parameters of electron spin resonance probes. There is no defined mathematical relation between the order parameter and a dynamical parameter which would afford a calculation of membrane "microviscosity". A Gaussian angular freedom parameter or cone angle of fluorophore motion has been calculated from the order parameters and shows in a geometric sense the limitations imposed on the angular displacements of TMA-DPH as compared to DPH.

In the preceding paper we have described the fluorescence depolarization of two molecules, diphenylhexatriene (DPH)[1] and a charged analogue thereof, 1-[4-(trimethylamino)-phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH) (Prendergast et al., 1981), when these are embedded in lipid bilayers. From these data we may infer the dynamic behavior of the probes' environment on the premise that the fluorophore does indeed provide a reliable measure of the response of its environment to a perturbation. But while we can now reasonably quantify some of the parameters of motion of probes such as DPH and TMA-DPH, the interpretation of these parameters in terms of membrane structure and mobility is not simple. We have grown accustomed, for example, to using the term "membrane fluidity" and the associated quantity "microviscosity", but the effective definition of these terms that would allow translation of one into the other is, in fact, elusive, especially in those situations where microviscosity values are determined through use of steady-state fluorescence anisotropy (r_{ss}) values. Several recent publications have shown the inappropriateness of as-

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[1] Abbreviations used: DPH, 1,6-diphenylhexa-1,3,5-triene; TMA-DPH, 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene; DMPC, 1,2-dimyristoylglycero-3-phosphocholine; DPPC, 1,2-dipalmitoylglycero-3-phosphocholine; DOPC, 1,2-dioleoylglycero-3-phosphocholine; LOPC, lysooleoylglycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoylglycero-3-phosphocholine; ESR, electron spin resonance; NMR, nuclear magnetic resonance; PC, phosphatidylcholine.

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signing quantitative significance to such microviscosity values. Yet it is clear that whether we employ measurements of r_{ss} or of the hindered limiting anisotropy (r_∞) in our definition of probe motion, the depolarization of the fluorescence signal does provide a measure of the extent of disorder in a lipid bilayer or membrane. The recent work of Jähnig (1979), Heyn (1979), Lipari & Szabo (1980), and Engel and Prendergast (unpublished results) has been focused on theoretical considerations of significance of r_{ss} and r_∞ as indicators of disorder; the findings are that r_∞ provides a measure of the order parameter, S (Lipari & Szabo, 1980). Fulford & Peel (1980) have used r_{ss} to calculate a cone angle of fluorophore rotation through application of (what we have termed) the "hard cone" model of Kinosita et al. (1979) or of a model that assumes a Gaussian static angular distribution for DPH in lipid bilayers. In this work we also have analyzed data for DPH and TMA-DPH embedded in various lipid bilayers in terms of a Gaussian cone model and have provided the rigid formalisms used in the development of the model. The foundation for the formalism developed is, however, to be found in the work of Lipari & Szabo (1980).

Theory

Fluorescence depolarization data from any randomly oriented system (except for cases like multiphoton interactions or nonradiative energy transfer) can be characterized by a single function: the δ-function excitation response of the quantity $\langle P_2(\cos\theta_{12})\rangle = (3\cos^2\theta_{12} - 1)/2$. θ_{12} is the angle between the absorption and emission dipoles, and $P_2(\cos\theta_{12})$ is the second Legendre polynomial in $\cos\theta_{12}$; the broken brackets denote an ensemble average. The anisotropy δ-function response, $r(t)$, is related to $\langle P_2(\cos\theta_{12})\rangle$ by

$$r(t) = (2/5)\langle P_2(\cos\theta_{12})\rangle \quad (1)$$

Formulas for steady-state anisotropy and for differential phase fluorometric observables follow from integral transforms of eq 1 and knowledge of the intensity δ-function response $I(t)$. [See, for example, Lakowicz et al. (1979a,b), Weber (1977), and L. W. Engel and F. G. Prendergast (unpublished results).]

Let us consider a probe in a hindered environment such as a lipid vesicle or a micelle. Define a coordinate system $(x_A y_A z_A)$, fixed in the hindering environment local to the fluorophore, and fix coordinate system $(x_B y_B z_B)$ in the fluorophore. The A system is rotated into the B system by the Euler angles $(\alpha\beta\gamma)_1$ and $(\alpha\beta\gamma)_2$ at instants of absorption and of emission, respectively (see Figure 1). If we assume that the vesicle or micelle rotates isotropically in the lab, which should often be the case, then the δ-function response of $P_2(\cos\theta_{12})$ is given by

$$\langle P_2(\cos\theta_{12})\rangle = \langle e^{-6Rt}\rangle \sum_{jkl} \langle D^{(2)}_{jk}(\alpha\beta\gamma)_1 D^{(2)*}_{jl}(\alpha\beta\gamma)_2\rangle \times D^{(2)}_{k0}(\phi_{B1}\theta_{B1}) D^{(2)*}_{l0}(\phi_{B2}\theta_{B2}) \quad (2)$$

where $(\theta_{B1}\phi_{B1})$ and $(\theta_{B2}\phi_{B2})$ are the spherical polar coordinates of the absorption and emission dipoles, respectively, the $D^{(2)}_{pq}(\alpha\beta\gamma)$ are elements of the Wigner rotation matrices (Brink & Satchler, 1961), and R is the rotational diffusion coefficient of the vesicles or micelles. The sums extend from -2 to $+2$. The mathematical techniques for deriving and using expressions like eq 2 are discussed in detail and were used for membrane fluorescence calculations by Lipari & Szabo (1980) and by Szabo (1980).

If the vesicles or micelles are not of uniform size, the initial bracketed factor is evaluated as

$$\langle e^{-6Rt}\rangle = \int_0^\infty e^{-6Rt} P(R)\, dR \quad (3)$$

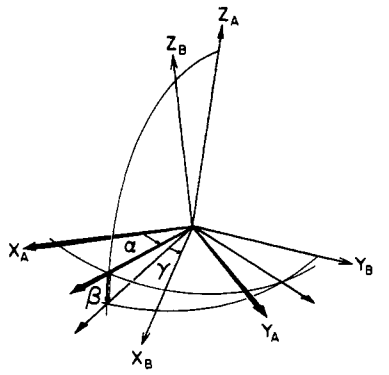

FIGURE 1: The Euler angles $(\alpha\beta\gamma) = (AB)$ that rotate the A axes into the B axes.

where $P(R)$ is the probability distribution in the rotational diffusion coefficient R. If the vesicles or micelles are of uniform size and have the rotational diffusion coefficient R_0, then $\langle e^{-6Rt}\rangle \simeq e^{-6R_0 t}$. Usually, for fluorescent probes in lipid vesicles the rotation of the vesicle during the probe's lifetime is negligible. In this case eq 2 becomes

$$\langle P_2(\cos\theta_{12})\rangle = \sum_{jkl} \langle D^{(2)}_{jk}(\alpha\beta\gamma)_1 D^{(2)*}_{jl}(\alpha\beta\gamma)_2\rangle D^{(2)}_{k0}(\phi_{B1}\theta_{B2}) D^{(2)*}_{l0}(\phi_{B2}\theta_{B2}) \quad (4)$$

The question of cone angles refers to the equilibrium distribution of the probe's orientations as measured in the local fixed coordinate system A, $P(\alpha\beta\gamma)$. The limit for times t much greater than the time scale of fluorophore rotations within the hindering environment of eq 2 becomes

$$\langle P_2(\cos\theta_{12})\rangle = \langle e^{-6Rt}\rangle \sum \langle D^{(2)}_{jk}(\alpha\beta\gamma)\rangle \langle D^{(2)*}_{jl}(\alpha\beta\gamma)\rangle \times D^{(2)}_{k0}(\phi_{B1}\theta_{B1}) D^{(2)*}_{l0}(\phi_{B2}\theta_{B2}) \quad (5)$$

where

$$\langle D^{(2)}_{jk}(\alpha\beta\gamma)\rangle = \int_0^{2\pi} d\alpha \int_0^{\pi} \sin\beta\, d\beta \int_0^{2\pi} d\gamma P(\alpha\beta\gamma) D^{(2)}_{jk}(\alpha\beta\gamma) \quad (6)$$

and $\langle D^{(2)*}_{jl}(\alpha\beta\gamma)\rangle$ is similarly evaluated. With the assumption that $P(\alpha\beta\gamma)$ is independent of α and of γ, integrals of the form of eq 6 vanish except when $j = k = 0$. If the probe is rod shaped, we choose the z_A axis parallel to the average chain direction in the micelle or membrane near the probe and choose the z_B axis along the long axis of the probe; then eq 5 becomes

$$\langle P_2(\cos\theta_{12})\rangle = \langle e^{-6Rt}\rangle |\langle D^{(2)}_{00}(\alpha\beta\gamma)\rangle|^2 \times D^{(2)}_{00}(\phi_{B1}\theta_{B1}) D^{(2)*}_{00}(\phi_{B2}\theta_{B2}) = \langle e^{-6Rt}\rangle \langle P_2(\cos\beta)\rangle^2 P_2(\cos\theta_{B1}) P_2(\cos\theta_{B2}) \quad (7)$$

a generalization of a formula obtained for the case of negligible "substrate" rotations by Lipari & Szabo (1980). It is important to consider the validity of the assumption of azimuthal symmetry of the equilibrium distribution $P(\alpha\beta\gamma)$ when approximating angular freedom parameters from fluorescence depolarization data. For the rod-shaped probes DPH and TMA-DPH, the transition dipoles are nearly aligned with the long axis of the probe; thus, one can use the approximate relation

$$\langle P_2(\cos\theta_{12})\rangle = \langle e^{-6Rt}\rangle \langle P_2(\cos\beta)\rangle^2 P_2(\cos\epsilon) \quad (8a)$$

where ϵ is the angle between the absorption and emission transition moments when the fluorophore is motionless. In more familiar notation eq 8a reads

$$r_\infty = \langle e^{-6Rt}\rangle S^2 r_0 \quad (8b)$$

where r_∞ is the anisotropy at times long compared to the fluorescence lifetime, r_0 is the zero-time anisotropy, and $S = \langle P_2(\cos\beta)\rangle$; S is known as the order parameter. Equations 8a and 8b are precisely equivalent to eq 7 when either θ_{B1} or θ_{B2} equals zero [see Lipari & Szabo (1980) and L. W. Engel and F. G. Prendergast (unpublished data)].

We may now consider systems for which eq 7 can be assumed valid and for which θ_{B1} and θ_{B2} may be estimated or eq 8 approximately holds. In such systems S, a weighted integral of the probability distribution $P(\alpha\beta\gamma)$, can be derived from fluorescence depolarization data. If rotation of the substrate during the fluorescence lifetime is negligible, then one can determine S by using $\langle e^{-6Rt}\rangle \simeq 1$ and the observed values of r_∞ in eq 7. Techniques for observing r_∞ through use of steady-state anisotropy data, differential phase fluorometry, or time-dependent depolarization measurements have previously been discussed in several works (Lakowicz et al., 1979a,b; Kawato et al., 1977; Dale et al., 1977). Observation of S may be more complicated for systems in which one cannot assume that $\langle e^{-6Rt}\rangle \simeq 1$ during the fluorescence lifetime. If $\langle e^{-6Rt}\rangle$ can be estimated, from the data or from other considerations, time-resolved measurements provide enough information to allow direct determination of S from eq 7 or 8. However, to use steady-state or differential phase data, one must evaluate time integrals that describe the time dynamics of fluorescence depolarization (like eq 5). We can rewrite eq 5 to explicitly show a term C_∞ representing $P_2(\cos\theta_{12})/\langle e^{-6Rt}\rangle$ in the long time limit where eq 7 holds.

$$\langle P_2(\cos\theta_{12})\rangle = \langle e^{-6Rt}\rangle(C(t) + C_\infty) \tag{9}$$

$C(t)$ is zero in the limits where eq 7 holds. A commonly adopted function for approximation of $C(t)$ is $(C_0 - C_\infty)e^{-t/\phi}$ where ϕ is a relaxation time of depolarization due to dynamics of the fluorophore within the substrate. If (i) $\langle e^{-6Rt}\rangle \simeq e^{-6R_1 t}$ [which means $P(R)$ can be approximated by a δ function], (ii) the approximation function can be assumed for $C(t)$, and (iii) $I(t) \propto e^{-\Gamma t}$, one can use (for example) a combination of differential phase and unquenched steady-state anisotropy measurements to determine ϕ and C_∞ by an adaptation of the analysis used by Lakowicz et al. (1979a,b) for the case when $e^{-6Rt} \simeq 1$. We merely carry through the integral transforms to evaluate tan Δ and the steady-state anisotropy, and then combining the formulas for these two quantitites, we determine ϕ and C_∞ for the case when eq 8 holds, so that $C_0 = (5/2)r_0$. With $\gamma = \phi^{-1}$, $I(t) \propto e^{-\gamma t}$, $\gamma_s = 6R_1$, and $E = (5/2)r_{ss}(\Gamma)$, we have

$$A_0 + A_1\gamma + A_2\gamma^2 = 0 \tag{10}$$

where

$$A_0 = C_0gh(10 - 8C_0) + 25g^2 + 4h(\gamma_s + \Gamma)^2(Ev - C_0)[5 - 2(C_0 + Ev)] + (10\Gamma + X)[C_0g\gamma_s + (C_0 - Ev)(\gamma_s + \Gamma)(h - \gamma_s^2)] \tag{11a}$$

$$A_1 = gX(C_0 - Ev) + 10\Gamma C_0g + (\gamma_s + \Gamma)[(50 - 10E)g + (3Ev - C_0)(10h + \gamma_s[10\Gamma + X]) - 16hE^2v^2] \tag{11b}$$

$$A_2 = 25g + Ev(10 - 8Ev)h + \gamma_s(10\Gamma + X)Ev \tag{11c}$$

in which

$$v = (\gamma_s + \Gamma)/\Gamma \qquad g = \omega^2 + (\Gamma + \gamma_s)^2$$
$$h = \omega^2 + \Gamma^2 \qquad X = (-30\omega)/\tan\Delta \tag{11d}$$

and

$$C_\infty = Ev + (\gamma_s + \Gamma)(Ev - C_0) \tag{12}$$

We have recently discussed this type of analysis in greater detail in a discussion on the use of fluorescence depolarization for study of probe motion in isotropically rotating proteins.

Table I: Values for Gaussian Cone Angles (β_0) Corresponding to Order Parameters (S) for Fluorescence Probes

β_0 (deg)	S	β_0 (deg)	S	β_0 (deg)	S
1	0.9995	33	0.6166	63	0.1960
3	0.9960	36	0.5642	66	0.1702
6	0.9837	39	0.5129	69	0.1472
9	0.9637	42	0.4634	72	0.1268
12	0.9365	45	0.4162	75	0.1087
15	0.9028	48	0.3717	78	0.0929
18	0.8634	51	0.3301	81	0.0789
21	0.8194	54	0.2918	84	0.0668
24	0.7716	57	0.2567	87	0.0561
27	0.7212	60	0.2247	90	0.0468
30	0.6692				

Having established that S is an observable quantity related to $P(\alpha\beta\gamma) = (1/4\pi^2)p(\beta)$, which is the equilibrium probability distribution function in probe orientation, we wish to relate an observed quantity to an angular freedom parameter in angular units. To do this, we need to assume a functional form of $p(\beta)$; the integral

$$S = \int_0^\pi p(\beta)P_2(\cos\beta)\sin\beta\, d\beta \tag{13}$$

cannot yield a parameter in angular units without such an assumption. Using an infinite conical well (Kinosita et al., 1977), we assume that

$$p(\beta) = (1 - \cos\beta_0)^{-1} \text{ if } 0 \le \beta \le \beta_0 \text{ or } 0 \le \pi - \beta \le \beta_0$$
$$0 \text{ otherwise} \tag{14}$$

so that

$$S = (1/2)(\cos\beta_0)(1 + \cos\beta_0) \tag{15a}$$

or

$$\cos\beta_0 = (1/2)[-1 + (1 + 8S)^{1/2}] \tag{15b}$$

where β_0 is an angular parameter. A second possible distribution would be Gaussian, with

$$p(\beta) = \frac{e^{-(\beta/\beta_G)^2} + e^{-[(\pi-\beta)/\beta_G]^2}}{\int_0^\pi [e^{-(\beta/\beta_G)^2} + e^{-[(\pi-\beta)/\beta_G]^2}]\sin\beta\, d\beta} \tag{16}$$

We performed integral 13 numerically for values of β_G to obtain the relationship of this angular parameter to S (Table I). Note that both of our choices for $p(\beta)$ obey the condition $p(\beta) = p(\pi - \beta)$. This is necessary for invariance of the probability distribution or under inversion of the z_A axis. Fulford & Peel (1980) also considered these models for obtaining the cone angle parameter although their derivations are somewhat less formal.

An algorithm was developed for a Hewlett-Packard 9835 calculator that utilized the theory developed above to calculate the order parameter (S) and cone angle (β) for the motions of 1,6-diphenylhexa-1,3,5-triene and 1-[4-(trimethylamino)-phenyl]-6-phenylhexa-1,3,5-triene embedded in lipid bilayers. The values for the limiting hindered anisotropy (r_∞) used for the calculation of S and β were those given in the preceding paper and in the work of Lakowicz et al. (1979a,b) and E. Taswell, L. W. Engel, and F. G. Prendergast (unpublished results).

Results

The data in Figure 2 show the relationship between the square of the order parameter (S^2) and the cone angle (β_0)

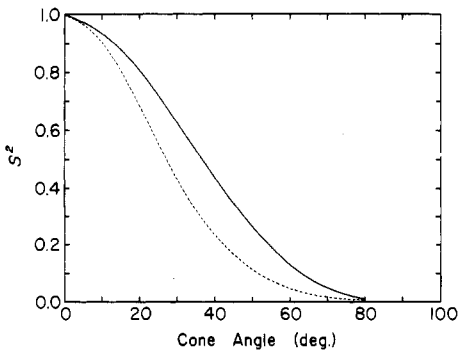

FIGURE 2: Plots of the square of the order parameter (S) vs. cone angle (β_0) of fluorophore rotation as calculated for the Gaussian (—) and hard-cone models (- - -) (Kinosita et al., 1977).

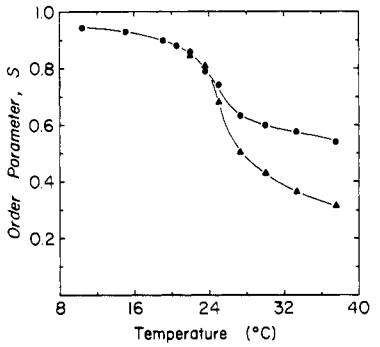

FIGURE 3: Variation of order parameters for TMA-DPH (●) and DPH (▲) in lipid bilayers prepared from DMPC as temperature is varied.

as calculated for the Gaussian and hard-cone models, respectively. (In Table I we have provided the numerical values of S and β_0 derived from the integration of eq 16 and which may be useful for interpolation of β_0 values.) Our rationale for choosing a Gaussian model was based simply on the notion that the diffusive motions of the probe, determined as they are by stochastic processes, would more likely approximate to a Gaussian distribution. In general, the cone model of Kinosita et al. (1976) gives larger values from the data on r_∞ values given in the preceding two papers, and through use of the theory outlined above values for the order parameters and cone angle for DPH and TMA-DPH in lipid bilayers were calculated and are presented in Figures 2–7. For DMPC and DPPC (Figures 3 and 4) the patterns of movement of both probes are essentially the same, with β_0 approaching 10° at temperatures $\ll T_c$, showing a gradual increase as temperature increases toward T_c and then a marked increase at T_c. Initially, the values for DPH and TMA-DPH are virtually identical but begin to deviate markedly at T_c. Finally, at temperatures $> T_c$ the cone angle for DPH distribution is in excess of 55° (r_∞ approaches 0) whereas β_0 for TMA-DPH appears to become essentially independent of temperature at a value of about 35°. The fundamental similarity in the profiles for the probes in the two different lipids indicates the similarity not only in the pattern of motion but also in the extent of freedom of motion of the probe at temperatures $< T_c$. Undoubtedly, the curves would show more cooperative changes in cone angle if multilamellar systems had been studied. It is instructive to note that the angles calculated by the method of Kinosita et al. (i.e., hard-cone model) are substantially larger and even for TMA-DPH approach 55° at temperatures $> T_c$ (Figure 4). When these probes are embedded in a mixed acid–phospholipid such as α-palmitoyl-β-oleoylglycerophosphocholine (POPC), the pattern of motion is essentially the same as that for the probes in DMPC at $T > T_c$, which

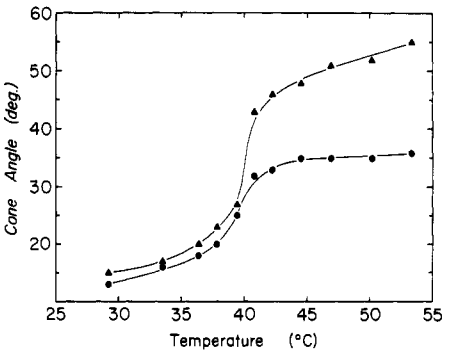

FIGURE 4: Gaussian cone angles of fluorophore [TMA-DPH (●) and DPH (▲)] motion in DPPC bilayers.

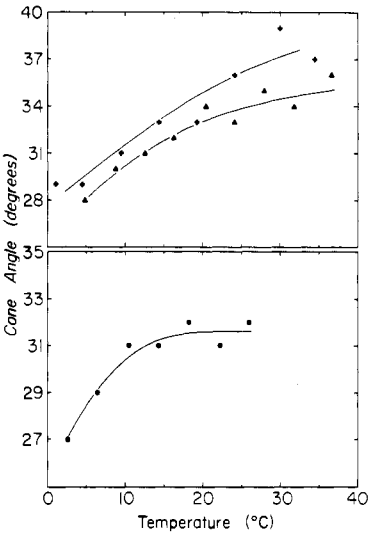

FIGURE 5: Cone angles for TMA-DPH in vesicles of DOPC (+) and egg lecithin (▲) (upper panel) and micelles of lysooleoylphosphatidylcholine (lower panel) as temperature is varied. The data show considerable scatter at the higher temperatures, which we attribute to the very short lifetimes of TMA-DPH measured under these conditions.

is reasonable since at all temperatures used here T is $> T_c$ of POPC. There are no changes in β_0 that would suggest a phase transition; rather β_0 changes smoothly with temperature for both TMA-DPH and DPH. The presence of a single unsaturated fatty acid in the 2 position of the glycerol backbone is clearly sufficient to markedly increase the orientational disorder in phospholipid bilayers prepared from such lipids. At low temperatures the cone angles are very similar to each other, but then the values deviate progressively as the temperature is increased and attain values of ~35° and greater than 60°, respectively, at $T \gg T_c$. This deviation in values at low temperatures, given the behavior of the probes in other systems, may reflect the occurrence of a phase transition for this lipid at a temperature <0 °C. Cone angle values for the TMA-DPH in egg lecithin and DOPC are very similar, one to the other and to those in POPC.

Lysooleoylphosphatidylcholine is not expected to form vesicles under the conditions of our experiments but rather should form micelles. The data in Figure 5 show that the effect of temperature on the motion of TMA-DPH in micelles of lysolecithin is very similar to that for the probe in DOPC, egg lecithin, and POPC (Figure 6), which implies the similarity of packing of the head group and upper portions of the fatty acyl chain in micelles made of lysooleoylphosphatidylcholine and lipid bilayers of largely unsaturated systems. Figure 7 clearly demonstrates the restricting effects of cholesterol on the extent of motion of diphenylhexatriene in lipid bilayers

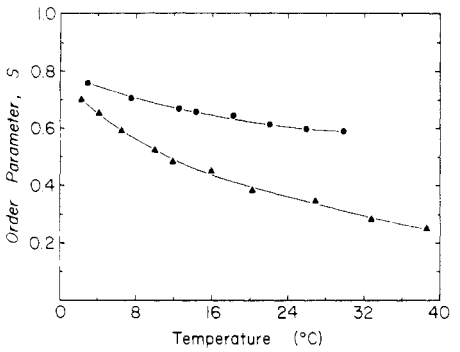

FIGURE 6: Order parameters of fluorophore motion in POPC bilayers for TMA-DPH (●) and DPH (▲) as temperature is varied.

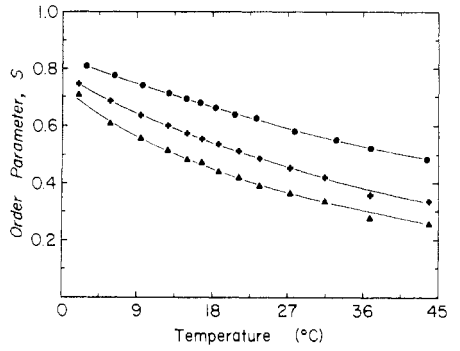

FIGURE 7: Influence of cholesterol on the order parameter of DPH. The vesicles were composed of POPC/cholesterol in molar ratios of 3:1 (●), 7.5:1 (+), and 20:1 (▲).

as the order parameters remain high (and β_0 values are therefore low).

Discussion

In this work and in the preceding paper we have provided comprehensive fluorescence depolarization data for diphenylhexatriene and the charged surface-interactive analogue 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene embedded in a variety of lipid bilayers. Our fundamental interest was to determine whether data derived from fluorescence probes allow us to infer the structure and dynamics of the systems that host the probes, in this instance, phospholipid membranes. In this paper we have sought to present fluorescence depolarization data in a form already familiar to ESR and NMR spectroscopists, i.e., as plots of order parameters, but also to translate the latter into cone angles of fluorophore rotation. Below we have divided our discussion into three parts. First, we consider the data presented in this paper; second, we discuss the nature and significance of order parameters, mainly in an effort to delineate what we might infer about membrane structure and dynamics from fluorescence data and from the models of fluorophore motion developed from those data. Third and finally, we consider the significance of steady-state anisotropy data and broach the issue of the relationship between r_{ss} and r_∞. In all instances we refer implicitly or explicitly to the data contained in this manuscript but do not exclude consideration of other data extant in the literature.

However, before proceeding to these topics we need to briefly discuss the significance of the r_∞ term as measured by differential phase fluorometry. The parameter r_∞, as applied here, is defined as that obtained by tan Δ and r_{ss} measurements ($r_{\infty,\Delta\tau}$), not as the long time limit value of real time anisotropy decay, which we denote $r_{\infty,t}$. The method we used to obtain $r_{\infty,\Delta\tau}$ was developed by Lakowicz et al. (1979a) using formulas of Weber (1977) for the case when the anisotropy and intensity decay as

$$r(t) = (r_0 - r_{\infty,t})e^{-t/\phi} + r_{\infty,t}$$

$$I(t) \propto e^{-t/\tau}$$

under δ-function excitation. For this case only then, $r_{\infty,\Delta\tau} = r_{\infty,t}$. tan Δ and r_{ss} values may then be combined to derive values of $r_{\infty,\Delta\tau}$ [just as they are here to derive anisotropic rotation parameters for the more complex anisotropy decay of the anisotropically rotating fluorophore; see Lakowicz et al. (1979a) for details], and steady-state anisotropies and tan Δ are relatively insensitive to details of the anisotropy decay for times much exceeding the fluorescence lifetime. We may then expect $r_{\infty,\Delta\tau}$ to approximate the value of $r(t)$ for a wide range of anisotropy decays at $t > \tau$.

It is important to realize that the equivalence of $r_{\infty,\Delta\tau}$ and $r_{\infty,t}$ has been shown empirically (Lakowicz et al., 1979a) to hold for probes such as DPH embedded in lipid bilayers but need not necessarily hold for all situations (L. W. Engel and F. G. Prendergast, unpublished data).

Order Parameters and Cone Angles for DPH and TMA-DPH in Lipid Bilayers. The data presented in Figures 2–7 illustrate that for each probe (i) the pattern of changes in order parameters in saturated phospholipids is the same; (ii) the values of the order parameters are also similar in all lipids at $T < T_c$, through the phase transition, and at $T > T_c$; (iii) the order parameters and hence the cone angles for DPH or TMA-DPH are similar for all lipids at $T > T_c$, irrespective of fatty acyl chain length or degree of unsaturation; and (iv) cholesterol increases the order of the system in proportion to the concentration of cholesterol in the membrane. Obviously, the same qualitative information is inherent in values of r_{ss} or r_∞, but the translation of the latter into order parameters allows more facile comparison with other spectroscopic techniques (see below). There are, however two aspects (of our mathematical analysis and of the data) that need to be considered.

First, the mathematical formalism is, as we have stated, similar in principle to that of Lipari & Szabo (1980) and Kinosita et al. (1977). The formalisms in this paper are developed with an emphasis on differential phase fluorometry, i.e., to allow calculation of $r_{\infty,\Delta\tau}$. Second, we also wished to illustrate mathematically the significance of substrate rotation on the calculated order parameter of a probe buried in that substrate. We have ignored substrate rotation in the studies reported here because the rotational correlation times of the vesicles under study (estimated molecular weight of $>2.5 \times 10^6$) are long in comparison to the lifetimes of DPH or TMA-DPH. We will soon present a detailed analysis of the effects of substrate rotation on measurement of differential phase lifetime (L. W. Engel and F. G., Prendergast, unpublished results).

We have assumed a Gaussian distribution model for calculation of cone angles simply on the basis that this seemed the most simple and likely statistical distribution of probe molecules. On this basis, there would be good reason to use the same probability distribution function to describe TMA-DPH and DPH. In fact, if DPH does "translate" in the membrane in the manner suggested below, the probability distribution would be invalid for DPH also. The model is obviously imperfect but is the best we presently can offer. Finally, the argument may be made that the inherently greater symmetry of DPH as compared to TMA-DPH inevitably means greater probability of "wobble". This argument is well taken, but we propose that the differences noted in the order

parameters are too great to be ascribed solely or even largely to the differences in symmetry between the two fluorophores. In any event, the extent to which symmetry considerations may influence the order parameters requires an understanding of the excited-state geometry of the fluorophores, and these have not yet been reported.

Nature and Significance of Order Parameters and Cone Angles. The original model of Kinosita et al. (1977) assumed that a unique symmetry axis of a cylindrical probe was free to diffuse (wobble) within a cone of semiangle θ_0 (β_0 in our notation). As a consequence of this model, the orientational distribution of the symmetry axis at equilibrium is constant for $0 \leq \theta \leq \theta_0$ or is otherwise zero. For a molecule in which either dipole is collinear with the symmetry axis, Kinosita et al. (1971) derived an expression that allowed interpretation of a measured parameter r_∞ (the anisotropy at times long compared to the fluorescence lifetime or hindered limiting anisotropy) in terms of θ_0. Subsequently, we (Lakowicz et al., 1979a,b) pointed out that the interpretation of Kinosita et al. was model dependent and instead sought to interpret r_∞ in a model-independent manner proposing that

$$r_\infty/r_0 = (3\cos^2\theta - 1)/2$$

Lipari & Szabo (1980) have recently pointed out the error in this simple interpretation since

$$r_\infty/r_0 = \lim_{t\to\infty} (3/2)[\cos^2\theta(t) - 1] - \tfrac{1}{2}$$

which requires evaluation of the limit for the derivation of the correct value of the ratio r_∞/r_0. When this limit is evaluated, then $r_\infty/r_0 = S^2$ (Lipari & Szabo, 1980). Our derivation given above precisely corroborates the conclusions of Lipari and Szabo and those of Jähnig (1979) and Heyn (1979). The evaluation of the static equilibrium distribution of a fluorescent probe in a bilayer in terms of the order parameter makes it possible to compare fluorescence depolarization data for such probes with the data obtained by ESR and to some extent NMR experiments. Heyn (1979) did a limited comparison along these lines for DPH in lipid bilayers prepared from DPPC. To do the comparison, Heyn used order parameters calculated for the C_{10} and C_{12} methylene carbons of DPPC from NMR data (Seelig & Seelig, 1974) and the average order parameter along the chain determined for DPPC (Davis & Jeffrey, 1977; Davis, 1979). Heyn showed that S calculated from the r_∞ data of Lakowicz et al. (1979a,b) fit the NMR data reasonably well. Necessarily, because of the size of a fluorescent probe and the mutual ordering effects of probe and lipid fatty acyl chains, the order parameter measured by a probe such as DPH can only indirectly reflect the order parameter of the fatty acyl tails in the bilayer and even then only the average order parameter over a length of fatty acyl chain at least equivalent to the length of the DPH molecule (ca. 13 Å). The latter statement is, however, subjected to the condition that the fluorophore must inevitably perturb the bilayer and need not do so only in the immediate vicinity of the probe (Podo & Blaisie, 1972). The variation in order parameter along the methylene groups from C_1 to C_{12} is such that it cannot be justified to merely compare S for DPH to that of the methylene units C_{10} and C_{12} of a palmitoyl fatty acyl chain, and so the reasonable fit obtained by Heyn must be considered fortuitous. Another, and somewhat more subtle, reason for not making such comparisons is related to the location of diphenylhexatriene in the bilayer. The high order parameter for DPH at temperatures $<T_c$ indicates a significant degree of orientational constraint if we assume that under these conditions DPH is oriented largely normal to the plane of the bilayer [cf. Mely-Goubert & Freedman (1980); E. Taswell, L. W. Engel, and F. G. Prendergast, unpublished results] and if we consider the cone angles, the symmetry axis at $T \ll T_c$ being displaced on average about 10°. On the other end of the scale, at $T \gg T_c$, the order parameter approaches a value of ~0.21 (corresponding cone angle ~60°), and the experimental error in determination of these values through determination of r_∞ grows increasingly large (>20%). A physical interpretation of these data would be that at $T \gg T_c$ the DPH molecule is able to redistribute itself in the bilayer during its fluorescence lifetime in much the manner implied by Figure 8. (The figure, in fact, implies that DPH may even translate to some extent and in such a manner as to further influence—decrease—the measured r_∞.) In any event, it is unlikely that the order parameter determined from these data could reasonably be compared to one or two methylene moieties of a fatty acyl chain. Furthermore, when we state that DPH is located in a plane normal to the plane of the bilayer and intercalated between the fatty acyl moieties of the phospholipids, we cannot assume a specific relation to unique methylene units of the fatty acyl chains. It is rather more likely that DPH occupies only some "average" position relative to the fatty acyl chains due to the "vertical" diffusion of the DPH molecule (E. Taswell, L. W. Engel, and F. G. Prendergast, unpublished results). In fact, such positional averaging normal to the plane of the bilayer would go a long way toward explaining the findings of Podo & Blaisie (1972) that probe molecules perturb the lipid bilayers over areas in excess of those determined by the dimensions of the probe. We might propose that such "longer range" perturbation in the sense of the data of Podo and Blaisie should be considerably less for a probe molecule that is tethered, either by charge interactions at the interface or by covalent bonding, say, to a phospholipid, than for freely mobile probes such as DPH. TMA-DPH clearly senses a different region of the bilayer, and the difference in the behavior of S is obviously related to the fact that the molecule is tethered to the interface. From the data we have, we see no reason to assume other than that the DPH portion of the molecule is oriented normal to the plane of the bilayer, and so the order parameter for this probe provides a reasonable measure of the orientational constraint imposed partially by electrostatic interaction at the interfacial region and by the ordering effects of the glycerol backbone region and the upper portions of the fatty acyl moieties of the phospholipids. It may, however, be entirely reasonable to compare the order parameters calculated for TMA-DPH with those obtained from

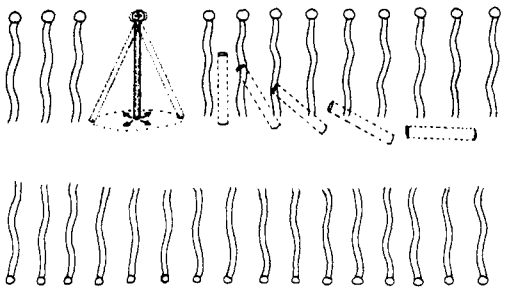

FIGURE 8: Models of motion of TMA-DPH (cationic moiety) tethered at the interface and DPH (represented as a dashed cylinder) embedded in lipid bilayers. It is proposed that TMA-DPH cannot penetrate into the hydrocarbon regions of the bilayer and so in its average motions carves out a true "conical" volume as depicted. DPH, when largely restricted, may exhibit similar motion in a cone but in a disordered membrane can also redistribute in the bilayer by a type of translational motion that may be responsible for some depolarization of the fluorescent signal. Diffusion of the probe in a plane normal to that of the bilayer should not affect the apparent polarization of the probe.

NMR experiments that define the behavior of the more proximal portions of the phospholipids. The observations made by Brown (1979) and Brown et al. (1979) of "an approximately constant effective correlation time over almost half the length of the fatty acyl chains, i.e., a plateau is observed in correlation times from chain segments C_3 to about C_9", are apposite. They suggest that the segmental motions in this region of the bilayer are rather strongly correlated, which gives credence to the calculation of an "average" order parameter over this region. However, perhaps the most useful comparison of fluorescence order parameters with those of NMR may be found in the model of Peterson & Chan (1977). These authors provided an elegant analysis of proton and deuterium order parameters measured for the liquid-crystalline phase of lipid bilayers in terms of chain reorientation and chain isomerization occurring via kink diffusion. The angles estimated for the chain deflection were of the order of 50°, which are attractively close to those that may be calculated from the Gaussian cone angle model presented here for the deflections of DPH (an indirect reflection of the fatty acyl chain motion). By analogy we may estimate from the TMA-DPH that the maximum angular deflection of the upper region of the chains/glycerol backbone is <35°. But neither intuitive nor mathematical analysis of the fluorescence data allows evaluation of fatty acyl chain dynamics specifically in terms of chain isomerizations and/or rates of kink diffusion.

TMA-DPH cannot "dive" as DPH can because of the quaternary ammonium moiety, and so the order parameter for this probe is unlikely to be influenced by translational motion. For these reasons, the "cone model" of fluorophore rotation is likely to be most effectively applied to the motions of this probe and others like it such as parinaric acid rather than to probes of the "hydrocarbon" region of the bilayer such as DPH. Heyn (1979) has also made this argument. Indeed, the most realistic measurements of the extent of chain motion will come only from direct measurements of chain dynamics, say, with a parinaroyl phospholipid.

It may be reasonable to use the cone angles to estimate the approximate (average) volume in which the probe is free to "rotate" as a function of temperature or to calculate the average separation of fatty acyl chains that would provide this volume at least in the vicinity of the fluorophore, and this free volume obviously may be unique to the fluorophore. Nevertheless, Fulford & Peel (1980) have performed calculations on free volumes of DPH in bilayers at various temperatures and used the changes in them to estimate the lateral pressure in the bilayer. The values obtained agreed rather well with those calculated by others [for discussion see Fulford & Peel (1980)].

Finally, we need to emphasize that the order parameter is a static distribution parameter. Consequently, while there is an apparent relation between disorder and microviscosity (η) in bilayers, this relation is not mathematically definable at present, and thus values of η calculated from r_{ss} data are quantitatively significant only on a relative basis. The problem is more fully discussed by E. Taswell, L. W. Engel, and F. G. Prendergast (unpublished results).

On the Relationship between r_∞ and r_{ss}. There would be an obvious advantage to be able to estimate r_∞ (and hence S) from values of r_{ss}. Recently, Fulford & Peel (1980) have used data of Hildebrand & Nicolau (1979) to deduce the empirical relation

$$r_\infty = 1.198 r_{ss} - 0.055$$

which implies a linear relation between r_{ss} and r_∞. In Figure 9 we have plotted r_{ss} vs. r_∞ from our data and show that for

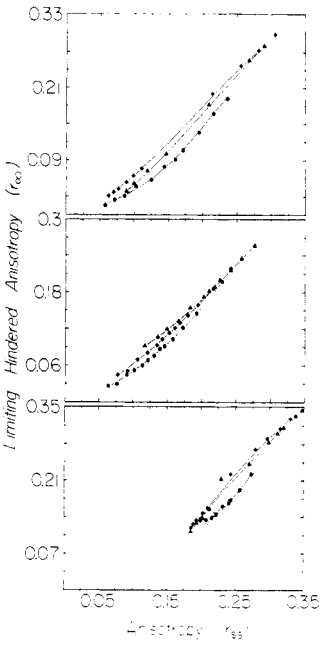

FIGURE 9: Plots of r_{ss} vs. r_∞ for DPH and TMA-DPH in lipid bilayers. Data are provided for DPH in DMPC (▲), DPPC (+), and POPC (●) (upper panel), for DPH in vesicles containing POPC and cholesterol in molar ratios of 3:1 (▲), 7.5:1 (+), and 20:1 (●) (middle panel), and for TMA-DPH in SOPC (+), POPC (▲), DMPC (○), and DPPC (●) (lower panel).

both TMA-DPH and DPH, there is indeed a fairly linear relation between the two parameters for the data from saturated PC vesicles but that neither the slope nor the intercept agree with the equation given by Fulford & Peel (1980). The relation is, however, decidedly nonlinear for the probes in POPC. While it would be of obvious value to be able to calculate r_∞ from r_{ss}, we caution against use of semiempirical formalisms until there are more data to support such estimations [cf. Fulford & Peel (1980) and Heyn (1979)]. The plots of r_∞ vs. r_{ss} do, however, serve to emphasize that steady-state anisotropies mainly measure order, not rotational dynamics per se.

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References

Brink, D. M., & Satchler, G. R. (1961) *Angular Momentum*, 2nd ed., Oxford University Press, London.

Brown, M. F. (1979) *J. Magn. Reson. 35*, 203.

Brown, M. F., Seelig, J., & Häberlen, U. (1979) *J. Chem. Phys. 70*, 5045.

Dale, R. E., Chen, L. A., & Brand, L. (1977) *J. Biol. Chem. 252*, 7500.

Davis, J. H. (1979) *Biophys. J. 27*, 339.

Davis, J. H., & Jeffrey, K. R. (1977) *Chem. Phys. Lipids 20*, 87.

Fulford, A. J. C., & Peel, W. E. (1980) *Biochim. Biophys. Acta 598*, 237.

Heyn, M. P. (1979) *FEBS Lett. 108*, 359.

Hildebrand, K., & Nicolau, C. (1979) *Biochim. Biophys. Acta 553*, 365.

Jähnig, F. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6361.

Kawato, S., Kinosita, K., Jr., & Ikegami, A. (1977) *Biochemistry 16*, 2319.
Kinosita, K., Jr., Mitaku, S., Ikegami, A., Ohbo, N., & Kunii, T. L. (1976) *Jpn. J. Appl. Phys. 15*, 2433.
Kinosita, K., Jr., Kawato, S., & Ikegami, A. (1979) *Biophys. J. 20*, 289.
Lakowicz, J. R., Prendergast, F. G., & Hogen, D. (1979a) *Biochemistry 18*, 508.
Lakowicz, J. R., Prendergast, F. G., & Hogen, D. (1979b) *Biochemistry 18*, 520.
Lipari, G., & Szabo, A. (1980) *Biophys. J. 30*, 489.
Mely-Goubert, B., & Freedman, M. H. (1980) *Biochim. Biophys. Acta 601*, 315.
Peterson, N. O., & Chan, S. I. (1977) *Biochemistry 16*, 2657.
Podo, F., & Blasie, J. K. (1972) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1032.
Prendergast, F. G., Haugland, R. P., & Callahan, P. J. (1981) *Biochemistry* (preceding paper in this issue).
Szabo, A. (1980) *J. Chem. Phys. 72*, 4620.
Weber, G. (1977) *J. Chem. Phys. 66*, 4081.

Structural Similarities between Human Receptors for Somatomedin C and Insulin: Analysis by Affinity Labeling†

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ABSTRACT: Human placental receptors for insulin and somatomedin C (Sm-C) were affinity labeled with [^{125}I]insulin and [^{125}I]Sm-C by using the bifunctional cross-linking agent disuccinimidyl suberate. Analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis demonstrated that both labeled hormones were specifically cross-linked to three protein species with apparent molecular weights of 240 000, 310 000, and 330 000. Following disulfide bond reduction, subunits of approximately 140 000 daltons were evident. Partial reduction of disulfide bonds yielded intermediate-sized species with apparent molecular weights of 180 000, suggesting the existence of an additional, smaller subunit attached to the 140 000-dalton subunit. Limited proteolysis of the hormone–receptor complexes with chymotrypsin, trypsin, and *Staphylococcus aureus* V-8 protease gave similar but not identical results for each labeled receptor. The distinction between the two receptors was further documented by inhibition of affinity labeling with graded amounts of the native hormones. These data demonstrate a substantial structural similarity between the human Sm-C and insulin receptors paralleling the homology of the native hormones and their actions.

The somatomedins (Sm's)[1] are small (approximate M_r 7500), growth hormone dependent, single-chain polypeptides which possess growth-promoting and insulin-like actions in vitro (Van Wyk & Underwood, 1978; Zapf et al., 1978; Phillips & Vassilopoulou-Sellin, 1980). At concentrations of 10^{-9}–10^{-10} M, Sm's are mitogenic for a variety of cultured cell lines, whereas 100-fold higher concentrations are required to elicit insulin-like responses. Two forms of human somatomedin have been substantially characterized. The basic form (p*I* 8.2–8.4) is known variously as somatomedin C (Sm-C) (Van Wyk et al., 1974; Svoboda et al., 1980), insulin-like growth factor I (IGF-I) (Rinderknecht & Humbel, 1978a), or "basic somatomedin" (Bala & Bhaumick, 1979). The neutral form of somatomedin has been termed insulin-like growth factor II (Rinderknecht & Humbel, 1978b).[2] Both the basic and neutral forms of Sm are structurally similar to human proinsulin (Blundell et al., 1978). Multiplication stimulating activity (MSA), a peptide isolated from the conditioned medium of rat hepatocyte cultures (Dulak & Temin, 1973; Moses et al., 1980), appears to be the rat equivalent of human IGF-II since the amino acid sequences of the two peptides are identical except for conservative substitutions at five positions (Marquardt et al., 1981).

The growth-promoting actions of Sm are thought to result from their interaction with specific somatomedin receptors, whereas the insulin-like effects are believed to occur by cross-reactivity with the insulin receptor (Clemmons et al., 1974; Van Wyk et al., 1975; Rechler et al., 1977; Zapf et al., 1981). Conversely, the mitogenic effects of insulin, usually demonstrable only at supraphysiologic concentrations, are believed to be mediated via the Sm receptor. Although cross-reactivity of Sm and insulin with their respective receptors may be due to the structural homology of the hormones, it may also reflect structural similarity of the receptors.

The structure of the insulin receptor has been studied by using a variety of methods and tissues sources (Sahyoun et al., 1978; Jacobs et al., 1979; Pilch & Czech, 1979, 1980; Wisher et al., 1980; Yip et al., 1980; Harrison & Itin, 1980; Van Obberghen et al., 1981). These data have shown that the native insulin receptor is a large (>300 000-dalton) glycoprotein composed of four disulfide-linked subunits. It appears that two identical 125 000–140 000-dalton peptide sub-

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[1] Abbreviations used: DSS, disuccinimidyl suberate; DTT, dithiothreitol; IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; INS, insulin; MSA, multiplication stimulating activity; NaDodSO$_4$, sodium dodecyl sulfate; Sm, somatomedin; Sm-C, somatomedin C.

[2] Somatomedin A (Fryklund et al., 1974) is not included in this classification since this peptide remains to be fully characterized. It is described as a neutral peptide, but its biological properties are more similar to those of Sm-C/IGF-I.

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