The Concept of Lipid Domains in Membranes

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In the past two decades or so there has been considerable advance in our knowledge of membrane structure. The fluid mosaic model of Singer and Nicolson (1) has had great heuristic value in thinking about membrane topology. In this model both proteins and lipids are free to diffuse in the bilayer, implying a random organization of protein and lipid. However, experimental evidence in diverse types of membranes and for several protein entities indicates that the lateral motion of most proteins is not determined primarily by free diffusion through a two-dimensional viscous fluid, but is constrained, probably by mechanisms such as interactions with cytoskeletal components. We will here present our evidence that lipids may also have nonhomogeneity in their lateral distribution, i.e., the lipid may be organized in domains, and that such organizational heterogeneity may have functional as well as structural significance. Although somewhat controversial, it is not inconceivable that proteins could order the packing of surrounding lipids by cooperative types of interactive forces (2), perhaps to a distance of ~50 Å. Domains could also be created by the segregation of the lipid components themselves. Lateral phase separations have been demonstrated in liquid-liquid and liquid-gel mixed systems (3), and cholesterol may play a role in such phase separations (4). Another suggested cause of lateral phase separation of lipids is a physical alteration in the membrane structure, such as a change in the radius of curvature (5). Transmembrane lipid asymmetry (6) in itself could create anisotropic forces that could induce imperfect lipid mixing.

Evidence for heterogeneity of lateral organization has been adduced in a variety of studies, including x-ray diffraction (7), electron microscopy (8), lateral diffusion measurements (9), differential partitioning of lipid probes (10, 11), differential scanning calorimetry (12), and spin-label measurements (13). We here present our own studies in support of the concept of membrane lipid domains.

Site Heterogeneity in Membranes

An obvious consequence of a lipid domain model is that there should be site heterogeneity in membranes. We have used 1,6-diphenyl-1,3,5-hexatriene (DPH) as a suitable probe for detecting site heterogeneity by studying its fluorescence lifetime decay. DPH locates in the hydrophobic acyl region of the membrane (14); as shown by differential scanning calorimetry, it does not perturb membrane structure (15). Most importantly, it weights equally fluid and less-fluid regions (5), and the lifetime decay is sensitive to the phase of the lipid constituents (16), i.e., the lipid environment in which the probe resides. The modulation and phase lifetimes at various modulation frequencies in both model and biological membranes were measured (16). The analysis of the heterogeneity that corresponds to these phase and modulation lifetimes was performed using a non-linear least squares algorithm in which the χ^2 value was used as a marker for the goodness of fit. The decay was analyzed in terms of two components characterized by the intensity and rate of decay. Thus, the fluorescence decay from the excited state depends on the type of environment in which the probe resides: in homogeneous systems the decay is monoexponential, in heterogeneous systems, multiexponential; furthermore, in fluid phases the lifetimes are shorter than in more solid phases.

The studies on single phase systems showed that the decay of DPH either in isotropic fluid solvents or in single phase lipid systems, either above or below the phase transition, is homogeneous; that is, the decay could be well described by essentially a single exponential or single lifetime (Fig. 1). In fluid systems (e.g., dilauroyl lecithin [DLL] vesicles at 25°C), the lifetime is shorter than in less fluid phases (e.g., dipalmityl lecithin [DPL] vesicles at 25°C). Thus this difference of decay of DPH in liquid or more solid phases should allow for detection of heterogeneity in membrane systems. In a mixture of single-phase lipid vesicles (one set fluid [DLL] and one set more solid [DPL]) held at 25°C, in which the fluorescence intensity from each was equal, two components of decay were detected, corresponding to the single-phase values (Fig. 1). However, when the decay was measured in mixed-lipid, mixed-phase systems (that is, vesicles composed of DLL and DPL) held at 25°C, in which there is, according to Shimshick and McConnell (17), coexistence of fluid and gel phases, it was found that although two components provided an excellent fit, one of the two lifetimes (corresponding to roughly half the intensity) was extremely short—about 4 ns. Since this short component is not

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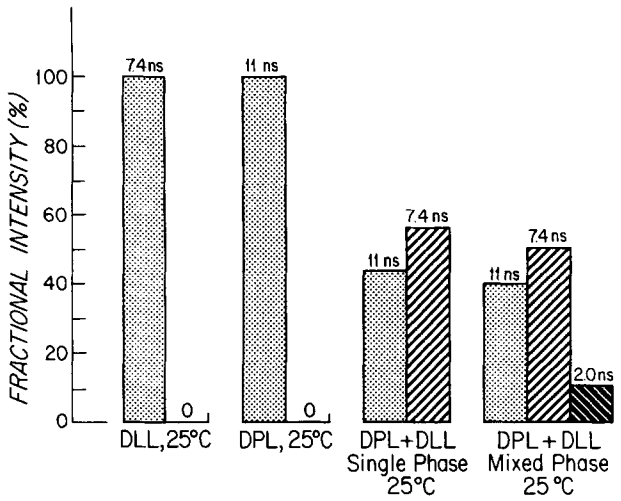

FIGURE 1 DPH lifetimes in single- and mixed-phase systems. The differences in the lifetime values shown for each system are significant. The fractional intensity is that proportion of the intensity represented by each lifetime. DLL and DPL liposomes show a single lifetime. A mixture of DPL and DLL single-phase liposomes at 25°C shows the two lifetimes characteristic of each of the constituent liposomes. Mixed fluid-gel phase liposomes consisting of DPL and DLL display heterogeneity. See text and reference 16 for further details. *DLL*, dilauroyl phosphatidylcholine; *DPL*, dipalmitoyl phosphatidylcholine.

observed in any single phase lipid system, we decided that a two-component description of the decay was probably inadequate. The next level of complexity, a three-component fit, cannot be performed uniquely. We therefore decided to model the decay by three components, with two of the lifetimes fixed a priori to the pure single phase values, allowing only the third lifetime and the amplitudes to vary. The most intriguing feature of this analysis was that the lifetime of the third component, comprising roughly 10–20% of the intensity, was quite short—about 2–3 ns. It was suggested (16) that the short lifetime may well represent an interface between domains where DPH is quenched due to the binding of a small amount of water in this boundary. Whatever the short lifetime represented, the system was clearly heterogeneous.

Thus, having shown that it was possible to detect site heterogeneity, we next examined biological membranes, and showed that in a variety of isolated and purified biological cell surface membranes there was distinct site heterogeneity, in that lifetime decays were multi-exponential (Fig. 2). In the biological systems we imposed, because of technical limitations, a two-component analysis on what may be inherently a much more heterogeneous system. The lifetimes given do not reflect the proportions of the lifetime domains, but clearly reflect heterogeneity. In similar studies, others have found that the purple membrane of *Halobacterium halobrium* (18), mitochondrial membranes (18), and L1210 cell membranes (19) are heterogeneous. The only exception we found in the biological membranes investigated was the erythrocyte membrane, which appears to be homogeneous in lipid organization (16).

A second method for obtaining evidence for heterogeneity would be to determine whether lipophilic molecules exhibit lateral phase partitioning preference. We derived from colligative solution theory that lipophilic molecules should exhibit such lateral phase partitioning preference (16). We used, for reasons that will be evident later, free fatty acids as probes of membrane lipid structure, and tested whether different classes of free fatty acids preferentially partitioned into either fluid or gellike lipid phases. In simple terms, we used the basic thermodynamic principle that certain types of molecules preferentially inserted into a fluid phase would decrease the melting temperature, whereas those inserted into a gel phase would increase the melting temperature; thus the direction of change in melting temperature would indicate whether the test fatty acid prefers the fluid or gel phase. We found (Table I) that the phase transition of dimyristoyl lecithin (DML) vesicles could be shifted either downward or upward, corresponding to the preferential partitioning into fluid or gel phases. The fatty acids fell into two classes: the *cis*-unsaturated (group A), which decreased the melting temperature and were thus inferred to have a fluid-phase preference, and the *trans*-unsaturated or saturated fatty acids (group B), which increased the melting temperatures and were thus inferred to have shown a gel-phase preference. It should be emphasized that partitioning is preferential in each case, but not necessarily exclusive, i.e., the different types of fatty acids probably partition to different extents into both types of phases.

Because of the differential behavior of group A and group B free fatty acids described above, it was of interest to see whether there was similar differential behavior in terms of perturbation of membrane lipid structure. To this end we used fluorescence polarization of DPH to monitor structural changes in lipid. Basically, in the fluorescence polarization technique

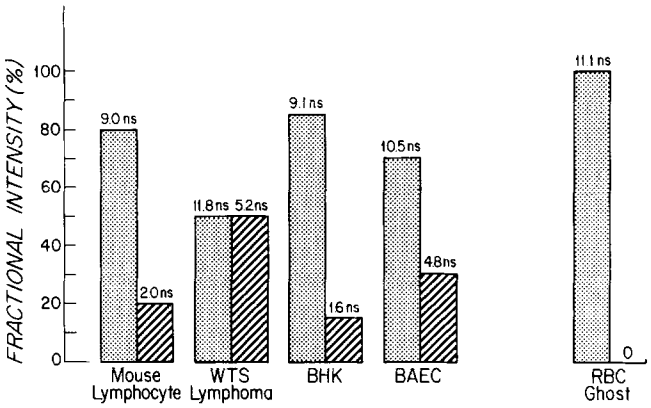

FIGURE 2 DPH lifetimes in isolated biological membranes. All the membranes show heterogeneity to a significant degree, with the exception of the erythrocyte ghost, which has a single lifetime. *WTS lymphoma*, CHI lymphoma; *BHK*, baby hamster kidney; *BAEC*, bovine aortic endothelial plasma membranes.

TABLE I

Phase Partition of Free Fatty Acids

Fatty acid	ΔT_m°C	Preferentially partitions into
Group A		
Oleic	−.8	Fluid
Linoleic	−1.7	Fluid
Arachidonic	−2.5	Fluid
Group B		
Elaidic	+2.95	Gel
Stearic	+1.9	Gel
Nonadecanoic	+5.2	Gel

The value ΔT_m represents the shift in the midpoint of the DPH polarization monitored melting curve for dimyristoyl phosphatidylcholine. An equation derived from colligative solution theory was used to relate the shift direction to the phase of the region into which the free fatty acids partition. From Klausner et al. (16).

the fluorophore, in this case DPH, is introduced into the bilayer and is excited with polarized light. The probe will emit polarized light at a characteristic angle to the direction of the exciting light, depending on the range of freedom of rotation of the probe in the membrane. The polarization value, p, is obtained by measuring the light emitted perpendicular and parallel to the direction of the exciting light. The degree of polarization thus reflects the local environmental constraints on the rotation of the probe during its excited state lifetime, i.e., the packing order of the lipids in the environment of the probe. In a gellike environment, there will be high constraints on the rotation of the probe and the p value will be high; the opposite applies to a fluid environment.

We examined multilamellar liposomes, which were exposed to either group A or group B free fatty acids. In fluid systems, for instance egg phosphatidylcholine liposomes, little or no change in p value was produced by either class of free fatty acid. However, when single-phase gel lipids were tested, a distinction between the two groups was apparent: the group A free fatty acids disordered the lipid packing to a much greater degree than did the group B. In mixed-phase vesicles, group A markedly disordered, while group B had either no effect or slightly increased p values. This is parallel to findings on spin label order parameters using long-chain alcohols as probes (20), and on the influence of fatty acids on endothermic phase transitions (21).

When we examined the effects of inserting free fatty acids into biological membranes we found, without exception, that the polarization changes produced paralleled those found in mixed-phase, gel-fluid vesicles, i.e., the group A free fatty acids caused DPH polarization to decrease, whereas the group B had little or no effect (Table II). We can surmise that group A, although preferentially partitioning into fluid phases, essentially disorder gel phases, whereas group B, preferentially partitioning into gel phase, have little overall effect.

TABLE II

DPH Polarization Values in Selected Plasma Membrane

Plasma membranes	Free fatty acids	p (DPH)	% Δp
Mouse lymphocyte	Control	0.264	0
	Linoleic	0.209	−21
	Stearic	0.265	0
CHI lymphoma	Control	0.313	0
	Linoleic	0.260	−17
	Stearic	0.315	+1
BHK cell	Control	0.265	0
	Linoleic	0.244	−8
	Stearic	0.265	0
Bovine aortic endothelial cell	Control	0.286	0
	Linoleic	0.263	−8
	Stearic	0.286	0
Human platelet	Control	0.294	0
	Linoleic	0.258	−10
	Stearic	0.305	+2
Human PMN*	Control	0.305	0
	Linoleic	0.273	−11
	Stearic	0.313	+3
RBC ghosts	Control	0.310	0
	Linoleic	0.240	−23
	Stearic	0.262	+2

Plasma membranes were labeled with DPH and the fatty acids were added to a final concentration of 10 μg/ml. After the incubation at 25°C, the preparations were washed and the p values measured.

* Intact cells.

Taking the lifetime heterogeneity measurements, the preferential partitioning data, and the selective perturbation data all into account, it seems not unreasonable to state that the biological membranes examined to date are heterogeneous in their lipid structure, with the exception of the erythrocyte membrane, which decays monoexponentially. In this light, the perturbation experiments on p values would also suggest that the erythrocyte membrane is gellike, i.e., the group A disorder, whereas the group B have no effect or slightly increase order; this conclusion is especially cogent in view of the long monoexponential lifetime of 11 ns.

One might ask whether the perturbation by the free fatty acids produced in biological membranes was due to a global fluidization. Kleinfeld et al. (22) measured the lateral diffusion rates of lipids and proteins in biological membranes perturbed by group A free fatty acids and found no change in the diffusion coefficients, although there were marked decreases in p values. Converting the p values into microviscosity units, which is frequently done (but which, in our view, is erroneous and misleading) showed a decrease of as much as 50% in microviscosity. Hence there is no correlation between the change in p value, which primarily reflects the static disordering of the acyl chains, and the lateral diffusion of membrane constituents. Additionally, these results may suggest that the fatty acid perturbation is local, since the p value change presumably reflects the disordering of the gel regions.

Functional Effects of Membrane Lipid Structure Perturbation

On the basis of the concept of lipid domain structure, it was of interest to see whether we could selectively perturb functional events in membranes with the different free fatty acids that we used as probes for the selective perturbation of membrane lipid structure.

FREE FATTY ACID EFFECTS ON CAPPING: It was found that the anti-IgG antibody-induced capping of SIg (but not patching) on murine B lymphocytes was selectively and almost completely inhibited by group A free fatty acids, but not affected by group B (Fig. 3) (23) when the cells were exposed for a few minutes to low concentrations (5–10 μg/ml) of the fatty acids. The partitioning of the free fatty acids into the surface membrane was about the same for both types of free fatty acids: they achieved a concentration of ~5 mol % phospholipid, and were present in the membrane essentially nonesterified (>90%). We have recently shown (24) that this inhibition of capping is not due, as has been claimed (25), to decreased ATP levels and uncoupling of oxidative phosphorylation, nor is it related to prostaglandin metabolism (23). There was no change in the lateral mobility of the cross-linked SIg patches as shown by fluorescence photobleaching recovery (23). The inhibition of capping was reversed by raising the external calcium concentration, whereas magnesium had no such effect. As the sweeping of IgG patches into a cap is dependent on metabolism and activities of cytoskeletal elements, it was postulated that selective perturbation of membrane lipid structure had interrupted some Ca^{++}-dependent transmembrane, transductive link between membrane moieties and the cytoskeleton (23).

FREE FATTY ACID EFFECTS ON CYTOSKELETON: It was thus of some interest to examine the short-term effects of free fatty acids per se on the organization of cytoskeletal elements in lymphocytes. Treatment of mouse lymphocytes with group A free fatty acids, under the same conditions as inhibited

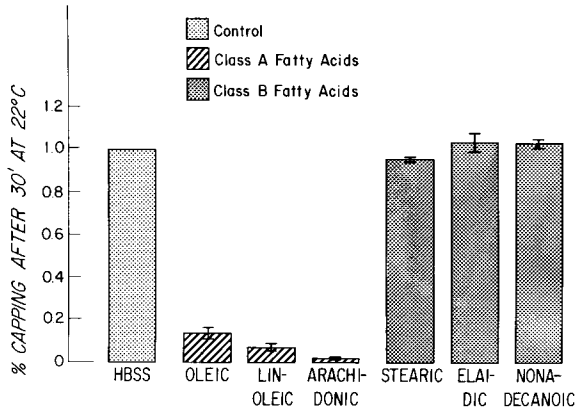

FIGURE 3 Inhibition of SIg on murine B cells capping by different free fatty acids. 10 μg of each fatty acid/ml was added to 8×10^6 cells per ml at 22°C. Shown is the fraction of cells capped relative to control cells in Hanks' balanced salt solution (HBSS); SEM is given. From Klausner et al. (23).

capping of SIg, produced dramatic alterations in the immunofluorescence patterns of the cytoskeleton and contractile proteins (26). In untreated control cells, the microtubular pattern exhibited microtubules radiating from an organizing center, resembling the spokes of an umbrella (Fig. 4). The addition of group A free fatty acid produced a single large submembranous aggregate of antitubulin fluorescence at one pole of the cell (Fig. 4). By electron microscopy intact microtubules were found to be present in this aggregate. Under control conditions, staining for actin revealed a diffuse pattern over the entire cell, but the addition of group A free fatty acids also caused the formation of a submembranous aggregate (Fig. 5). The pattern for alpha-actinin normally showed perinuclear staining on a diffuse background. Group A free fatty acids similarly caused submembranous aggregate formation. Group A free fatty acid treatment also caused the pattern for myosin to change from diffuse staining to uniform submembranous patching around the periphery of the cell. For all of these entities, the effects of the group A free fatty acids were partially reversed by external Ca^{++}, but not by Mg^{++}. Group B free fatty acids had no effect on the distribution of tubulin, actin, alpha-actinin, or myosin. The parallelism between the effects of the free fatty acids on capping and on cytoskeletal distribution patterns, as well as the reversal of the effects specifically by Ca^{++}, suggests that the thesis of an alteration in the interaction of surface receptors with the cytoskeleton through perturbation of specific lipid domains may well be tenable. Evidence has recently been obtained suggesting a conformational change due to a free fatty acid–induced perturbation of lipid domains in a Ca^{++}-binding cytoplasmic facing membrane protein (27). In these studies, using Tb^{3+} as a Ca^{++} analogue, it was found that group B, but not group A, free fatty acids caused increase of the tryptophan-mediated fluorescence of membrane-bound Tb^{3+}.

ROLE OF CHOLESTEROL IN IgG CAPPING: We have examined the effects of membrane cholesterol levels on IgG capping in mouse lymphocytes (28). As cholesterol was depleted from the cells by exposing them to phosphatidyl choline/phosphatidic acid vesicles, capping was decreased proportionately. Concomitant with the decrease in cholesterol levels, there was a decrease of DPH fluorescence polarization values, indicating a progressive disordering of lipid packing. This, as well as capping, was restored to normal values by replenishing

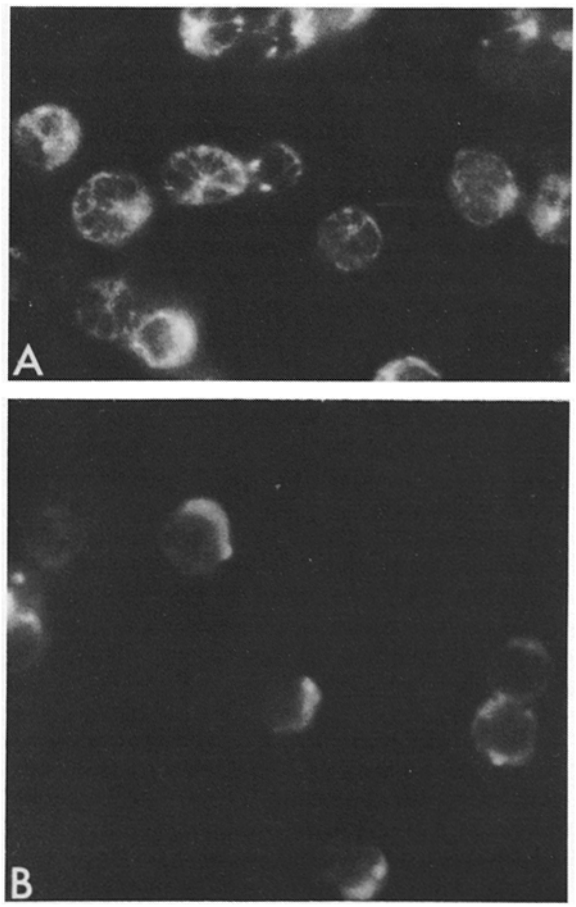

FIGURE 4 Immunofluorescence pattern of antitubulin antibody applied to mouse lymphocytes. (*A*) Control; (*B*) linoleic acid–treated (10 μg/ml). From Hoover et al. (26). × 800.

membrane cholesterol. Remarkably, stearic acid as a representative group B free fatty acid could also restore normal levels of polarization values and capping after cholesterol depletion. This would suggest that depletion of cholesterol makes gellike domains more fluid, and that stearate, partitioning into these domains, restores their gellike qualities. Furthermore, it may be postulated that group A free fatty acids, although preferentially partitioning into fluid domains, exert their biological effects in the capping system by also partitioning into gellike domains and fluidizing them. However, group B free fatty acids cannot reverse the effects of group A, possibly because there is little or no available volume in the pertinent domains for their insertion; however, removal of the cholesterol leaves "space" in the domains, either for replenishment of cholesterol or for a group B free fatty acid to enter the requisite domains and render them more gellike.

FREE FATTY ACID EFFECTS ON PLATELET AGGREGATION: Brief exposure of human platelets to group A free fatty acids completely abolished the aggregatory effects of ADP thrombin and collagen (29). It was established that primary as well as secondary aggregation, shape change, and release reactions were all inhibited. Thromboxane production in response to thrombin was halved, though not abolished, but the platelets were nevertheless unresponsive. The inhibitory effects could be overcome by the calcium ionophore A23187, which bypasses the initial receptor occupancy stage in aggregation. The inhibition of aggregation also occurred in indomethacin-treated platelets, indicating that inhibition was not due to

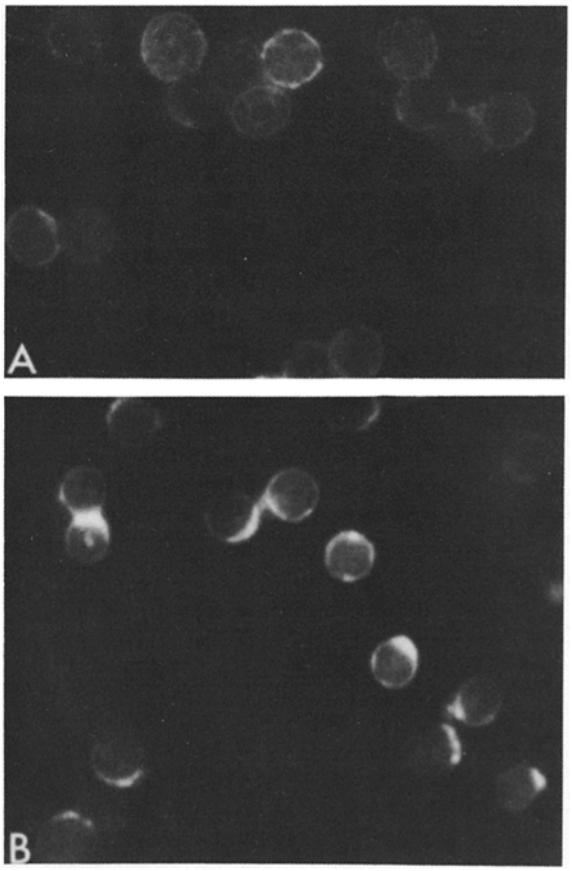

FIGURE 5 Immunofluorescence pattern of antiactin antibody applied to mouse lymphocytes. (*A*) Control; (*B*) linoleic acid-treated (10 μg/ml). From Hoover et al. (26). × 800.

prostaglandins, and the inhibitory effect was obtained as well in platelets treated with an inhibitor of adenylate cyclase, 2′,5′-dideoxyadenosine, indicating that the inhibition of aggregation was not due to raised cyclic AMP levels. Group B free fatty acids had no inhibitory effect on aggregation.

The endoperoxide analogue U46619 induces primary aggregation and platelet shape change; these effects were completely abolished by group A free fatty acids, suggesting a primary effect due to perturbation of membrane lipid structure at the cell surface itself.

OTHER EXAMPLES OF GROUP A AND GROUP B FREE FATTY ACID EFFECTS: When we had established that group A and group B free fatty acids produced different biological effects in the systems we had studied, effects that accord with and can be interpreted in the light of the lipid domain model, we reviewed the quite extensive literature on the biological effects of free fatty acids on various biological functions to see whether similar patterns of behavior prevailed. In a number of published studies it is clear on reexamination that the fatty acids can once again be grouped into A (*cis*-unsaturated) and B (*trans*-unsaturated and saturated) in terms of the effects produced. Some of these examples are given in Table III.

Although in general terms the differential effects can be related to group A and group B free fatty acids, subtleties such as number of double bonds, position of double bonds, and chain length introduce variations into the pattern. We will not discuss these stereochemical relationships here, although they are of obvious import to the mode of perturbation of membrane lipid environment. Furthermore, the precise mode for each

TABLE III

Functional Differentiation of Group A and Group B Free Fatty Acids

Function	Group A	Group B	Reference
Temperature agglutination in 3T3 cells			
Wheat germ agglutinin	No effect	Inhibit below 32°C	30
Concanavalin A	Inhibit below 5°C	Inhibit below 25°C	
Adhesion of BHK cells	Inhibit	No effect	31
Adhesion of mouse lymphocytes	Inhibit	Stimulate	32
Chloride transport in isolated frog cornea	Stimulate	No effect	33
D-Glucose transport in isolated plasma membranes from rat adipocytes	Stimulate (− insulin) No effect (+ insulin)	No effect (− insulin) Inhibit (+ insulin)	34
Glucose-6-phosphate transport in rat liver membranes	Inhibit	No effect	35
Brain Na,K-ATPase	Inhibit more	Inhibit less	36
Fusion of chromaffin granules	Stimulate	No effect	37
Superoxide production by leukocytes	Stimulate	No effect	38

system whereby the free fatty acids affect specific biological functions is at present unknown. Nevertheless, it is felt that the functional experiments provide indirect but quite compelling support for the concept of membrane lipid structural heterogeneity, i.e., lipid domains.

The concept of the organization of the lipid components of membranes into domains raises several questions, which can be briefly mentioned. Firstly, do specific membrane proteins reside in specific lipid domains, and can perturbation of the specific domain structure affect protein structure and function? Several of the biological studies mentioned above have been interpreted in this light (e.g., 23, 28–30). Secondly, do lipophilic molecules and drugs preferentially partition and segregate into specific domains rather than into a bulk lipid phase, and may such unique partitioning predicate specific functional effects? Thirdly, since the very concept of domains implies domain boundaries or interfaces, what is the possible biological significance of such interfaces? Lastly, what forces underlie the formation, mainentance, and fluctuation of lipid domains?

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