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Membrane Bilayer Assembly in Neural Tissue of Rat and Squid as a Critical Phenomenon: Influence of Temperature and Membrane Proteins

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Summary. Cell membrane bilayers have been reconstructed in vitro utilizing total lipid extracts from rat neural tissue (forebrain, cerebellum, brainstem and spinal cord) and from the optic lobe and fin nerve of the squid *Loligo pealei*. In agreement with the critical state theory of bilayer assembly (Gershfeld, N.L. 1986. *Biophys. J.* **50**:457–461; Gershfeld, N.L. 1989. *J. Phys. Chem.* **93**:5256–5261), these lipid extracts spontaneously formed purely unilamellar structures in aqueous dispersion, but only at a critical temperature, T^*, which was species dependent. For all the rat tissues $T^* = 37 \pm 1$°C; for squid neural extracts $T^* = 15.5 \pm 1.4$°C. These values correspond to 'physiological' temperatures for both organisms, implying that their lipid metabolism is geared to permit spontaneous assembly of unilamellar membranes at the ambient temperature in the tissues. Membrane protein composition had little or no effect on critical bilayer formation.

Key Words bilayer assembly · neural membranes · critical temperature

Introduction

Lipid bilayer assembly in cellular membranes has been characterized as a spontaneous process occurring only at a critical temperature, namely, the membrane's physiological temperature (Gershfeld, 1986, 1989*a,b*). Supporting this concept is direct correspondence between the temperature at which cell-free membrane lipids spontaneously form a pure unilamellar state and the ambient temperature of the original membranes in vivo. Thus, aqueous dispersions of total lipid extracts from different species of bacteria form a pure unilamellar state but only at a critical temperature T^* which was found to be identical to the growth temperature of the source organisms. This relationship held for bacteria cultured at temperatures varying between 20 and 60°C; similarly $T^* = 37$°C for dispersions of lipids extracted from human erythrocytes (Gershfeld, 1986). The corollary to these observations is that the lipid composition of cellular metabolic pools is controlled such that unilamellar membrane assembly will occur spontaneously at the cell's ambient temperature (Gershfeld, 1986, 1989*b*).

The thermodynamic basis for describing biological membrane bilayer assembly as a critical phenomenon was developed in a systematic study of phospholipid dispersions in which it was demonstrated that a pure unilamellar state will form spontaneously only at a singularity in temperature T^* (Tajima & Gershfeld, 1985; Gershfeld, Stevens & Nossal, 1986; Gershfeld, 1989*a*). When T exceeds or falls below T^* the unilamellar state does not form; the equilibrium structure is generally a multibilayer. Assembly of the unilamellar critical state conforms to conditions imposed by the phase rule. Thus T^* depends on lipid composition and the phase relations in the equilibrium dispersion. In general, the unilamellar state forms only as the liquid crystal (Gershfeld, 1989*a*). Once formed, the critical bilayer exhibits predictable responses to changes in ambient temperature. When $T > T^*$ the bilayer transforms to the state characteristic of the new equilibrium conditions, the multibilayer; when $T < T^*$ the unilamellar state supercools and remains stable until nucleating conditions for the transformation to the equilibrium multibilayer appear (Gershfeld & Murayama, 1988; Gershfeld, 1989*b*). The formation of a multibilayer at the expense of the unilamellar state in cell membranes is potentially catastrophic (Gershfeld, 1989*b*).

To establish whether critical bilayer assembly occurs in more complex membranes we here report measurements of T^* for lipids from neural tissues of rat and squid. In these systems we again demonstrate a striking correspondence between T^* and the core temperature of the parent organism. A subsidiary question concerns the influence of proteins on bilayer assembly. Critical temperatures were therefore obtained both with crude total neural lipid extracts, contaminated with membrane protein, and with extracts manipulated to eliminate any protein effects. Values

for T^* were identical in the two preparations, suggesting that membrane bilayer assembly is an intrinsic lipid property, independent of this protein composition.

Materials and Methods

Tissue Preparation and Lipid Extraction

The following tissues were dissected from an adult Wistar rat immediately after sacrifice: whole forebrain, whole cerebellum, brainstem and cervical spinal cord. The optic lobe and fin nerve of the squid *Loligo pealei* were obtained fresh at the Marine Biological Laboratory, Woods Hole, MA. Eight medium-sized squid were dissected using standard techniques (Gilbert, Adelman & Arnold, 1989), and the neural tissues were pooled. Immediately after removal, all tissues were homogenized and macerated in 2-propanol at 4°C. Chloroform was added to the homogenates to produce a final 2-propanol/chloroform ratio of 11 : 7 (Rose & Oklander, 1965); this solvent composition yields a homogenous organic phase. Extractions were conducted in screw-topped test tubes. Each tube was vortexed and incubated at room temperature for 1 hr, after which the homogeneous organic phases containing the lipid were separated from particulate material by low-speed centrifugation. The supernatants were evaporated to dryness and the lipids then stored as chloroform solutions at −20°C in test tubes wrapped in aluminum foil to shield them from light. Lipids were kept under nitrogen at all stages.

Removal of Protein from Lipid Extracts

Chloroform/methanol extracts of myelin contain two major classes of protein: myelin basic protein and proteolipid protein (Autilio, Norton & Terry, 1964; Gonzalez-Sastre, 1970). These same classes were found in our crude rat neural lipid extracts, and proteolipid protein was assumed also to be present in the squid preparations. We were concerned that these proteins might influence lipid bilayer assembly and therefore aimed to study the assembly process in their presence and (relative) absence. A portion of each of the chloroform stock solutions (rat and squid) was therefore purified by a solid phase extraction (SPE) technique in an attempt to remove the bulk of the protein which had co-extracted with lipid.[1] The solutions were first evaporated under nitrogen, and the dried extracts redisssolved in methanol, heating to 60°C for 10 min to facilitate protein denaturation. Undissolved material was separated by low-speed centrifugation at room temperature. The supernatants were then added directly to silica gel SPE tubes (Supelclean LC-Si, 1 ml: Supelco, Bellefonte, PA) which had been conditioned with 2–3 ml methanol just prior to sample application. Typical sample volumes were 0.2 ml containing up to 0.5 mg lipid. After application, extracts were drawn into the matrix of the column and allowed to equilibrate on the gel for 10 min before elution. The upper surface of the silica column was kept under nitrogen. Elution was achieved by drawing 2 ml methanol through the silica gel into a gas-tight syringe. This closed system prevented exposure of lipid to oxygen at any stage. There were minor lipid losses (~10%) but the relative amounts of the major phospholipid classes were the same before and after SPE treatment as shown by comparison of lipid fractions separated by high performance liquid chromatography and thin-layer chromatography.

To determine the efficacy of the SPE technique we utilized conventional protein assays (Lowry et al., 1951; Smith et al., 1985) and, for the lowest protein concentrations, a method sensitive at nanogram levels based on the interaction of protein with colloidal gold (Stoscheck, 1987).[2] From these assays we estimate that the original lipid extracts in 2-propanol/chloroform contained ≤10% protein by weight. In methanol (pre-SPE), protein was ≤1% of the weight of dissolved material, and the weight fraction fell below 0.1% post-SPE. Although protein continued to denature slowly in methanol (pre-SPE) these solutions were sufficiently stable to obtain T^* for comparison with post-SPE samples. The efficacy of the SPE method was confirmed by two further experiments: (*i*) after one passage through an SPE tube, myelin basic protein and proteolipid protein were no longer detectable in the eluate by silver staining following sodium dodecyl sulfate-polyacrylamide gel electrophoresis; (*ii*) SPE tubes were stained after use with ninhydrin: a dense band of ninhydrin-positive material appeared in the silica gel of the column matrix.

Measurement of the Critical Bilayer Assembly Temperature, T*

The experimental basis for identifying T^* was previously established in a thermodynamic analysis of aqueous lipid dispersions in equilibrium with adsorbed lipid film at the water surface (Tajima & Gershfeld, 1985; Gershfeld, 1989*a*). In principle, the chemical activities of all the components in a lipid dispersion are reflected in the behavior of the surface film with which bulk lipid is in equilibrium (Defay et al., 1966; Gershfeld, 1976). Surface properties are more sensitive indicators of chemical activity than bulk measurements for lipids which generally possess low solubilities in water (Gershfeld, 1989*c*). Thus, a surface pressure method has been developed for measuring T^*. The presence of a gas/liquid interface in the experimental system merely aids identification of T^*; the same value would be obtained in the absence of this interface (Gershfeld, 1986, 1989*a*).

[1] The standard method for removing myelin basic protein involves precipitation by electrolytes (Gonzalez-Sastre, 1970) but this may lead to lipid losses through partition into the aqueous phase. Proteolipid protein may be fractionated by virtue of its insolubility in diethyl ether (Gonzalez-Sastre, 1970; Poduslo, Everly & Quarles, 1977). However, use of this solvent proved unsatisfactory in three respects: (*i*) only ~60% by weight of the lipid was ether soluble, as previously reported (Gonzalex-Sastre, 1970); (*ii*) traces of protein were still detectable in pilot experiments where a precipitate-free ether solution of rat neural lipids was evaporated to dryness, the lipid redissolved in methanol and the resulting solution assayed for protein by a method sensitive at nanogram levels (*see below*); and (*iii*) diethyl ether may contain trace peroxide contaminants raising the possibility of inducing lipid decomposition.

[2] Conventional protein assays based on interactions with the Folin-Ciocalteau reagent (Lowry et al., 1951) or bicinchoninic acid (Smith et al., 1985) are restricted to aqueous solution, necessitating use of sodium dodecyl sulfate to solubilize membrane proteins. They are also relatively insensitive and subject to interference by lipid (Kessler & Fanestil, 1986). Methanol did not interfere with the more sensitive colloidal gold method and lipids, if anything, slightly reduced the absorbance measured at 595 nm.

The surface pressure-temperature phase diagram for a lipid dispersion readily identifies T^* as the temperature where the surface pressure reaches a maximum. Thermodynamic arguments which led to this simple conclusion have been detailed elsewhere (Tajima & Gershfeld, 1985; Gershfeld, 1989*a*). In summary, at the temperature of the surface pressure maximum, thermodynamic quantities for each component of the bulk lipid dispersion (partial molar entropies and partial molar heat contents) become equal to the corresponding surface thermodynamic quantities. Furthermore, the compositions of the surface and bulk states become identical, as confirmed experimentally in radiotracer studies (Tajima & Gershfeld, 1985). At this temperature, the surface and bulk states are therefore identical. When the bulk lipid consists of lamellar bilayers, the surface film will also be in a bilayer state. As radiotracer studies indicate that the surface film density at the temperature of the surface pressure maximum is that of a single bilayer (Gershfeld & Tajima, 1979; Tajima & Gershfeld, 1985), the dispersion must be in a unilamellar state, too. Experiments with synthetic lipids support this contention (Gershfeld et al., 1986). The surface pressure maximum is a critical point at which the surface and bulk lipid phases converge; at this temperature, T^* the entire lipid system is in a uniform unilamellar state. In addition to the radiotracer studies revealing a surface film density equal to that predicted for a bilayer at T^*, the water permeability of the surface film falls to a level consistent with the presence of a bilayer at that same temperature (Ginsberg & Gershfeld, 1985). Each of these types of measurement may be used to identify T^* for a particular lipid composition, as in principle may examination of the bulk dispersion (Gershfeld et al., 1986). But the surface pressure method remains the most accessible experimentally. Thus, critical temperatures for bacterial and erythrocyte lipid extracts (Gershfeld, 1986) were obtained from surface pressure measurements, and a similar approach has been adopted here for neural lipids.

Surface Pressure Measurements

Surface pressures, π_e, for the neural lipid extracts were measured on a horizontal float film balance by the equilibrium spreading pressure method. The design of the film balance and theoretical basis for the method have been discussed elsewhere (Gershfeld, 1976, 1986, 1989*b*). This technique entails confining a large amount of anhydrous lipid directly on the water surface within a flexible enclosure of variable area; the quantity of lipid generally far exceeds the amount which is necessary to form a saturated surface film. The equilibrium surface pressure is a characteristic property of the equilibrium that exists between the excess hydrated lipid phase and the surface film (Gershfeld, 1976). The film balance method yields identical results for equilibrium surface pressure as those obtained with a Wilhelmy plate on dilute aqueous dispersions of phospholipids (Gershfeld & Tajima, 1979; Tajima & Gershfeld, 1985). We have used the film balance method primarily because it is easier to establish the conditions of equilibrium.

Experiments were conducted in a constant temperature chamber (Gershfeld, 1986). The whole film balance system was equilibrated under nitrogen before adding lipid. Oxygen levels within the chamber were monitored with a probe electrode (model YSI 5739) connected to an oxygen meter (model YSI 51B: Yellow Springs Instrument, Yellow Springs, OH). Nitrogen levels >99% were maintained throughout all experiments. The solvent-free dried lipid was applied to the water surface using clean disposable glass loops inserted through a small aperture in the darkened, thermally insulated box which covered the film balance. Care was taken to ensure lipid was methanol free before application by drying it under nitorgen for 30 min after solvent evaporation ceased to be visible; further attempts at solvent removal with freeze-drying apparatus did not affect surface pressure results. The equilibrium spreading pressure method requires that bulk lipid be present on the water surface in equilibrium with lipid surface film. This was achieved empirically. Criteria for equilibrium were satisfied as experimental results were independent of surface area, amount of lipid added and time.

Two sets of experiments were performed, the protocol depending whether pre-SPE lipid extracts were being used or whether the lipid had been purified by SPE to remove protein. Thus, two sets of results were obtained representing our experimental extremes of protein concentration; these will be termed pre- and post-SPE. The protocol for the pre-SPE extracts was as described previously for bacterial and erythrocyte lipids (Gershfeld, 1986).

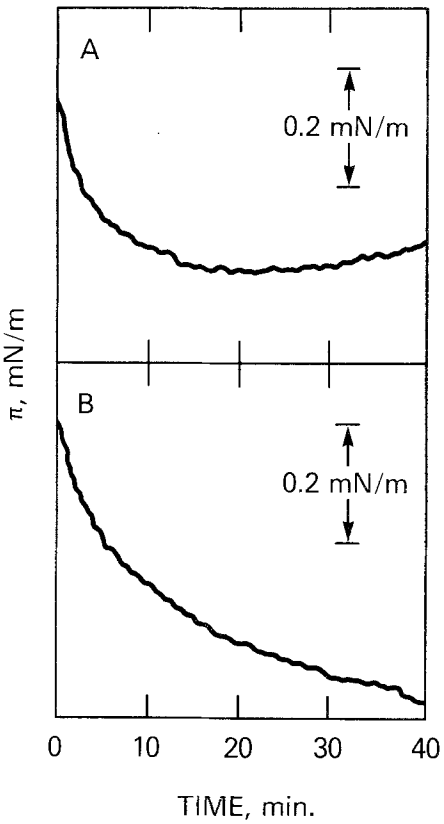

Fig. 1. Recorder tracings of surface pressure, π, as a function of time. (*A*) At time = 0 the lipid film area had been decreased to give a value of π less than the equilibrium value, π_e. After an initial drop, due principally to mechanical relaxation of the film balance float in response to the compression, the tracing reversed, at time ≈20 min, and a continuous increase in π towards π_e was then recorded. (*B*) π at time = 0 exceeded π_e and a continuous fall in surface pressure towards π_e was recorded. When $\pi < \pi_e$, reversals in the surface pressure tracings generally occurred within 15–20 min (*see A*). If no reversal was seen, tracings were routinely followed for at least 1 hr to ensure π_e had been exceeded. By monitoring π as a function of time for each step compression, π_e could be estimated for a particular temperature by interpolation between a tracing of type *A* and one of type *B*

Pilot experiments with post-SPE lipids indicated that films prepared from different SPE eluates of a particular stock solution could yield minor differences in π_e at the same temperature. We attributed this to varying residual protein concentrations even after SPE. To improve standardization, we developed a technique whereby an entire π_e-temperature curve could be obtained using a single film. After lipid application, the film was allowed to equilibrate for at least 12 hr at a surface area where the surface pressure, π, was negligible. This served the triple purpose of permitting the bulk lipid to become fully hydrated, eliminating any remaining organic solvent effects and favoring the surface denaturation of residual protein traces (Cheesman & Davies, 1954; Gaines, 1966). At this initial area, the film's surface concentration was likely to be less than the equilibrium value. Attainment of equilibrium by simply allowing the bulk lipid to spread would, however, be very slow for such complex multicomponent systems. At each temperature point we therefore determined π_e by the following time-saving method. The film was first compressed to a value of π that was assuredly greater than π_e for 30 min, leading to collapse of the surface film and therefore ensuring the presence of an adequate bulk lipid phase. The film was then re-expanded to the initial large area (where $\pi \approx 0$) for a further 30 min. This re-expansion period had the dual purpose of simulating the initial conditions of lipid adsorption from bulk dispersion and of eliminating hysteresis effects due to metastable states that arise when lipid films are overcompressed (Gershfeld, 1974). The film area was subsequently decreased stepwise, pausing after each step to record π as a function of time. At areas where $\pi < \pi_e$, a continuous increase in π was observed (towards π_e), whereas when $\pi > \pi_e$, a continuous fall was recorded until π_e was reached (Fig. 1). The rate of change of π with time depended on several factors including the type and amount of excess lipid, temperature and the absolute difference between π and π_e at any given time. Once π_e had been determined for a particular temperature, the entire film balance system was heated or cooled to a new temperature with the film remaining *in situ*, and the above method was repeated to obtain the new π_e value. A single film could be maintained on the balance for 4–5 days. Provided the film remained under nitrogen throughout, there was no evidence of lipid decomposition as shown by comparing values of π_e obtained early in a film's history with those obtained at similar temperatures several days later. Reproducibility of results was also established by examining films made from identical lipid in two separate film balance systems; similar π_e-temperature curves were obtained.

Precision of π_e measurements was ±0.3 mN/m; temperature control was ±0.2°C. The reported temperatures are those of the water surface, as monitored by a thermistor. There is approximately ±0.5°C uncertainty for these temperatures as they represent an average of the actual surface, bulk water and nitrogen temperatures. At high temperatures (>10°C above room temperature), the surface temperature may be ≥ 0.5°C below the bulk water temperature due to surface cooling from evaporation (Barnes & Hunter, 1982; Ginsberg & Gershfeld, 1985).

Microscopy

The morphology of the neural lipid extracts in aqueous dispersion was examined by depositing a sample of each of the solvent-free dried extracts on clean microscope slides, allowing the lipid to swell in water sealed under a coverslip, and viewing under phase-contrast (Zetopan microscope, A/O Reichert, Buffalo, NY, equipped with a constant-temperature stage, Cambion, Cambridge, MA).

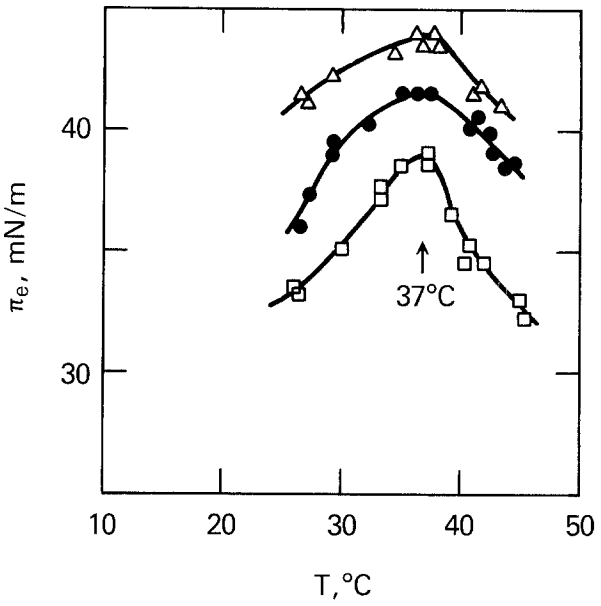

Fig. 2. Equilibrium surface pressure, π_e, as a function of temperature, T, for aqueous dispersions of total lipid extracts from rat forebrain (●), cerebellum (△) and brainstem (□). These lipid extracts had not been subjected to rigorous maneuvers to eliminate protein effects (*see* text). Each data point was obtained independently with a fresh lipid preparation

Results

Two conditions must be satisfied to confirm that a particular lipid dispersion is capable of forming the critical unilamellar state spontaneously: (*i*) the π_e-temperature phase diagram must show a maximum, and (*ii*) in the temperature range encompassing the surface pressure maximum, the bulk lipid must form a lamellar liquid-crystalline phase (Tajima & Gershfeld, 1985; Gershfeld, 1986, 1989*a*). The temperature of the surface pressure maximum then gives T^*.

Figure 2 shows the π_e-temperature relations for pre-SPE lipid extracts from rat neural tissues. Equivalent curves for post-SPE purified lipid extracts are shown in Fig. 3. Figures 4 and 5 show the results for pre- and post-SPE squid optic lobe and fin nerve extracts. Each of these curves (Figs. 2–5) passes through a maximum.

Phase-contrast microscopy confirmed that the bulk lipid was in a lamellar liquid-crystalline state in the temperature regions of the surface pressure maxima (Figs. 2–5), as evidenced by the appearance of liposomes, myelin figures and thin-walled vesicles when each of the dried neural lipid extracts was allowed to swell in water on a microscope slide at an appropriate temperature. Although the formation of unilamellar vesicles has been inferred in studies of synthetic lipids in bulk dispersion (Gershfeld et

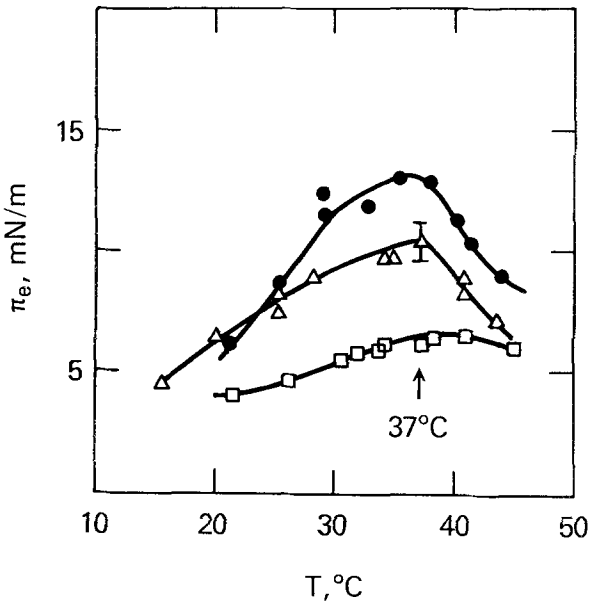

Fig. 3. Equilibrium surface pressure-temperature relations for lipid extracts from rat forebrain (●), cerebellum (△) and spinal cord (□) dispersed in water. These extracts were purified (by SPE) and manipulated on the film balance to minimize protein effects (*see* text). Note change in ordinate from Fig. 2; the (relatively) protein-free films generally gave lower values for π_e than the nonpurified (pre-SPE) films. The data point with the error bar represents the mean of three separate measurements

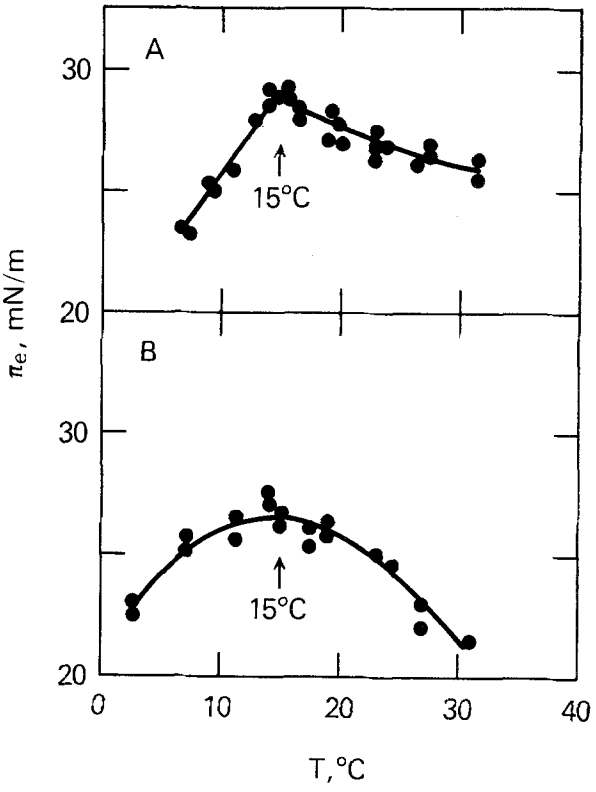

Fig. 4. Equilibrium surface pressure-temperature relations for aqueous dispersions of squid optic lobe (*A*) and fin nerve (*B*) total lipid extracts (pre-SPE, not manipulated to eliminate protein effects). Each data point was obtained independently with a fresh preparation of lipid

al., 1986), it was not possible to identify T^* except by the surface pressure method in the current series of experiments, largely due to the inability of phase-contrast microscopy to distinguish between thin-walled vesicles and those with walls only one bilayer thick.

Since each of the curves of Figs. 2–5 passes through a maximum at a temperature where the bulk lipid forms a lamellar liquid-crystalline phase, the necessary conditions for spontaneous assembly of a unilamellar state in each of the lipid extracts shown in the figures have been satisfied. The maxima in Figs. 2–5 are broad, as might be predicted for multi-component systems as complex as our lipid extracts; more pronounced surface pressure maxima are seen only for monocomponent lipid systems or simple mixtures (Tajima & Gershfeld, 1985; Gershfeld, 1989*a*). We have chosen the midpoints of these broadened maxima to obtain values of T^*.

The variation in T^* for both rat and squid tissues are well within our limits of locating the surface pressure maxima, and therefore we report only the average values and standard deviations of T^* for these tissues. We conclude that $T^* = 37 \pm 1$°C for rat neural lipids, both in the absence of protein (Fig. 3, *see* Materials and Methods) and with up to 1% protein present in the lipid extracts (Fig. 2). Comparison of Fig. 2 with Fig. 3 shows that the presence of protein affects the absolute magnitude of π_e but not the temperature of the surface pressure maximum. Similarly, $T^* = 15.5 \pm 1.4$°C for squid optic lobe and fin nerve extracts regardless of protein content within our experimental limits. The error in the T^* values arises in part from uncertainty about temperature measurement (*see* Materials and Methods) but also from constraints due to the shape of the π_e-temperature curves.

Discussion

The first objective of this study was to demonstrate that total lipid extracts from nerve membranes can form the critical unilamellar state in vitro. From thermodynamic arguments (*see* Materials and Methods and Results), each extract fulfilled the criteria for such an assembly process (Figs. 2–5). In the following discussion, the relevance of these findings to the mechanism of membrane bilayer formation in neural tissue is examined. In a final section, the

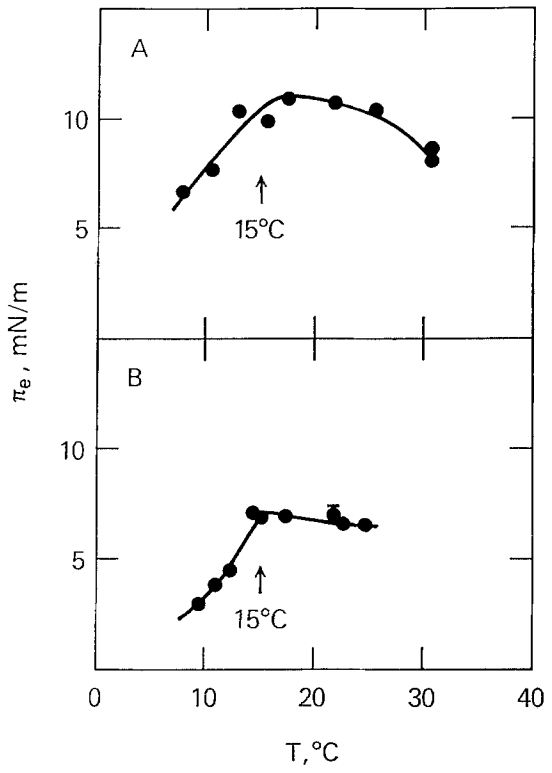

Fig. 5. Equilibrium surface pressure-temperature relations for squid optic lobe (*A*) and fin nerve (*B*) lipid extracts dispersed in water (post-SPE, manipulated on film balance to minimize protein effects; again note change in ordinate from pre-SPE (Fig. 4) extracts). The data point with the error bar represents the mean of three separate measurements

influence of protein on membrane bilayer assembly is analyzed.

Rat Neural Tissue

The striking correspondence between T^* for the rat samples (37 ± 1°C) and the normal core temperature of these organisms (37–38°C) indicates that the critical bilayer state can form under the existing in vivo conditions in rat neural tissue, and suggests that this process is fundamental to the mechanism of membrane bilayer assembly. The alternative view that this agreement is mere coincidence is rendered less tenable by analogous studies with bacterial and erythrocyte lipids (Gershfeld, 1986) where T^* was again shown to be identical to the temperature of the cells from which the lipids had been extracted spanning a 40°C range of growth temperature.

If neural membrane bilayer assembly is a critical phenomenon, the rules which have been deduced from studies of phospholipid dispersions must apply in these cells. The physical theory indicates that the unilamellar state is optimally stable only in a narrow temperature range of perhaps ±0.001°C around T^* (Ginsberg & Gershfeld, 1985), in common with other critical points (Rowlinson, 1969). Outside this range the critical bilayer will necessarily degrade to a different equilibrium state, the multibilayer. Degeneration of the unilamellar state in cell membranes is potentially lethal due to creation of membrane structural defects (*see* next section). How can this stringent thermal stability requirement be reconciled with the degree of temperature control found even in the rat, a homeotherm? The solution to this apparent problem lies in a consideration of the relative rates of formation and degradation of the critical bilayer state. Estimates based on the kinetics of micelle formation indicate that bilayer self-assembly is very rapid (Gershfeld, 1989*b*). Indeed, this must be the case if equilibrium between the lipid metabolic pool and the membrane bilayer is to be established. When temperatures shift from T^*, the stability of the critical bilayer is unlikely to depend on adjustments in lipid metabolism, adapting to the new ambient temperature, as lipid turnover rates are slow, particularly in neural tissue (Smith, 1967; Hayes & Jungalwala, 1976). Stability depends rather on the requirement that degeneration of the critical unilamellar state into multilamellar structures is slower than thermal fluctuations. In general, if temperature deviates from T^* for a particular lipid composition, critical bilayer assembly will cease and the membrane must ultimately conform to the new equilibrium conditions by forming a multilamellar state. But the unilamellar → multibilayer transformation is impeded by two main factors, namely the ability of the unilamellar state to supercool at temperatures below T^* (Gershfeld, 1989*b*) and, at $T > T^*$, an estimated activation energy of 25 kcal/mol for the transition, derived largely from the heat of solution of the bilayer lipids (Gershfeld & Murayama, 1988).

No attempt was made in the present study to separate the different types of membrane present in the rat neural tissue samples. Thus, the extracts contained lipids derived from myelin, intracellular membranes, even nonneural membranes in addition to neuronal plasma membranes. The meaning of T^* for such a complex mixture is clarified by application of the phase rule which predicts for multicomponent lipid systems that more than one composition may form the critical unilamellar state at a given temperature (Gershfeld, 1989*a*,*b*). Thus, the presence of membranes with different lipid compositions within a single cell, necessarily at the same T^*, is compatible with the theory. Furthermore, T^* for a mixture of the total lipids from all the membranes in a tissue will be identical with values obtained for extracts from each individual membrane present. We have

confirmed these consequences of the phase rule by measuring T^* for a purified neuronal membrane fraction, using hamster brain synaptosomes and microsomes, and again obtain a value of 37°C (*unpublished*).

Other aspects of the critical unilamellar state as they related to well-known properties of biomembrane lipids, including bilayer asymmetry and the 'fluidity' concept, are not relevant to the present experimental results and are discussed elsewhere (Gershfeld, 1989*b*).

Squid Nerve Extracts

What physiological significance may one attach to a value of 15.5 ± 1.4°C for T^* in squid optic lobe and fin nerve? Although nominally poikilothermic, these pelagic organisms are only exposed to a limited temperature range, with a probable maximum at 15–17°C (*see below*). Applying the same stability concepts to squid membranes as argued for the case of homeotherms, the following critical membrane behavior may be postulated in vivo. Assuming lipid metabolism is geared to permit spontaneous assembly of the unilamellar state at ~15°C, cell growth and division, and therefore development of the organism will presumably be restricted to this temperature. Thermal fluctuations may induce changes in lipid metabolism but phospholipid biosynthetic rates are likely to be slow (Larrabee & Brinley, 1968; Gould et al, 1983). Survival of the cephalopods in deep Atlantic waters ($T < 10$°C) is more readily explained in terms of preservation of membrane structural integrity by supercooling of bilayers at $T < T^*$. Such a mechanism is compatible with the behavior of marine molluscs whose life history has been better characterized than *L. pealei*. Thus, the oyster *Ostrea edulis* will survive in seawater below 10°C but only spawns at 15–16°C (Nelson, 1928, quoting Orton), the highest temperature to which it is likely to be exposed (Nicol, 1960). Although less information is available for *L. pealei,* these organisms are most plentiful and fertile at Woods Hole annually in late May/early June, at which time the seawater temperature varies between 15 and 17°C (data of the Woods Hole Oceanographic Institution).

If a squid nerve is exposed to sustained temperatures exceeding T^*, the critical bilayer theory predicts that its unilamellar membrane will slowly degrade to a multilamellar state, the rate of this transformation increasing as the difference between ambient temperature and T^* widens. The formation of a multibilayer at the expense of the unilamellar state would be expected to produce membrane structural defects (Gershfeld & Murayama, 1988) with concomitant development of pathways for passive leakage of ions and small molecules, and potentially lethal results. Such a sequence of events is compatible with anecdotal information on the decreased viability of squid axons in electrophysiological studies performed at room temperature, i.e., $T > T^*$. Indeed, the survival of the intact organisms in captivity is limited at temperatures above 22°C (Arnold, 1989) and is improved if the seawater in their holding tanks is cooled below 18°C (D.L. Gilbert, *unpublished observations*).

Reported effects of temperature on the electrophysiological properties of squid axons are not amenable to the kinetic analysis which would be required to test the critical bilayer theory quantitatively for two main reasons. First, these studies have generally been conducted on a much briefer time scale than that needed to detect the consequences of lipid bilayer degeneration (Gershfeld & Murayama, 1988). Second, electrophysiological parameters such as nerve conduction velocity, or even resting membrane potential, are complex functions of multiple variables, each of which may show a characteristic temperature dependence and from which it may be difficult to extract the influence of bilayer destabilization at temperatures above or below T^* on the parameter in question.

An idealized experiment would involve measurement of the rate of wholly passive membrane leakage at various temperatures. This would permit comparison of the activation energy for leakage, calculated via the Arrhenius equation, with the estimated activation energy for the unilamellar → multibilayer transformation (Gershfeld & Murayama, 1988). An indication that individual ion fluxes conform to predictions of the critical state theory is provided by radiotracer studies of squid axons (Landowne & Scruggs, 1976). An increase in passive membrane leakage with time at temperatures exceeding T^* would be expected from the theory due to progressive breakdown of membrane bilayer structure with formation of multibilayers. Such an increase is in fact observed both for resting chloride efflux at 20°C and resting potassium efflux at 26°C. yet both fluxes remain virtually constant with time on cooling to 4 or 8°C, i.e., below T^*.

Influence of Protein on Membrane Bilayer Assembly

Although our lipid extracts even pre-SPE contained only a small proportion of the total protein originally present in the neural membranes (*see* Materials and Methods), they yielded values for T^*, the critical temperature for assembly of the unilamellar state,

which closely corresponded with "physiological" temperatures for the source membranes (*see* Discussion above). This implies that lipid bilayer assembly in the intact membranes is largely independent of protein composition, as might have been anticipated from previous in vivo studies where membrane bilayer formation was observed to continue after protein synthesis had been suppressed (Kahane & Razin, 1969; Mindich, 1970; Benjamins et al., 1971). A possible explanation for the lack of influence of proteins on bilayer assembly would be that most of the protein is located in a separate phase from the lipid bilayer in these membranes. Under such circumstances protein concentration should not affect T^*, which would depend only on the lipid composition of the unilamellar phase. The failure of most membrane protein to dissolve in organic solvents is consistent with this two-phase model.

But what of the small fraction of protein which did co-extract with lipid into organic solution? This was largely proteolipid protein (*see* Materials and Methods), for which a degree of miscibility with membrane bilayer lipids might have been expected, both from its solution properties (Folch-Pi & Stoffyn, 1972) and from its structure (Stoffel et al., 1983). Our experiments suggest that this protein fraction does indeed form a homogeneous phase with bilayer lipid because π_e varies with protein concentration, as would be predicted from the Gibbs adsorption equation (*compare* Figs. 2 and 4 with Figs. 3 and 5, respectively). Yet when this lipid-miscible protein fraction is eliminated (post-SPE and by the maneuvers on the film balance described under Materials and Methods) there is still no change in T^*, implying that proteolipid, like the lipid-immiscible proteins, has little or no influence on membrane bilayer assembly. It remains possible that there is a minor effect, but that the removal of proteolipid produces only a small change in T^*, beyond the limit of detection in our experimental setup. The lack of influence of proteolipid on T^* is not an isolated example of the apparently anomalous situation where proteins (and other polymers) clearly interact with lipids (and other surfactants) without affecting phase relations both in dispersions and in solution. Thus, polymers have been reported to interact with nonionic micelles yet not affect the critical micelle concentration (Brackman, van Os & Engberts, 1988). This has been interpreted in terms of the interaction producing compensatory enthalpy and entropy changes, leaving the free energy of micellization of the surfactant unaltered. Proteolipid protein itself does not affect the temperature of the main gel-liquid crystal phase transition in dipalmitoyl lecithin bilayers even at high molar ratio despite evidence of protein/lipid mixing (Papahadjopoulos, Vail & Moscarello, 1975).

In summary, total lipid extracts from rat and squid neural membranes form a unilamellar state but only at a "physiological" temperature which is species dependent. This finding suggests that cellular lipid metabolic pools maintain a critical composition which permits the unilamellar state to assemble spontaneously at the organism's internal temperature. Membrane protein composition has little or no influence on this equilibrium. The biochemical mechanism linking intermediary lipid metabolism to temperature and hence membrane structural integrity remains unknown. Lipid turnover in microorganisms is sufficiently rapid to allow appropriate adjustments to temperature shifts, such that intracellular lipid composition remains in step with changing equilibrium conditions for bilayer assembly (Gershfeld, 1986, 1989*b*). Metabolic rates are relatively slower in higher organisms and a sustained change in temperature from T^* will necessarily destabilize the bilayer (Gershfeld & Murayama, 1988). Similarly, if lipid metabolism is defective, membrane instability may again result if a cell is no longer able to synthesize lipids appropriate to its ambient temperature. The role of such a mechanism in the pathogenesis of neurodegenerative disease is currently under investigation.

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