tem are slow, as might be expected for a rigid tridentate ligand; however, we have observed other cases where the rapid exchange limit obtains. In the case of such rapid exchange any attempt to achieve temporal resolution in the excitation spectrum, such as that described below, will fail. The excitation experiment thus has the potential for yielding qualitative and perhaps quantitative information regarding chemical exchange and interconversion processes.

In systems with multiple Eu(III) ion environments, each with a characteristic lifetime, it is possible to record excitation spectra in a time-resolved mode, provided the individual lifetimes are sufficiently different. The signal from the photomultiplier tube, after amplification and suitable conditioning, is fed into a boxcar signal averager. The boxcar samples the decay curve for a preset time interval and delay following the trigger pulse. For example, if the sample contains two species, one with a short τ and one with a long τ, setting the boxcar delay very close to the trigger (coincident with the laser pulse) will result in luminescence emission from both long- and short-lived species being recorded. If a longer delay is set, the short-lived species will have decayed away before the averager is activated and only the spectrum of the long-lived species will be recorded. This is illustrated for the DPA^{2-}-Eu(III) system in Fig. 1B. At a DPA^{2-}/Eu(III) ratio of 1.7 significant quantities of both $[Eu(DPA)]^+$ and $[Eu(DPA)_2]^-$ exist in equilibrium. The τ for $[Eu(DPA)]^+$ (169 μsec) is sufficiently shorter than the τ for $[Eu(DPA)_2]^-$ (304 μsec) that as the boxcar delay time following the laser excitation pulse is increased from 20 to 800 μsec (ascending Fig. 1B), the spectrum of the former ion disappears almost completely. Such temporal resolution experiments may be useful for simplifying complex excitation spectra that are due to solution equilibria or the presence of multiple metal binding sites in macromolecules.

To assess the usefulness of time-resolved Eu(III) excitation spectroscopy (TREES) for the study of Eu(III) binding to macromolecules, we applied this technique to the structurally well-characterized (*12*) zinc endoprotease thermolysin, which binds one Zn(II) and four Ca(II) ions in the native state. This enzyme is known (*13*) to bind a Ln(III) ion strongly at calcium site 1 of a double site when Ln(III) ions are added to solution, even in the presence of Ca(II). Furthermore, it has been shown (*6*) by x-ray crystallographic techniques that soaking crystals of thermolysin in the presence of Ln(III) ions under specified conditions results in the isomorphous replacement of Ca(II) by Ln(III) at three distinct binding sites (sites 1, 3, and 4). The spectrum of thermolysin to which three equivalents of Eu(III) are bound was recorded in a time-resolved mode and is shown in Fig. 2. The lowest trace in Fig. 2 was recorded with a short (10 μsec) delay after the laser trigger. It appears to consist of a strong peak at a low energy with a feature, probably consisting of two peaks, at a higher energy. As the delay time is progressively increased (ascending Fig. 2), the high-energy feature becomes progressively less and less prominent, and it is virtually undetectable at a delay time of 1000 μsec. This uppermost trace belongs to a long-lived Eu(III) species and is identical to the excitation spectrum obtained from samples of thermolysin to which less than one equivalent of Eu(III) was added; it therefore corresponds to Eu(III) in Ca(II) site 1. The short-lived peaks at higher energies are consequently assigned to Eu(III) bound at sites 3 and 4. The energies and individual τ values measured in H_2O and D_2O solutions are recorded in Table 1, along with the estimated numbers of coordinated water molecules. These results are in reasonable accord with the crystallographic findings (*3*) that the Eu(III) ion at Ca(II) site 1 has one coordinated water molecule, while the Eu(III) ions at sites 3 and 4 have three each.

These initial results suggest that the TREES technique has considerable potential for elucidating the details of Eu(III) binding to macromolecules. Questions regarding the characterization of distinct binding sites, sequential binding, the numbers of metal-coordinated water molecules, complex equilibria in solution, and even chemical exchange processes may be addressed by this method. The technique should be particularly valuable for applications requiring selective excitation of ions in specific sites—for example, to monitor Förster-type energy transfer from a particular bound Eu(III) ion to a bound energy acceptor ion (*13*).

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The Dielectric Constant of Phospholipid Bilayers and the Permeability of Membranes to Ions

Abstract. *The Born charging equation predicts that the permeability of a phospholipid bilayer membrane to ions should depend markedly on the dielectric constant of the membrane. Increasing the dielectric constant of an artificial bilayer increases its permeability to perchlorate or thiocyanate by a factor of 1000, to a value comparable to that of mitochondrial membranes.*

The permeability of the inner mitochondrial membrane to thiocyanate and perchlorate ions (*1*) is three to four orders of magnitude higher than the permeability of decane-containing lipid bilayers to these ions (*2*), even when the bilayers are formed from mitochondrial lipids (*3*). This disparity could be due to the presence of proteins in the biological membrane, which could increase the dielectric constant of the lipid bilayer. We present evidence that the dielectric constant of artificial lipid bilayers can be increased with 1-chlorodecane, and show that this increase in dielectric constant enhances the perchlorate or thiocyanate permeability of lipid bilayers to a value commensurate with that of mitochondrial membranes.

We assume the bilayer to be an iso-

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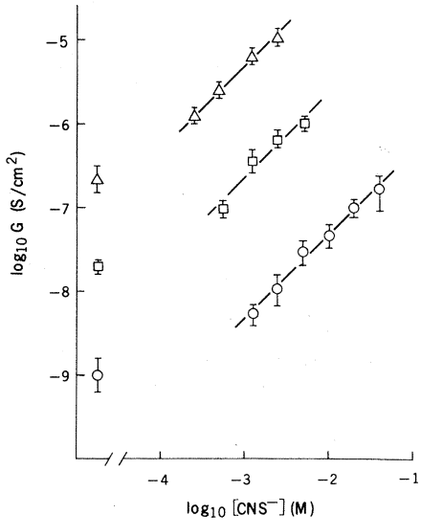

Fig. 1. Steady-state conductance of (△) PC-chlorodecane, (□) solvent-free PC, and (○) PC-decane bilayers in the presence of thiocyanate ions. The vertical bars through the points represent the standard deviations of at least four measurements. The aqueous solutions contained 0.1*M* NaCl and 1 m*M* 3-(*N*-morpholine) propanesulfonic acid (MOPS) buffer at *p*H 7.5 The lines are drawn with a slope of 1.

tropic slab of hydrocarbon with a dielectric constant ϵ_2 and the aqueous phases to be homogeneous media of dielectric constant ϵ_1. The electrostatic energy, W, required for the transfer of a nonpolarizable, spherical, monovalent ion of radius a and charge e from the aqueous phase into the center of the bilayer is given by the Born charging energy (*4*)

$$W = \frac{e^2}{8\pi\epsilon_0 a}\left(\frac{1}{\epsilon_2} - \frac{1}{\epsilon_1}\right) - \frac{e^2}{4\pi\epsilon_0\epsilon_2 d}\ln\left(\frac{2\epsilon_1}{\epsilon_1 + \epsilon_2}\right) \quad (1)$$

where ϵ_0 is the permittivity of free space and d is the thickness of the bilayer. The permeability, P, of the bilayer to ions of either sign is approximately proportional to $\exp(-W/kT)$, where k is Boltzmann's constant and T is absolute temperature. Either a decrease in d or an increase in ϵ_2 should increase P. Other factors that affect the permeability will be considered below.

Lipid bilayers made from diphytanoylphosphatidylcholine (PC) were formed by dissolving 12.5 mg of the lipid in 1 ml of either *n*-decane (dielectric constant = 2.0 at 20°C) or 1-chlorodecane (dielectric constant ≃ 4.5 at 25°C) and depositing the mixture on an orifice in a Teflon chamber (*5*, *6*). The resulting bilayers retain some of the alkane solvent. Essentially "solvent-free" bilayers were made by bringing together two monolayers of lipid to form a bilayer over a hole in a thin sheet of Teflon treated with either squalene or Vaseline (*7*). Steady-state conductance measurements were made at 20° to 22°C by applying 10 mV across the bilayer. X-ray diffraction measurements on multilamellar arrays of PC and PC-alkane bilayers were performed by methods described previously (*8*). Surface potential measurements were made with an ionizing electrode by standard methods (*9*).

In the presence of thiocyanate, the conductance of PC-chlorodecane bilayers (Fig. 1, top curve) is three orders of magnitude larger than the conductance of PC-decane bilayers (Fig. 1, bottom curve). A linear conductance-concentration relationship was also observed when perchlorate was the permeant ion (*10*); the conductance of PC-chlorodecane bilayers was 3.5 orders of magnitude higher than that of PC-decane bilayers.

The available evidence suggests that these large increases in conductance are at least partially due to changes in the dielectric constant, ϵ_2, of the lipid bilayer. The specific capacitance, which is porportional to ϵ_2/d, is nearly twice as large for PC-chlorodecane bilayers (0.73 μF/cm²) as it is for PC-decane bilayers (0.39 μF/cm²) (*11*). This increase could be due to either an increase in ϵ_2 or a decrease in d. X-ray diffraction experiments, however, indicate that there is little difference in repeat period between PC-decane and PC-chlorodecane bilayers, which suggests that the thickness of the two bilayers is similar (*12*). Furthermore, the conductance of solvent-free PC bilayers is only a factor of 40 higher than that of PC-decane bilayers (Fig. 1, middle curve), a result consistent with the prediction of Eq. 1 (*13*). It has been argued that the dielectric constants of decane-containing and solvent-free bilayers are both about 2.1 to 2.2 (*14*). The high specific capacitance of the solvent-free (0.70 μF/cm²) compared to the decane-containing (0.39 μF/cm²) PC bilayers is thus due to their reduced thickness. It follows that the large conductance of PC-chlorodecane bilayers cannot be due entirely to a thickness change.

The possibility that chlorodecane enhances the permeability of bilayers to thiocyanate and perchlorate by merely producing a more positive electrostatic potential in the interior of the bilayer is ruled out by two control experiments. First, the surface potentials of PC-decane and PC-chlorodecane monolayers were 498 ± 22 and 468 ± 19 mV [mean ± standard deviation (S.D.), N = 6], respectively. The difference between these surface potentials is small and in the wrong direction to account for the effect of chlorodecane on the permeability of bilayers to thiocyanate and perchlorate. Second, the conductance of PC bilayers due to the positively charged nonactin-potassium complex is also enhanced by chlorodecane.

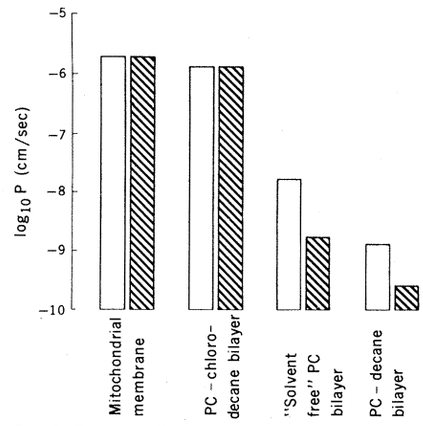

Fig. 2. Permeability of submitochondrial particles (*1*) and various lipid bilayers to perchlorate (striped bars) and thiocyanate (open bars).

It is unlikely, for several reasons, that chlorodecane produces an increase in the fluidity of the lipid bilayer sufficient to account for the increase of three orders of magnitude in ion permeability. For example, the relative increase in conductance produced by chlorodecane is largest for the smallest ions. Relative to PC-decane bilayers, the increase is a factor of 1000 to 3000 for thiocyanate and perchlorate, a factor of 100 for the anionic form of 5,6-dichloro-2-trifluoromethylbenzimidazole (DTFB), and a factor of 10 for both the dimeric form of DTFB (*6*) and the nonactin-potassium complex. These results are consistent with chlorodecane producing an increase in the dielectric constant (Eq. 1) but are inconsistent with it producing an increase in fluidity (*15*). Furthermore, the permeabilities of PC-decane and PC-chlorodecane bilayers to the neutral form of DTFB, as determined by the method of LeBlanc (*16*), are the same within experimental error.

The permeabilities of the various bilayers were determined from the conductance data (for example, Fig. 1) by means of

$$P = GRT/cF^2 \quad (2)$$

where G is the specific conductance, c is the aqueous concentration of the perchlorate or thiocyanate ions, R is the gas constant, and F is the Faraday. The results are compared, in Fig. 2, with the

permeabilities of submitochondrial particles to perchlorate and thiocyanate (*1*). The histogram illustrates the large disparity between the permeability of the biological membrane and that of PC-decane bilayers to these ions. Differences in bilayer thickness may account for some of the discrepancy, as shown by the permeability of solvent-free PC bilayers. The increased dielectric constant of the PC-chlorodecane bilayers, however, has a much greater effect on permeability, especially when perchlorate is the permanent ion.

To reconcile the ability of weak acids to transport protons across artificial phospholipid bilayers with their ability to uncouple oxidation from phosphorylation in mitochondria, we previously postulated (*6*) that some fraction of the lipid bilayer component of the inner mitochondrial membrane has a dielectric constant greater than 2.2. The data presented in Fig. 2 are consistent with this postulate.

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3. Some experiments were performed with a mixture of lipids chosen to mimic the lipid composition of the inner mitochondrial membrane; 40 percent phosphatidylethanolamine, 40 percent phosphatidylcholine, and 20 percent cardiolipin (by weight). The thiocyanate permeabilities of solvent-free, decane-containing, and chlorodecane-containing bilayers made with these lipids are within a factor of 2 of the conductances of the analogous bilayer made with PC (see Fig. 1).
4. The first term in Eq. 1 is the Born expression for two semi-infinite media; the second term is a correction for the finite thickness of a lipid bilayer [A. Parsegian, *Nature (London)* **221**, 844 (1969)].
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8. The PC was dried from a chloroform solution with nitrogen, mixed with an appropriate volume of decane or chlorodecane, then combined with a saline solution containing 0.1*M* NaCl adjusted to *p*H 7.4. The lipid-alkane-saline suspensions were thoroughly mixed, equilibrated for several hours, sealed in quartz-glass x-ray capillary tubes, and mounted in a flat-plate x-ray camera. Measurements and analysis were performed as described by T. J. McIntosh [*Biochim. Biophys. Acta* **513**, 43 (1978)].
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12. Diphytanoylphosphatidylcholine swells in saline in a manner similar to the swelling of egg lecithin [D. M. Small, *J. Lipid Res.* **8**, 551 (1967)]. The lamellar x-ray repeat period increased with increasing concentration of either decane or chlorodecane, up to a hydrocarbon mole fraction of 0.8. The repeat period did not increase further, indicating that excess alkane is present when the mole fraction of hydrocarbon is greater than 0.8. The average lamellar repeat period for PC in excess saline is 64 ± 1 Å, whereas the repeat period with PC with excess saline and excess (0.9 mole fraction) alkane is 104 ± 6 Å for decane and 97 ± 3 Å for chlorodecane (three and four experiments, respectively). Additional evidence that decane and chlorodecane affect the bilayer structure in a similar manner was obtained by differential scanning calorimetry. At equal concentrations, the two organic solvents had similar effects on the dipalmitoyl lecithin phase transition: they lowered and broadened the transition.
13. The permeabilities of solvent-free PC and PC-decane bilayers to perchlorate, on the other hand, differ by less than a factor of 10 (Fig. 2). Others have also noted that the dependence of permeability on thickness predicted by Eq. 1 is not observed with all ions [A. D. Pickar and R. Benz, *J. Membr. Biol.* **44**, 353 (1978)]. Factors not included in Eq. 1, such as the polarizability of the ions and the inhomogeneity of the dielectric constant, must also be important.
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Microwaves: Effect on Thermoregulatory Behavior in Rats

Abstract. *Rats, with their fur clipped, pressed a lever to turn on an infrared lamp while in a cold chamber. When they were exposed to continuous-wave microwaves at 2450 megahertz for 15-minute periods, the rate at which they turned on the infrared lamp decreased as a function of the microwave power density, which ranged between 5 and 20 milliwatts per square centimeter. This result indicates that behaviorally significant levels of heating may occur at an exposure duration and intensities that do not produce measurable changes in many other behavioral measures or in colonic temperature. Further study of how microwaves affect thermoregulatory behavior may help us understand such phenomena as the reported "nonthermal" behavioral effects of microwaves.*

Reports of "nonthermal" behavioral effects of microwaves have contributed to debates about the safety of microwaves. There is no question that microwaves can affect behavior (*1*). Microwaves heat tissue, and heat itself can affect behavior (*2*). When microwaves increase colonic temperature, concomitant behavioral changes are often attributed to the thermal burden (*3*). When microwaves produce no observable changes in colonic temperature, concomitant behavioral changes are sometimes attributed to "nonthermal" actions of microwaves, especially by Soviet and Eastern European investigators (*4*). Numerous biological processes, however, are affected by local temperatures that are not highly correlated with the core temperature (*5*); indeed, many help ensure its constancy. Thermoregulatory behaviors, those behaviors that directly affect, and are often controlled by, the thermal environment of the subject, generally respond to skin and hypothalamic, rather than colonic, temperatures (*6–8*). It seems plausible, then, that microwaves might alter behavior as a consequence of thermal stimulation in the absence of measurable core temperature changes.

Current techniques for measuring temperature changes in animals exposed to microwaves are inadequate for several reasons: (i) sensitivity is limited to about 0.1°C; (ii) ongoing thermoregulation serves to dissipate heat; (iii) "hotspots," that is, localized increases in temperature, can occur at locations not being monitored; (iv) most sensors distort the microwave field. Finally, since any absorption produces some temperature increase, it is still necessary to determine its functional significance. One way to avoid these difficulties is to use the organism's behavior as the thermometer, so to speak.

Six male Long-Evans hooded rats (325 to 450 g) were individually trained to press a small lever in order to turn on an infrared lamp for 2 seconds. Responses made during the 2-second period produced no programmed consequences. Test sessions, each of approximately 24 hours, started late in the afternoon. At this time, the fur of the rat was clipped, and the rat was placed in a chamber (*9*) located in a dark, refrigerated room (see Fig. 1). After a few such sessions, the rat generally pressed the lever at a nearly constant rate for several hours. This performance provided a baseline for study-

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