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Is Ubiquinone Diffusion Rate-Limiting for Electron Transfer?

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Abstract

The different possible dispositions of the electron transfer components in electron transfer chains are discussed: (a) random distribution of complexes and ubiquinone with diffusion-controlled collisions of ubiquinone with the complexes, (b) random distribution as above, but with ubiquinone diffusion not rate-limiting, (c) diffusion and collision of protein complexes carrying bound ubiquinone, and (d) solid-state assembly. Discrimination among these possibilities requires knowledge of the mobility of the electron transfer chain components. The collisional frequency of ubiquinone-10 with the fluorescent probe 12-(9-anthroyl)stearate, investigated by fluorescence quenching, is 2.3×10^9 M^{-1} sec^{-1} corresponding to a diffusion coefficient in the range of 10^{-6} cm^2/sec (Fato, R., Battino, M., Degli Esposti, M., Parenti Castelli, G., and Lenaz, G., *Biochemistry*, **25**, 3378–3390, 1986); the long-range diffusion of a short-chain polar Q derivative measured by fluorescence photobleaching recovery (FRAP) (Gupte, S., Wu, E. S., Höchli, L., Höchli, M., Jacobson, K., Sowers, A. E., and Hackenbrock, C. R., *Proc. Natl. Acad. Sci. USA* **81**, 2606–2610, 1984) is 3×10^{-9} cm^2/sec. The discrepancy between these results is carefully scrutinized, and is mainly ascribed to the differences in diffusion ranges measured by the two techniques; it is proposed that short-range diffusion, measured by fluorescence quenching, is more meaningful for electron transfer than long-range diffusion measured by FRAP, or microcollisions, which are not sensed by either method. Calculation of the distances traveled by random walk of ubiquinone in the membrane allows a large excess of collisions per turnover of the respiratory chain. Moreover, the second-order rate constants of NADH-ubiquinone reductase and ubiquinol-cytochrome *c* reductase are at least three orders of magnitude lower than the second-order collisional constant calculated from the diffusion of ubiquinone. The activation energies of either the above activities or integrated electron transfer (NADH-cytochrome *c* reductase) are well above that for diffusion (found to be ca. 1 kcal/mol). Cholesterol incorporation in liposomes, increasing bilayer viscosity, lowers the diffusion coefficients of ubiquinone but not ubiquinol-cytochrome *c* reductase or succinate-cytochrome *c* reductase activities. The decrease of activity by ubiquinone dilution in the membrane is explained by its concentration falling below the K_m of the partner enzymes. It is calculated that ubiquinone diffusion

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is not rate-limiting, favoring a random model of the respiratory chain organization. It is not possible, however, to exclude solid-state assemblies if the rate of dissociation and association of ubiquinone is faster than the turnover of electron transfer.

Key Words: Ubiquinone; diffusion; diffusion control; electron transfer; mitochondria; fluorescence quenching; respiratory chain; membrane viscosity; NADH-ubiquinone oxidoreductase; ubiquinol-cytochrome *c* oxidoreductase.

Introduction

The passive lateral mobility of membrane-bound molecules is essential for many biological functions (Edidin, 1974; Cherry, 1979; Axelrod, 1983; Wiegel, 1984; McCloskey and Poo, 1985; Peters, 1985), although the role of diffusion has seldom been quantified. The diffusion of membrane-bound molecules is driven by the lateral motion of the lipids, which in turn is activated by the very fast movement of defects along the hydrocarbon chains (Galla *et al.*, 1979).

Adam Delbrück (1968) proposed that organisms resolve some of the problems of timing and efficiency of diffusion of certain molecules by reducing the dimensionality in which diffusion takes place from three-dimensional space to two-dimensional surface. Even if the efficiency of two-dimensional vs. three-dimensional diffusion has been questioned (cf. McCloskey and Poo, 1985), membrane-bound diffusion may well compete with transport inside soluble compartments of the cell, which is expected to be not so effective due to the high viscosity of the cytoplasmic matrix (Keith and Snipes, 1973; Keith and Mastro, 1983). Biological membranes are known to exist in a fluid state (Aloia, 1983; Shinitzky, 1985), and are endowed with relatively free mobility of lipids and of the molecules dissolved in the lipid bilayer, unless restrictions of different kinds hinder the molecular motions (Nicolson, 1976).

In mitochondrial and other energy-conserving membranes the mobility of the electron transfer complexes has received particular attention (Dixit and Vanderkooi, 1983) to resolve the question whether lateral diffusion is fast enough to account for the observed electron transfer rates.

The pathways for efficient electron transfer between redox carriers in energy-conserving membranes is the topic of this review.

Organization of Electron Transfer Chains in Energy-Conserving Membranes

There is little reason to believe that the organization of electron transfer chains of the inner membrane of mitochondria, the plasma membrane of bacteria, and the thylakoid membrane of chloroplasts is widely different (Kell

and Westerhoff, 1985), so we will assume that evidence accumulated in one system may generally be applicable to the others.

The types of molecular organization possible in theory for the multiprotein complexes and the smaller connecting molecules which constitute the biological electron transfer chains have been discussed in detail by Rich (1984). Four alternative views are possible, without excluding that mixed situations or different states exist, varying as a function of physiological conditions (Lenaz *et al.*, 1986).

(a) The chain is organized in a liquid state; the large multiprotein complexes are randomly distributed in the plane of the membrane, where they move freely by lateral diffusion; ubiquinone (or plastoquinone) and cytochrome *c* (or plastocyanin) are also mobile electron carriers, possessing faster diffusion than the bulkier protein complexes, so that their diffusion-coupled collision frequencies are greater than the maximal turnover numbers of the chain.

(b) The components of the chain are randomly distributed in a liquid state system, but all the rates are diffusion-controlled, so that the diffusions of the fastest components (ubiquinone and cytochrome *c*) represent the rate-limiting steps for integrated electron transfer.

(c) Electron transfer is ensured by diffusion and collision of protein complexes, each carrying bound quinone as a prosthetic group; the bound quinone may be in equilibrium with the pool by very fast association–dissociation, but the free pool does not directly participate in electron transfer.

(d) The components of the chain are organized in solid-state assemblies, where electron transfer occurs via the fixed association of the redox components of the membrane.

Some of the states outlined above may not be completely clear-cut: for example, solid-state clusters of electron transfer complexes could occur temporarily, allowing several turnovers to take place during their lifetimes. Furthermore, a heterogeneous disposition of the redox components may also exist in the plane of the membrane, with random distribution of an aliquot of the units and patches of others.

Some of the situations described above are amenable to kinetic scrutiny, whereas others may not be distinguishable by kinetic analysis, so that other kinds of experimental evidence are required.

Kinetic Evidences

Following the pioneering intuition of Green (1962), Kröger and Klingenberg (1970, 1973a, b), from a kinetic analysis of the rate of electron input to ubiquinone (NADH-Q reductase and succinate-Q reductase) and of

electron output from reduced ubiquinone (ubiquinol oxidase), under a wide range of input and output rates (V_{red} and V_{ox}, respectively), established that ubiquinone in mitochondria exists as a homogeneous mobile pool, shuttling electrons from each dehydrogenase molecule to each molecule of the enzyme oxidizing the quinol (ubiquinol-cytochrome *c* reductase). The overall observed rate through the respiratory chain (V_{obs}) follows the relation

$$V_{obs} = \frac{V_{red} \cdot V_{ox}}{V_{red} + V_{ox}} \quad (1)$$

This expression, known as the homogeneous pool equation, states that the current through the quinone pool is determined by the combination of electron influx and efflux. Such behavior has been confirmed in a large variety of experimental situations (Gutman, 1985).

The "pool behavior" requires that diffusion of ubiquinone is not rate-limiting for electron transfer. Deviations from the pool behavior are expected if diffusion becomes rate-limiting. Gutman (1985) has introduced a diffusion term in the pool equation:

$$\frac{1}{V_{obs}} = \frac{1}{N}\left(\frac{[dH]}{k_{ox}[Q_t]} + \frac{2}{DP[Q_t]}\right) + \frac{1}{k_{red}[dH][Q_t]} \quad (2)$$

where k_{ox} and k_{red} are the rate constants for ubiquinone oxidation by bc_1 complex and of reduction by dehydrogenases (dH), respectively, $[Q_t]$ is total ubiquinone concentration, N is the concentration ratio of bc_1 complex to dehydrogenase, D is the diffusion coefficient, and P is a proportionality factor related to average diffusion distance between dehydrogenase and bc_1 complexes. This equation predicts a linear function of $1/V_{obs}$ vs. $1/N$ with intercept of $1/V_{red}$ ($= 1/k_{red}[dH][Q_t]$): the slope of the line will vary with the magnitude of D. For preparations where diffusion of ubiquinone is limiting, the small value of D will increase the slope of Eq. (2). This behavior has been verified by Gutman (1985) on previous experiments by Heron *et al.* (1978) and Ragan and Heron (1978), where reconstituted Complex I : Complex III preparations in varying molar ratios behaved in contrast with the pool behavior at low phospholipid : protein ratios.

A more detailed formalism of the pool equations is described by Kröger and Klingenberg (1973a), Gutman (1985), and Ragan and Cottingham (1985), and is discussed by Ragan in this volume. It is important to conclude, however, that the pool equation appears equally valid in a number of arrangements of the respiratory chain, provided that the diffusion rate is *not* limiting for electron transfer.

A different approach to the demonstration of pool behavior was employed by Schneider *et al.*, 1980a, b, 1982a, b); by increasing the phospholipid content of mitochondrial membranes, a decrease of electron flow

was observed between the enzymes reducing and oxidizing the ubiquinone pool; the inhibition was reversed by adding ubiquinones together with the phospholipids. This observation was taken to mean not only that the rate of electron transfer is diffusion-coupled, but also that diffusion of ubiquinone is the rate-controlling step of the overall process (cf. Hauska and Hurt, 1982). Similar results were obtained in phospholipid-enriched chromatophores from photosynthetic bacteria (Casadio *et al.*, 1984; Snozzi and Crofts, 1984). The decreased rate of electron transfer as a consequence of ubiquinone dilution, however, is not a proof *per se* that diffusion is the limiting step in the reaction rate (see later).

By investigating the second-order reduction of cytochrome b_{561} in chromatophores by the quinol produced by photoreduction in the reaction center at different redox poise of the quinone pool, Crofts and Wraight (1983) calculated a diffusion coefficient $D = 10^{-10}$ cm^2/sec assuming that diffusion was rate-limiting. The possibility was discussed that the rate-limiting steps were quinol release from the reaction center and its binding to the bc_1 complex, but it was suggested that these steps should constitute only a minor contribution to the order rate constant for the reaction, also in view of the fact that the lag in cytochrome *b* reduction increased at low quinol concentration or when the distance between reaction centers and bc_1 complexes was increased by dilution with phospholipids (Snozzi and Crofts, 1984; Crofts and Wraight, 1983; Crofts *et al.*, 1983).

In general, the kinetic evidence points out that Q diffusion is not rate-limiting, but is unable *per se* to throw light on the arrangement of the electron transfer complexes in the membrane.

Structural Evidences

The ultrastructural study by Fleischer *et al.* (1967) on the fine structure of lipid-depleted mitochondria showed that the microscopic appearance of the inner mitochondrial membrane did not change after lipid removal, indicating that protein–protein contacts are sufficient to keep the membrane *in situ*; of course, this evidence only means that lipid–protein interactions may be replaced by hydrophobic protein–protein interactions. This behavior of mitochondrial membranes is the result of the very high protein : lipid ratio, which is shared by other energy-conserving membranes (Tzagoloff, 1982; John and Whatley, 1977). The high protein : lipid weight ratio, however, does not mean that there is a high protein : lipid area ratio, since most protein complexes completely span the lipid bilayer and extend beyond it on both sides, so that the actual membrane area occupied by phospholipids may well be rather high (Kell and Westerhoff, 1985; Schneider *et al.*, 1985). Differential scanning calorimetry, detecting the thermotropic behavior of lipids removed

from direct contact with proteins (Blazyk and Steim, 1972), showed that the majority of phospholipids in the inner mitochondrial membrane behave as a free bilayer (Höchli and Hackenbrock, 1976); this is confirmed by NMR evidence (Cullis *et al.*, 1980; De Kruijff *et al.*, 1982) and by the rotational correlation times of lipid-soluble spin labels (Lenaz *et al.*, 1983). Freeze-fracture electron microscopy showed that the intramembrane particles, which are related to proteins (Segrest *et al.*, 1974), are randomly distributed in the inner mitochondrial membrane above the phase transition, which occurs at subzero temperature (Höchli and Hackenbrock, 1976, 1977; Schneider *et al.*, 1985). Although this can be taken as evidence for a random distribution of protein complexes in the lipid bilayer under the conditions of investigation, it is not possible from microscopy, by just evaluating the number and diameter of the particles, to attain a quantitative evaluation of the relative area of the membrane occupied by proteins and lipids.

Additional evidence for a random distribution of the electron transfer complexes in the inner membrane stems from the fact that antibodies against cytochrome oxidase and Complex III aggregate these complexes separately (Hackenbrock and Hammon, 1975). Furthermore, the rotational correlation time of cytochrome oxidase was found to be the same whether Complex III and cytochrome *c* are present or not in the same reconstituted membrane (Kawato *et al.*, 1981).

In the native mitochondrial membrane, however, part of the cytochrome oxidase appears strongly immobilized (Kawato *et al.*, 1981). A similar immobile fraction was found for the ATP-ADP translocator (Müller *et al.*, 1982). In general, it appears that in reconstituted vesicles the mitochondrial enzymes are largely mobile and therefore probably not aggregated (Dixit and Vanderkooi, 1983). The question is, however, whether the proteins in reconstituted vesicles share the organization they have in the native membrane.

Circumstantial evidence against a random distribution of respiratory complexes comes from the isolation of Complex I–Complex III (Hatefi *et al.*, 1962; Fowler and Richardson, 1963; Hatefi and Rieske, 1967) and Complex II–Complex III supercomplexes (Yu *et al.*, 1974; Yu and Yu, 1980) and also of b_6f-photosystem I units (Boardman, 1971), indicating that such units may be preferentially associated in the native membrane.

An additional complication is the notion of permanently bound quinone. The existence of Q-binding proteins has been postulated on the basis of some experimental evidence (Vinogradov *et al.*, 1980; Yu and Yu, 1981; Suzuki and Ozawa, 1984; King, 1985), and of the fact that ubiquinone is usually isolated together with the respiratory complexes (Lenaz *et al.*, 1985a, b). The bound quinone could be in rapid equilibrium with the pool, and would therefore be kinetically indistinguishable from it. Yu and Yu (1981), however, claimed to have demonstrated the existence of permanently

bound quinone, with no exchange between free and bound forms. Such quinone in a solid-state assembly should be clearly distinguishable kinetically; no kinetic evidence exists for participation of bound quinone in *intercomplex* electron transfer, although it cannot be excluded that it participates in *intracomplex* electron transfer, for example in a Q-cycle (Mitchell, 1976) or *b*-cycle (Wikström and Saraste, 1984) mechanism. The notion of a special bound quinone (Q_z) in the bc_1 complex of bacterial chromatophores (Crofts *et al.*, 1983) has recently been abandoned in favor of a Q_z *site* where the quinone pool rapidly exchanges (Snozzi and Crofts, 1984).

Biophysical Evidence

Much of the uncertainty still existing on the mechanism of electron transfer in the quinone region of electron transfer chains could be clarified by direct measurements of the lateral diffusion coefficients of the quinone and of the protein complexes in the membranes. In spite of the physiological relevance of such determinations, there are as yet only few data on the lateral diffusion coefficients of redox components in energy-conserving membranes. A critical analysis of the experimental facts on such topic requires some previous discussion on the experimental and theoretical implications of diffusion in biological membranes.

Lateral Diffusion in Biological Membranes

The method of choice for measuring lateral diffusion of proteins is fluorescence recovery after photobleaching (FRAP) (Cherry, 1979), whereas several additional methods have been used for lipids and small molecules, such as EPR line broadening of spin labels (Marsh and Watts, 1981), NMR (Cornell and Pope, 1980), pyrene excimer formation (Galla *et al.*, 1979), fluorescence collisional quenching (Lakowicz and Hogen, 1980), and others (Galla *et al.*, 1979; Wade, 1985).

FRAP was first used for rhodopsin in the outer segment of rod disc membranes (Poo and Cone, 1974), yielding $D = 3.5 \times 10^{-9}$ cm^2/sec. Since few proteins contain natural chromophores, the method can be applied to nonchromophoric proteins by attaching covalent probes to them (Cherry, 1979; Peters, 1985). The diffusion coefficients can be calculated by the recovery curves, since D is inversely proportional to the half-time of fluorescence recovery. The range of diffusion coefficients which can be measured by this method is between 5×10^{-7} and 10^{-11} cm^2/sec. The disadvantage of chemical modification of the diffusing molecule may be negligible for bulky

proteins, but of major importance for smaller molecules. A limitation of the method is that D measured by FRAP in nonplanar membrane surfaces, as is often the case, is underestimated, representing mobility in projected flat planes of real nonflat membranes (Aizenbud and Gershon, 1982). The method is unsuitable for membranes of small diameter, like subcellular organnelles, unless they are modified to increase the size by fusion or other means.

As pointed out by McCloskey and Poo (1985), the possibility that biomembranes are laterally inhomogeneous over short distances (Jain, 1983) makes FRAP only suited for measuring long-range ($\geqslant 1\ \mu$m) lateral diffusion. Since chemical reactions and other collision-dependent interactions are probably more directly related to local than to long-range diffusion (McCloskey and Poo, 1985), this represents a real shortcoming of the FRAP method.

The study of short-range lateral diffusion by other techniques widely used for lipids (Razi-Naqvi *et al.*, 1974; Marsh and Watts, 1981; Cornell and Pope, 1980; Lakowicz and Hogen, 1980) has not yet been extended to proteins. It is likely that dielectric spectroscopy (Kell and Harris, 1985) is able to measure *both* short-range and long-range diffusion, but the applicability of the method has yet to be defined to measure reliable diffusion coefficients. Long-range protein diffusion is also detected by a method combining electrophoresis and freeze-fracture electron microscopy (Poo, 1981; Sowers and Hackenbrock, 1981).

The diffusion coefficients of membrane proteins usually range between 10^{-9} and 10^{-12} cm^2/sec, although there are reports of higher values (Kell and Harris, 1985; Small *et al.*, 1984), particularly in liposome-reconstituted systems (McCloskey and Poo, 1985).

The diffusion coefficients of lipids, investigated by a wide variety of methods (Galla *et al.*, 1979; Marsh and Watts, 1981; Cornell and Pope, 1980; Lakowicz and Hogen, 1980; Razi-Naqvi *et al.*, 1974), usually range between $\approx 10^{-7}$ and $> 10^{-8}$ cm^2/sec (Wade, 1985), and are therefore more uniform than those of proteins.

Theory

The translational diffusion coefficients of proteins in membranes are usually related to the physical parameters of the system by the equation of Saffman and Delbrück (1975) for a cylindrical object moving in a viscous continuum fluid sheet bounded by an aqueous fluid:

$$D = \frac{k_B T}{4\pi\eta h}\left(\ln\frac{\eta h}{\eta' a} - \gamma\right) \tag{3}$$

where k_B is Boltzmann's constant, T is absolute temperature, η is membrane viscosity, η' is the water phase viscosity, h is the height and a the radius of the protein, and γ is Euler's constant (0.5772). The model assumes that the viscosities of the fluid bathing the membrane as well as that of the lipid phase itself determine protein diffusion. The system is characterized by a dimensionless parameter $\varepsilon = \eta' a/\eta h$, and the equation is only valid if $\eta' \ll \eta$ and $a < h$, so that $\varepsilon \ll 1$ results. Hughes *et al.* (1982) have extrapolated true values for ε, confirming that only values of $\eta' = 1$–2 P and $\eta = 0.1$–0.2 P can give diffusion coefficients approaching the experimentally determined ranges of values for proteins (see later).

The Saffman–Delbrück model has been tested (Peters and Cherry, 1982) by studying the mobility of bacteriorhodopsin in bilayers of dimyristoyl lecithin, allowing one to calculate a molecular radius near 20 Å and a membrane viscosity between 1.1 and 3.5 P at molar lipid : protein ratios between 210 and 90. Furthermore, the viscosity of the aqueous phase was varied between 0.76 and 9.54 cP by addition of sucrose, which yielded a twofold decrease of D, in accordance with the Saffman–Delbrück equation.

As for lipids, Vaz and Hallmann (1983) have produced evidence against the applicability of the Saffman–Delbrück model, since D was found independent of the height of the diffusing species. In the case of a diffusant comparable in size to the solvent, the free volume theory of Cohen and Turnbull (1959) and Montroll (1969) seems to apply best (Galla *et al.*, 1979; Vaz *et al.*, 1984). According to this theory, the diffusion of a molecule in a fluid system may be divided up into a three-step process: (a) creation of local free volume by density fluctuations which open up a hole within the cage where a solute molecule is situated; (b) the jump of the diffusing molecule into this hole, creating a void at the previous position; and (c) the filling of the void by another solvent molecule. The lateral mobility of amphipathic molecules will be determined by the free area in the outer polar region of the bilayer, whereas nonpolar molecules would diffuse according to the free area of the central region, which has much larger fluidity, with expected higher probabilities of forming void volumes. This is why oxygen (Vandegriff and Olson, 1984), benzene (Rigaud *et al.*, 1972), and pyrene (Galla *et al.*, 1979) may diffuse more rapidly than the lipid molecules.

Restrictions

The observation that the mobility of membrane proteins appears largely dependent on the type of membrane and on the type of protein, and is usually lower than theoretically expected, suggested that mobility may be hindered by several physiological restrictions (Cherry, 1979). The elements of the cytoskeleton, in particular the microfilaments (Smith *et al.*, 1979) or other

peripheral proteins, produce a marked reduction of the measured diffusion coefficients. Other restrictions are represented by the regions of specific membrane junctions (Edidin, 1982).

Another possible restriction is the increase of membrane viscosity (Nicolson, 1976). The lateral diffusion coefficients of proteins are usually decreased by two to three orders of magnitude below the lipid phase transitions (cf. Vaz *et al.*, 1981). However, the correspondence between viscosity and lateral diffusion of proteins is often quite poor. The changes in lipid microviscosity as measured by the *rotational* mobility of a fluorescent or paramagnetic lipid probe do not necessarily correspond to changes in the lateral mobility of proteins embedded in the lipid bilayer (Kleinfeld *et al.*, 1981). This lack of correlation is consistent with the suggestion that lateral mobility of membrane proteins *in situ* is not modulated by the lipid viscosity but by the constraints from the cytoskeleton and the aqueous matrix in general. Analysis of experimental results (Koppel *et al.*, 1981) by a theoretical model for protein diffusion retarded by steric hindrance in a labile matrix yields an effective matrix surface viscosity consistent with the viscoelastic properties of the membrane. Agents destabilizing the matrix *in vitro* also increase the lateral mobility of integral proteins (Schindler *et al.*, 1980a).

Another factor that appears to strongly modify protein diffusion is their concentration in the membrane. The dependence of the lateral distribution of membrane proteins on the protein : lipid ratio has been modeled by Monte Carlo calculations (Freire and Snyder, 1972) and shown to vary from random to aggregated in a continuous network. It was calculated that long-range diffusion is relatively sensitive to the area fraction of impermeable patches, representing the membrane proteins, and, at a critical area fraction, diffusion is completely blocked (Saxton, 1982). It was shown that in a bacteriorhodopsin/dimyristoyl lecithin system (Peters and Cherry, 1982) the diffusion coefficient of bacteriorhodopsin decreased from 3.4 to 0.15×10^{-8} cm^2/sec when the molar lipid/protein ratio was decreased from 210 to 30, whereas lipid mobility was much less affected. In reconstituted membranes from *E. coli* over a range of protein concentrations of 0–60 % by weight, D for lipopolysaccharide decreased 10-fold, whereas D for phospholipid remained constant (Schindler *et al.*, 1980b).

Although the diffusion coefficients are relatively insensitive to the size of the diffusing molecule, it has been shown that aggregation, by increasing the size of the mobile unit, results in lowered mobilities of membrane antigens (Barisas, 1984).

Another factor that can affect the values for diffusion coefficients is the concentration gradient of the moving objects. By a careful study of the distribution of intramembrane particles in the growing olfactory axons, Small *et al.* (1984) have derived diffusion coefficients in the range of 10^{-7} cm^2/sec,

Table I. Factors Affecting Lateral Diffusion Coefficients in Membranes

	Proteins		Lipids	Hydrophobic molecules
	Long-range (> μm)	Short-range (nm)	Short-range (nm)	
Theoretical (25°C) η (P)	⩾1		⩾1	⩽0.1
Theoretical (25°C) η' (P)	0.01		—	—
Theoretical (25°C) D (cm²/sec)	10^{-8}–10^{-9}[a]		10^{-7}–10^{-8}[b]	⩽10^{-7}[b]
Increased protein concentration	Slower	Little effect	Little effect	Little effect
Increased size (protein aggregation)	Slower	Slower	—	—
Viscosity of outer medium	Slower	Slower	No effect	No effect
Viscosity of membrane	Slower	Slower	Slower	Slower
Temporary protein associations	Slower	Little effect	—	—
Protein gradient	Faster	Little effect	No effect	No effect

[a] Using the Saffman–Delbrück relation [Eq. (3)].
[b] According to Eq. (5) or derivatives therefrom (Berg, 1983).

which are linearly dependent upon the inverse particle diameter in accordance with the Stokes–Einstein equation. Nonequilibrium processes depend upon the rate of entropy production (Onsager, 1931), and this is a major driving force of diffusion in a chemical gradient, as in the growing neuron.

The factors affecting diffusion of proteins and lipids are summarized in Table I.

Diffusion Control of Chemical Associations

All association processes in solution are ultimately limited by the time it takes to bring reactants together by diffusion. Most macromolecular reactions also require that the molecules attain a correct mutual orientation so that potentially reactive groups are properly aligned. Collisions between potentially reactive molecules are named *encounters*, but not every encounter brings about a reaction; usually the molecules have to collide many times before the reaction takes place.

If the rate of a chemical reaction is limited by the time it takes to bring the reactive groups together via diffusion, the reaction is considered *diffusion-controlled*; on the contrary, if subsequent chemical processes are rate-limiting, the rate is *reaction-controlled* (Berg and Von Hippel, 1985).

The most straightforward experimental way to distinguish between diffusion-controlled and reaction-controlled processes is to examine the viscosity dependence of the reaction. A diffusion-limited association rate constant is proportional to the diffusion coefficient and therefore inversely

proportional to the viscosity of the solvent; furthermore, the temperature dependence is weak and determined by the temperature dependence of the solvent viscosity.

The simplest geometry for a diffusional association is that of two spheres in a three-dimensional solution. According to Smoluchowsky (1917) the bimolecular association rate constant, k_a, for two spherical molecules A and B is

$$k_a = 4\pi(D_A + D_B)(r_A + r_B) \tag{4}$$

where D_A and D_B are the diffusion constants and r_A and r_B the radii of the two molecules. According to the Stokes–Einstein relation:

$$D_A = k_B T/6\pi\eta r_A \quad \text{and} \quad D_B = k_B T/6\pi\eta r_B \tag{5}$$

where k_B is Boltzmann's constant, T is absolute temperature, and η is solvent viscosity. Therefore, if $r_A \approx r_B$, we can approximate

$$k_a = \frac{8k_B T}{3\eta} \tag{6}$$

which corresponds to $k_a \approx 10^9$–$10^{10}\,M^{-1}\,sec^{-1}$ under normal aqueous solution conditions. The association could be faster if one molecule is small and diffuses rapidly while the other is large and provides a large target.

If the association reaction depends on a chemical step, the rate constant will be

$$1/k_a = 1/[4\pi(D_A + D_B)(r_A + r_B)] + 1/k \tag{7}$$

where k is the rate constant of the chemical step. When intermolecular forces are included, $(r_A + r_B)$ is replaced by an effective target radius which depends on the intermolecular potential between the two molecules. Since macromolecules are not reactive over their entire surfaces, but on restricted active sites, a full description of the diffusion-limited association process must consider the establishment of the relative positions and orientations of the molecules that are needed for the reaction to occur (Sole and Stockmayer, 1971). In contrast with diffusion in gases, in solutions it can be assumed (Berg and Von Hippel, 1985) that a molecule, owing to the erratic nature of the diffusional path (*random walk*), will come close to its starting point a large number of times prior to achieving an appreciable separation from its origin (*microcollisions* as distinguished from *macrocollisions*); molecules that have come together by diffusion will experience a large number of microcollisions, with changes in orientation, facilitating the occurrence of the *useful* collision. For two spherical molecules, assuming one molecule to be completely reactive, and the other having a reactive path over its surface, limited by an angle

θ_A with the center of the molecule, the diffusion-limited association will be roughly proportional to sin θ_A. Thus

$$k_a \approx \pi(D_A + D_B)(r_A + r_B) \sin(\theta_A/2) \tag{8}$$

If the steric constraint is severe (i.e., θ_A is very small), there can be a difference of orders of magnitude with respect to the simple Smoluchowsky relation. In real macromolecular associations, however, it is likely that long-range and short-range interaction forces will facilitate and prolong the macrocollisions, giving the molecules ample opportunity to seek out orientations for reaction. Thus electrostatic or hydrophobic interactions are sources of useful adhesion interactions (cf. Chou and Zhou, 1982). The reduction of dimensionality, as usually happens in membrane-mediated reactions, is considered to enhance the rate constants (Adam and Delbrück, 1968; Berg and Purcell, 1977); there is some empirical evidence in favor of guided diffusion by reduced dimensionality (Welch and Gaertner, 1975; Mosbach, 1976; Overfield and Wraight, 1980), but the rate constants for two-dimensional diffusion have yet to be rigorously defined (McCloskey and Poo, 1985). The equation developed by Hardt (1979), relating reaction rates with diffusion in two dimensions, and often taken as a basis for calculations of diffusion-limited rate constants in membranes, has been severely criticized (McCloskey and Poo, 1985) in that it does not take into account that the rate "constants" for two-dimensional diffusion-controlled reactions decline continuously with time (Emeis and Fehder, 1970; Razi-Naqvi *et al.*, 1974).

The lateral translational rate of a diffusing molecule is usually calculated by the Einstein–Smoluchowksy relation

$$d^2 = 4Dt \tag{9}$$

for a bidimensional path, where d is the distance run by the diffusing molecule and t is time. However, the mean time τ required to reach a small target of radius r in the middle of a cell of radius L ($L \gg r$) is (McCloskey and Poo, 1985)

$$\tau = (L^2/3D)(L/r) \qquad \text{in three dimensions} \tag{10}$$

and

$$\tau = (L^2/2D)\ln(L/r) \qquad \text{in two dimensions} \tag{11}$$

Thus, the diffusional search for a small target is much more efficient in two dimensions than in three, assuming D to be of comparable magnitude in three and two dimensions.

Short-Range and Long-Range Diffusion

First of all, we have to distinguish between true diffusion leading to macrocollisions and the local random walks leading to multiple microcollisions (McCloskey and Poo, 1985). Only macrocollisions are meaningful for establishing the diffusion limit; the duration of a diffusional macrocollision, i.e., the mean time that the molecules remain in proximity of one another and experience these multiple microcollisions, is $\approx R^2/D$, where R is the collision radius. Such time would range from nanoseconds to milliseconds, depending on the size of the molecule and its diffusion coefficient. We may name microdiffusion the diffusion leading to multiple microcollisions between the same molecules.

Nevertheless, very long-range diffusions ($\geqslant 1\ \mu$m) may also be distinguished from the paths normally leading to molecular interactions. The distances between reacting molecules in membranes (which is the meaningful subject of our discussion) usually range from 10 to 100 nm.

In practice, microdiffusion and short-range and long-range diffusion might be numerically different. This depends on several factors, including the fact that the solvent, the lipid bilayer, is anisotropic and itself consists of molecules of large size (700–1000 daltons for most phospholipids). Another reason may be in the discontinuous nature of diffusion. Diffusion is a stepwise process in which the molecules stay in one position before jumping to another one. The jump is the fast part of the process, but the overall time of diffusion takes into account also the time spent at the starting point. The jump frequency of phospholipid molecules is of the order of $\approx 10^8\ \text{sec}^{-1}$ (Galla *et al.*, 1979; Pace and Chan, 1982); this means that only few jumps take place during the lifetime of fluorescent or spin probes. On the other hand, if we measure diffusion over long distances, we need innumerable such steps before the diffusing molecules can reach the final point, so that the time spent between each jump may play an important role in the total diffusing time.

Additional reasons may be considered for natural membranes; thus, any lateral heterogeneity, including high density of proteins (Kawato and Kinosita, 1981; Peters and Cherry, 1982; Freire and Snyder, 1982; O'Shea, 1984), may lead to differences in short- and long-range diffusion; furthermore, if the diffusing molecules undergo reversible aggregation phenomena, the longer the path considered, the smaller would be the apparent diffusion coefficient.

Different techniques to probe diffusion are based on different physical principles, and might detect different ranges of the diffusion process. We have already discussed that FRAP, by measuring the recovery of fluorescence over a bleached area in the fluorescence microscope, is usually able to measure only long-range diffusion (cf. also Kawato and Kinosita, 1981). The spectroscopic

techniques, on the other hand, are based on collisional encounters; do they measure short-range diffusion or microdiffusion?

Let us take the fluorescence quenching method, which we have employed for measuring the diffusion of ubiquinone. The quenching process of an excited fluorophore F* to its ground state F by a quencher Q is a typical bimolecular collisional process which competes with the fluorescence emission of the fluorophore

$$F^* + Q \xrightarrow{k} FQ$$

where k is the bimolecular collisional quenching constant. The phenomenon of collisional quenching is adequately expressed by the Stern–Volmer relation:

$$I_0/I = 1 + \tau_0 k[Q] = \tau_0/\tau \tag{12}$$

where I_0, I, τ_0, τ are respectively the intensities and the lifetimes of fluorescence in the absence and in the presence of quencher Q, and k is the quenching constant. Deviations from the Stern–Volmer law can derive from different mechanisms of quenching, the most common being a positive deviation in the plot due to the presence of static quenching, when the quencher molecules are in the sphere of action of the fluorophore forming a nonfluorescent ground-state complex (Lakowicz, 1983).

The lifetime of the excited state for most fluorophores employed in quenching studies is within 10 nsec. It is likely that in this time, being shorter than the time R^2/D (McCloskey and Poo, 1985) in which two colliding molecules remain in contact with one another, many such microcollisions take place. What is the probability that the quenching method detects such microcollisions rather than the diffusion-controlled macrocollisions between fluorophore and quencher?

The problem may be simply approached by asking what is the probability that the same molecule, once quenched by a macrocollision, is reexcited by the incident light in the spectrofluorimeter and quenched again, during a time corresponding to the lifetime of the excited state. A simple comparison between the number of photons entering the optical path in the cuvette and the number of fluorophores in the same path allows one to solve the problem. For example, under the experimental conditions employed in our laboratory, 2×10^{-9} mol photons per minute traverse the optical path (as measured directly by an actinometer), corresponding to 3.3×10^{-19} mol of photons in 10 nsec, whereas 10^{-10} mol of fluorophore are contained in the same volume. Thus, in the lifetime of the excited state about one molecule out of 10^9 is excited, or, in other words, the probability that one molecule is reexcited is $1/10^9$. It is therefore clear that fluorescence quenching, as well as similar spectroscopic methods, do not measure microdiffusion, but rather meaningful short-range diffusion.

Diffusion Coefficients of Electron Transfer Components

The diffusion of protein complexes in mitochondrial membranes was measured by Sowers and Hackenbrock (1981) by a combination of postfield relaxation and freeze-fracture electron microscopy to quantitate the distribution of intramembrane particles; a D of 8.3×10^{-10} cm^2/sec was calculated from these experiments for the particles in spherical mitoplasts of rat liver mitochondria. The same group later found D values near 4×10^{-10} cm^2/sec using FRAP on labeled complexes I–IV in fused megamitochondria (Gupte *et al.*, 1984), whereas Hochman *et al.* (1985) also using FRAP, obtained $D = 1 \times 10^{-10}$ cm^2/sec for cytochrome oxidase in megamitoplasts from cuprizone-fed mice.

The discrepancy between the electrophoretic relaxation method and FRAP may be accounted for by the fact that in the former a particle gradient is established, leading to possible faster diffusion (Small *et al.*, 1984).

As stressed before, the diffusion coefficients of integral proteins protruding into aqueous compartments are strongly affected by the viscosity of the aqueous matrices. Mitoplasts lose substantial portions of matrix proteins (Caplan and Greenwalt, 1966), so the true long-range diffusion coefficients in intact mitochondria *in vivo* could be significantly lower than those measured. On the other hand, however, the long-range diffusion measured by FRAP could be slowed down, with respect to short-range diffusion, by the density of proteins and by macromolecular associations leading to temporary clusters of greater size. It is significant, as we have previously stressed, that the diffusion coefficients of proteins in reconstituted systems are at least one order of magnitude higher than those found in mitochondria (cf. McCloskey and Poo, 1985).

In the same study quoted above, Gupte *et al.* (1984) have measured the diffusion coefficient of a fluorescent derivative of 2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)benzoquinone by FRAP, reporting a D value of the order of 3×10^{-9} cm^2/sec. On the other hand, by exploiting fluorescence quenching of 12-(9-anthroyl)stearic acid (and other probes) inserted in liposomes and mitochondrial membranes by unmodified ubiquinone homologs, including the physiological Q_{10}, we have calculated D values close to 10^{-6} cm^2/sec (Fato *et al.*, 1985, 1986; Lenaz *et al.*, 1986).

The discrepancy between the results obtained by the two methods is so large (about three orders of magnitude) and the kinetic consequences on the turnover of the respiratory chain are so compelling, that we must carefully analyze both experimental conditions in order to seek an explanation.

The possibility that either data are the result of obvious technical artifacts is remote. Membrane damage by the potent laser beam in FRAP is usually discounted, and other factors as fluorescence energy transfer or

uncertainties in the mathematical treatment in relating distance to diffusion, which is based on the Einstein–Smoluchowsky relation [Eq. (9)] could affect the results by no more than one order of magnitude (M. Fragata, personal communication).

As for fluorescence quenching, we have carefully eliminated possible artifacts arising from static quenching and the possibility that high quenching constants are the result of proximity effects of quinones and probes within aggregates either in water or in the membrane phase (Fato *et al.*, 1986). The contribution of static quenching (Lakowicz, 1983), in particular, was avoided by measuring the fluorescence lifetimes by time-resolved fluorescence.

A pseudo-static quenching effect may ensue when the quencher happens to be close to the fluorophore at the moment of excitation; in such a case a high probability exists that quenching will occur before these molecules diffuse apart (Lakowicz, 1983). The modified form of the Stern–Volmer equation describing this situation is (Lakowicz, 1983)

$$I_0/I = (1 + k[\mathrm{Q}] \exp [\mathrm{Q}] VN/1000) \tag{13}$$

where V is the volume of the sphere (the sphere of influence comprising the two species). The percentage of "complexed" fluorophores increases as the mole fraction of the quencher Q increases, leading to an upward deviation of the Stern–Volmer plot simulating static quenching. Upward inflections of the Stern–Volmer plots have been observed in some of our experiments at high ubiquinone concentrations (Fato *et al.*, 1986) and are most probably the result of such form of static quenching; when necessary, the "static" component was subtracted, but no significant differences were observed when the Q concentrations were kept very low, indicating that this mechanism under our conditions is negligible. Moreover, this effect also does not affect time-resolved fluorescence (Fig. 1).

A somewhat different problem (Nemzek and Ware, 1978), however, can arise when the lifetime of the excited state is very short; then the quenching is not an ideal diffusion-controlled process any more, because of the so-called *transient effect* (Nemzek and Ware, 1978; Andre *et al.*, 1978). When the lifetime of the excited state is too short, the quenching can occur before the system attains equilibrium, and the Smoluchowsky relation for fluorescent quenching encounters is modified by a time-dependent term (Andre *et al.*, 1978):

$$k(t) = 4\pi N' \gamma (r_\mathrm{A} + r_\mathrm{B})(D_\mathrm{A} + D_\mathrm{B}) \left(1 + \frac{r_\mathrm{A} + r_\mathrm{B}}{\sqrt{(D_\mathrm{A} + D_\mathrm{B})t}}\right) \tag{14}$$

where N' is Avogadro's number per millimole and γ is quenching efficiency.

For lifetimes $\ll 10$ nsec and/or slow diffusion coefficients the transient term can become significant. For example, for $(r_\mathrm{A} + r_\mathrm{B}) = 10$ Å,

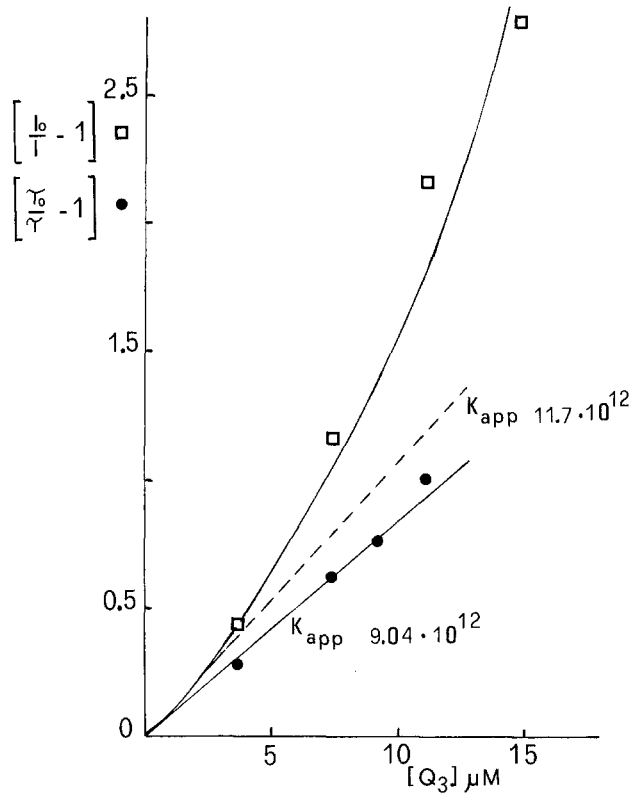

Fig. 1. Contribution of static quenching to the Stern–Volmer plots of the probe 12-(9-anthroyl)stearate in asolectin vesicles (0.5 mg/ml) by Q_3; (□) I_0/I; (●) τ_0/τ. We have purposely reported one of the experiments where the upward inflection in the I_0/I plots was most accentuated. k_{app} is the apparent quenching constant obtained from [Q] concentration in the total medium; from Stern–Volmer plots at different phospholipid concentrations the true quenching constant in the membrane was obtained (Fato *et al.*, 1986). The quenching constants in the membrane derived from the complete experiment were $6.4 \times 10^9\ M^{-1}\ sec^{-1}$ for the I_0/I experiment and $4.6 \times 10^9\ M^{-1}\ sec^{-1}$ for the τ_0/τ experiment (Fato *et al.*, 1986).

$D = 10^{-9}$ cm²/sec, and $\tau = 10$ ns, the time-dependent term would increase k by a factor of ≈ 10, i.e., a theoretical k of $1.4 \times 10^6\ M^{-1}\ sec^{-1}$ would be matched by an experimental value of $\geqslant 10^7\ M^{-1}\ sec^{-1}$. It is clear that the transient effect cannot explain the discrepancy between the results of Gupte *et al.* (1984) and ours (Fato *et al.*, 1986). On the other hand, for $D = 10^{-7}$ cm²/sec, the transient term would be reduced to a factor of ≈ 2. In practice, however, we have observed no difference in the quenching constants using probes having different lifetimes.

An energy-transfer mechanism may lead to a diffusion-controlled quenching (Streyer *et al.*, 1982) under conditions in which $D\tau_0/d^2 \geqslant 1$ (where τ_0

is the fluorescence lifetime and d is the average distance between donors and acceptors). Under the conditions used in our studies, however, the term is $\ll 1$ by several orders of magnitude, and the efficiency of such diffusion-controlled energy transfer would practically be zero. Moreover, energy transfer is excluded by the almost complete lack of overlapping of the wavelengths of emission of the fluorophores employed (> 400 nm) with that of absorption of ubiquinone (Mayer and Isler, 1971).

The use by Gupte *et al.* (1984) of a quinone derivative which is admittedly more polar than the physiological ubiquinone and lies in the polar phase of the membrane may cast doubts on the significance of their results toward natural ubiquinone. Using a polar derivative of Q_2, we have found a diffusion coefficient about four-fold lower than that of the parent quinone (Fato *et al.*, 1986). This difference, however, can hardly explain the large discrepancy observed. The problem raised by Aizenbud and Gershon (1982) that diffusion coefficients for nonplanar membrane surfaces are underestimated does not probably hold for the membranes used by Gupte *et al.* (1984): mitochondrial cristae are highly convoluted membrane surfaces, but the fused mitoplasts used by Gupte *et al.* (1984) are swollen spherical organelles.

The discrepancy should therefore mainly reside in the intrinsic differences by which the two methods measure diffusion, that is to say, short-range diffusion (nm) by fluorescence quenching and long-range diffusion (μm) by FRAP. If this is the case, irrespective of the reasons for the differences, we feel that short-range diffusion has greater physiological significance for mitochondrial electron transfer (cf. Kawato and Kinosita, 1981).

A close scrutiny of the reasons leading to retarded diffusion in the long-range FRAP experiments, however, could cast light on the organization of the electron transfer components. In fact, besides hindrance by the high protein concentration of the inner mitochondrial membrane, the presence of transient reversible associations of the ubiquinone molecules with the protein complexes could also result in retarded long-range diffusion, since in the average time spent in association with proteins, ubiquinone would move at the slower pace of protein diffusion. This topic will be developed later.

Is a diffusion coefficient of 10^{-6} cm^2/sec (or within an order of magnitude of this value) compatible with the known facts about ubiquinone location and membrane structure and dynamics?

The localization of ubiquinone in the mitochondrial membrane is still a matter of much debate. Many studies support the view that ubiquinone is distributed in a hydrophobic environment but a large aliquot of it is located near the membrane surface (Kingsley and Feigenson, 1981; Stidham *et al.*, 1984). Chatelier and Sawyer (1985), by using a set of anthroyloxy fatty acid derivatives, found some evidence for two pools of ubiquinone-10 in the

transverse membrane plane in mitochondria, one near the surface and the other located in the bilayer midplane. Similar results were obtained in our laboratory (Fato *et al.*, 1986). The interchange between the two compartments, if any, must be slower than the nanosecond time scale on which the fluorescence quenching occurs. Transmembrane rates for ubiquinones were measured by Kingsley and Feigenson (1981) by proton NMR, using lanthanide shift reagents, and were found to range between 20 and 300 sec^{-1}, depending on the homolog considered. With these rates, hardly any movement would be detected on the time scale of the fluorescence decay. By studies of the accessibility of ubiquinones to sodium borohydride and of the methoxy proton resonances in phospholipid bilayers, Ulrich *et al.* (1985) also concluded that Q_{10} is localized in two pools of comparable size, a superficial one and a deep one (cf. also Alonso *et al.*, 1981; Katsikas and Quinn, 1982a, b, 1983). Studies on partition and determination of critical micelle concentrations for the Q homologs in aqueous media (Fato *et al.*, 1986; Battino *et al.*, 1986) showed that the free energy for transfer on the quinone ring from water to hydrophobic medium is rather negative, allowing its significant localization only in the hydrophobic portion of the membrane. From the micellization properties of mixed lecithin–Q dispersions in ethanol–water (Lenaz and Degli Esposti, 1985), the CMC appeared largely independent of the quinone hydrophobicity, contrary to pure Q micelles in the same system. The results suggest that, if the ubiquinone molecules stack between the phospholipid molecules, they do so by keeping the quinone ring in a nonpolar phase, and the critical length of the isoprenoid chain for stacking is 3–4 units, the remainder of the Q side chain being accommodated in such a way that it does not contribute to the net thermodynamics of the system (Ferri *et al.*, 1982; Lenaz and Degli Esposti, 1985; Battino *et al.*, 1986). The localization of the quinone ring in a hydrophobic environment is also suggested by the spectroscopic properties of ubiquinone having side chains $\geqslant 2$ units, resembling those in fluid hydrocarbons (Stidham *et al.*, 1984; Lenaz and Degli Esposti, 1985; Degli Esposti *et al.*, 1981a, b).

It may be inferred from all these results that ubiquinone may be located in the hydrophobic core of the membrane, with the quinone ring switching its position back and forth toward the membrane surfaces, but always remaining in a hydrophobic environment. The thermodynamic driving force for such a disposition of the quinone ring would not be polarity, since the quinone ring behaves as a hydrophobic group, but rather the geometry of the system which would favor a disposition of the ring with the first few isoprenoid units fitting between the hydrocarbon chains of the fatty acids (as is the case with the hydrophobic molecule diphenylhexatriene) (Thulborn, 1981). A roughly discoid molecule having an average radius of 15 Å would describe the shape of the moving object (Fig. 2).

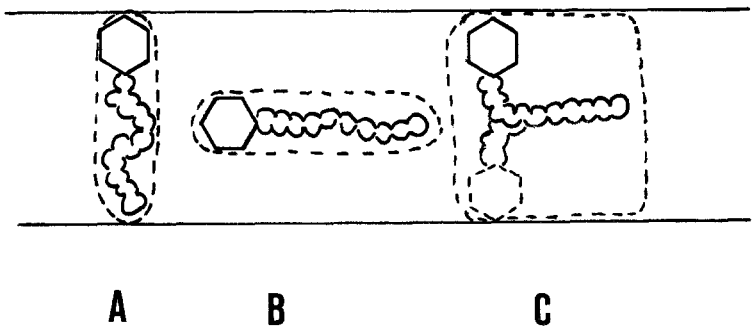

Fig. 2. Possible models for the localization of Q_{10} in the lipid bilayer. The model favored by the authors is the one in C (see text).

Membrane viscosities reported in the literature range from one to several cP (Dix *et al.*, 1978; Brown *et al.*, 1979) up to 1 P (Hare and Lussan, 1978) or more (Shinitzky and Inbar, 1976). The discrepancies are due to several reasons, but the main one is that there is a fluidity gradient perpendicular to the plane of the bilayer (Seelig and Seelig, 1980). We may add that microviscosity determined by fluorescence polarization receives contributions from both the mobility and the order parameter of the probe (Brown *et al.*, 1979; Jähnig, 1979; Zannoni, 1981; Meier *et al.*, 1982).

The viscosity of the bilayer midplane is usually assumed to be a few centipoise; direct measurement of the viscosity of mitochondrial membranes from the rotational correlation time of the spin probe 16-doxylstearate (Lenaz *et al.*, 1983; Fato *et al.*, 1986) yields between 5 and 20 cP, depending on the molecular radius of rotation assumed. Since the Saffman-Delbrück model [Eq. (3)] cannot be applied a molecule moving within the membrane interior (Vaz *et al.*, 1984), approximations can be obtained by modifications of the Stokes–Einstein law [Eq. (5)] for two dimensions (Berg, 1983). Assuming the Q_{10} headgroup sweeping the membrane thickness, the molecule can be depicted as a disc of 40 × 30 Å perpendicular to the plane of the membrane. By assuming that the disc moves laterally sideways, a viscosity around 10 cP, as experimentally found, would correspond to a diffusion coefficient between 10^{-7} and 10^{-6} cm^2/sec, as from our results: in fact, application of the Smoluchowsky equation [Eq. (4)] to the quenching constants found by Fato *et al.* (1986) for a molecule of the above dimensions will yield a diffusion coefficient of 7.6×10^{-7} cm^2/sec. The agreement is therefore quite good, considering the uncertainties in the numerical parameters used to fit the equations.

A criticism that can be raised concerning a diffusion coefficient higher than 10^{-7} cm^2/sec is that it would be higher than that of the solvent molecules, i.e., the phospholipids. The D values for phospholipids usually range

between 10^{-8} and 10^{-7} cm^2/sec, with some reports of values higher than 10^{-7} cm^2/sec (cf. Wade, 1985). The diffusion coefficient of the spin label 5-doxylstearate was found by the quenching method to be 2.5×10^{-7} cm^2/sec (Fato *et al.*, 1986; Blatt and Sawyer, 1985). On the other hand, D for the spin label analog 16-doxylstearate was found to be 8.5×10^{-7} cm^2/sec. The discrepancy between the two values might derive from a true difference in *lateral* displacement of the two, otherwise like, molecules, since the shape and location of 16-doxylstearate in the membrane could be different from that of 5-doxylstearate. The explanation provided by Blatt and Sawyer (1985) is that the flipping up motion of the doxyl group in the distal position (16-doxylstearate) provides a better chance of collisions with the fluorophore by allowing faster segmental motions. A similar explanation could be invoked for the long-tailed Q_{10} molecule (but not for the short-chain homologs, which have about the same quenching constants).

The anisotropic nature of the lipid solvent makes it possible for a solute to have short-range diffusional motions faster than those of the lipids, as pointed out in a previous section (cf. Galla *et al.*, 1979; Rigaud *et al.*, 1972; Vandegriff and Olson, 1984), without having to invoke particular mechanisms or artifacts.

From all of the above considerations we can reasonably conclude that the diffusion coefficients of short-chain Q homologs in the membrane are truly close to 10^{-6} cm^2/sec whereas for Q_{10} a true translational diffusional motion can be described by a diffusion coefficient between 2×10^{-7} and 10^{-6} cm^2/sec.

Ubiquinone Diffusion and Turnover of Ubiquinone-Requiring Enzymes

A diffusion coefficient for ubiquinone of not less than 10^{-7} cm^2/sec must be compared with the kinetic constants of the ubiquinone-requiring enzymes; several approaches are possible and some of them have been experimentally tested.

Calculation from the Displacement Rates

The lateral displacement rate, calculated from the diffusion coefficients by the Einstein–Smoluchowsky relation [Eq. (9)], allows one to measure the time employed to cover a given distance in the membrane. If we assume that the average distance between Complex I and Complex III in the inner mitochondrial membrane is 30 nm (Capaldi, 1982), this distance would be covered in between 0.022 msec for $D = 10^{-7}$ cm^2/sec and 0.0022 msec for $D = 10^{-6}$ cm^2/sec.

For diffusion of a particle to a small target of diameter r, the time necessary for a displacement d allowing to hit the target is given by (Berg and Purcell, 1977):

$$t = \frac{d^2}{2D}\left(\ln\frac{d}{r} - \frac{3}{4}\right) \tag{15}$$

For a distance of 30 nm, and assuming a diameter for mitochondrial Complex III of 4.5 nm (Rieske and Ho, 1985), taking a $D = 10^{-7}$ cm^2/sec, the time for a ubiquinone molecule leaving Complex I to reach Complex III would be 0.05 msec (0.005 msec for $D = 10^{-6}$ cm^2/sec).

The turnover of mitochondrial redox complexes may exceed 1000 sec^{-1} (1 msec per turnover), even if lower turnovers are expected for the integrated electron transfer between complexes according to the mobile pool equation [Eq. (1)]; but even an exceedingly high turnover of 5000 sec^{-1}, corresponding to 0.2 msec/turnover, would still be lower than the probability of collisions. Under normal coupled conditions, the turnover of the respiratory chain should not be beyond 50 sec^{-1} (20 msec/turnover), leaving *at least* a 400-fold excess for the lateral displacement rate. There would be wide allowance even for distances larger than 30 nm; for example, assuming dilution to a distance between Complex I and III of 300 nm, with $D = 10^{-7}$ cm^2/sec, the time to reach Complex III would be 15 msec, only approaching the turnover of the chain under coupled conditions. This is obviously an extreme case showing that, in theory, the diffusion limit *can* be reached, particularly for long distances and under uncoupled conditions.

Calculations from the Second-Order Rate Constants

It may be demonstrated that the interaction of ubiquinone with Complex I and ubiquinol with Complex III is not diffusion-limited, that is to say, no contribution of diffusion is given to the second-order rate constant of enzyme substrate interaction. The bimolecular collision constants may be obtained from the Smoluchowsky equation using the diffusion coefficients of the interacting partners (ubiquinone and complexes); thus, for ubiquinol and the bc_1 complex, we would have:

$$k_{+1} = 4\pi N'(D_Q + D_{bc_1})(r_Q + r_{bc_1}) \tag{16}$$

Assuming a molecular collisional radius of 15 Å for ubiquinol-10 and 45 Å for Complex III, and using $D_Q = 10^{-7}$ cm^2/sec (a conservative value) and 10^{-10} cm^2/sec for D_{bc_1} (which can be neglected), we obtain a bimolecular collision constant of 4×10^8 M^{-1} sec^{-1} (4×10^9 M^{-1} sec^{-1} for $D_Q = 10^{-6}$ cm^2/sec).

The second-order rate constant of substrate enzyme interaction k_{+1} is indirectly calculated as $k_{+1} = k_{cat}/K_m$ (Rawn, 1983). For the bc_1 complex such calculation has been applied to the physiological substrate ubiquinol-10

Table II. Kinetic Constants for Isolated Complex I and Complex III in Phospholipid Vesicles (25°C)

	Complex I		Complex III	
	Q_1	Q_{10}	Q_1H_2	$Q_{10}H_2$
k_{cat} (sec^{-1})	370	700	200	5,000
K_m (in PL) (mM)	53	32	8	2.3
k_{+1} (k_{cat}/K_m) ($M^{-1}\,sec^{-1}$)	7×10^3	2.2×10^4	2.5×10^4	2.1×10^6
Activation energy (kcal/mol)	—	—	8.0	—

(Fato *et al.*, 1986) (Table II). Using a value of k_{cat} as high as 5000 sec^{-1} (Lenaz *et al.*, 1985a; Zhu *et al.*, 1982) and an average K_m of 8 mol/mol complex (Zhu *et al.*, 1982; Poore and Ragan, 1982), equivalent to 1.68 nmol/mg protein in mitochondria or roughly 3.34 mM in the phospholipids, we can calculate

$$k_{+1} = 5 \times 10^3/3.34 \times 10^{-3} = 1.5 \times 10^6\,M^{-1}\,sec^{-1}$$

This value is not far from the second-order rate constant directly obtained by Snozzi and Crofts (1984) of $1\text{–}2 \times 10^5\,M^{-1}\,sec^{-1}$ for endogenous Q_{10} in phospholipid-enriched *R. sphaeroides* chromatophores. The second-order rate constants found with ubiquinol-1 as substrate is $4 \times 10^4\,M^{-1}\,sec^{-1}$ in beef heart (Lenaz *et al.*, 1985b, 1986) and $\approx 10^6\,M^{-1}\,sec^{-1}$ in *S. cerevisiae* mitochondrial Complex III (Palmer *et al.*, 1985).

Similar calculations have been performed for ubiquinone reduction by NADH, catalyzed by Complex I (Fato *et al.*, 1986). The value of k_{cat} of NADH-Q_{10} reductase may be calculated in the range of 700 sec^{-1} (Ragan, 1985). The K_m for Q_{10} in NADH-Q reductase is extrapolated from the saturation of reactivation of NADH oxidase by Q_{10} (Kröger and Klingenberg, 1973a) to 15 nmol/mg protein, or 32 mM in the phospholipids. Calculation of k_{+1} as above yields

$$k_{+1} = 7 \times 10^2/3.2 \times 10^{-2} = 2.18 \times 10^4\,M^{-1}\,sec^{-1}$$

The discrepancy between the second-order rate constants and the diffusion-controlled collisional constants exceeds a factor of at least 10^2, probably $\geqslant 10^3$ for Complex III, and $\geqslant 10^4$ for Complex I. Thus it seems clear that diffusion cannot be a limiting factor, unless gross steric constraints are present in the interaction between ubiquinone and the enzymes.

Let us assume, for discussion, that the second-order rate constant for ubiquinol-10 + Complex III in the range of 2×10^6 is diffusion-limited; this would correspond, from the Smoluchowsky relation, to $D = 4.4 \times 10^{-10}\,cm^2/sec$ for ubiquinol + Complex III. Likewise, for Complex I–ubiquinone interaction, we would calculate from $k = 2.18 \times 10^4\,M^{-1}\,sec^{-1}$ a $D = 2.7 \times 10^{-12}\,cm^2/sec$ for ubiquinone + Complex I. The diffusion coefficients of ubiquinol + Complex III and ubiquinone + Complex I should be equated to those of ubiquinol and ubiquinone, respectively, since the

protein complexes are expected to be at least one order of magnitude slower than the quinone. Even neglecting that the diffusion coefficients calculated by this procedure are even lower than those measured by FRAP for ubiquinone (Gupte *et al.*, 1984), it is unrealistic to suppose that the diffusion of ubiquinol and ubiquinone could differ by two orders of magnitude. Thus, if the reactions are coupled to diffusion of ubiquinone (ubiquinol) to the complexes, only ubiquinol-cytochrome *c* reductase would be diffusion limited (by diffusion of ubiquinol), whereas NADH-Q reductase could *only* be reaction-limited.

The most reasonable conclusion from these calculations, in our opinion, is that *both* enzymes are reaction-limited. Thus, for example, in the case of ubiquinol-cytochrome *c* reductase, a second-order rate constant of $2 \times 10^6\ M^{-1}\ sec^{-1}$ for cytochrome *b* reduction by ubiquinol-10 could be limited by the chemical redox reaction of the quinone ring with the heme group, which could take place over a great distance, or by exchange of exogenous with endogenous ubiquinone (Snozzi and Crofts, 1984), or by reduction of endogenous ubiquinone by exogenous ubiquinol (from the pool) (Palmer *et al.*, 1985), or finally by steric factors as reorientation of the quinone in the active site of the complex.

Effect of Viscosity and Temperature

Diffusion-limited reactions are characterized by their low activation energy and by being viscosity dependent (Berg and Von Hippel, 1985). The temperature dependence of mitochondrial enzymes is characteristically biphasic, with breaks in Arrhenius plots occurring at near 18–20°C, with activation energies in the range of 8–10 kcal/mol above the break and $\geqslant 20$ kcal/mol below the break (Lenaz, 1979; Lenaz and Parenti Castelli, 1985; McMurchie *et al.*, 1983). The reason for the break is beyond the scope of this article, and is usually ascribed to phase transitions or other temperature-dependent properties of the lipids. No striking differences exist between the activation energies of the overall oxidase activities (succinate oxidase, NADH oxidase) and those of individual complexes using short-chain ubiquinones as substrates [NADH-Q_1 reductase; ubiquinol-1 (or -2) cytochrome *c* reductase (Lenaz *et al.*, 1986; Lenaz, 1979; Hackenbrock *et al.*, 1986)]. This means that, if ubiquinone (or ubiquinol) diffusion in the lipids is the rate-limiting step, the same step would apply also to short-chain ubiquinones, which therefore would reach their active sites by diffusion through the lipid phase. There is wide evidence that short-chain ubiquinones reach their active sites by diffusing in the lipids (Lenaz *et al.*, 1985a). In addition, the second-order rate constant of ubiquinol-1–Complex III interaction, calculated from the k_{cat}/K_m relation, is in the range 2.5×10^4 and $2 \times 10^5\ M^{-1}\ sec^{-1}$, using for K_m the concentration of ubiquinol-1 in the phospholipids [1–8 mM, depending on the conditions (Fato *et al.*, 1986)]; using for the K_m the total ubiquinol-1 concentration in the aqueous medium (1–8 μM, cf. Degli Esposti and Lenaz,

1982) would yield k_{+1} from 2.5×10^7 to $2 \times 10^8\ M^{-1}\ sec^{-1}$. The constant determined with ubiquinol-1 by direct stopped flow experiments (Lenaz *et al.*, 1985b; Palmer *et al.*, 1986) ranges between 4×10^4 and $10^6\ M^{-1}\ sec^{-1}$, in agreement with the former calculation but not with the latter.

The activation energy for diffusion has been measured in mitochondria in temperature-dependent quenching experiments, and found to be close to 1 kcal/mol (Fato *et al.*, 1986). This low value agrees with the somewhat higher value of 3–4 kcal/mol found for the activation energy of viscosity (calculated from Lenaz *et al.*, 1983), still significantly lower than the activation energy of any mitochondrial redox enzyme.

The effect of viscosity on the activity of mitochondrial Q-enzymes has not been widely investigated independently of temperature. Viscosity may be changed by varying the lipid composition, but other, conformational, effects may be involved in the changes of enzyme kinetics (Lenaz, 1979; Lenaz and Parenti Castelli, 1985). Cholesterol is widely used as a membrane rigidifying agent (Shinitzky, 1984). Addition of cholesterol to lipid vesicles has been found to decrease the diffusion coefficient of ubiquinones (Fato *et al.*, 1986). Cholesterol has been incorporated in bc_1 proteoliposomes, and the activity of ubiquinol-1 cytochrome *c* reductase has been found to be *increased* (Lenaz *et al.*, 1986); moreover, a temperature dependence study showed that the break normally found in the Arrhenius plot of bc_1 proteoliposomes was abolished, but the activation energy was not lowered above the break. It may be concluded therefore that, at least above 20°C, the diffusion of ubiquinol-1 is not the rate-limiting step, and that this step has the same activation energy in the presence and absence of cholesterol. For the reason outlined previously, the diffusion of ubiquinol-1 in the water phase is the prerequisite for its partition in the membrane, but it is its diffusion in the membrane that assures interaction with the active site.

Integrated Electron Transfer (NADH Cytochrome c Reductase and Succinate Cytochrome c Reductase)

Ragan and Cottingham (1985) have elaborated the pool equation [Eq. (1)] kinetically under conditions in which the Q concentration is not saturating for the activity of the individual enzymes. Under such conditions we have

$$V_{obs} = \frac{\dfrac{V_{m_{red}} \cdot V_{m_{ox}}}{V_{m_{red}} + V_{m_{ox}}}[Q_t]}{\left\{\left[V_{m_{red}}\left(K_{s.2} + K_{s.2}\sum_1^n \frac{c_n}{K_{sn}}\right) + V_{m_{ox}}\left(K_{s.1} + K_{s.1}\sum_1^n \frac{c_n}{K_{sn}}\right)\right] \Big/ [V_{m_{red}} + V_{m_{ox}}]\right\} + [Q_t]} \tag{17}$$

where $[Q_t]$ is the total ubiquinone concentration, $V_{m_{red}}$ and $V_{m_{ox}}$ are the maximal velocities of Q reductase and Q oxidase, respectively, $K_{s,1}$ and $K_{s,2}$ are the dissociation constants for Q_{ox} and Q_{red} from the dehydrogenase and bc_1 complex, respectively, and K_{sn} and c_n are the dissociation constants and concentrations, respectively, of all Q-reactive enzymes.

The equation can be approximated for the integrated electron transfer between, let us say, Complex I and Complex III, by the following:

$$V_{obs} = \frac{\dfrac{V_{m_{red}} \cdot V_{m_{ox}}}{V_{m_{red}} + V_{m_{ox}}} [Q_t]}{\dfrac{(V_{m_{red}} \cdot K_{m2}) + (V_{m_{ox}} \cdot K_{m1})}{V_{m_{red}} + V_{m_{ox}}} + [Q_t]} \tag{18}$$

where K_{m1} and K_{m2} are the K_m of Complex I for ubiquinone and of Complex III for ubiquinol, respectively.

The concentrations of Q_t are usually in the range of the K_m of Q-reactive enzymes. For example, the K_m of the bc_1 complex for ubiquinol-10 is 3.34 mM in the phospholipids and the K_m of Complex I for ubiquinone-10 is ca. 32 mM in the phospholipids (see above), while $[Q_t]$ in mitochondria is ca. 8 mM. This means that Q_t is not saturating for either V_{red} or V_{ox}, and hence for V_{obs}. Using values for $V_{m_{red}}$ or 45 nmol sec^{-1} mg^{-1} and for $V_{m_{ox}}$ of 78 nmol sec^{-1} mg^{-1} (Kröger and Klingenberg, 1973b) and a value for $[Q_t]$ of 8 mM in the phospholipids, a V_{obs} of 7.72 nmol sec^{-1} mg^{-1} for NADH oxidase can be readily calculated, compared with a maximal value at infinite $[Q_t]$ of 28.4 nmol sec^{-1} mg^{-1}. This means that under physiological conditions the ubiquinone content of the inner mitochondrial membrane is not sufficient to yield maximal turnover of electron transfer.

As pointed out by Lenaz and Parenti Castelli (1984), Ragan and Cottingham (1985), and Fato *et al.* (1986), the decreased electron transfer activity upon dilution of the membrane with phospholipids (Schneider *et al.*, 1982) need not be related to the increased diffusion path for ubiquinone or ubiquinol, but simply reflects the decrease of saturation of the enzymes by ubiquinone and ubiquinol. This is clearly shown in Fig. 3. If we account for an aliquot of bound quinone, not participating in the pool, of 25% of the total quinone (and this could be a conservative value) (Suzuki and Ozawa, 1984), we would find for a 2.5-fold dilution of the membrane a decrease from 6.22 to 1.5 nmol sec^{-1} mg^{-1}, i.e., to 24% of the original value. This decrease is well comparable with the fall in activity described by Schneider *et al.* (1982a) upon phospholipid enrichment of mitochondria.

The temperature dependence of V_{obs} (NADH to cytochrome *c* or succinate to cytochrome *c*) does not show any striking difference with respect to the temperature dependence of $V_{m_{red}}$ and $V_{m_{ox}}$ (NADH to Q or succinate to Q,

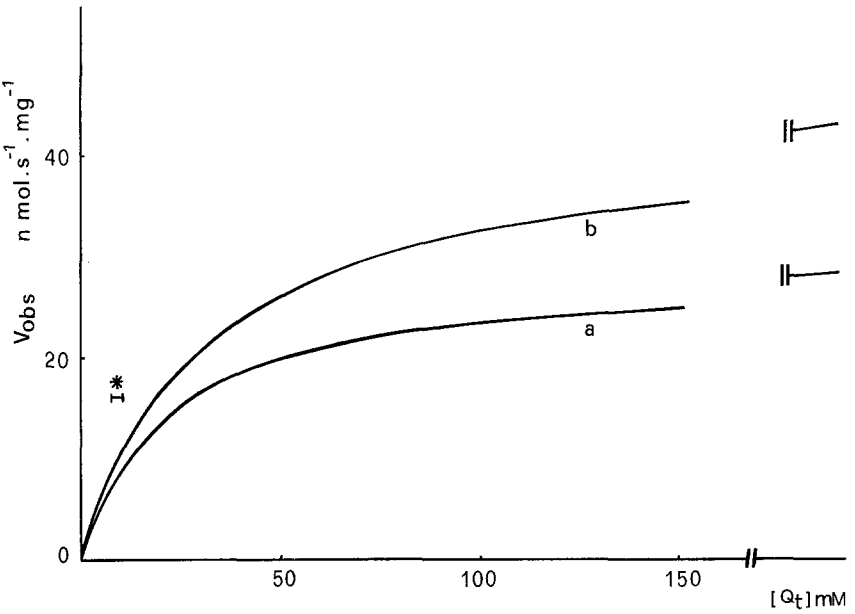

Fig. 3. The relation between V_{obs} and the concentration of the ubiquinone pool in the inner mitochondrial membrane. The curves were plotted using Eq. (18) and the K_m values listed in the text. Curve a is obtained using $V_{m_{ox}}$ of 78 nmol sec^{-1} mg^{-1} for ubiquinol oxidase and $V_{m_{red}}$ of 45 nmol sec^{-1} mg^{-1} for NADH-Q reductase at 25°C in uncoupled submitochondrial particles (Kröger and Klingenberg, 1977). Curve b is obtained using as $V_{m_{ox}}$ the value of 1050 nmol sec^{-1} mg^{-1} for ubiquinol cytochrome *c* reductase (from a turnover number of 5000 sec^{-1}) (Lenaz *et al.*, 1985; Zhu *et al.*, 1982). Using a K_m of 5 nmol/mg protein for Q_{10} in NADH dehydrogenase (Norling *et al.*, 1974), the saturation of NADH oxidase (V_{obs}) at physiological Q_{10} concentration is still *ca.* 50%.

and ubiquinol to cytochrome *c*); the activation energies above 20°C are close to 10 kcal/mol in all cases. It seems therefore unlikely that a diffusion-limited step exists between $V_{m_{red}}$ and $V_{m_{ox}}$ under normal conditions.

The viscosity dependence of V_{obs} is under investigation in our laboratory. Preliminary studies have shown that in a crude preparation of succinate cytochrome *c* reductase inlayed in liposomes, the incorporation of cholesterol, while increasing the viscosity of the lipids, induces no decrease but rather an increase of V_{obs} (succinate cytochrome *c* reductase activity) (Table III). Titration with exogenous Q_2 of such proteoliposomes shows that V_{obs} increases to a V_m higher in the presence than in the absence of cholesterol, but with the same K_m. If diffusions of Q were the rate-controlling reaction, we would expect a *decrease* of V_m. The observed increase of V_m could be due to a conformational effect of cholesterol on the enzymes, as suggested for other mitochondrial enzymes (Krämer, 1982; Coleman and Lavietes, 1981; Lenaz *et al.*, 1984). An alternative explanation could be, however,

Table III. Effect of Cholesterol on the Kinetic Constants of Succinate-Cytochrome *c* Reductase in Proteoliposomes Enriched with Q_2

	− Cholesterol	+ Cholesterol (2 : 1 PL/cholesterol mol/mol)
k_{cat} (s^{-1})	20	42
K_m for Q_2 (μM)	0.45	0.40
Polarization of fluorescence (DPH)	0.10	0.19

cholesterol-induced clustering of the Q-enzymes (cf. Schneider *et al.*, 1982b) with decreased diffusional path for ubiquinone and ubiquinol, not compensated by the viscosity increase; for a diffusion-controlled reaction, this would indeed result in a V_m increase.

Conclusions

It is not yet possible to draw any firm conclusion on the state of organization of the electron transfer chain in the ubiquinone region. Direct measurements indicate that the diffusion coefficient of ubiquinone in the mitochondrial membrane is very high; comparison with the turnovers of electron transfer makes it unlikely that ubiquinone diffusion represents the rate-limiting step for electron transfer. Obedience of electron transfer to the pool kinetics of Kröger and Klingenberg (1973a) favors a picture of a random distribution of the electron transfer complexes, with their redox interactions coupled to, but not limited by, ubiquinone diffusion.

Similar pool kinetic behavior, however, would be obtained by a clustered organization of the respiratory complexes (either transient or fixed), but only if ubiquinone dissociation and association is faster than any one step in the turnover of electron transfer. Evidence for a transient association of the complexes is accumulating and is discussed by Ferguson-Miller (Hochman *et al.*, 1985).

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