Proc. Natl. Acad. Sci. USA
Vol. 78, No. 10, pp. 6246–6250, October 1981
Cell Biology

Rate of lateral diffusion of intramembrane particles: Measurement by electrophoretic displacement and rerandomization

(lateral diffusion coefficient/integral proteins/freeze-fracture electron microscopy)

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Communicated by Walther Stoeckenius, June 8, 1981

ABSTRACT **A method combining electrophoresis and freeze-fracture electron microscopy is described; the method was used to determine the lateral diffusion coefficient of intramembrane particles (integral proteins) in the mitochondrial inner membrane. An electric current was passed through microsuspensions of purified, spherical inner membranes at pH 7.4, which caused an electrophoretic migration of intramembrane particles in the membrane plane into a single, crowded patch facing the positive electrode. The membrane microsuspensions were quick-frozen at specified times after the packed particles were released from the electrophoretic force and while the particles were diffusing back to a random distribution. Observed concentration gradients of intramembrane particles during this time were quantitatively compared with and found to follow a mathematical model for Fickian diffusion of particles on a spherical membrane. The results determine the kinetics of free diffusion of integral proteins at the resolution of individual proteins. The diffusion coefficient of the integral proteins in the mitochondrial inner membrane was determined to be 8.3×10^{-10} cm^2/sec at 20°C, from which a root-mean-square displacement of 57 nm in 10 msec is predicted.**

The capacity of lipids and some proteins of cell membranes for free lateral diffusion in the membrane plane has been reported from a number of laboratories (for reviews see refs. 1–5). Lateral diffusion of these two membrane components is modulated by membrane composition (protein, lipid, and carbohydrate), physiological conditions (e.g., ligand binding, cytoskeletal elements), and physical and chemical conditions (e.g., temperature, pH, ionic strength). The lateral motion of integral membrane proteins may play an important role in catalytic sequences in which an overall reaction rate is limited by lateral diffusion of interacting proteins. Catalytic sequences in which lateral motion may be of significance include those in electron transfer (6, 7) and hormone receptor–adenylate cyclase systems (8).

A number of methods have been developed to measure the lateral diffusion of lipid and protein components in both natural and artificial membranes (1–5). The majority of these methods employ foreign molecules that serve as detectable labels. These labels may be bridged by crosslinking agents to ligands that bind to membrane components, bridged directly to membrane components, or may themselves be inserted into the membrane. No methods have been developed in which the lateral diffusion of integral membrane proteins can be followed ultrastructurally at the resolution of proteins themselves.

We describe here an ultrastructural method for studying lateral diffusion of integral proteins in membranes that permits direct monitoring of the location of the integral proteins during diffusion without the use of an added label. In this method an electrophoretic force is used to laterally displace the integral proteins in the membrane plane. Quick-freezing the membranes at specified times after the laterally displaced integral proteins are released from the electrophoretic force followed by analyzing concentration gradients of intramembrane particles (integral proteins in the membrane plane) by freeze-fracture electron microscopy permits the lateral diffusion coefficient of the proteins observed to be determined. As a model system we have used preparations of purified mitochondrial inner membranes because earlier results from this laboratory indicate that the integral proteins of this membrane have a high capacity for free lateral diffusion (6, 9–12).

METHODS

Membrane Preparation. Purified mitochondrial inner membranes were prepared by a controlled digitonin incubation of isolated rat liver mitochondria (13). The purified inner membranes (0.4 ml at 50 mg of protein per ml) were transferred to a hypotonic buffered medium (3.6 ml) to convert the highly convoluted inner membrane to a simple spherical configuration (14). The hypotonic medium was 29.2 mM D-mannitol/9.3 mM sucrose/2.7 mM Hepes buffer/30% (vol/vol) glycerol/0.1% methylcellulose (Fisher stock M-281). The pH was adjusted to 7.4 with NaOH. The spherical inner membranes were then centrifuged at 10,000 × *g* for 15 min into a pellet. Such spherical membranes are completely active in electron transfer (12).

Electrophoresis and Sample Freezing. Aliquots (1–2 μl) of the membrane pellet were placed into the well of a standard Balzers freeze-fracture gold specimen holder held by fixed fiberglass forceps. Wire electrodes (Ag/AgCl), which were made from 1.0-mm-diameter silver wire and had flat faces perpendicular to the axis of the wire, were micropositioned into contact with the aliquot of inner membrane suspension to give an expected electric field with nearly parallel vectors in the upper locations of the sample (Fig. 1). The electric field in the membrane microsuspension was developed by a current pulse applied to these electrodes. The voltage of the pulse followed a square waveform. The membrane sample was subjected to the electric current pulse and quick-frozen from room temperature at known times before or after the electric current pulse ended. This was accomplished by using a system composed of an electromechanical quick-freezing device, constant-voltage power supply, and a double interval timer (Fig. 2). The electromechanical quick-freezing device was composed of a well of liquid nitrogen-cooled propane on a spring-loaded vertically movable carrier. Upon triggering, springs elevated the carrier at a velocity limited by a viscous damper. The surface of the propane was elevated from a position about 3 mm below the specimen holder to a position about 2 mm above the membrane sample within a period of 0.4–0.6 sec. A double interval timer initiated and terminated the current pulse and released the propane carrier for quick freezing. Both the current pulse and instant of quick freezing were monitored by a dual trace Tektronix 564B

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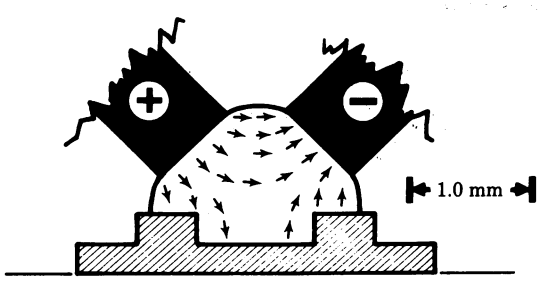


FIG. 1. Relationship of membrane microsuspension of a standard Balzers gold freeze-fracture specimen holder to Ag/AgCl wire electrodes and the expected electric field as represented by vectors.

storage oscilloscope. The freezing rates and exact times at which the membranes were frozen were determined from an iron/constantan thermocouple inserted directly into the microsample or attached to the fixed fiberglass forceps at a vertical level identical to that of the center of the membrane microsample.

After the membrane sample was frozen, the forceps were opened with the frozen sample held below the propane surface. Precooled metal forceps were used to break the frozen sample away from the electrodes in such a manner as to leave the AgCl electrode coatings attached to the frozen specimens. Such coatings on the frozen samples provided visual cues for alignment and vertical location for freeze-fracturing, which was carried out in a Balzers BA 360 freeze-fracture unit. The gold specimen holders were positioned so that the axis of the electric field vector created in the sample by the electric current was parallel to the fracturing knife travel direction and perpendicular to the Pt/C shadowing direction. Micrographs of freeze-fracture replicas were made with a JEOL-100CX electron microscope operated at 80 KV.

Calculation of the Lateral Diffusion Coefficient. Calculation of the lateral diffusion coefficient is based on Huang's mathematical analysis of a theoretical model of particles diffusing laterally on a spherical membrane according to Fick's law (see figure 2 of ref. 15). Huang's analysis relates the instantaneous changes in particle concentration at all locations on the surface of a spherical membrane as a function of time between a specific initial nonrandom distribution and a final random distribution. With regard to the present study, the calculation requires: (*i*) the measurement of the radius of the spherical inner membrane under study, and (*ii*) simultaneous measurement of (*a*) the gradient of intramembrane particle concentration along a meridian parallel to the electric field vector that existed during the current pulse, and (*b*) the time interval between the cessation of the current and the instant of freezing of the sample (hereafter referred to as the OFF interval). The diffusion coefficient, D, is related to the relaxation time constant, τ, and the sphere's radius, R, according to the equation $D = R^2/2\tau$.

The actual radius, R, of a spherical membrane partially embedded in ice was estimated from calculation of the height of the membrane exposed above the ice (Fig. 3*a*). The height, h, was determined by measuring the displacement, d, of a reference detail on the highest part of the spherical membrane after rotation on an axis perpendicular to the electron optical axis through an angle θ. The height was found by the equation $h = d/\sin\theta$. Any spherical membrane will have a measurable radius, r, defined by the intersection of the sphere and the ice surface (Fig. 3*b*). The actual radius, R, is related to the height and the apparent radius, r, by the equation $R = h + (r^2 - h^2)/2h$.

The relaxation time constant, τ, of the diffusion process was determined for intramembrane particle concentration gradients on fractured membranes frozen after OFF intervals of 2.0 sec. Intramembrane particle concentration gradients were measured by counting the particles in three adjacent square sample areas. To minimize errors in counting intramembrane particles and errors in the location of adjacent sample area boundaries, each membrane was micrographed at tilt angles of +20°, 0°, and −20°, so that the center of each of the three adjacent sample areas would be perpendicular to the electron optical axis (Fig. 4). Felt-tip pen dots were made over all intramembrane particles on transparent film laid over micrographs. Particle concentrations in the three adjacent sample areas were plotted in rectangular coordinates with particle concentration on the ordinate and tilt angle, θ, of the sample areas on the abscissa (Fig. 8). Huang's mathematical model (equations 10 and 11 in ref. 15)

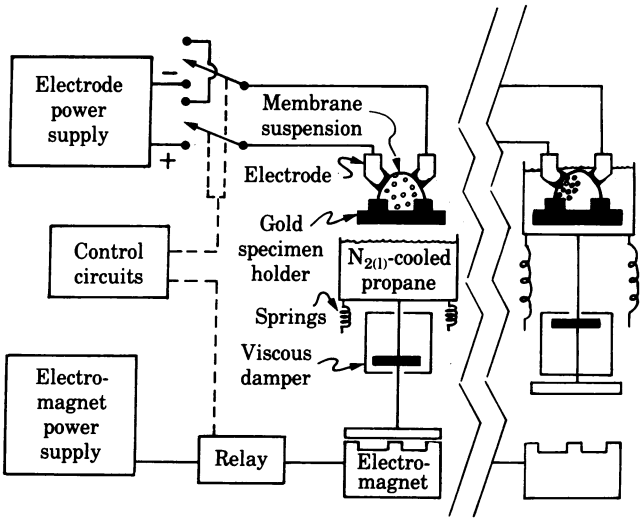


FIG. 2. Diagram of apparatus used to subject a membrane suspension to an electric current pulse with a known voltage and duration (*Left*) followed by freezing of the suspension at a known time after the termination of the current pulse (*Right*). See text for details.

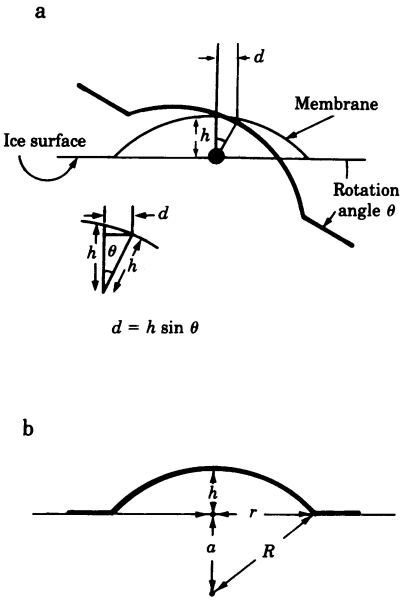


FIG. 3. Determining the actual radius of a spherical membrane partially embedded in ice by use of specimen rotation. (*a*) The height, h, of the membrane exposed above the ice is determined by measuring the displacement, d, after rotation on an axis (●) by angle θ, as given by $h = d/\sin\theta$. (*b*) Actual radius, R, is determined by the relationship of R to h and apparent radius, r, as given by $R = h + (r^2 - h^2)/2h$.

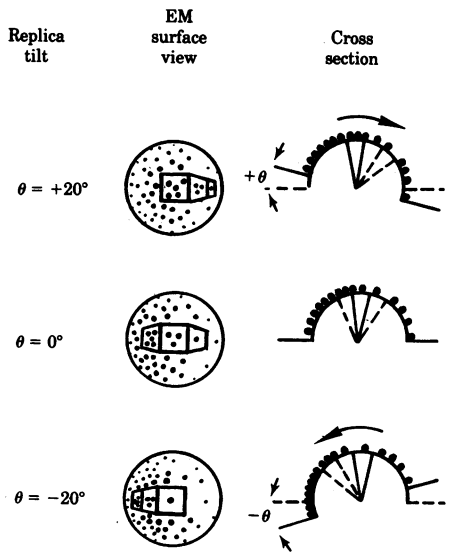


FIG. 4. Illustration showing how the planes of three adjoining square areas were oriented perpendicular to the optical axis of the electron microscope (EM) by rotation of the membrane replica to minimize foreshortening errors and short-counting of intramembrane particles located by happenstance behind other particles. Centers of square areas are along a meridian that is in the direction of the intramembrane particle concentration gradient.

was programmed and run on a PDP-1160 minicomputer for large ranges of values for θ and τ. Example computer-generated slopes are plotted in Fig. 8. Experimental slopes were compared with the computer-generated slopes to obtain relaxation time constants (Fig. 8).

RESULTS

Fig. 5 shows a typical oscilloscope trace of the waveform of the current that passed through the membrane microsuspension throughout the duration of the voltage pulse. During this pulse, the average current was about 30–60 μA. Simultaneous traces of the signal of the thermocouple indicated that the membrane suspensions were frozen with an OFF interval reproducibility of $\pm$0.05 sec. Traces from microthermocouples located in the center of the membrane suspension volume showed freezing rates of 1000°C/sec or greater.

When the membrane microsuspensions at pH 7.4 were frozen at the end of the 3.0-sec electric current pulse (i.e., an OFF interval of zero), intramembrane particles were found to have migrated into a single particle-rich patch revealed on convex (Fig. 6 *Upper*) and concave (Fig. 6 *Lower*) fracture faces. Migration was not always clearly obvious on the concave face. At maximal packing the patch of particles was approximately hemispherical and occupied only 40–50% of the hydrophobic surface area of the membrane (Fig. 6 *Upper*). Freezing the samples at the end of current pulses longer than 3.0 sec (up to 240 sec) did not cause intramembrane particles to pack into a smaller patch. The remaining area of the fractured membrane was usually devoid of intramembrane particles. The particle-rich patch always faced the positive electrode and the particle-poor area always faced the negative electrode. The packed intramembrane particle concentration (number of particles per unit surface area) in the convex fracture face was about double the density of the randomly distributed particles. The membranes were not deformed significantly from sphericity by the electric field or polar migration of the intramembrane particles, although all membranes migrated towards the positive electrode at pH 7.4 during the current pulse.

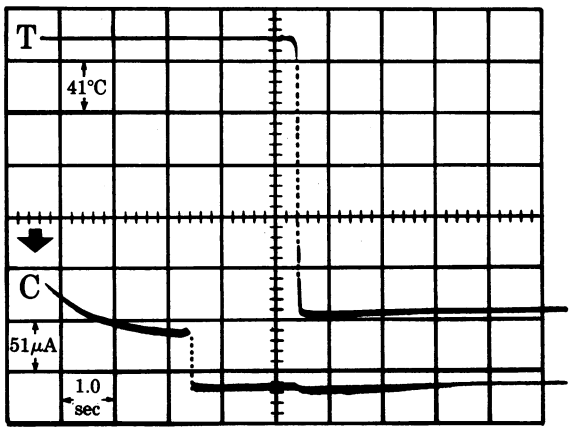


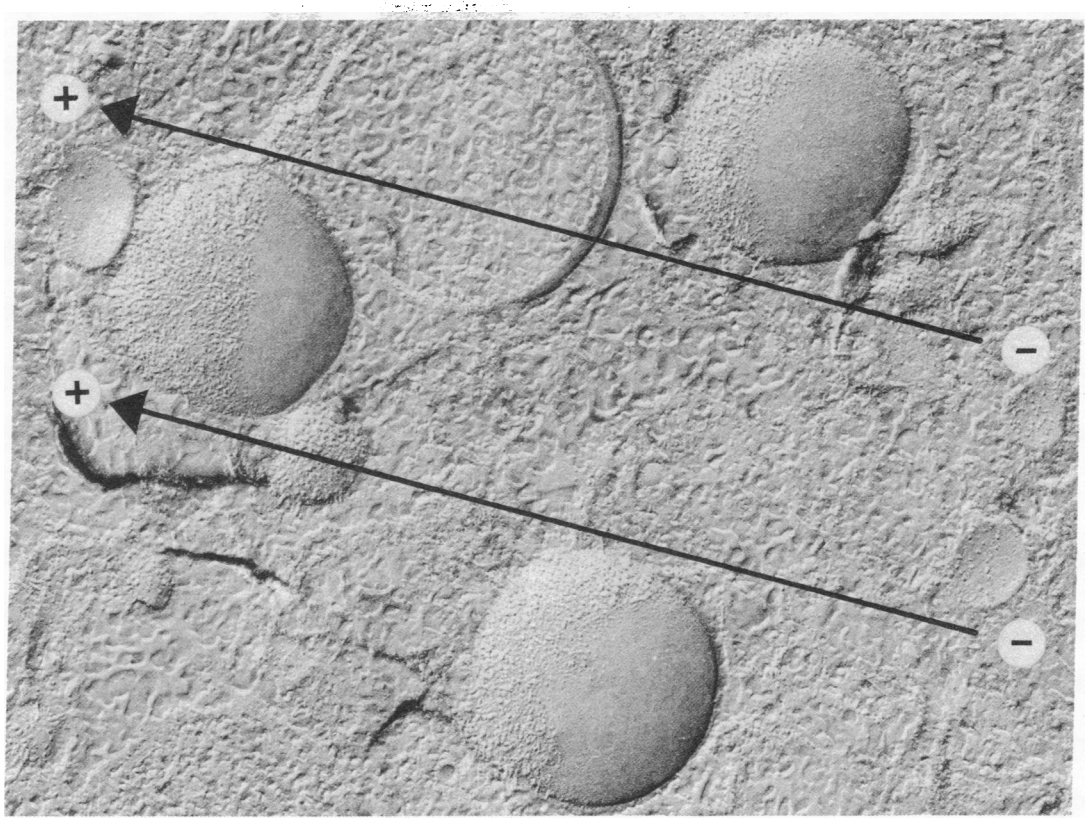
FIG. 5. Oscillograph showing kinetics of electric current pulse (*C*) and kinetics of temperature change during rapid freezing (*T*) of a membrane microsuspension on a freeze-fracture specimen holder. Temperature curve starts at 22°C and ends at liquid nitrogen temperature. Oscilloscope sweep initiated on start of current pulse (arrow).

Membrane microsuspensions frozen at an OFF interval of 2.0 sec—i.e., 2.0 sec after the end of the 3.0-sec current pulse—resulted in visually observable gradients of intramembrane particle concentrations parallel to the direction of the electric field (Fig. 7). Gradients were never observed for OFF intervals greater than 10 sec. Some intramembrane particles appear to be in contact because of inevitable and statistical collisions. To arrive at a value for the lateral diffusion coefficient, five membranes were quantitatively analyzed as follows. The slopes of the measured intramembrane particle concentration gradients of each of the five membranes were matched to the computer-generated, theoretically derived slopes to determine the value of the relaxation time constant, τ, associated with the OFF interval, t, of 2.0 sec. As an example (Fig. 8), the experimentally determined intramembrane particle concentration gradient in one of the membranes matched the slope in the family of computer-generated concentration gradients that corresponds to 1.7 time constants. The same membrane was found to have a radius, R, = 0.44 μm. Because $1.7\ \tau = 2.0$ sec, $\tau = 2.0/1.7 = 1.18$ sec. Thus the diffusion coefficient, $D = R^2/2\tau = (4.4 \times 10^{-5}\ \text{cm})^2/2(1.18\ \text{sec}) = 8.3 \times 10^{-10}\ \text{cm}^2/\text{sec}$. The values of D for each of the four other membranes were 2.9, 4.8, 7.3, and $18.5 \times 10^{-10}\ \text{cm}^2/\text{sec}$, giving an average value for D of $8.3 \times 10^{-10}\ \text{cm}^2/\text{sec}$ for the five sample membranes.

DISCUSSION

This report demonstrates the feasibility of (*i*) electrophoretically displacing the intremembrane particles of the mitochondrial inner membrane laterally into a particle-rich patch and (*ii*) estimating the lateral diffusion coefficient of the intramembrane particles as they return to equilibrium by free lateral diffusion. It was anticipated that a unidirectional electrophoretic force would cause integral proteins to migrate in the plane of the inner membrane, because earlier work showed that the integral proteins migrate laterally in the membrane as a result of thermotrophic lipid–protein separations (9), antibody crosslinking of cytochrome *c* oxidase (10), and crosslinking of membrane integral proteins generally with artificial peripheral proteins (16).

To a first approximation the patch of intramembrane particles when maximally packed is hemispherical and occupies 40–50% of the total surface area of the spherical membrane. This result is consistent with the fractional area occupied by intramembrane particles crowded into particle-rich areas by thermotropic lipid phase transitions induced in mitochondrial inner membranes (9). This indicates that the integral proteins of the inner membrane occupy only 50% or less of the total available mem-



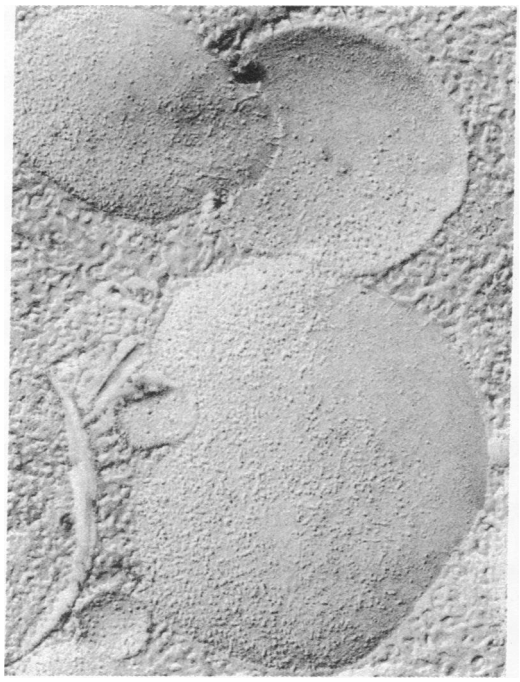


FIG. 6. Membranes frozen after a 3.0-sec exposure to an electric field of about 65 V/mm. Arrows indicate direction of migration of intramembrane particles towards the positive electrode. Convex (*Upper*), and concave and convex (*Lower*) fracture faces reveal migration of intramembrane particles toward the positive electrode. (*Upper*, ×55,000; *Lower*, ×62,000.)

brane surface area and that considerable space exists for free lateral diffusion in the native membrane.

Membranes frozen 2.0 sec after the termination of a current pulse showed continuous concentration gradients of intramembrane particles indicative of their equilibration from a patched to a random distribution. The fact that these gradients of intramembrane particles follow a mathematical model for Fickian diffusion of particles on a spherical membrane is a further extension of and consistent with several earlier studies which indicate that the integral proteins of the mitochondrial inner membrane are capable of free lateral diffusion (9–12, 16). In the present study, the average diffusion coefficient of the integral proteins represented by intramembrane particle was determined to be 8.3×10^{-10} cm^2/sec, which allows one to predict a root-mean-square displacement of 57 nm in 10 msec. Thus the integral proteins in the inner membrane can diffuse an average of 57 nm during the time it takes for approximately one turnover (one catalytic event) or more of the integral redox proteins during electron transfer activity. The diffusion coefficient determined for the intramembrane particles is consistent with and within the range of the majority of reported values for a variety of proteins of various natural membranes (1–5).

Presumably a number of factors could contribute to either an underestimation or overestimation of the lateral diffusion

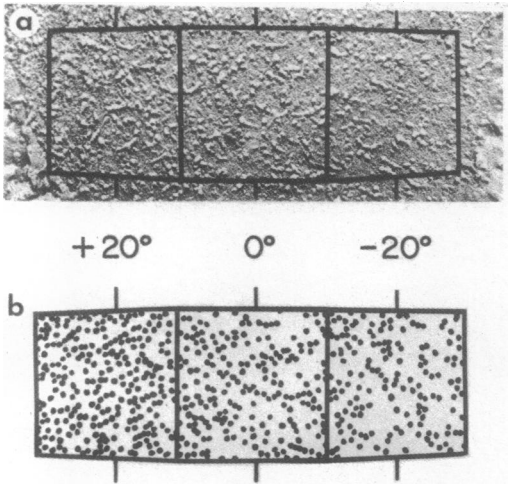


FIG. 7. Determination of intramembrane particle concentration gradient. (*a*) Micrograph of a membrane frozen 2.0 sec after the termination of a 3-sec current pulse; (*b*) counted intramembrane particles on adjacent equal-area squares with centers located at tilts of +20°, 0°, and −20° (see Fig. 4).

coefficient estimated by the method presented. Although a complete discussion of all of the possible factors that could be considered and their relative contribution to this question is beyond the scope of this report, several will be briefly discussed here. One factor to be considered is the possible influence of an increased viscosity in both the mitochondrial matrix and extramatrix aqueous compartments caused by the presence of glycerol (added for cryoprotection) and an increase in viscosity of the extramatrix compartment caused by methylcellulose (added to suppress Brownian motion-induced tumbling of the membranes). Many mitochondrial inner membrane integral proteins are transmembranous, and a number of these are oriented with approximately 75% of their mass external to the membrane lipid bilayer (17, 19). Thus diffusion of these proteins could be affected by viscous drag from the aqueous phases external to the bilayer. External to the spherical inner membrane, the viscosity of the electrophoresis medium was measured in separate experiments to be 5.05 centipoise (1 centipoise = 1

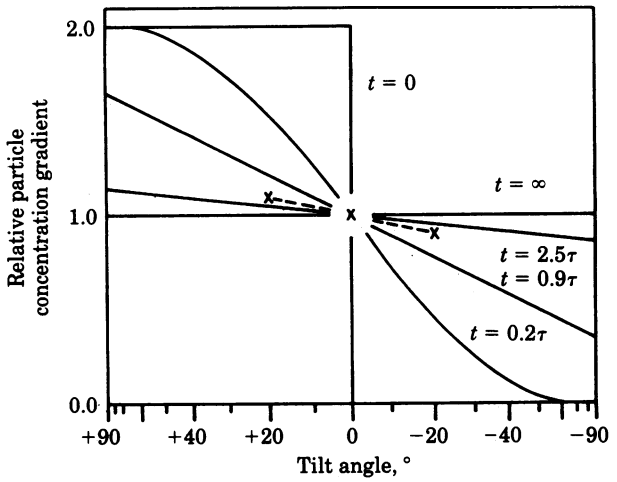


FIG. 8. Determination of the relaxation time constant from an intramembrane particle concentration gradient. Included are computer-generated particle gradients (solid lines) for several relaxation time constants obtained from equations for Fickian diffusion on a sphere. The broken line shows experimental intramembrane particle gradients in one membrane with a slope that corresponds to 1.7 time constants (see text for details). OFF time was 2.0 sec.

mPa·sec). Internal to the spherical inner membrane, the matrix protein, which is about 13% [and assumed to behave like aqueous hemoglobin solutions (20)], together with the glycerol gives a viscosity of 6.5–7.5 centipoise. These values are to be compared with the published estimates of the viscosity of the mitochondrial lipid component of 90 centipoise (21). Thus the inner membrane viscosity is much greater than the viscosities of the two aqueous compartments under the conditions of the experiments, eliminating viscous drag external to the bilayer as a significant factor in restraining protein diffusion.

Another factor to consider is that, during the process of electrophoresing the membrane proteins, the lateral distribution of lipids as well as the proteins may be significantly altered from a random distribution. Hence at early times after the release from the electrophoretic force, lateral heterogeneity in the distribution of the membrane lipids might present a barrier that hinders the initial stages of free diffusional rerandomization of proteins and may lead to an underestimation of the diffusion coefficient.

Reports showing evidence for the lateral electrophoretic displacement and free rerandomization of proteins of the plasma membrane of mammalian cells attached to a substratum have been published previously. In these studies the distribution of such proteins was determined by fluorescent labels or by the use of iontophoretic mapping of local membrane depolarization along the plasma membrane (22, 23). Our study ultrastructurally demonstrates the electrophoretic mobility and free rerandomization of integral proteins. It also demonstrates the applicability of an electrophoretic technique to (*i*) small vesicular membranes in suspension, and (*ii*) protein diffusion at the resolution of the electron microscope.

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