Light Scattering from Suspensions of Membrane Fragments Derived from Sonication of Beef Heart Mitochondria†

B. T. Storey,*,‡ C. P. Lee, S. Papa,§ S. G. Rosen, and G. Simon

ABSTRACT: The intensity of light scattering from suspensions of membrane fragments prepared by sonication of beef heart mitochondria in the presence of EDTA at alkaline pH (ESMP) was determined at 45, 90, and 135° with light of wavelength 546 nm. The dissymmetry ratio $Z = I_{45°}{}^{c}/I_{135°}{}^{c}$, where $I_{45°}{}^{c}$ and $I_{135°}{}^{c}$ are the scattering intensities at 45 and 135° extrapolated to zero particle concentration and corrected for reflectance effects, was used to calculate particle size from the Rayleigh-Gans-Debye theory. An average particle diameter D of 184–190 nm was obtained, within the range of particle diameter 50–300 nm determined previously by electron microscopy. This average diameter determined by light scattering is a useful parameter for characterization of ESMP particle size. We propose the term: light scattering average particle diameter, $\bar{D}_{LS}$, for this parameter. The refractive index of ESMP was determined to be 1.443 by measurement of scattering intensity in buffer solutions of varying sucrose concentration. The value of Z was independent of sucrose concentration in this determination, showing that the particles are osmotically inactive toward sucrose. The values of average particle diameter $\bar{D}_{LS}$ and of refractive index fall within the range of validity of the Rayleigh-Gans-Debye theory, for which light scattering changes are attributable solely to dimension change, rather than to change in particle refractive index. Uptake of water accompanying energy-linked salt uptake in ESMP was calculated from light scattering changes to be 0.18 μl of H_2O/mg of protein, compared with 0.49 μl of H_2O/mg of protein measured by dextran inaccessibility. Measurement of light scattering changes provides a rapid and sensitive method for determining volume changes of ESMP. The magnitude of the volume change observed during energy-linked water and salt uptake and the initial degree of hydration suggests that ESMP are analogous to polyelectrolyte gels with regard to sorption of strong electrolytes and that the Donnan formulation for ion exchange equilibria may be usefully applied to these processes in ESMP.

Sonication of beef heart mitochondria in the presence of EDTA at alkaline pH yields a preparation of membrane fragments in which the energy conservation capacity of the inner mitochondrial membrane is preserved and can be expressed by treatment with oligomycin (Lee and Ernster, 1966). These are designated EDTA submitochondrial particles (ESMP[1]). One diagnostic of energy coupling in ESMP is a greatly enhanced uptake of H^+ from the appropriate medium during respiration in the presence of oligomycin (Mitchell and Moyle, 1965; Chance and Mela, 1967; Chance et al., 1967; Papa et al., 1970; Papa et al., 1972; Papa et al., 1973a; Papa et al., 1973b). This uptake of H^+ is accompanied by uptake of NH_4^+ as measured by the NH_4^+ electrode or by uptake of 9-aminoacridine (9AA[1]) as measured by fluorescence quenching (Rottenberg and Lee, 1975), from which values of ΔpH between suspending medium and particles ranging from 2.2 to 3.6 were calculated. The higher values of ΔpH were calculated when anions near the top of the Hofmeister or lyotropic series (Jencks, 1969; Taylor and Kuntz, 1972) were present in the medium. Papa et al. (1973c) have shown that salts of these particular anions are taken up by ESMP under conditions of energy conservation and that this uptake is accompanied by water uptake and a decrease in light scattering from the particles. The light scattering decrease could be due to either an increase in particle diameter corresponding to swelling, or to a decrease in refractive index of the particle, or both (Van de Hulst, 1957; Latimer and Pyle, 1972). Light scattering changes in cells or subcellular organelles have generally been attributed to swelling or shrinking (Tedeschi and Harris, 1958; Packer, 1960; Koch, 1961; Packer, 1963), although the analysis of Latimer and Pyle (1972) indicates that, with entities of mitochondrial size or larger, considerable caution should be exercised. Light scattering changes reported by Packer and Tappel (1960) in mitochondrial membrane fragments, which were derived from digitonin treatment (Devlin and Lehninger, 1958) and were not osmotically active toward sucrose, were postulated to be protein conformational changes; a similar conclusion was reached more recently by Lundberg (1975) from results obtained with beef heart submitochondrial particles prepared by sonication in the presence of Mg^{2+} and ATP (Löw and Vallin, 1963).

Measurement of light scattering intensity and of changes in scattered intensity are easy to carry out, and the method is potentially very useful. Determination of particle diameter D from light scattering intensities at different angles,

† From the Department of Obstetrics and Gynecology, Biophysics and Physical Biochemistry, and Physiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19174. *Received May 5, 1975.* This work was supported by the National Science Foundation, Grant GB-23063 and the National Institutes of Health, Grant GM-19636.

‡ Present Address: Division of Reproductive Biology, Department of Obstetrics and Gynecology, Medical Labs 338/G3, University of Pennsylvania, Philadelphia, Pennsylvania 19174.

§ Permanent Address: Istituto di Chimica Biologica, Facolta di Medicina e Chirurgia, Universita di Bari, Via Amendola 165-A, 70126 Bari, Italy.

[1] Abbreviations: ESMP, mitochondrial membrane fragments (submitochondrial particles) prepared by sonication of beef heart mitochondria in the presence of EDTA at alkaline pH; 9AA, 9-aminoacridine.

and changes in D from light scattering changes, can be difficult both to calculate and to interpret; however, if D is not less than the wavelength of light used for the measurements, the particle refractive index is much different from that of the suspending medium. Latimer and Pyle (1972) have computed light scattering intensities from the equations of the Mie (1910) theory at 10, 45, 90, 135, and 170° for particles of different sizes and found that the scattering intensities with light of 500-nm wavelength decrease monotomically with increasing particle volume only for particles of D = 150 nm and relative refractive index m = 1.05. Jennings and Jerrard (1965) have shown with butadiene-acrylonitrile copolymer latex samples of uniform particle sizes suspended in water that the Rayleigh-Gans-Debye (1944) theory gives particle diameters within 5% of those calculated from the more exact and less restricted Mie theory for values of D up to 210 nm and m up to 1.15, corresponding to particle refractive index n_p = 1.53. The work of Jennings and Jerrard (1965) therefore sets limits within which the scattering intensity vs. particle volume curves are "well behaved" ones of the type calculated by Latimer and Pyle (1972) for D = 150 nm and m = 1.05.

Both the Rayleigh-Gans-Debye (1944) and the Mie (1910) theories are strictly applicable only to suspensions of spheres of uniform diameter. However, one can calculate D from measured light scattering intensities as an average value which functions as a single parameter to characterize the particle size of any suspension which contains a distribution of particle sizes. In this respect, D provides a convenient measure of an important physical property of particle suspensions, namely size, much as the weight average or number average molecular weight of a polymer provides a convenient measure of polymer chain length. Examination of ESMP preparations with the electron microscope showed membrane fragments of assorted shapes, with the majority of the particles being roughly spherical (Huang et al., 1973). The particle diameters calculated from the electron micrographs fell in the range 50–300 nm. These particles are large enough to show dissymmetry of the angular scattered light intensity (Heller and Nakagaki, 1959), but small enough to fall near the limit determined by Jennings and Jerrard for equal applicability of the Mie (1910) and Rayleigh-Gans-Debye (1944) theories. In this paper, we show that both the calculated average particle diameter D and relative refractive index m of ESMP suspended in aqueous media fall within the limits determined by Jennings and Jerrard (1965). This provides the physical basis for a facile determination of particle size for ESMP suspensions and quantitation of changes in particle volume under different experimental conditions.

Materials and Methods

ESMP were prepared by sonication of beef heart mitochondria as described by Lee and Ernster (1967). Light scattering measurements were carried out in a Brice-Phoenix Series 2000 Universal light scattering photometer, using light at 546 nm obtained with the filter supplied with the instrument. The sample cuvette was made from Vycor tubing 7-mm o.d., 5-mm i.d., and 6-mm high, sealed at one end; the cuvette was mounted in a shielded holder closely patterned after the cell described by Jerrard and Sellen (1962). Their design provides maximal shielding from stray light while allowing full access to the scattered light over the range of angles 45 to 135°. Readings were corrected for the blank obtained with medium alone in the cuvette. Simultaneous readout of changes in the redox state of cytochrome b and 90° light scattered was obtained with a dual wavelength spectrophotometer (Chance, 1957) compensated for light source fluctuations (Chance et al., 1970). For this determination, the reaction cuvette was an absorbance cell with all sides polished; the optical path was 10 mm and the chamber width 3 mm to minimize self-absorbance by the sample of the scattered light, which was detected by a photomultiplier tube with axis set at 90° to the incident light beam. The wavelength pair used to monitor the absorbance change due to redox state changes of cytochrome b was 566–540 nm, with the reference beam at 540 nm being used to monitor light scattering at 90°.

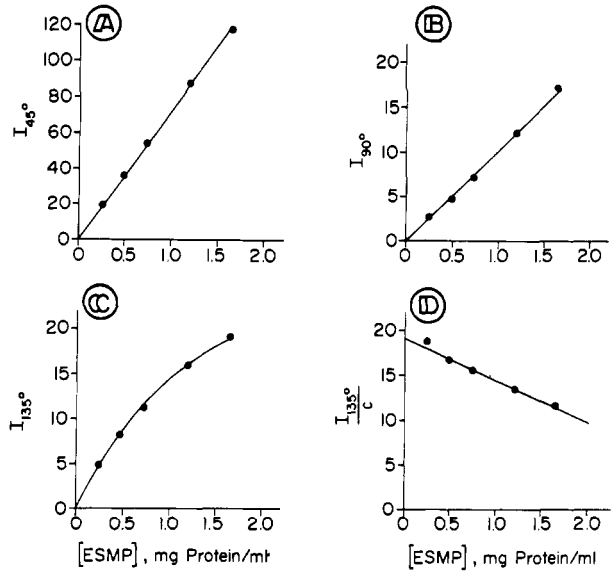

FIGURE 1: Light scattering intensities from ESMP suspensions in 0.24 M sucrose, 25 mM Tris-Cl buffer at pH 7.5 at 26 °C as a function of particle protein concentration at 45 (A), 90 (B), and 135° (C). The data for the light scattering intensity at 135°, $I_{135°}$, are replotted as $I_{135°}/C$ vs. C, where C is protein concentration in D.

Results

The light scattering intensities observed with suspensions of ESMP from the angles 45, 90, and 135° to the incident beam are shown as a function of particle protein concentration in Figure 1. The scattering intensity is linear at 45 and 90° up to 1.6 mg of protein/ml (Figures 1A and 1B), but the scattering intensity increase with protein concentration at 135° falls away from linearity (Figure 1C). The observed scattering intensity is comprised of the light scattered directly from the particles at the given angle and from reflectance of light scattered at 180° from this angle due to refractive index differences at the air-glass and glass-medium interfaces (Sheffer and Hyde, 1952; Oth et al., 1953). At 135°, the reflectance component of the scattering intensity from ESMP suspensions is about 20% of the total, and this reflected light is, in turn, absorbed by the particles. This absorbance of the reflected light leads to the nonlinearity in scattering intensity with increasing particle concentration, but should, to a first approximation, yield a linear plot with negative slope of $I_{135°}/C$ vs. C, where $I_{135°}$ is scattered intensity at 135° and C is particle concentration. This is the case, as shown in Figure 1D. The problem does not arise with the light scattered at 45 and 90° since the reflectance component at these angles amounts to only 1 and 5% of the total, respectively: absorbance effects would not be detectable, and the plots are linear. From the plot of

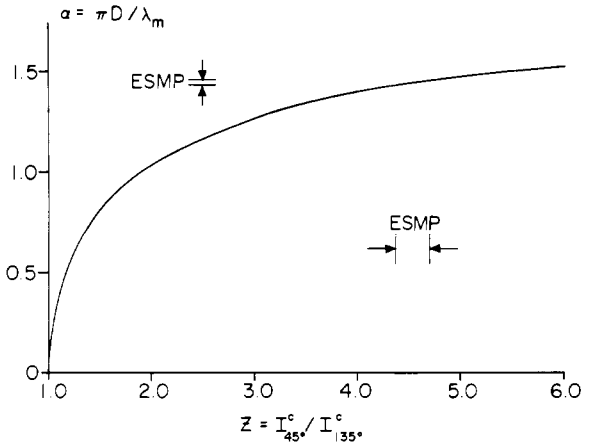

FIGURE 2: Plot of the dimensionless ratio, $\alpha = \pi D/\lambda_m$, where D is particle diameter and λ_m is wavelength of light in the medium, vs. the dissymmetry ratio $Z = I_{45°}{}^c/I_{135°}{}^c$, where $I_{45°}{}^c$ and $I_{135°}{}^c$ are the corrected scattering intensities at 45 and 135° as described in the text. The plot is taken from the calculated figures given in Heller and Nakagaki (1959). The spread of Z values for ESMP preparations and the corresponding spread in values of α are shown.

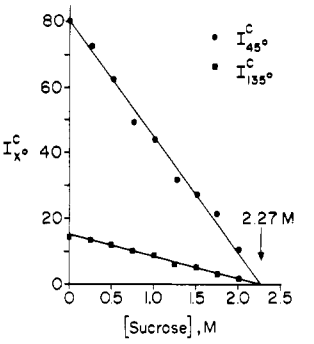

FIGURE 3: Plot of $I_{45°}{}^c$ and $I_{135°}{}^c$ from ESMP suspensions in 25 mM Tris-Cl buffer containing sucrose as a function of sucrose concentration. Extrapolation point of the lines on the abscissa is indicated.

$I_{135°}/C$ vs. C, the value of $I_{135°}$ extrapolated to zero particle concentration may be obtained. This value is required by both the Rayleigh-Gans-Debye and the Mie theories, which postulate that the particles scatter light independently without mutual interference (Van de Hulst, 1957). The values of the scattered intensities at 45 and 90°, extrapolated to zero particle concentration, are given directly by the slopes of the lines in Figures 1A and 1B. The intensities plotted in Figure 1 have not been corrected for the extra intensity from the reflectance of scattered light mentioned above. This correction at 45 and at 90° can be neglected, but at 135° must be included. The corrected, extrapolated value at 135° is designated $I_{135°}{}^c$, and the extrapolated value at 45° is designated $I_{45°}{}^c$.

The dissymmetry factor, Z, defined as $Z = I_{45°}{}^c/I_{135°}{}^c$, is a function of the size parameter, $\alpha = \pi D/\lambda_m$, where D is the particle diameter and λ_m is the wavelength of the light in the suspending medium. The function has been calculated from the Rayleigh-Gans-Debye theory by Heller and Nakagaki (1959). The plot of α as a function of Z is shown in Figure 2, along with the range of Z values obtained with four separate ESMP samples prepared at intervals of about 1 month. The range of values is 4.37 to 4.70, with a mean value of 4.45. The corresponding range of α values is also shown; from these one calculates a range for D of 184 to 190 nm, with a mean of 185 nm. This value for particle diameter falls within the range of 50 to 300 nm found for ESMP by Huang et al. (1973) and, thus, can be used as an average particle diameter which is readily obtained experimentally. We propose the term *light scattering average particle diameter*, with the designation $\bar{D}_{LS}$, for the value of D for ESMP suspensions calculated from the dissymmetry ratio Z obtained by direct measurement of light scattering intensities at 45 and 135°, as in Figure 1. The value of $\bar{D}_{LS}$ is near the high end of the particle size range, as would be expected because of the greater dissymmetry of scattering by the larger particles in this range of particle diameters. In this respect, $\bar{D}_{LS}$ resembles more the weight average molecular weight of a polymer obtained by viscometry than the number average molecular weight obtained by osmometry. Full characterization of the particle size distribution of ESMP would require either determination of diameters from electron micrographs and/or use of the forward scattering lobe method described by Wims (1973), a task of considerable magnitude with marginal return of further useful information. Of great utility to the experimentalist is the fact that $\bar{D}_{LS}$ falls within the limit determined by Jennings and Jerrard (1965) for equivalence of the Mie (1910) and Rayleigh-Gans-Debye (1944) theories so that $\bar{D}_{LS}$ can be readily obtained from the measured light scattering intensities by means of the tabulation provided by Heller and Nakagaki (1959).

The refractive index of ESMP was determined by measuring the light scattering intensities at 45 and 135° at varying concentrations of sucrose in the medium. The refractive index of sucrose solution increases essentially linearly with sucrose concentration in this region. At the point where the refractive index of the medium matches that of the membrane fragments, the scattering intensities are zero (Van de Hulst, 1957). A plot of $I_{45°}{}^c$ and $I_{135°}{}^c$ vs. sucrose concentration is shown in Figure 3. The scattering intensities decrease linearly with sucrose concentration and both lines extrapolate to zero at 2.27 M sucrose. The scattering intensity at 90°, not shown for the sake of clarity, also extrapolates to the same point. This sucrose concentration corresponds to a refractive index $n_p = 1.443$, which is within the limit for this property determined by Jennings and Jerrard (1965) for valid application of the Rayleigh-Gans-Debye theory to aqueous suspensions of particles.

Application of this theory to light scattering changes observed during energy-linked salt and water uptake by oligomycin-treated ESMP with succinate as energy source is shown in the following experiment. The light scattering decrease at 90° and absorbance change due to reduction of cytochrome *b*, which acts as monitor of the energy state, are obtained simultaneously during this reaction in the trace shown in Figure 4. Uptake of salt is facilitated by addition of nigericin to mediate K^+/H^+ exchange and provision of NO_3^- as "permeant" anion (Montal et al., 1969a; Cockrell and Racker, 1969; Montal et al., 1969b; Montal et al., 1970; Papa et al., 1973a). Addition of succinate gives a rapid reduction of cytochrome *b* (Figure 4B) followed by a slower reduction with a time course similar to the 90° light scattering decrease (Figure 4A). The redox state of cytochrome *b* in the aerobic steady state corresponds to 50% reduction; 20% is the fast component and 30% the slow component. At the wavelength pair 566–540 nm, changes due to cytochrome *b*-566 in ESMP predominate (Papa et al., 1972; Brandon et al., 1972), and the slow reduction, which is energy-linked, is attributed to this component. Upon anaerobiosis, the cytochrome becomes rapidly reduced, and

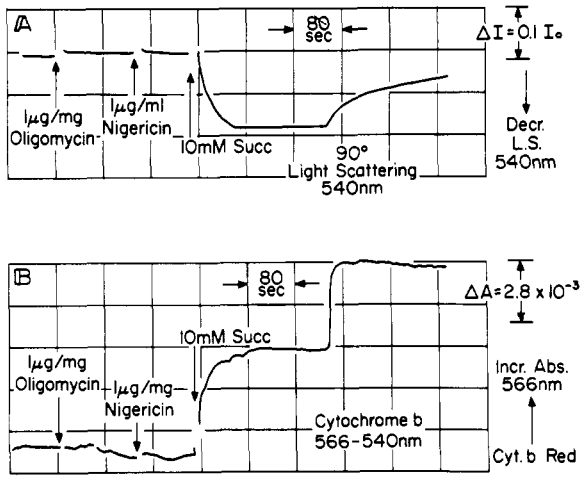

FIGURE 4: Simultaneous readout of change in light scattering intensity at 90° from ESMP (A) and change in redox state of cytochrome *b* in ESMP monitored at 566–540 nm (B), using the dual wavelength spectrophotometer. The 540 nm is used for the light scattering measurement. The ESMP are suspended in 0.24 M sucrose, 10 mM Tris-Cl buffer at pH 7.4 and 26°, containing 25 mM KNO_3. Downward deflection of the trace in A indicates a decrease in light scattering intensity; upward deflection of the trace in B indicates cytochrome *b* reduction.

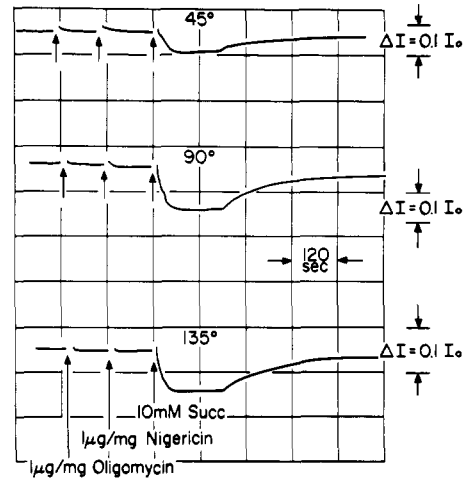

FIGURE 5: Parallel experiments showing the light scattering intensity changes at 45, 90, and 135° with the light scattering photometer under the same experimental conditions as in Figure 4.

the light scattering change reverses with a slow time course. The decreases in light scattering intensity observed at 45, 90, and 135° for the same ESMP samples in parallel experiments under the conditions of Figure 4 are compared in Figure 5. The time course of the scattering decrease is the same at all three angles of observation, but the decrease at 45° as a percentage of initial intensity is less than at 135°, corresponding to an increase in Z and, hence, of average particle diameter $\bar{D}_{LS}$. Taking the average Z value of 4.45 for the initial state, the Z value calculated for the aerobic steady state is 4.88. The calculated increase in $\bar{D}_{LS}$ is from 185 to 189 nm, corresponding to an average volume increase from 6.38×10^{-3} to 6.80×10^{-3} μm^3.

Discussion

The angular distribution of light scattering intensity recorded from ESMP suspensions yields a dissymmetry factor Z with an average value of 4.45, and determination of the particle refractive index yields a value of 1.443, both within the range shown by Jennings and Jerrard (1965) to be valid for size determination by the Rayleigh–Gans–Debye theory, in the sense that one obtains values essentially equal to those obtained with the Mie theory. Not only is this theory simpler to use than the Mie theory, but the light scattering intensities derived from it depend on particle size, and not explicitly on particle refractive index. Changes in intensity of light scattering from ESMP suspensions may be attributed with confidence to dimensional changes caused by swelling or shrinking. Since the scattering intensity at 90° is linear in ESMP protein concentration in the useful experimental range and changes in scattering intensity at 90° follow those at 45 and 135° faithfully, it is valid to use scattering measurements at 90° which are readily made simultaneously with absorbance measurements.

It is a useful property of ESMP that their characteristic light scattering average particle diameter $\bar{D}_{LS}$ determined from the dissymmetry factor Z is in a range where Z is sensitive to small changes in α. The volume increase calculated from the experiment of Figure 5 is 6.6% of the initial volume, yet is readily observed. This volume change may be compared with the water uptake by ESMP measured as an increase in dextran-inaccessible water under the same experimental conditions (Papa et al., 1973c) by making the following assumptions. The mitochondrial membrane is taken to be 70% protein with a density of 1.2 mg/ml and 30% lipid with a density of 0.8 mg/ml. In the initial state, ESMP contain 1.40 μl of H_2O/mg of protein (Papa et al., 1973c). The total volume of protein plus lipid plus water in the particles is calculated to be 2.77 μl/mg of protein, and an increase of 6.6% would correspond to 0.18 μl of H_2O/mg of protein, assuming additive volumes. The value determined by dextran inaccessibility was 0.46–0.49 ml/mg of protein. Volume-inaccessibility measurements include water occluded by aggregates of small particles and thus may overestimate the volume change. Measurements of changes in $\bar{D}_{LS}$ not only exclude this extra water, but also reflect the swelling characteristics of the larger particles in the dispersion and, hence, may underestimate the volume change. The two values obtained by the two different methods may, therefore, be regarded as upper and lower limits.

Two observations concerning the physical nature of ESMP emerge from this study. The first observation is that the plots in Figure 3 are linear and intersect at the abscissa, showing that the Z value does not change with sucrose concentration. The particles are therefore osmotically inactive toward sucrose, implying that sucrose is nearly as permeable to the membrane space as water. This is in line with the observation that dextran of molecular weight less than 15 000 is accessible to water in the particles (Azzone and Massari, 1972), while dextran of molecular weight in the range of 60 000–90 000 is not accessible (Papa et al., 1973c). It is also in agreement with the observation of Packer and Tappel (1960) that digitonin particles from rat liver mitochondria are freely permeable to sucrose. The free permeability of sucrose ensures that the refractive index matching of suspending medium and ESMP, obtained by extrapolation to zero scattering intensity (Figure 3), is free from complications due to compartments excluding sucrose, unlike intact mitochondria which are osmotically active toward sucrose (Tedeschi and Harris, 1958). The value of 1.443 would thus seem to represent a valid determination of particle refractive index. Since ESMP comprise mostly fragments of the inner mitochondrial membrane (Huang et al., 1973), the overall refractive index of the protein-rich inner mitochondrial membrane can be taken to be 1.443. To

the authors' knowledge, this value is the first quantitation of the refractive index of this membrane. The second observation is that the water content of the particles is low and increases little even under optimal energy-linked uptake of salt with accompanying particle swelling. The value of 1.4 μl of H_2O/mg of protein corresponds to a water content of 50% by weight, increasing to 57% by weight with uptake of 0.5 μl/mg of protein, using the assumptions described to calculate particle volume/mg of protein. This water content is very similar to that encountered with solid-gel, ion-exchange resins (Kunin, 1958; Helfferich, 1962) and is reasonable for hydrated proteins; it is less reasonable if some water is needed for formation of an aqueous phase in the interior of the particle (Chance et al., 1967; Skulachev, 1971; Papa et al., 1973a).

Analogy of ESMP with ion-exchange resins is of interest with regard to ion uptake if one considers ESMP to be hydrated polyelectrolyte gels containing fixed charges, as are ion-exchange resins. In ESMP, the fixed charges are either attached to membrane proteins or to membrane phospholipids. This consideration enables one to focus on a peculiar property of these gels suspended in a medium containing salts of strong electrolytes: the phase boundary between the gel phase and the suspending medium phase has the properties of a restrictive membrane, which acts precisely like the membrane in a Donnan system (Bauman and Eichhorn, 1947; Boyd et al., 1947; Gregor, 1948; Glueckauf, 1952). Detailed treatments of this formalism are given by Kunin (1958) and Helfferich (1962). A potential exists across the phase boundary "membrane" in the presence of salts of strong electrolytes, which originates in the uneven distribution of the ions between suspending medium and gel due to the concentration of fixed charge in the gel phase. This is calculable as a true membrane potential, whose orientation and magnitude are a function of the sign and concentration of fixed charges in the gel phase. If the concentration of fixed charges increases, the gel will swell by taking up water along with salt. As the gel hydration increases to the point where it is offset by increased swelling pressure imposed by the crosslinks in the gel, a new Donnan equilibrium is established. We postulate that energization of ESMP increases the fixed charge concentration of the membrane proteins in the membrane phase, and that salts and water are taken up in accordance with the Donnan formulation by hydration of these membrane proteins.

The formulation of Azzone et al. (1972, 1974) for the mitochondrial membrane in submitochondrial particles, based on studies of cationic dye accumulation, differs from the formulation above in that the binding sites are considered to be nucleophilic and are either hindered or exposed, depending on energy state. The notion of fixed electrostatic charge, required by our formulation, is missing here, but the concept of change in sites with energy state resembles our concept of change in fixed charge with energy state.

Formulation of ion uptake by energized ESMP in terms of a change in the Donnan equilibrium state of hydrated polyelectrolyte gels may provide a new approach to assessment of the transmembrane (Papa et al., 1973a) and intramembrane (Lee, 1971, 1973; Papa et al., 1973c; Azzone and Massari, 1973) hypotheses of energy-linked salt and water uptake by these particles. The intramembrane hypothesis postulates that uptake occurs within the mitochondrial membrane phase due to energy-linked changes in charged groups in the membrane proteins. The transmembrane hypothesis postulates that uptake occurs by transport of salt and water across the membrane to a phase within the particle, due to energy-linked changes in the electrochemical potential across the membrane resulting from vectorial proton translocation. The latter hypothesis requires only that there be a restrictive membrane at a phase boundary relative to which ion movements can occur in a vectorial manner. This membrane need not be the mitochondrial membrane per se. The phase boundary membrane of the Donnan formulation could serve as this membrane since it is restrictive, and an electrostatic potential is maintained across it. Vectorial ion movements can occur across the phase boundary membrane as postulated by the transmembrane hypothesis, yet the movements would be in response to events occurring in the mitochondrial membrane phase, as postulated by the intramembrane hypothesis. The analogy between polyelectrolyte gels and ESMP may be thus useful in describing energy-linked uptake of salts and water by the particles in terms of the well-established Donnan formulation for polyelectrolyte gels.

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