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THE RELATIONSHIP BETWEEN THE SIZE OF MITOCHONDRIA AND THE INTENSITY OF LIGHT THAT THEY SCATTER IN DIFFERENT ENERGETIC STATES

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The intensity of light scattered at 90° to the incident beam and the effective hydrodynamic radii of mitochondria incubated under a variety of conditions have been measured. Addition of high concentrations of uncouplers to respiring mitochondria resulted in a decrease in scatter which was not due to swelling. Addition of valinomycin to mitochondria depleted of substrate in K^+-free medium produced an increase in scatter that was not due to shrinking. It is concluded that changes in the intensity of scattered light are not reliable indices of changes of volume of mitochondria, and that changes in conformation with changes in metabolic state dominate changes in light scatter. A molecular mechanism for the effect of metabolic state upon the scattered intensity is suggested.

Introduction

Mitochondria are known to swell or shrink in response to changes in conditions of incubation. The volume changes are commonly followed by observing either the intensity of light scattered at 90° to the incident beam, or the absorbance of a suspension, the assumption being that as mitochondria swell their relative refractive index decreases and so does the intensity of light that they scatter. This report cites examples of changes in light scatter that do not correlate in that simple fashion with either the K^+ contents of the mitochondria or with their effective hydrodynamic volumes measured by self-beat laser spectroscopy. The results suggest that changes in light scatter are often due to changes in refractive index which are brought about by generation or discharge of the energised state without changes in volume.

Methods

Rat liver mitochondria were prepared by the method of Parsons et al. [1] and finally suspended in a solution containing: 70 mM sucrose, 210 mM mannitol, 0.1 mM EDTA, 1 mM Tris, pH 7.2 (mannitol medium). The ADP : O ratio was measured using a Y.S.I. Model 53 oxygen monitor [2] and, unless otherwise stated, only tightly coupled mitochondria were used for the subsequent experiments. Solutions other than the stock solutions of valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) which were in ethanol were made up in dust-free water. Protein was measured by a biuret method [3]. For determination of K^+ content mitochondria were rapidly centrifuged, the pellets washed in cold isotonic sucrose and then extracted in HNO_3 (0.1 M). K^+ in the extract was determined using an EEL flame photometer with external standards made up in HNO_3 (0.1 M). For experiments using the laser a suspension of mitochondria at a concentration of about 1 mg/ml was allowed to drip through a 1.2 μm Millipore filter, in order to remove aggregates and dust particles and to produce a population more

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uniform with respect to size. The slight change in the ATP : O ratio (1–2%) suggested that the mitochondria were not damaged by this procedure.

Scatter was measured using an MPF-4 Perkin-Elmer fluorescence spectrophotometer at wavelengths of 600 and 488 nm. In addition, using an argon laser operated at 488 nm (Spectra Physics Model 156/03), light scatter was measured concurrently with the diffusion coefficient from which the effective hydrodynamic radius of the scattering particles was computed. Light scattered from the sample was detected and analysed by a Malvern system 4300 photon correlation spectrometer. Timing of the diffusion coefficient measurements was accomplished by allowing the correlator to collect $2 \cdot 10^5$ samples at a dwell time of 250 μs before data analysis was performed by an ALPHA-LSI mini-computer interfaced to the correlator. Details of the technique are given elsewhere [4]. The scattered intensity was obtained from the first channel of the Malvern correlator.

The degree of polydispersity in a sample preparation may be characterised by the 'Q' parameter [5] calculated from the coefficient in the quadratic term in a least-squares best-fit quadratic to the logarithmic plot of the autocorrelation function. In an ideal monodisperse system the average value of Q is zero. In the present experiments the average values were consistently greater than zero, indicating that the population was heterogenous; points for which $|Q| > 0.25$ were rejected; the overall mean and standard deviation of points that were accepted were 0.167 ± 0.112 ($n = 50$). The existence of a range of mitochondrial volumes in all preparations precludes precise quantitative treatment of the data; qualitative changes with time and with additions should, however, be reliable. For calculation of effective volume density, viscosity and refractive index of the incubation media were measured using a Westphal specific gravity balance, an Ostwald viscometer and an Abbé 3L refractometer, respectively. All measurements were made at 21°C.

Viscosities were: mannitol medium, 1.166 cP; 0.125 M KCl, 10 mM Tris (isotonic KCl), 0.976 cP. Refractive indices were: mannitol medium, 1.3416; isotonic KCl, 1.3345.

Results

Concentrations of mitochondria at which particle-particle interactions were negligible were found by determining the effective hydrodynamic radius over a range of concentrations. It was found that while the mean and standard deviation of the apparent radius were 496 ± 22 nm ($n = 8$) at a concentration of 0.25 mg/ml, there were no significant differences among values measured at 0.125 (456 ± 9 nm, $n = 5$), 0.0625 (461 ± 10 nm, $n = 4$) and 0.0312 (440 ± 13 nm, $n = 4$) mg/ml. All subsequent measurements were therefore made at concentrations lower than 0.125 mg/ml.

First, changes in volume and light scatter accompanying passive swelling of mitochondria were followed. Fig. 1 shows that addition of sodium acetate (which can cross the mitochondrial membrane passively) in mannitol medium resulted in swelling and a decrease in scatter. When, however, sodium acetate was added to mitochondria already swollen in KCl, the mitochondria swelled still more but the intensity of scattered light increased. This

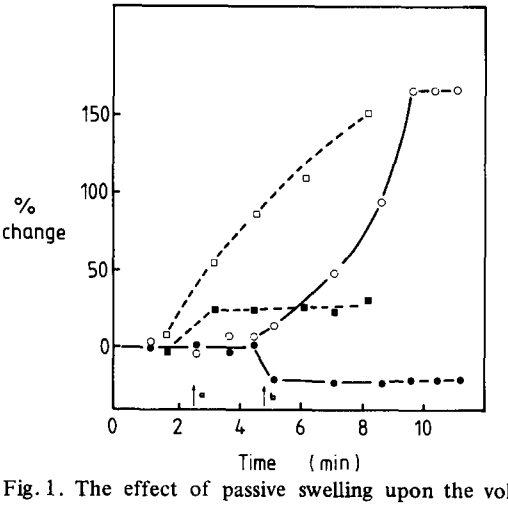

Fig. 1. The effect of passive swelling upon the volume of mitochondria and the intensity of light that they scattered at 90°. (a) 0.05 mg/ml in isotonic KCl; 20 μl sodium acetate were added at the arrow a to a final concentration of 10 mM. Wavelength 488 nm; (■- - - - -■) scatter, (□- - - - -□) volume. The initial volume was 384 μm^3. (b) 0.05 mg/ml in mannitol medium; 20 μl sodium acetate were added at the time of the arrow b to a final concentration of 10 mM. Wavelength 488 nm; (●———●) scatter, (○———○) volume. The initial volume was 0.140 μm^3.

illustrates the serious difficulty in the way of correlating changes in light scatter with changes in particle size. When the wavelength of the scattered light is of the same order of magnitude as a dimension of the scattering particle, interference can result in either an increase or a decrease of light scattered at a single angle as the volume of particle increases [6]. Latimer and Pyle [7] used the Mie theory of scattering to predict how changes in particle volume, with no change in dry weight, should influence light scattering for various scattering angles. They found that as mitochondria swelled from a volume of 0.25 to about 0.4 μm^3, the intensity of light of wavelength 500 nm scattered at 90° decreased. But for a range of volumes from approx. 0.4 to 0.6 μm^3 scatter at 90° increased with swelling. In the present experiments scatter decreased with passive swelling in the range 0.144–0.375 μm^3 and increased with passive swelling in the range 0.384–0.579 μm^3 (Fig. 1). Even passive swelling of mitochondria, therefore, results consistently in a decrease in scatter at 90° of light of wavelength 500 nm only if the mitochondria are less than 0.4 μm^3 in volume. Use of a double-beam spectrophotometer to measure absorbance does not solve the problem, because in most commercial spectrophotometers the photocell collects both transmitted light and light that has been scattered at small angles [8].

Fig. 2a shows the effect upon light scatter, measured by conventional techniques, of adding the uncoupler CCCP at high concentrations to a suspension of mitochondria in isotonic KCl. Before addition of CCCP scatter was falling slowly; possibly because the mitochondria were slowly swelling. Addition of the uncoupler resulted in a rapid decrease in scatter. When CCCP was added in sequential, smaller aliquots scatter decreased in a step-wise fashion. When mitochondria, incubated under the conditions of Fig. 2a, were analysed for K^+ it was found that there was no significant difference between the K^+ contents of mitochondria incubated in the presence of CCCP (288 nmol/mg protein) or in its absence (294 nmol/mg protein); i.e., the uncoupler-induced decrease in scatter was not due to an influx of KCl solution. Moreover, a similar result was obtained in mannitol solution which contained few small ions or molecules which could contribute to a mitochondrial swelling. This initial

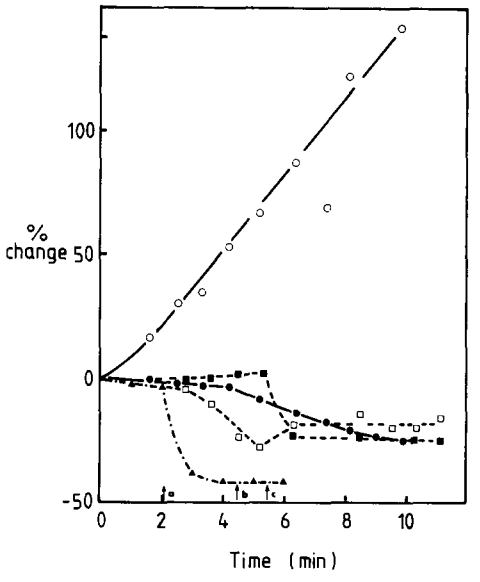

Fig. 2. The effect of uncouplers upon the volume of mitochondria and the intensity of light that they scatter at 90°. (a) 1.25 mg/ml respiring in isotonic KCl; 10 μl CCCP were added at the time of the arrow a to a final concentration of $2.5 \cdot 10^{-4}$ M. Wavelength 600 nm; (▲- - - - -▲) scatter. (b) 0.05 mg/ml respiring in isotonic KCl; 10 μl valinomycin were added at the time of the arrow B to a final concentration of $5 \cdot 10^{-6}$ M. Wavelength 488 nm; (●———●) scatter, (○———○) volume calculated from the effective hydrodynamic radius assuming that the mitochondria were spherical. The initial volume was 0.187 μm^3. (c) 0.05 mg/ml respiring in mannitol medium; 4 μl CCCP were added at the arrow c to a final concentration of 10^{-4} M. Wavelength 488 nm; (■ · · · · · ■) scatter, (□ · · · · · □) volume. Initial volume was 0.308 μm^3. Neither valinomycin nor CCCP absorbed light of wavelength 488 or 600 nm.

result suggested that discharge of the energised state per se might result in a change in relative refractive index of the mitochondrion.

The relationship between light scatter and particle size was therefore investigated further, using laser self-beat spectroscopy. Fig. 2b shows the result of an experiment similar to that of Fig. 2a, in which the uncoupler valinomycin was added to mitochondria in isotonic KCl. Before addition of the uncoupler, scatter was falling and effective volume increasing; i.e., the mitochondria were indeed swelling, and more drastically than was suggested by the relatively slight change in scatter. With addition of valinomycin the volume continued to increase steadily without a detectable change in gradient,

but scatter fell relatively sharply. This result confirms that a fall in light scatter does not necessarily reflect an increase in size. Fig. 1b also illustrates a feature common to all these measurements: light scatter is a rather insensitive index of the swelling process. Before addition of valinomycin the effective volume increased by 54% while the intensity of scattered light decreased by only 3%. Fig. 2c shows the effect upon scatter and volume of discharging the energised state in mitochondria incubated in mannitol medium. For the first 5 min the mitochondria decreased in volume by 28% while their scatter increased by only 2%. On the same scale, if the 25% decrease in scatter induced by CCCP had been due to swelling, the volume should have increased by 350% instead of the observed 20%. Again, therefore, most of the uncoupler-induced decrease in scatter reflected a change in refractive index unrelated to swelling.

Fig. 3 shows the reverse process. Mitochondria were incubated in mannitol medium until they were depleted of endogenous substrate. Both scatter and volume were constant. Addition of valinomycin under these conditions has been shown to induce a membrane potential and generate an energised state of the mitochondrion capable of synthesising ATP [9]. Fig. 2 shows an increase in scatter and

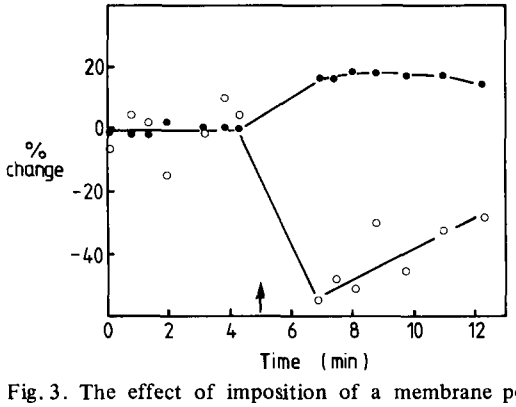

Fig. 3. The effect of imposition of a membrane potential upon the volume of substrate-depleted mitochondria and the intensity of light that they scattered at 90°. Protein concentration 0.1 mg/ml in mannitol medium; 10 μl valinomycin were added at the time of the arrow to a final concentration of $5 \cdot 10^{-3}$ M. Wavelength 488 nm; (●———●) scatter, (○———○) volume. The mean and standard deviation of the volume before addition of valinomycin were 0.388 ± 0.29 μm^3.

initial decrease in volume, presumably as K^+, anion and water left. Again, however, the increase in scatter was larger than would be expected for the corresponding decrease in volume, and scatter remained high while the volume returned toward its initial value. All these results (Figs. 2, 3) were qualitatively reproducible, but the magnitude of the changes observed with addition of uncoupler or ionophore depended upon the age of the mitochondria. All the results were consistent, however, with a change in relative refractive index of the mitochondrion independent of changes in volume.

Discussion

Bryant et al. [10] showed that large changes in light scatter can accompany a change of conformation in which the particle size remains constant, but the internal structure and relative refractive index change. The changes in scatter induced by uncouplers reported here are therefore consistent with a change in internal structure of the mitochondrion or of some of its components. The nature of the conformational changes that accompany synthesis and hydrolysis of ATP in mitochondria are not clearly defined. In the simpler, better understood $(Na^+ + K^+)$-ATPase and Ca^{2+}-ATPase, there is relatively detailed information about the reaction sequences, the properties of the intermediates, and the thermodynamics and kinetics of the transport and catalytic processes [11,12]. Racker [13] has pointed out that, although a phosphorylated intermediate of the mitochondrial ATPase has not been identified, the possibility that there might be an acyl phosphate intermediate cannot be ruled out. It might therefore be profitable to assume that the mitochondrial ATPase has, indeed, phosphorylated intermediates similar to those of the other ATPases, and to consider in those terms the effect of uncouplers upon conformation.

Both the $(Na^+ + K^+)$-ATPase and Ca^{2+}-ATPase [11,12] have two phosphorylated intermediates, one of which is ADP sensitive, donating its phosphate to ADP with synthesis of ATP. The other phosphoenzyme is ADP insensitive; it is the actively transporting conformation, and its lifetime is terminated by hydrolysis. Both phosphoenzymes are conformationally distinct from the unphosphorylated enzyme. The ADP-insensitive phosphoenzyme can be

synthesised from P_i when the solution bathing the phosphorylation site contains Mg^{2+} and extremely low concentrations of the activating cation (Na^+ or Ca^{2+}). The actively transporting conformation is converted into the ATP-synthesising conformation when the solution bathing the opposite side of the membrane contains a high concentration of the activating cation. The ATP-synthesising conformation is transformed into the actively transporting conformation when the solution bathing the phosphorylation site contains Mg^{2+}, and the concentration of activating cation on the opposite side of the membrane is below an upper limit.

By analogy, there might be two phosphorylated intermediates of the mitochondrial ATPase: the ADP-sensitive form, which does not actively transport H^+, but which does synthesise ATP; and the ADP-insensitive form which pumps H^+ outward, but which does not synthesise ATP. Conversion of ADP-sensitive to ATP-insensitive forms then should accompany a sufficient decrease in the effective H^+ activity at the external surface of the membrane. This might be accomplished by the relatively low concentrations of uncouplers which are commonly found to inhibit ATP synthesis. If the conformations of the two phosphorylated intermediates are similar, concentrations of uncouplers which inhibit ATP synthesis need not necessarily cause large changes in scatter.

In the present experiments, and in those of Manella and Parsons [14], concentrations of uncoupler necessary to change scatter significantly were much higher than those found to uncouple oxidative phosphorylation. In terms of the proposed scheme, high concentrations of uncoupler not only decrease the effective H^+ activity at the external surface of the membrane, but also increase its activity at the internal surface. Neither the $(Na^+ + K^+)$-ATPase nor the Ca^{2+}-ATPase is phosphorylated by P_i when the concentration of the activating cation (Na^+ or Ca^{2+}) is increased to or above physiological levels at the phosphorylation site. By analogy, the mitochondrial ATPase should not be phosphorylated by P_i when the activity of H^+ is too high at the phosphorylation site. Therefore, in the presence of sufficiently high concentrations of uncoupler, the mitochondrial ATPase assumes its unphosphorylated conformation, which is sufficiently different in density of scattering elements to involve a large decrease in scatter. Manella and Parsons [14] pointed out that one of the effects of high concentrations of uncouplers was a stimulation of ATPase activity. In the present scheme, the ATPase predominantly in its unphosphorylated conformation is all available to be phosphorylated by ATP. Therefore, added ATP is rapidly hydrolysed. It has been suggested [15–17] that both phosphorylated intermediates of the $(Na^+ + K^+)$-ATPase and those of the Ca^{2+}-ATPase contain within a cleft a small interfacial phase of highly ordered water of low density and high viscosity. The altered chemical potentials of all contained solutes then form a source of energy for either ATP synthesis or for active transport. Such fluctuations in density of liquid water cause anomalous light scatter [18] because of the change in electron density. One possible molecular mechanism for the observed changes in light scatter induced by uncouplers, therefore, is that there are two conformations of an intermediate of the ATPase, each of which contains a microaqueous phase of low density; their scattering properties are similar. Low concentrations of uncouplers convert the ATP-synthesising conformation into the actively transporting conformation, with inhibition of ATP synthesis, but no dramatic change in scatter. High concentrations of uncouplers prevent formation of either intermediate, and cause a change in light scattering power as all the water reverts to bulk-phase-like properties, and the scatter produced by gross fluctuations in its density disappears.

It is concluded that light scatter measured by conventional techniques is not a reliable method of following changes in mitochondrial volume, and that changes in the scattered intensity are often dominated by changes in internal structure of the mitochondrion independent of changes in particle size.

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References

1 Parsons, D.F., Williams, G.R. and Chance, B. (1966) Ann. N.Y. Acad. Sci. 137, 643–666
2 Estabrook, R.W. (1967) Methods Enzymol. 10, 41–47
3 Layne, E. (1957) Methods Enzymol. 3, 450–451
4 Bellamy, A.R. and Harvey, J.D. (1976) Virology 70, 28–36
5 Pusey, P.N., Koppel, R., Schaefer, D.W., Camerini-Otero, R.D. and Koenig, S.H. (1974) Biochemistry 13, 952–959
6 Kerker, M. (1969) The Scattering of Light and Other Electromagnetic Radiation, Academic Press, New York
7 Latimer, P. and Pyle, B.E. (1972) Biophys. J. 12, 764–773
8 Latimer, P. (1972) J. Opt. Soc. Am. 62, 208–211
9 Cockrell, R.A., Harris, E.J. and Pressman, B.C. (1967) Nature 215, 1487–1488
10 Bryant, F.D., Latimer, P. and Seiber, B.A. (1969) Arch. Biochem. Biophys. 135, 109–117
11 Robinson, J.D. and Flashner, M.S. (1979) Biochim. Biophys. Acta 549, 145–176
12 De Meis, L. and Vianna, A.L. (1979) Annu. Rev. Biochem. 48, 275–292
13 Racker, E. (1975) In Electron Transfer Chains and Oxidative Phosphorylation (Quagliarello, E., Pape, S., Palmieri, F., Slater, E.C. and Siliprandi, N., eds.), pp. 401–406, North-Holland, Amsterdam
14 Manella, C.A. and Parsons, D.F. (1977) Biochim. Biophys. Acta 460, 375–378
15 Wiggins, P.M. (1979) Bioelectrochem. Bioenerg. 6, 123–133
16 Wiggins, P.M. (1980) J. Biol. Chem. 255, 11365–11371
17 Wiggins, P.M. (1981) in Epithelial Ion and Water Transport (Macknight, A.D.C. and Leader, J.P., eds.), p. 307, Raven Press, New York
18 Stillinger, F.H. (1980) Science 209, 451–457