J. Membrane Biol. 18, 95–112 (1974)

Description by Quasi Elastic Laser Light Scattering of a Biological Preparation: Sarcoplasmic Reticulum Vesicles

B. Arrio, J. Chevallier, M. Jullien, J. Yon, and R. Calvayrac

Institut de Biochimie, Equipe de Recherche No. 118
and Laboratoire d'Enzymologie Physico-Chimique et Moléculaire,
Groupe de Recherche C.N.R.S. No. 13, Université de Paris Sud 91405 Orsay, France
and Laboratoire de Cytophysiologie de la Photosynthèse
du C.N.R.S, 91190 Gif sur Yvette, France

Received 26 November 1973; revised 7 March 1974

Summary. Quasi elastic laser light scattering (QELS) was used to describe the size and the homogeneity of a membrane preparation: vesicles from skeletal sarcoplasmic reticulum. The data were compared with results obtained by electron-microscopy. The advantages of each method are discussed. By electron-microscopy the average value for the diameter of the vesicles obtained after negative staining is 0.100 ± 0.040 μm. With the freeze-etching technique this value ranges from 0.13 to 0.25 μm. For the same biological preparation the analysis of the QELS recording curves displaying one correlation time (τ) gives an apparent diameter for the vesicles of 0.17 μm. Although it is difficult to describe by QELS the homogeneity of the preparation, the technique remains a very convenient method for evaluating variation of the size of the vesicles, and thus offers the possibility to follow general modification in the size of a vesicular preparation. The effect of trypsin and phospholipase C treatment on vesicle size is studied: an increase of vesicle size is clearly observed after trypsin treatment.

A direct evaluation of the homogeneity and the size of constituents of a membrane preparation after extraction and purification is a major problem. Presently, the only way to solve this problem is by electron-microscopy. However, recent developments in the field of quasi elastic light scattering techniques give a possibility, immediately after preparation, to describe the homogeneity and to study possible structural changes in the membrane structure.

Quasi elastic laser light scattering (QELS) [1, 28] has been already used to determine in aqueous medium, the size of large molecular organizations: for instance, viruses [6, 24, 25], muscular proteins [11], and so on. The purpose of the present paper is to study the possibilities offered by quasi

elastic laser light scattering to describe the state and to evaluate the size of a biological system: vesicles from sarcoplasmic reticulum (SR) of rabbit skeletal muscle. The function of the SR (the releasing and the accumulation of calcium ions during the muscular contraction-relaxation process) was studied by *in vitro* experiments on microsomal fractions isolated from SR by differential centrifugation of the homogenized muscle (for a general presentation of that problem *see* Martonosi [18]). A lot of ultrastructural studies of that material were done by electron-microscopy [3, 12, 17, 29]. So it seems possible to apply QELS to the study of the state of that well-described membrane system. Each experiment is performed on a microsomal preparation displaying a high calcium uptake ability. On the same sample we compare the results obtained by QELS with electron-microscopy pictures. The advantages and the limitations of the QELS technique are discussed.

Materials and Methods

Isolation of Sarcoplasmic Reticulum

The isolation of SR is performed by a procedure somewhat modified from Meissner and Fleischer [22]. Ground muscles are homogenized in 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 0.3 M sucrose, pH 7.5, for 30 sec in a Waring Blendor. The homogenate is centrifuged in a GSA rotor Sorvall 30 min at $8{,}000 \times g$. The supernatant is centrifuged again for 75 min at $95{,}000 \times g$ in a Spinco 30 rotor. The pellet is treated overnight by 0.6 M KCl in the cold room, then washed three times in 10 mM HEPES, pH 7.5, 0.3 M sucrose, 0.6 M KCl and finally resuspended in 5 mM HEPES, 0.3 M sucrose, pH 7.5. At that step the reticulum preparation can be stored for at least a month at -70 °C (fraction A).

A more highly purified preparation can be obtained after a step gradient centrifugation in 5 mM HEPES buffer, pH 7.5, and different concentrations of sucrose in each layer: from 37.1% (w/w) to 26.7% (w/w). Forty to fifty mg of fraction A is applied on the top of each tube and centrifuged during 170 min at $106{,}000 \times g$ in a Spinco SW 25.2 rotor at 5 °C. The fractions recovered [F_1 on the top, F_2 between 26.7 and 29.1% (density g/ml = 1.11 and 1.13), F_3 between 29.1 and 33.9% layer (density g/ml = 1.13 to 1.16) and a pellet] are diluted in 5 mM HEPES, pH 7.5, centrifuged down and resuspended in the same buffer. These fractions are kept no more than three days in ice before use. All experiments are carried out on fraction F_2.

Calcium Uptake Activity

Calcium uptake activity of SR preparation (30 to 10 µg per ml) is measured in 10 mM HEPES, pH 7.2, with 5 mM ATP, oxalate, $MgCl_2$ medium in 0.1 mM $^{45}CaCl_2$ 0.15 M KCl. The reaction is performed at 20 °C during 10 min and stopped by pressing 1 ml of the reacting solution through a Millipore filter (GS 0.22 µm) following the Martonosi technique [20].

The procedure of Lowry *et al.* [15] is used with bovine serum albumin as a standard to determine the protein concentration. Contamination by mitochondria is monitored

using cytochrome C oxidase activity of the preparation [26]. Phospholipase C (*C. Welchii* B grade from Sigma with no detectable proteolytic activity) and trypsin (Novo) treatments are performed as described in the literature [14, 17, 19].

Electron-Microscopy

SR microsomal preparation in 5 mM HEPES (pH 7.5) buffer are negatively stained by the Horne technique [13]. 200-mesh grids are coated with a Formvar film. The preparation is applied for 2 min on the grid and then dried on filter paper. Negative staining is carried out in a solution of 1% phosphotungstic acid (PTA), brought to pH 7.2 with potassium hydroxide, during 40 to 50 sec, then dried out again. Electron-micrographs are taken with a Hitachi H.S. 8 at 50,000 magnification. The distribution of sarcoplasmic reticulum vesicle diameters is analyzed by classical statistical methods. Polystyrene spheres of nominal diameter 0.109, 0.176, 0.234, and 0.312 μm (from Dow-Latex Feinbiochemica, Heidelberg) are used to calibrate the electron-microscope and the QELS apparatus. These spheres are the references for the evaluation of the average size of the vesicles in each experiment.

SR suspension (F_2 fraction) is centrifuged for 1 hr ($130{,}000 \times g$) in 5 mM HEPES, pH 7.5, 30% glycerol. The specimens are fractured in a Bendix-Blazers apparatus and shadowed at 30°. The pictures are given with 115,000 magnification.

Quasi Elastic Light-Scattering Apparatus

The apparatus used in the present work is almost the same as that already described by Dubin [6]. The 6,328 Å radiation of a Spectra Physics model 124 laser is focused on the scattering cell. The light scattered at an angle θ is focused on a 56 TUVP Radiotechnique photomultiplier, the output of which is amplified by an ATNE (0 to 10 MHz) amplifier and the signal analyzed with a Hewlett-Packard correlator. The autocorrelation function displayed on the oscilloscope is recorded on a Hewlett-Packard X-Y recorder. Diffusion coefficients are obtained by fitting the best exponential curve by the use of a Wang Electronic calculator, model 370–362 E. All the data are taken between 18 and 19 °C, with a protein concentration of 1 mg/ml (unless notified) in a 5 mM HEPES buffer, pH 7.5. The temperature in the cell is constant during the experiment (5 min maximum).

Theoretical

The underlying theory has been extensively presented by Dubin [5, 9] and by Clark *et al.* [2]. Whereas classical light scattering can provide information on the molecular weight of macromolecules in solution on the basis of the intensity of scattered light, quasi elastic light scattering considers the fluctuations of light. The intensity of the light scattered by a suspension of particles or by a solution of macromolecules at time t is determined by the superposition of the phases of the scattered waves. Since the phase of the light scattered by each particle is randomly changing as the particle undergoes a random motion, the intensity of the scattered light fluctuates. The scattered intensity at time t must reflect a concentration fluctuation

Δc, obeying the translational diffusion equation:

$$\frac{\partial(\Delta c)}{\partial t} = D_T \nabla^2(\Delta c) \tag{1}$$

where D_T is the translational diffusion coefficient and is characterized by a wave vector:

$$|\vec{K}| = |\vec{k}_o - \vec{k}_s| = \frac{4\pi}{\lambda/n} \sin\theta/2 \tag{2}$$

$\vec{k}_o$ and $\vec{k}_s$ being, respectively, the wave vectors of the incident and scattered light. λ is the wavelength of the incident light in vacuum, n the refractive index.

Solving Eq. (1) for a fluctuation having wave vector $\vec{K}$ at time 0, the time dependence of the fluctuation is given by

$$\Delta C_{(t)} = \Delta C_{(t=0)} e^{-K^2 D_T t}. \tag{3}$$

Experimentally, it is possible to define the time dependence of a fluctuation by an average of the fluctuations, at time t and $t+\tau$, where τ is a delay time. The correlation function of the random fluctuations is given by

$$\langle \Delta C_{(t)} \Delta C_{(t+\tau)} \rangle_K = \langle \Delta C^2_{(t)} \rangle e^{-K^2 D_T \tau}. \tag{4}$$

Eq. (4) has been verified in the simplest case of rigid isotropic, monodisperse spherical particles. The influence of particle length, rotational diffusion and polydispersity have been already discussed [4, 8, 23, 27]. The diffusion coefficient is obtained by the measurement of the time constant of the photocurrent correlation function [7, 10, 27]. The time constant τ_c is defined by

$$\frac{1}{\tau_c} = 2K^2 D_T \tag{5}$$

for homodyne detection; this is the case for our spectrometer. The particle radii are derived from the Stokes relation:

$$D_T = \frac{kT}{6\pi\eta r} \tag{6}$$

where η is the viscosity of the solvent, T the temperature in degrees K, k the Boltzman constant, and r the radius.

Results

The data showing the purification of fraction A by step sucrose gradient centrifugation appear in Table 1. All experiments are done on fraction F_2 which displays the highest calcium uptake ability and a very low cytochrome C oxidase activity.

Table 1. Activities of the different fractions obtained after step sucrose gradient centrifugation

Fraction	Density (g/ml, 5 °C)	Ca^{++} Uptake [a]	Cyto C oxidase activity [b]
A	–	1.5 – 2.6	0.052
F_1	top of the gradient	0.01 – 0.05	0.002
F_2	1.11 – 1.13	3.9 – 5.7	0.004
F_3	1.15 – 1.16	0.7 – 1.5	0.104

[a] μmoles Ca^{++} accumulated/mg protein at 20 °C, pH 7.3, after 10 min according to the Martonosi technique [20].
[b] μmoles cyto C oxidized/min/mg protein at 20 °C, pH 7.5 [26].

F_2 Fraction

Electron-micrographs of fragmented sarcoplasmic vesicles are shown in Figs. 1, 2 and 3. Fraction A (Fig. 1*A*) is composed of vesicles and tadpole-like structures, as already described in the literature [3, 12, 17, 29]. These tubular structures are eliminated after sucrose gradient centrifugation in the fraction F_2 (Fig. 2). A population of apparently spherical vesicles, the diameter of which ranges from 0.04 to 0.120 μm, is seen. The distribution of the vesicles as a function of their diameters (Fig. 4*A*) can be described as follows: average value $(\bar{x}) = 0.100$ μm and standard deviation $(\sigma) = 0.040$ μm.

The sizes of the vesicles can be evaluated by freeze etching (Fig. 3). They fall in the range from 0.13 to 0.25 μm: this is in agreement with the average size given by Deamer and Baskin [3] but different with the results obtained from the negative staining method.

This description of the fraction F_2 by electron-microscopic techniques, displaying a good homogeneity in size and shape, is very convenient to test the results obtained by the QELS technique. Electron-micrographs of latex spheres used as control samples for both the electron-microscope and the QELS apparatus are shown in Fig. 1 (*B–C*).

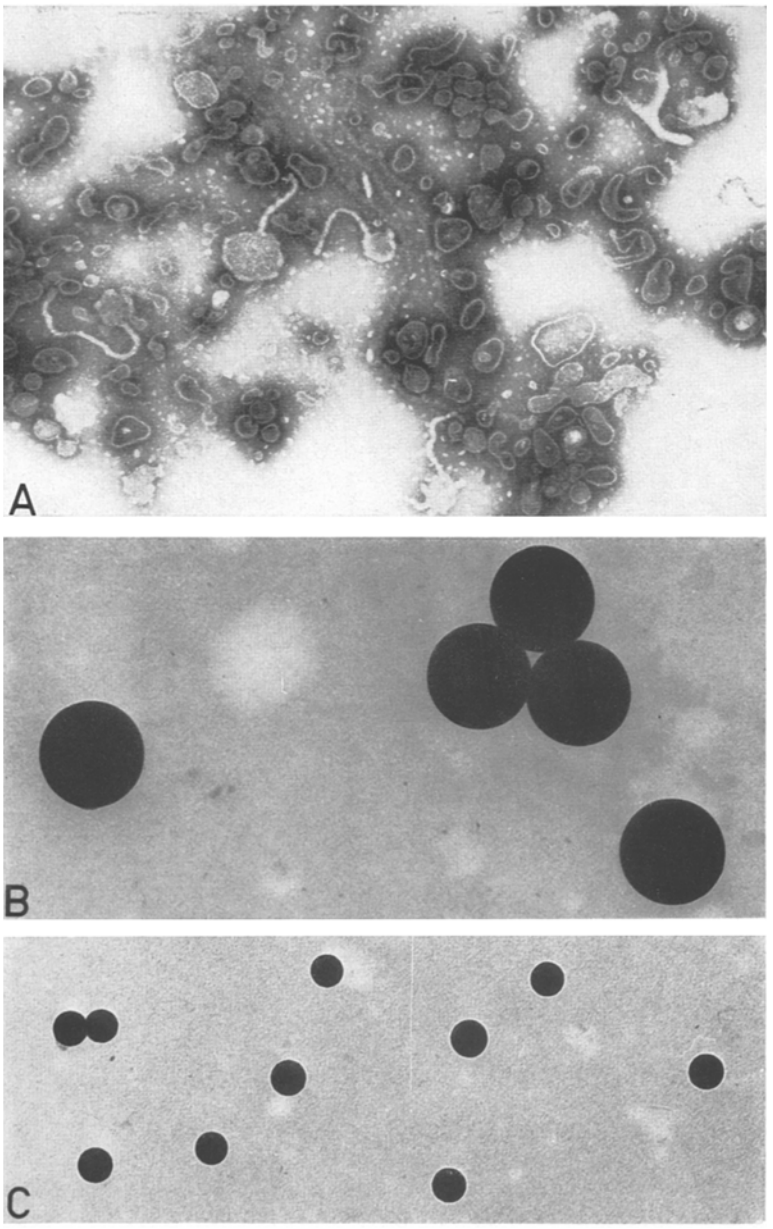

Fig. 1. Electron-micrographs of sarcoplasmic reticulum vesicles and latex spheres. (*A*) Crude sarcoplasmic reticulum vesicle preparation (fraction A) Ca^{++} uptake activity: 1.5 to 2.6 μmoles Ca^{++}/mg protein under standard conditions (*see text*). Negatively stained preparation with 1% potassium phosphotungstate, pH 7.2. Magnification $\times$ 50,000. Latex spheres of nominal diameter 0.312 μm (*B*) and 0.109 μm (*C*) suspended in aqueous medium. Magnification $\times$ 50,000. These spheres were used to calibrate the QELS apparatus

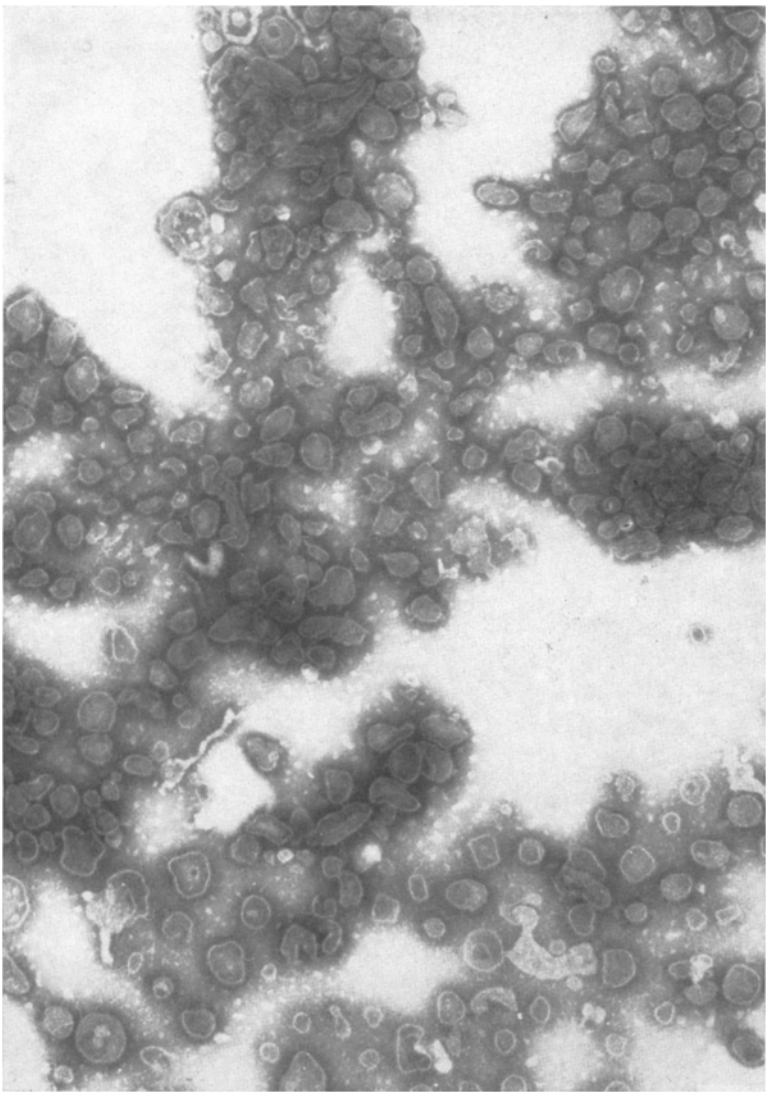

Fig. 2. Electron-micrograph of negatively stained purified sarcoplasmic reticulum preparation (fraction F_2). Calcium uptake activity: 3.9 to 5.7 μmoles Ca^{++}/mg protein under standard conditions (*see text*). Negatively stained as already described. Magnification × 50,000

The correlation time 590 ± 10 μsec for the fraction F_2 in 5 mM HEPES buffer, pH 7.5, obtained from Fig. 5, is not concentration dependent from 0.7 to 7.0 mg/ml (Fig. 6). This indicates the absence of aggregates in this range of concentration. So we evaluate the apparent diameter of the F_2

Fig. 3. Freeze-etch image of purified sarcoplasmic reticulum preparation (fraction F_2). The sarcoplasmic reticulum suspension is freeze-etched (*see text*). Magnification × 115,000

vesicular fraction as 0.170 μm. We have observed that the decay constant of the autocorrelation function [Eq. (5)] obeys the angular relation (3) between 33° and 135° (Fig. 7); this is in agreement with the absence of filaments or tadpole-like structures. The angular distribution of the intensity of the scattered light indicates that the vesicles exhibit a spherical shape.

F_2 after Trypsin and Phospholipase C Treatment

The same studies were performed with fraction F_2 previously treated with trypsin or with phospholipase C. In both cases the calcium uptake ability of sarcoplasmic reticulum vesicles is destroyed. Under the same

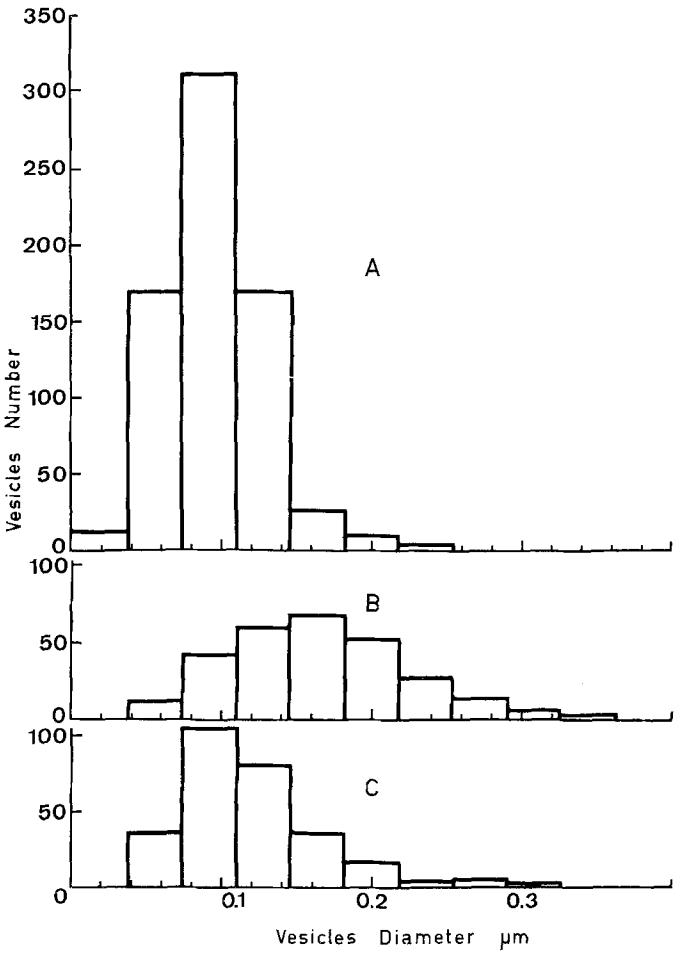

Fig. 4. Diameter distribution of sarcoplasmic reticulum preparations. (*A*) Fraction F_2 – The distribution is measured from the electron-micrograph presented in Fig. 2. The measured distribution has not been corrected since large vesicles (diameter higher than twice the average diameter) represented less than 1% of the total vesicle number. (*B*) Diameter distribution of highly purified sarcoplasmic preparation treated by trypsin. The distribution is measured from the preparation presented in Fig. 8*A*. (*C*) Diameter distribution of highly purified sarcoplasmic reticulum treated by phospholipase C. The distribution is measured from the preparation presented in Fig. 8*B*

experimental conditions, control preparations without enzyme treatment are able to actively accumulate calcium. As we can see by electron-microscopy (Fig. 8*A* and *B*), the different fractions are always vesicular, but the average size of the elements increases: notably in the case of the trypsin treatment where $\bar{x} = 0.18$ μm, and little for the phospholipase C-treated preparation

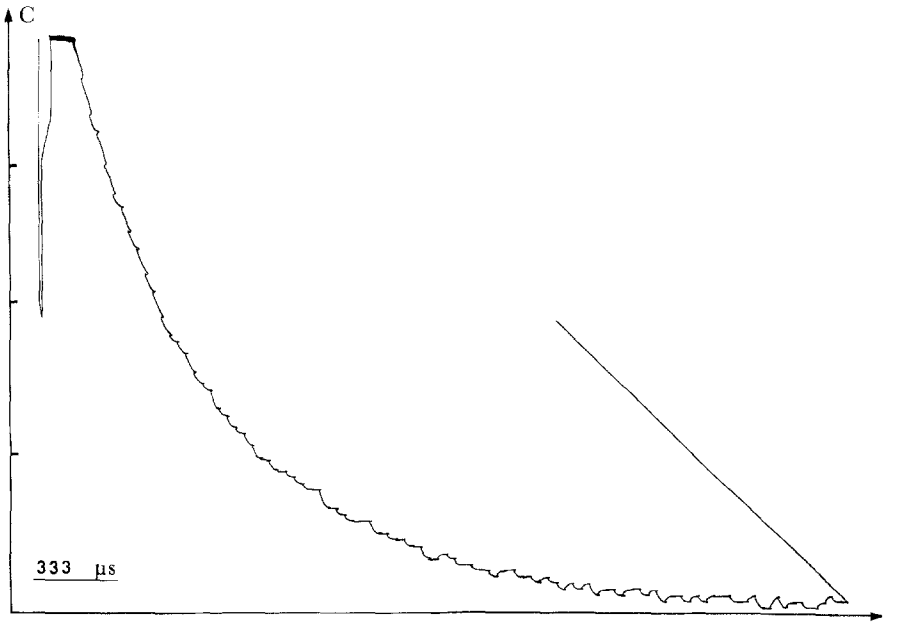

Fig. 5. Direct recording of the correlation function obtained for the fraction F_2. Fraction F_2: 1 mg/ml in HEPES buffer, pH 7.5, at 18 °C. The light is scattered at $\theta = 90°$

Table 2. Comparative results obtained by QELS and by electron-microscopy with the sarcoplasmic reticulum vesicles (F_2 fraction)

Sample	Electron-microscopy	QELS	
	Diameter, average value (µm) ($\bar{x}$)	Diffusion coefficient $\times 10^8$ cm^2 sec^{-1}	Diameter (µm)
Latex	0.120 0.109[a] 0.310 0.310[a]	3.87 ± 0.11 1.43 ± 0.07	0.110 ± 0.003 0.298 ± 0.008
F_2	0.100 ± 0.040	2.41 ± 0.04	0.175 ± 0.003
F_2 (trypsin-treated)	0.170 ± 0.060	1.64 ± 0.02	0.258 ± 0.003
F_2 (Phospholipase C-treated)	0.130 ± 0.055	1.86 ± 0.02	0.227 ± 0.003

[a] Nominal diameter given by Dow Chemical, cm^2 sec^{-1}.

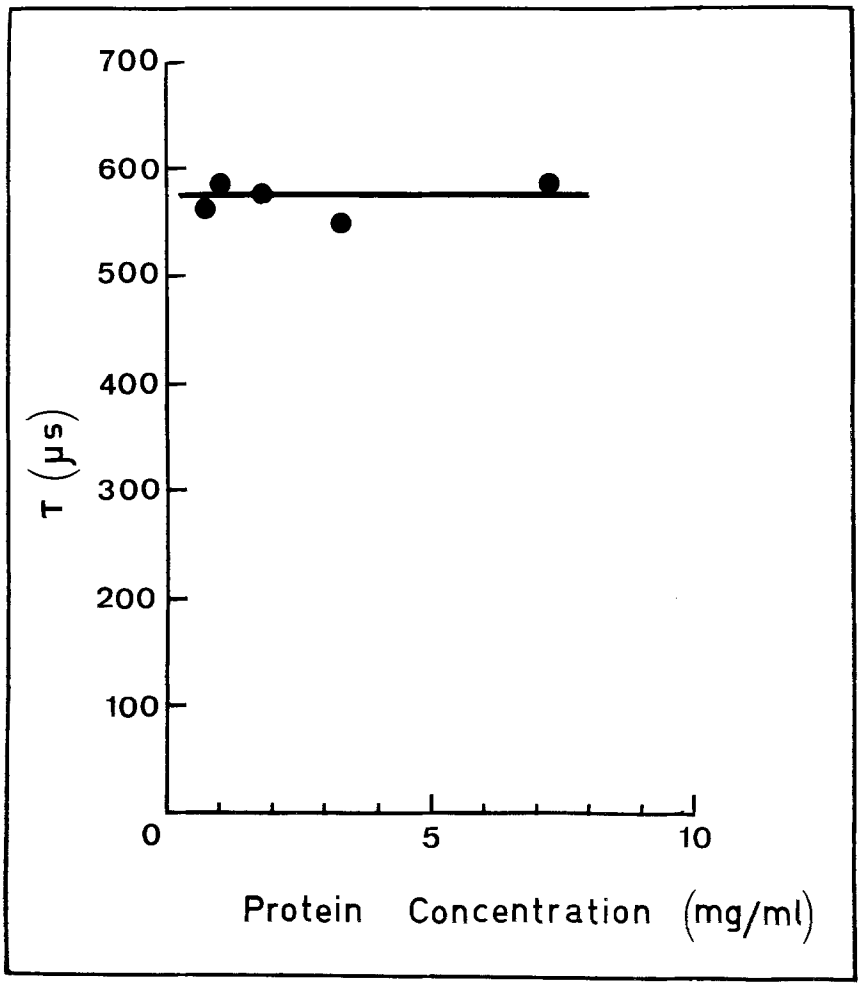

Fig. 6. Dependence of the correlation time on the concentration of sarcoplasmic preparations (F_2 fraction). The experiments are done as described in the text, in 5 mM HEPES, pH 7.5. $\theta = 90°$

where $\bar{x} = 0.12$ μm with a more pronounced heterogeneity (Fig. 4*B* and *C*). The statistical parameters describing these preparations are presented in Table 2.

The correlation times obtained with the QELS technique are 870 ± 10 μsec and 760 ± 10 μsec, respectively, for trypsin and phospholipase C-treated preparations. The apparent diameters of the vesicles obtained would be, respectively, 0.25 and 0.22 μm; so we can detect by this technique

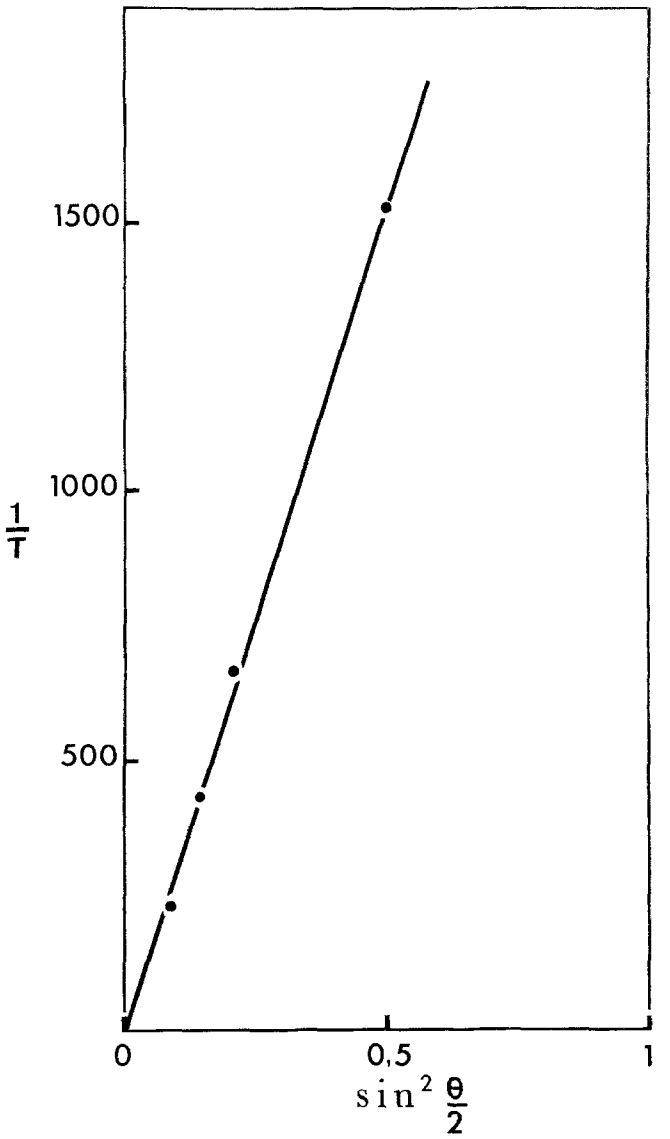

Fig. 7. Dependence of the opposite of the correlation time on the angle of the light scattered for the fraction F_2. Fraction F_2: 1 mg/ml in HEPES buffer, pH 7.5, at 18 °C. $35° < \theta < 90°$

a large increase of the vesicle size in the preparation, although, by electron-microscopy, this phenomenon is observed clearly only for the trypsin-treated SR.

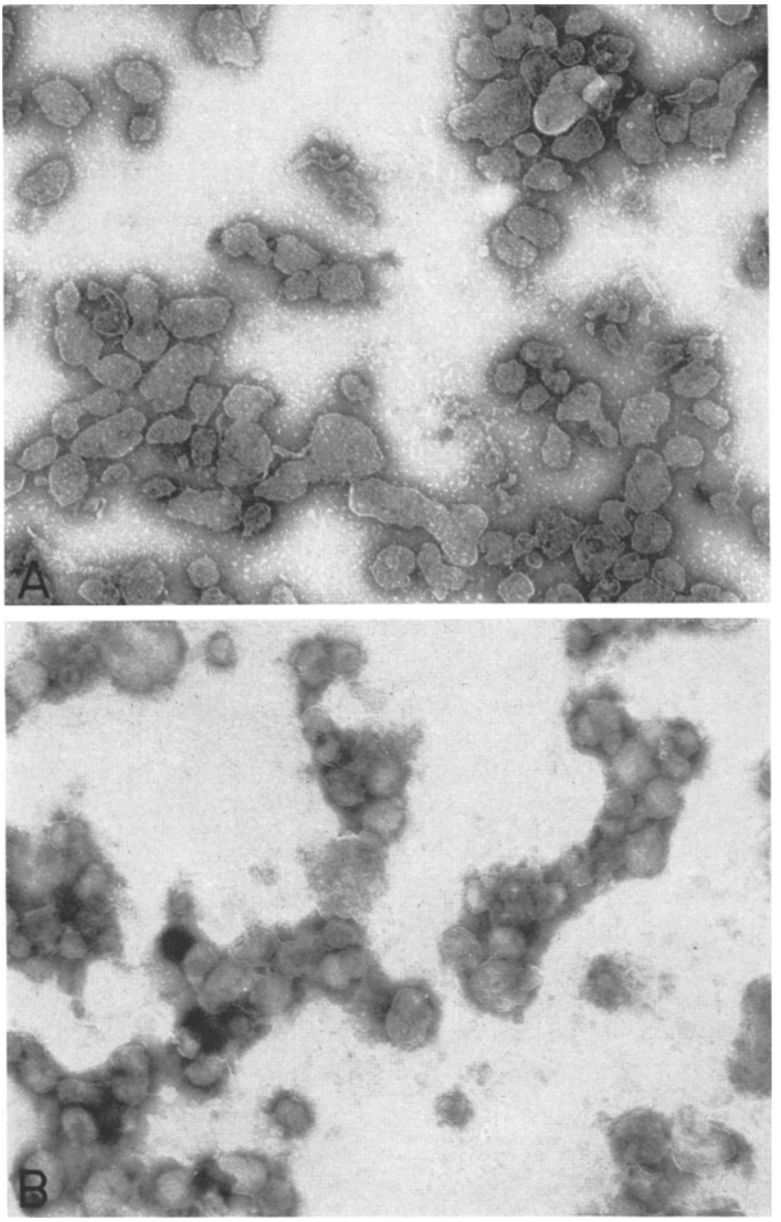

Fig. 8. Negatively stained sarcoplasmic reticulum vesicles after enzymatic treatments. (*A*) Effect of trypsin on F_2 fraction (4.15 mg/ml) was treated by trypsin (0.2 mg/ml), pH = 7.0, 22 °C during 20 min. The reaction was quenched by soybean trypsin inhibitor. (*B*) Effect of phospholipase C. F_2 fraction (6.8 mg/ml) was treated by phospholipase C (1.5 mg/ml), pH 7.3, 25 °C during 2 hr, then washed three times by 1% bovine serum albumin solution

Discussion

Description of the Fraction F_2 by Electron-Microscopy

The purification of fragmented sarcoplasmic reticulum by extensive 0.6 M KCl treatment and step sucrose gradient centrifugation gives a fraction F_2 displaying good calcium uptake activity under standard conditions. In a recent paper, Meissner *et al.* [21] described a more sophisticated method to purify sarcoplasmic reticulum vesicles. The calcium uptake activity of their preparations was the same as that which we obtain for the F_2 fraction. The absence of tadpoles in negative staining electron-micrographs and in freeze-etching pictures shows clearly that this kind of structure has nothing to do with the calcium transport activity of the biological material. The vesicle size for that fraction lay around 0.100 ± 0.040 μm. This result is in agreement with those of Yu *et al.* (0.1 to 0.16 μm) [29], Deamer and Baskin (0.12 ± 0.03 μm) [3] and Heuson-Stiennon *et al.* (0.120 μm) [12]. As we can see on Figs. 2 and 4, the preparation is very homogenous in size. The size obtained (0.13 to 0.25 μm) by freeze etching is greater than the average diameter measured by the negative staining technique, but the former is not accurate enough for the evaluation of the homogeneity since the number of vesicles observed is lower.

The average size obtained for the fraction F_2 is influenced by the intensity and the duration of the homogenization as well as by the selection which occurs during subsequent differential centrifugation. However, we have to point out two facts; first, the procedure we use to purify sarcoplasmic reticulum seems to be the most efficient one to obtain the highest specific calcium transport activity, mostly because mitochondrial fragments are easily eliminated; second, it appears that a modification in the vesicle size is more or less correlated with a destruction of the biological activity (*see below*). In view of these two remarks we can define artificially an "active vesicular size" which corresponds, for the purification procedure used, to the best specific activity.

Modification of Vesicle Size after Trypsin or Phospholipase C Treatment

After phospholipase C and trypsin treatment the vesicles are not destroyed. However, a variation in their size is observed. In both cases we notice an increase in the average diameter (Fig. 4 and Table 2), more pronounced for the trypsin-treated preparation than for phospholipase C, even though the action of these enzymes is completely different. As Martonosi *et al.* stated [18], an incomplete but specific hydrolysis of phosphati-

dylcholine of the membranes inhibited calcium transport activity without a comparable effect on the formation of phosphoprotein intermediate. The vesicles were not destroyed [13]. Since our experimental conditions for the hydrolysis by phospholipase C (*C. welchii* free of proteolytic activity) are identical to those described by Martonosi *et al.* [18, 19], we can assume that the slight modification in size observed is due to partial removal of phosphatidylcholine.

Since we work in absence of sucrose in the buffer used for trypsin digestion, the removal of both surface spheres and stalks occurs as presented by Ikemoto *et al.* [14] and Martonosi [17]. The treatment induces a partial removal of proteins. Again an increase in vesicle size is observed by the two different techniques. Therefore, qualitatively different treatments of the membrane seem to induce the same effect on the vesicle size.

This size increase seems to be a very general phenomenon. In our case, with aging vesicles which display at least 10% of the original calcium uptake ability or with sarcoplasmic reticulum preparations treated by some drugs (J. Chevallier, *unpublished results*), we observed an increase of the average size of the vesicles by QELS. The same phenomenon occurred with reconstituted membrane preparations. For instance, Mac Lennan *et al.* [16] obtained vesicle formation by reconstituted vesicles displaying activated ATPase activity, with a diameter of about 0.7 to 0.8 μm (from Fig. 2*A* of reference [16]). No clear explanation can be given for this general increase observed in this experiment.

Comparison between Electron-Microscopy and QELS Results

In discussing these results, it is tempting to consider two alternative proposals: the histograms obtained from electron-micrographs are the real image of the vesicle size and QELS cannot accurately describe it, or the opposite. If electron-micrographs reflect exactly the size heterogeneity of the F_2 fraction, it is clear that the best fit of the exponential autocorrelation function given by QELS, leading to a single correlation time, cannot describe the size distribution. At this step it is necessary to discuss this limitation and the difference observed between the diameters measured by both techniques. Assuming that the molecular weight of the vesicles varies as the cube of the radius and combining that with the F_2 fraction size distribution, one can observe that the contribution of the larger diameter vesicles increases the diameter measured by QELS. This leads to an apparent vesicle diameter of 0.17 μm. A direct simulation of the F_2 distribution performed with latex spheres (0.109, 0.176 and 0.234 μm) leads to a correlation time

of 586 μsec, comparable with that found for the F_2 fraction. We can point out also that the contribution of the larger vesicles cannot be extrapolated always using a cubic function of the radius, but it is diminished due to the increasing ratio between the particle diameter and the wavelength, as already shown by Mie [23]. Similar considerations of the histograms show that the differences between electron-micrographs and QELS data can be explained by these effects.

This is clearly supported in the case of the phospholipase C-treated preparation (Figs. 4*C* and 8*B*) where the weight of vesicles of higher diameter gives us by QELS an apparent diameter of 0.22 μm, very different from the average value obtained by EM (0.12 μm).

On the other hand, the freeze-etching data show that the vesicle size is greater than that observed by negative staining technique. Therefore, the negative staining histograms cannot be considered as an absolute description of the size of the constituents of the preparation.

Thus, QELS displays a severe limitation in describing the heterogeneity of a biological preparation, as has already been shown by Dubin [4, 8] on the problem of molecular weight for various viruses and on the lysozyme denaturation. However, we observe that this technique is a very useful and elegant one for following the gross structural behavior of reticulum vesicles by correlating QELS size determination and the modification in activity under various conditions, such as trypsin and phospholipase C treatment.

Conclusion

Generally, when membranes are prepared, the time delay in which activities and structural behavior can be observed is very narrow. As we show in this paper, QELS offers the possibility of a size determination under the same conditions as those of the activity measurements. Another interesting feature is the rapidity of the acquisition of information when correlators are used for the light-scattering fluctuations analysis. QELS is a good complement of electron-microscopy for the description of biological material, but the former can be used as a dynamic tool to evaluate the impact of chemical modifications and drug action on membrane structure.

The authors are greatly indebted to G. Durand and I. Smith from the Laboratoire de Physique des Solides (Orsay) for helpful discussions. They want to acknowledge Dr. G. Nemethy for reviewing the manuscript and Mrs J. Carette and A. Lacourly for skillful technical assistance. This work was supported by D.G.R.S.T. conventions 72.7.0490 and 72.7.0176.

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