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Effect of Electric Fields on Light-Scattering and Fluorescence of Chromaffin Granules

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Summary. Electric field pulses (in the 5 to 25 kV/cm range) were found to cause a transient increase in the intensity of scattered light (wavelength = 369 nm, scattering angle = 90°) from aqueous sucrose suspensions of chromaffin granules. Similar observations were made with the membranes of osmotically lysed chromaffin granules. Under the same experimental conditions the degree of polarization of the scattered light changed only very slightly. The fluorescence of the hydrophobic probe diphenyl-hexatriene, incorporated into the membrane of intact chromaffin granules, showed similar transient changes in the intensity. The calculated relaxation times for these changes in optical properties were approximately 150 μsec for the rising phase, and approximately 1 msec for the early stage of the decay. A further relaxation time of about 30 msec was also observed by using this probe. Essentially, all of these signals originated from the granule membrane, and could be attributed to rather small changes in particle size, membrane thickness or refractive index. Moreover, these signals were found to be completely reversible. Catecholamine release from intact granules, pulsed at voltages of 25 kV/cm, occurs already during the first few milliseconds of the transient membrane change.

The relation between the depolarization of cell membranes and release of biogenic amines is now well established in the adrenergic nerve cells and the chromaffin cells of the adrenal medulla. Recently, the possibility was considered that variations in electric potential, occurring across the cell membrane during depolarization could exert a triggering action on the amine storing vesicles. In fact, using bovine chromaffin granule suspensions *in vitro* as a model system, changes in membrane permeability leading to release of the biogenic amines stored, could be induced by short, electric field pulses in the 20 kV/cm range (Neumann & Rosenheck, 1972).

The present study was aimed at obtaining more detailed information on the release process induced by the electric field. In particular, it was tried to determine its time course in relation to the triggering pulse. Furthermore,

it was deemed important to obtain further data relating to the question whether or not the observed release was due to irreversible destruction of the vesicles, a possibility that was thought very unlikely on the basis of chemical data (Neumann & Rosenheck, 1972). Ultimately, it was intended to focus on some of the structural rearrangements occurring in the granules and their membranes, that could be at the basis of the observed release of biogenic amines stored in the granules. To this end, measurements of the fast transient changes occurring in the scattered light and the fluorescence from chromaffin granule suspensions during and immediately following application of an electric field pulse, were carried out. In the following we describe experiments carried out at field pulses of varying strength, as well as duration, in both the range below and above the minimum field necessary for release. Time constants characteristic of the transients are derived and an evaluation based on Mie scattering calculations of the structural changes in the granules, is carried out.

Materials and Methods

Chromaffin granules (CG) were prepared as in previous work (Neumann & Rosenheck, 1972) by isotonic density gradient centrifugation (Trifaro & Dworkind, 1970). The granule pellets were dispersed in isotonic sucrose (0.27 M) containing either tris or phosphate buffer at pH = 6.8 and ionic strength $I = 0.01$. Further ionic strength adjustments were made by addition of 1 M NaCl solution in appropriate amounts. The volume fraction of vesicles in suspension was calculated as described earlier (Neumann & Rosenheck, 1972).

Membranes (CGM) were prepared by subjecting the CG to an osmotic shock in dilute tris buffer (pH 6.8, $I = 0.01$). The membranes were washed and sedimented at $24{,}000 \times g$ for 20 min. This procedure was repeated four times. The washed membrane pellet was dispersed in the appropriate buffer to give the desired optical density. All manipulations were carried out at 0 to 4 °C. For fluorescence measurements 25 µliters of a 0.01 M diphenyl-hexatriene (DPH) solution in tetrahydrofuran were added to 50 ml of the final CG or CGM suspension and the mixture was left for four hours at 0 °C before use.

The electric field pulses were applied to the samples in a T-jump apparatus by which changes in fluorescence intensity and polarization as well as light scattering (LS) intensity and polarization could be measured at right angles to the incident beam (Rigler, Rabl & Jovin, 1974). The light source was a Hanovia 200 W Mercury/Xenon arc. A Bausch and Lomb high intensity monochromator was used to select the incident wavelength and the scattered or emitted light was filtered through a cut-off filter (Jenaer Glaswerk Schott Gen., Mainz) eliminating all light at wavelengths shorter than the cut-off value of the filter. For polarization measurements, Polacoat polarizing filters were used. The incident light was unpolarized.

In fluorescence experiments the exciting wavelength was 368 nm and the cut-off value was 435 nm; in light-scattering experiments, these values were 369 and 360 nm, respectively.

The transients in photomultiplier output were displayed on an oscilloscope (Tetronix 546) and photographs were taken with a Polaroid camera.

To compare the traces and initial prepulsed scattered light intensity of the parallel and perpendicular components of the scattered light from a given sample, an amplification factor G was introduced, accounting for the two different amplifications of the constant unpulsed output voltage A_0 of the photomultipliers. The deviations from this value following the electric field pulse were amplified five times before displaying on the oscilloscope. The degree of polarization is defined by the relation

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

where

$$I_{\parallel} = \frac{1}{G_{\parallel}} \left(A_{0\parallel} + \frac{1}{5} A_{\parallel} \right)$$

$$I_{\perp} = \frac{1}{G_{\perp}} \left(A_{0\perp} + \frac{1}{5} A_{\perp} \right)$$

and $A_{\parallel}$ and $A_{\perp}$ are the respective voltage changes displayed on the oscilloscope screen. In this way the degree of polarization before the application of the field, P_0, and at the time the change in light scattering reaches its maximum value, P_{max}, were calculated.

To compute the time constant for the decay of the applied electric field the resistance R of the cell filled with the sample was measured by means of an AC Wheatston bridge. The time constant of the field as well as the Joule heating could be varied by using different capacitors. Most measurements were carried out using a 0.05 μF capacitor giving a temperature rise of about 4 to 5 °C at 20 kV. The initial temperature was 4 °C, well under the temperature range where thermal release of the granule content occurs.

Results

Oscilloscope traces of the electric field-induced transient changes in the polarized scattered light intensities A from aqueous suspensions of intact CG and CGM, are shown in Figs. 1 and 2. It can be noted that both systems behave in a similar manner. In both cases the fast rise to a maximum value A_{max} is followed by a slower decay. Measurements over a longer time range show that A returns quantitatively to its initial value A_0 within a time of ~ 150 to 200 msec, except in those cases in which release from CG occurs. This observation indicates that the change in LS is essentially not a temperature effect, since the cooling back of the solution sets in after a few seconds. Further evidence excluding temperature change as a main direct cause of the LS transient will be given later. The maximum increase in the LS signal, $A_{max} - A_0$, is usually of the order of 3 to 10 percent of the steady value A_0, depending upon experimental conditions, such as ionic strength and capacitance of the discharge circuit. The fact that for both the parallel and the perpendicular components of the scattered light, $A_{max} - A_0$ is of

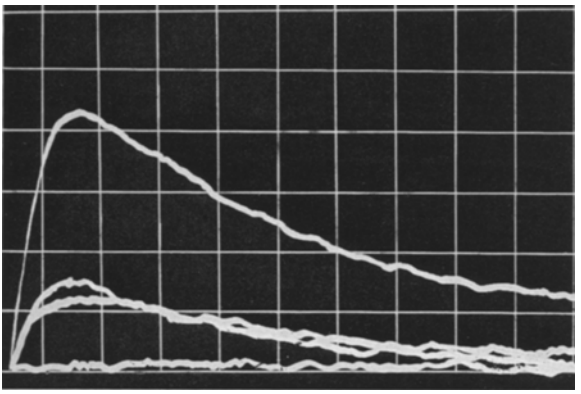


Fig. 1. Oscillograms of scattered light from a CG suspension subjected to a 20 kV discharge. Medium: 0.27 M sucrose in phosphate buffer, pH = 6.8, ionic strength $I = 0.01$. Oscilloscope: ordinate = 500 mV/large division; abscissa = 0.2 msec/large division. Upper curve: parallel component $G = 1$; $A_0 = 4$ V. Lower curve: perpendicular component $G = 1.75$; $A_0 = 2$ V. $P(A_0) = 0.554$; $P(A_{max}) = 0.575$

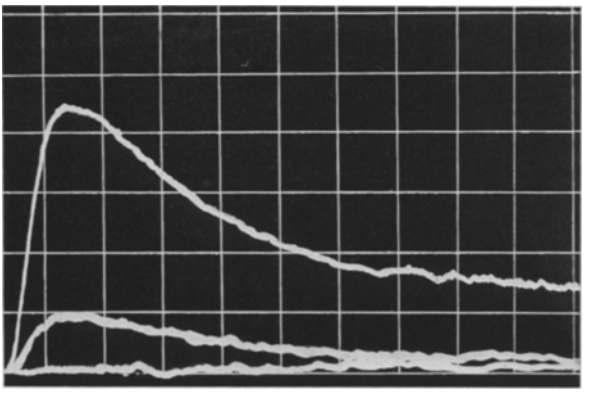


Fig. 2. Oscillogram of scattered light from CGM subjected to a 15 kV discharge. Medium: phosphate buffer, pH = 6.8, $I = 0.01$. Oscilloscope: same as in Fig. 1. Upper curve: parallel component $G = 1$; $A_0 = 4$ V. Lower curve: perpendicular component $G = 2.26$; $A_0 = 1.84$ V. $P(A_0) = 0.662$; $P(A_{max}) = 0.676$

the *same sign*, is a strong indication that no orientational effects are present. This is expected on the basis of the spherical shape of the particles we are dealing with.

The variation in A of a suspension of intact CG at varying values of the initial field strength $E_0 = V_0/d$, is shown in Fig. 3. Fig. 4 gives the dependence of A_{max} on V_0 at two different capacitances of the discharging circuit, as well as two different ionic strengths. The latter Figure does not include

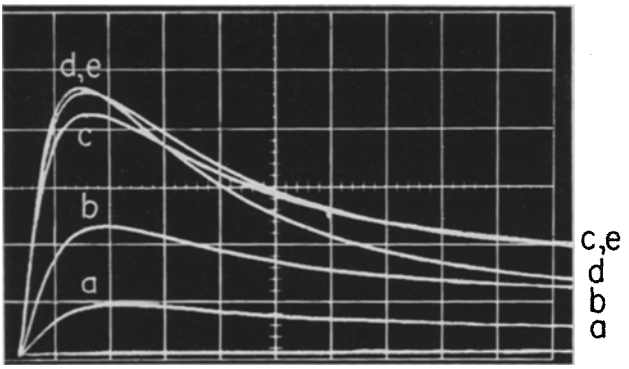


Fig. 3. A series of discharges of increasing voltages applied to a sample of CG. Medium: 0.27 M sucrose in tris buffer, pH = 6.8, $I = 0.01$. (*a*) 5 kV; (*b*) 10 kV; (*c*) 20 kV; (*d*) 25 kV; (*e*) 25 kV (second discharge)

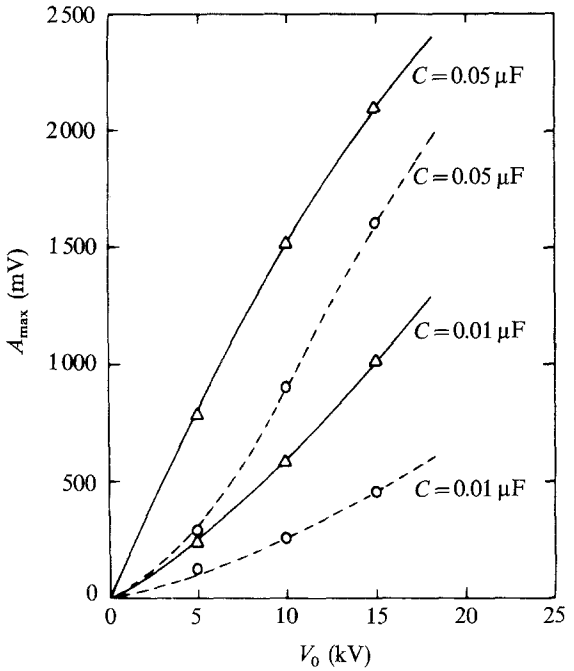


Fig. 4. Voltage dependence of the light-scattering signal amplitude A_{max} for two capacities and two ionic strengths. Solid curves: CG in isotonic sucrose containing phosphate buffer, pH = 6.8, $I = 0.01$. Dashed curves: same as before, $I = 0.02$

values of V_0 higher than 20 kV/cm at which the LS signal is affected by release. The data in Fig. 4 argue against a major role for the temperature change in causing these signals. In the first place, the increase in A_{max} at constant V_0 as a function of the capitance C, i.e. at different energy

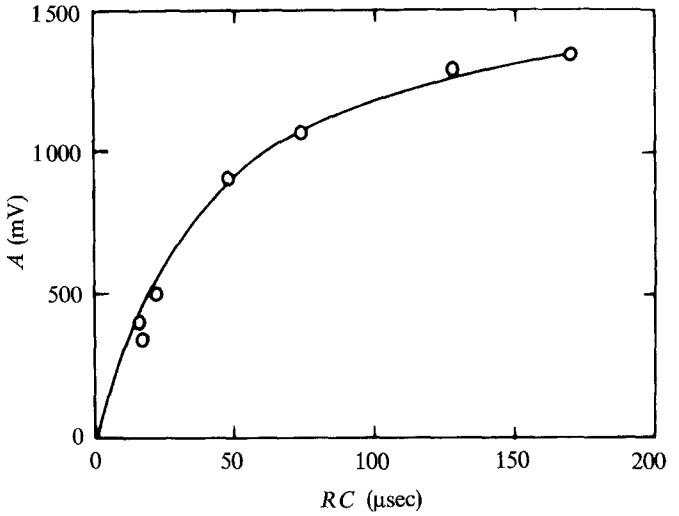
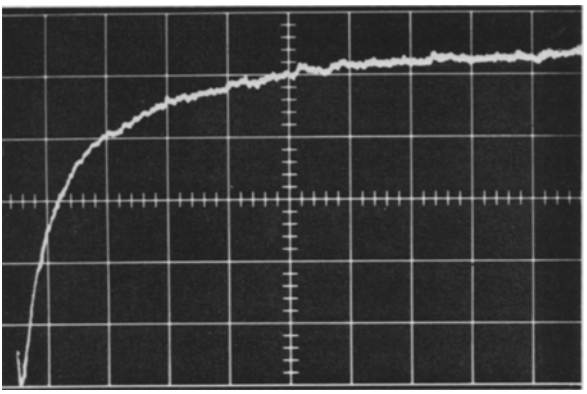


Fig. 5. Dependence of A_{max} on $\tau_E = RC$; $V_0 = 15$ kV

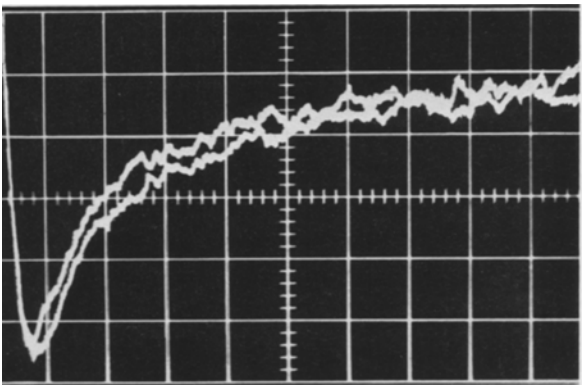
inputs, is much less than the increase in the latter. Furthermore, A_{max} decreases steeply with increasing ionic strength. The latter does not affect the temperature rise in the suspension, but markedly influences the decay time τ_E of the field. All these observations indicate that we are observing an essentially field-induced signal, though a limited contribution from the temperature rise is also present.

The magnitude of A_{max} is determined by the response of the granule and its membrane to the imposed electric field, as well as on the strength and duration of the latter. The rising phase of the signal reflects the rate of build-up of the changes occurring in the granule under the imposed field, while the latter decays exponentially with a time constant $\tau_E = RC$. While, as seen above, A_{max} increases with increasing V_0, the rising phase of A becomes shorter under the same conditions. Furthermore, both A_{max} and the rise-time decrease sharply with increasing ionic strength. It can thus be concluded that τ_E and the time constant for the response of the granule are of comparable magnitude. The dependence of A_{max} on τ_E, shown in Fig. 5, indicates that, at constant V_0, A_{max} becomes saturated as τ_E reaches values of approximately 150 μsec. This value then gives us an estimate of the time constant for the response of the granule to the electric field.

In contrast to the fast build-up of the LS signal, its decay is significantly slower and composed of several close lying relaxation times. The relaxation observed via fluorescence of the hydrophobic probe DPH, is shown in Fig. 6. It can be analyzed in terms of two relaxation processes characterized



a



b

Fig. 6. (*a*) Change of DPH total fluorescence in a CG suspension subjected to a 10 kV discharge. Medium: 0.27 M sucrose in tris buffer, pH = 6.8, $I = 0.01$. Oscilloscope: ordinate = 500 mV/large division; abscissa = 20 msec/large division. (*b*) Expansion of the initial portion of Fig. 6*a*. Oscilloscope: ordinate = 50 mV/large division; abscissa = 0.5 msec/large division

by the time constants $\tau_1 \sim 1$ msec, and $\tau_2 \sim 30$ msec. A more detailed analysis of the LS and fluorescence relaxation is in progress.

When E_0 reaches values at which catecholamine (CA) release is known to occur (Neumann & Rosenheck, 1972), the LS changes assume a somewhat different character. Fig. 3 shows that at $E_0 = 25$ kV/cm, A_{max} seems to level off. The decay is faster in the early phase of the relaxation than that at initial field values $E_0 \leqq 20$ kV/cm. It is also seen that when a second pulse of 25 kV/cm is applied, the recovery phase resembles that at pulses below the threshold for CA release. After complete relaxation of the LS transient it is found that the steady value of A, reached after a 25 kV/cm pulse, is

some 10 percent lower than the original one, A_0. When the pulse applied is 20 kV/cm or lower, A returns quantitatively to its original value, as was already mentioned. In the previously mentioned work, it was observed that a 25 kV/cm pulse induces maximum CA release from a CG suspension, while pulses of $\leqq 18$ kV/cm did not lead to CA release. The observations in the present work are thus consistent with the previous ones, since the *decrease in scattering* outside the CA absorption region (i.e. at 369 nm) is correlated with an *increase in absorbance* at the peak of the absorption band of CA (i.e. 198 nm). This, in fact, is the behavior expected on the basis of theoretical considerations of scattering from absorbing particles (Duysens, 1956).

Discussion

The questions that we have tried to answer in the present study are: what is the time course and extent of the structural changes induced in the chromaffin granule by the electric field pulse? Are the changes reversible? Are there qualitative differences between the changes produced by pulses below and above the field strength necessary for CA release?

The optical transients described are qualitatively the same under all experimental conditions, including those leading to CA release. The time course comprises a fast build-up of the optical changes, of the order of several hundred microseconds, followed by a slower relaxation, over a period of several hundred milliseconds. The apparently faster decay when the applied field exceeds the threshold value for CA release (i.e. 25 kV/cm) may be attributed to a reduction in the difference between the particle refractive indices and that of the immediate surrounding medium, superimposed on the changes observed at subthreshold values. Such a partial equalization of refractive indices would be a normal consequence of release. This is confirmed by the fact that when a second pulse of 25 kV/cm is applied, the signal assumes the character displayed at subthreshold pulses. If this is so, it enables us to locate the release event in time and indicates that the latter occurs already during and immediately following the period at which the recorded changes in light-scattering are at their maximum value.

The main part of the optical signal seems to originate from the particle membrane as indicated by the similarity in behavior of CG and CGM. Furthermore, it is observed that the time course of the fluorescence signal from the probe DPH is in line with that of the scattered light. DPH is a hydrophobic dye and is located in the lipid region of the membranes. This

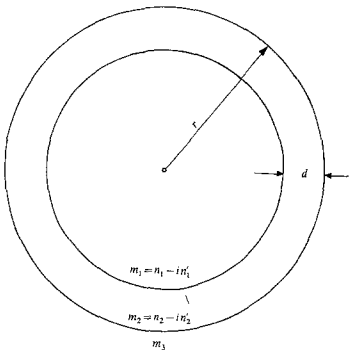


Fig. 7. Concentric sphere scattering model

then leads us again to the conclusion that it is the membrane in which most of the optically monitored changes occur.

The changes seem to be entirely reversible, as demonstrated by the complete return of the light-scattering as well as the fluorescence intensity to their original value before the pulse. From this fact, and from an evaluation of the extent of structural change, which is outlined in the following, it may be concluded that under none of the conditions used in these experiments is there any significant irreversible destruction of granules, such as might have been due to massive dielectric breakdown.

The kind and extent of the changes in granule properties leading to the light-scattering signals described have been evaluated by using the Mie type scattering functions for a coated sphere (Kerker, 1969). In this method (Fig. 7) the granule is approximated by two concentric spheres, each characterized by a complex refractive index. The inner sphere represents the core of the granule, namely soluble proteins, catecholamines and ATP, while the shell represents the granule membrane (Rosenheck & Schneider, 1973). Basically, two approaches seemed worthwhile examining. One was to assume *homogeneous* particle parameters, both before and during the field-induced transient. In this case the analysis deals with the determination of the changes in r, d, m_1 and m_2 that will lead to the observed changes in particle scattering efficiencies Q_s, intensities $I(\theta)$ and degree of polarization P. From such calculations we can obtain estimates of the changes in *average* particle properties, such as variations in size, (i.e. swelling or shrinking), in the thickness of the membrane, or in the refractive indices of the core and/or the membrane. The other approach was to focus on the membrane, where most, if not all, of the optical signal appeared to come from, and consider the possibility that the electric field was producing internal structure

Table 1. Ranges of variation in particle parameters used in the Mie calculations

r (Å)	d (Å)	n_1	n_2
1100 – 1300 (10)	90 – 110 (2)	1.035 – 1.045 (0.002)	1.080 – 1.120 (0.002)

Numbers in parentheses indicate step size.

changes *over limited regions*, leading to formation of domains and consequent *heterogeneity* in membrane refractive index, while other particle properties such as size would not vary. Domain formation with attendant increases in light scattering is a well-known occurrence in certain types of liquid crystals subjected to electric fields (Heilmeier, Zanoni & Barton, 1968). The effect of internal structure changes on light scattering has been theoretically worked out for the case of a homogeneous spherical particle, and it was shown from calculations for several model cases that the introduction of inhomogeneities could cause an increase in 90° scattering (Latimer, Moore & Bryant, 1968). An application of the latter type of analysis will be dealt with in future work. In the present we have carried out calculations according to the first approach mentioned.

Table 1 lists the values of the particle parameters used in the calculations. The particle size was obtained from published electron-microscopic work (Helle, Flatmark, Serck-Hanssen & Lönning, 1971). For membrane thickness, presently accepted values were taken from the literature. The refractive indices have been estimated previously in the visible and ultraviolet range (Rosenheck & Schneider, 1973). It should be noted that at the wavelength at which the measurements were made, CG have no absorption bands, and therefore only the real part of the refractive index exists. The calculations, carried out on the Golem computer (19 significant figures) selected those combinations of the parameter values that gave the observed static and transient values of the scattering efficiency, polarized scattering intensities and degree of polarization. These requirements restricted severely the number of possible combinations, so that, for the step size used in the calculations, only eight were found. The overall results are listed in Table 2, which also gives the experimental results expressed on a per particle basis. When the individual solutions are examined, it is found that all comprise the same kind of variations, differing only somewhat in magnitude. These are an increase in particle size and membrane thickness of approximately 10 Å in each of these parameters, a slight increase in core refractive index and a somewhat larger decrease in membrane refractive index.

Table 2. Range of values of the particle parameters leading to the observed static and transient values (all within limits of ± 10 percent)[a]

	r (Å)	d (Å)	n_1	n_2
Initial	1180 to 1220	90 to 104	1.037 to 1.041	1.088 to 1.118
Final	1190 to 1220	96 to 108	1.039 to 1.043	1.082 to 1.116

[a] Particle scattering efficiency factor: $Q_s = 3.7 \times 10^{-2}$ [from optical density measurements (K. Rosenheck & A. S. Schneider, *unpublished results*)]. Increase in parallel component of scattering intensity: $\Delta I_{\parallel} = +7.0 \times 10^{-5}$. Increase in perpendicular component of scattering intensity: $\Delta I_{\perp} = +1.5 \times 10^{-5}$. Degree of polarization ($p = 0.56$): $\Delta p = +0.015$.

Several points can be made on the basis of these results. The first is that the overall changes in particle properties are rather small. They indicate a slight swelling, or possibly deformation of the granules in the electric field. A decision in favor of either of these cannot be made on the basis of these calculations which are insensitive to slight shape changes for a suspension of randomly oriented particles. Further, the decrease in membrane refractive index could be indicative of a reorganization of membrane components, in conjunction with an increased perfusion or penetration of suspension medium and/or core components into the membrane, a process that eventually could lead to CA release at high enough field strengths. A point worthwhile investigating is whether such reorganization leads to an increased accessibility of lysolecithin molecules that were hidden in the absence of the field. Lysolecithin, an uncommon membrane lipid, characteristic of chromaffin granules (Blaschko, Firemark, Smith & Winkler 1967; Winkler, Streider & Ziegler, 1967), has been postulated to be involved in the exocytotic process which is part of CA release *in vivo* (Winkler, 1971). Finally, the calculated Q_s and p values are very sensitive to r, and therefore the near identity of these values before and after a CA releasing pulse indicates that the size distribution of granules does not change perceptibly. Granule destruction in an extent comparable with release, i.e. 40 to 50 percent of stored CA, would however, be expected to alter this distribution significantly. Thus irreversible breakdown of granules is not a factor in CA release, under the conditions of our experiments.

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