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SURFACE POTENTIAL AND SURFACE CHARGE DENSITY OF THE CEREBRAL-CORTEX SYNAPTIC VESICLE AND STABILITY OF VESICLE SUSPENSION

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Using a microelectrophoresis instrument employing the Lazer-Zee system, the electrophoretic mobility of synaptic vesicles isolated from Guinea-pig brain cortex was measured under several conditions. The mobility was found to depend on both pH and ionic concentration of the solution. The surface of the synaptic vesicle was shown to be negatively charged under physiological conditions. The isoelectric point was observed at pH 4.0 in 0.01 M NaCl solution. Effects of divalent cations were examined and reversal of surface charge was observed in 0.1 M $CaCl_2$ solution. Interaction of vesicles was also considered on the basis of the DLVO theory of colloid stability by using calculated values of surface charge density and surface potential of the synaptic vesicle.

Introduction

Surface potential or surface charge density of the synaptic vesicle is an important parameter which controls the movement of vesicles inside neurons, the interactions of vesicles and interactions between vesicles and the presynaptic membrane, as discussed by Bass and Moore [1] and Remler [2]. McLaughlin et al. [3] measured the electrophoretic mobility of synaptic vesicles of guinea-pig cerebral cortex under several conditions and studied the effects of pH, ionic strength and divalent cations. They showed that the synaptic vesicle has a negatively charged surface under physiological conditions.

Observations of nerve terminals by electron micro scope indicate that under usual physiological conditions a suspension of synaptic vesicles is a stable system: no aggregation, assemblage or rosette formation of synaptic vesicles occurs in spite of their high concentration [4–8]. Following the DLVO theory of colloid stability [9,10], which has already been applied to interpret biological phenomena such as cell interactions by several investigators [11–16], the repulsive electrostatic and van der Waals attractive interactions are expected to act between approaching synaptic vesicles. The electrostatic interaction is caused by the surface charge of the synaptic vesicles.

We shall here report extensive studies on the electrophoretic mobility of the synaptic vesicles isolated from guinea-pig brain cortex. The vesicles were isolated by a method of Ohsawa and Uchizono [6] modified by using galss-bead chromatography [8] and their mobility was measured by using a microelectrophoresis instrument employing the Lazer-Zee system [17]. Surface potential and surface charge density of the vesicle, and the interaction between the vesicles, are discussed.

Materials and Methods

Measurements of electrophoretic mobility

Isolation of synaptic vesicles. Synaptic vesicles were prepared by a method of Ohsawa and Uchizono [6] modified by using glass-bead chromatography

[7]. The cerebral cortices of two guinea-pigs (250 g) were removed, homogenized and centrifuged at 2000 × *g* for 10 min. Supernatant S_1 was recentrifuged at 10 000 × *g* for 30 min and then the P_2 fraction was suspended in 5 ml 0.1 M sucrose Hepes-Tris, pH 7.4, solution. The suspension was sieved with fine porous glass beads of column bedding 16 mm × 1 m in length. Fine porous glass beads of 300 nm porous diameter (Fuji Film Co., Ltd., Tokyo) were used. After the suspension had been eluted by glass-bead chromatography, the absorbance of the elutants was measured at 280 nm with a spectrophotometer (Hitachi No. 124), and the fraction giving the highest peak was separated and centrifuged at 105 000 × *g* for 20 min. The pellet was resuspended in several volumes of solution and this stock vesicle suspension was kept at 0°C, and the shape and size of one of such pellets were observed by electron microscopy (Akashi S-500).

Solution. In all experiments, 10 μl of the above-mentioned stock vesicle suspension were added to 2 ml suspending medium. The pH of 0.1 M sucrose medium was adjusted as follows: to pH 7.37 with 5 mM Hepes-Tris, to pH 2.38 with glycine-HCl, to pH 3.52, 4.52 and 5.31 with sodium acetate/acetic acid, and to pH 8.87 with glycine-NaOH. Several media of various ionic strengths (0.01 M to 0.2 M NaCl) were adjusted to pH 7.4 ± 0.1 with 5 mM Hepes-Tris.

Electrophoretic mobility. An automated particle electrophoresis instrument employing the Lazer-Zee system 3000, Pen Kem In., New York [17] was used. Typical performance parameters for the grating analyzer system were as follows: chamber volume, 0.1 ml; measurement time per 10^4 particles, 1 min; resolution of histogram, 0.25 $\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$ (S.D.). Each independent value was obtained by timing the movement of at least 10^3 particles over a distance of 100 μm with a reversal of polarity for each measurement, and the movement of synaptic vesicles was monitored with a binocular microscope during the measurements. All experiments were done at 20°C, and the conductuvity of each medium was indicated automatically.

Results

Electron micrography of purified synaptic vesicle

The ascending peak of glass-bead column fractionation corresponding to larger molecular weight was proved by electron microscopy to be overwelmingly contaminated with membrane debris of more than 50 nm in diameter.

Almost spherical and plain vesicles as shown in Fig. 1 appear at the desending peak of the column fraction.

The average diameter of the images of the homogeneous vesicles seen in electron micrograph was 44.6 ± 8.6 nm and little contamination occurred in this fraction (Fig. 1). Aggregation and contamination among the vesicles were not observed.

pH and ionic concentration effects

Fig. 2 and 3 give the pH dependence of the electrophoretic mobility of purified synaptic vesicles at a constant ionic concentration of 0.01 M. These indicate the presence of a mono-ionic group with a charge reversal point at pH 4.0 which is an isoelectric point of the synaptic vesicles. The mobility has a nearly constant value of about $-2\ \mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$ between pH 8.9 and 4.5 (Figs. 2 and 3).

Fig. 4 and 5 show the effects of ionic concentration on electrophoretic mobility in NaCl solution. The figures show relatively small changes in mobility at ionic concentration between 0.1 M and 0.2 M. No reversal point was observed.

Figs. 6 and 7 show the effects of divalent cations. Incubation of vesicle fraction with increasing concentration of $CaCl_2$ resulted in decreasing electrophoretic mobilities as shown in Figs. 6 and 7. The salt solution employed were buffered at pH 5.31 with sodium acetate/acetic acid. The charge reversal concentration for the cation was obtained by interpolation of zero mobility. It is seen in Fig. 7 that the surface charge of the vesicle was reversed in 0.1 M $CaCl_2$ solution.

The *ζ-potential* of a spherical particle of radius *r* which exhibits an electrophoretic mobility *U* in an electrolyte solution of relative permittivity ϵ_r and viscosity η is given [18] by

$$\zeta = \frac{3\eta}{2\epsilon_r \epsilon_0 f(\kappa r)} U \qquad (1)$$

where ϵ_0 is the permittivity of vacuum, and $f(\kappa r)$ is a function of κ, κ being the Debye-Huckel parameter, and is tablulated in Refs. 18 and 19. In accordance with experimental data, we used $r = 22.5$ nm, $\epsilon_r =$

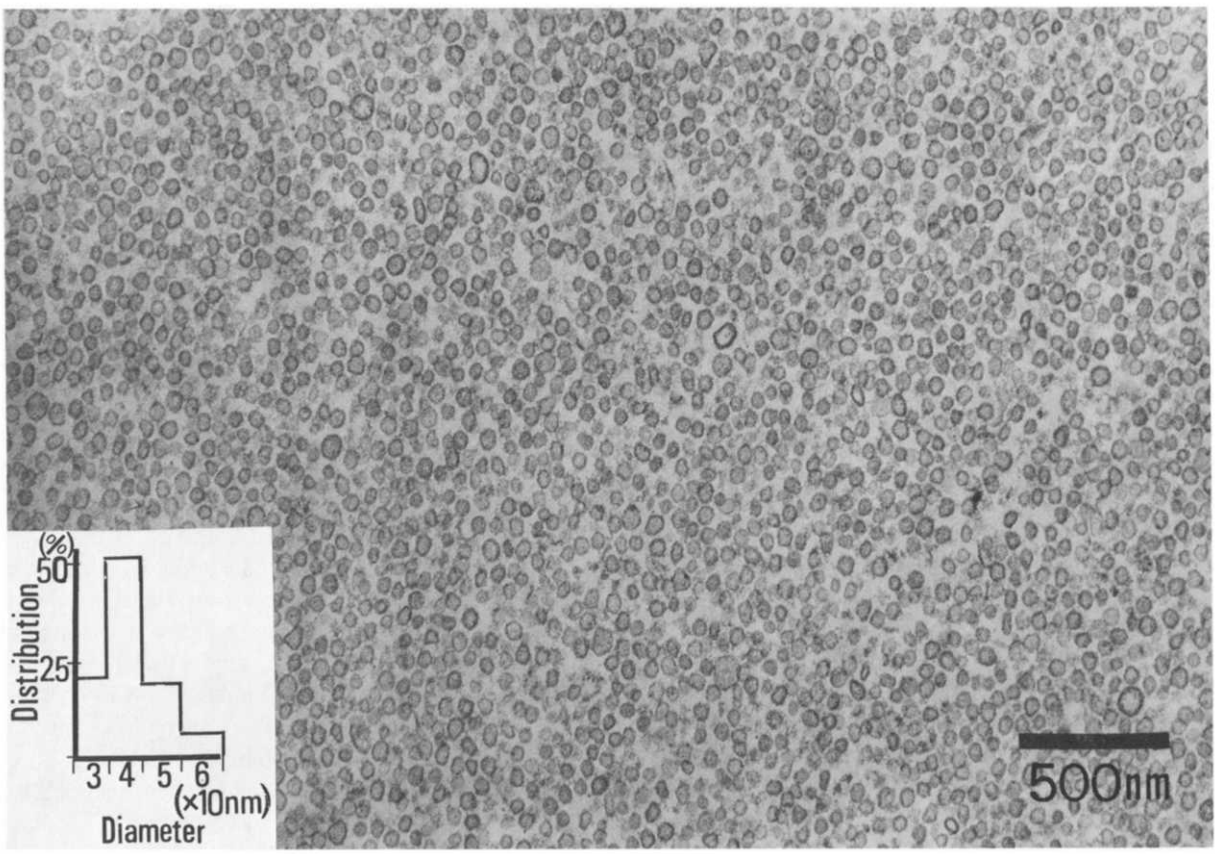

Fig. 1. Electron micrograph of synaptic vesicles from glass-bead column chromatography. Synaptic vesicles of guinea-pig, 44.6 ± 8.6 nm (mean ± S.D.) in diameter which was calculated from diameter distribution of inset, were purified from the cerebral cortex by pelleting with $1.05 \cdot 10^5 \times g$ centrifugation under the physiological conditions: phosphate buffer 10 mM and pH 7.2. No rosette formation or aggregation and/or assemblage occurs among the synaptic vesicles. The best fractionationed preparation thus obtained was used to determine electrophoretic mobility.

80.4 (20°C) [20] and $\eta = 0.001$ $N \cdot s \cdot m^{-2}$ [21]. With the above values Eqn. 1 reduces to

$$\zeta = -21.08\, U/f(\kappa r) \qquad (2)$$

where U is expressed in units of $m \cdot s^{-1} \cdot V^{-1} \cdot cm$. Results calculated using Eqn. 2 is shown in Figs. 8 and 9.

Surface charge density

The surface charge density σ is related to the surface potential φ_s, which can be considered to be approximately equal to the ζ potential.

In the SI units, σ can be expressed in terms of φ_s as follows: In a 1–1 type electrolyte (e.g., NaCl) of concentration n,

$$\sigma = \sqrt{2\epsilon_r\epsilon_0 kTn}\left\{\exp\left(\frac{2e\varphi_s}{kT}\right) - \exp\left(-\frac{2e\varphi_s}{kT}\right)\right\} \qquad (3)$$

and in a 2–1 type electrolyte (e.g., $CaCl_2$) of concentration n,

$$\sigma^2 = 2\epsilon_r\epsilon_0 kTn\left\{\exp\left(-\frac{2e\varphi_s}{kT}\right) + 2e^{e\varphi_s/kT} - 3\right\} \qquad (4)$$

where the sign of σ is chosen so that it coinsides with that of φ_s. Results calculated using Eqns. 3 and 4 are given in Figs. 8 and 9.

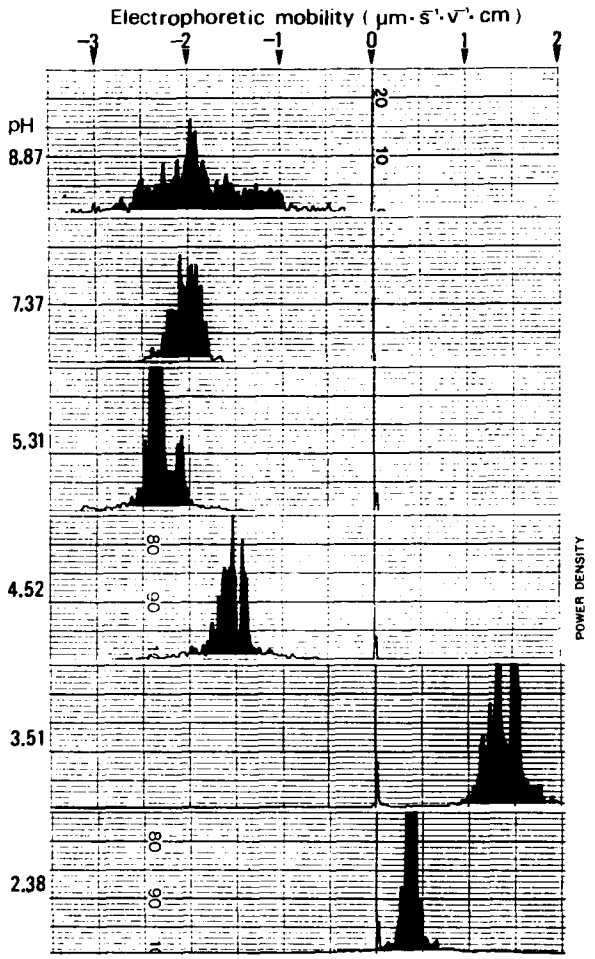

Fig. 2. Distribution and histogram of electrophoretic mobilities of purified synaptic vesicles. An instrument of the Lazer-Zee-tm system 3000 was capable of providing mobility histogram data for a wide variety of purified synaptic vesicles in six experiments, where all vertical axes of mobilities are proportional to the spectral power densities and represent the relative number of synaptic vesicles. Experimental conditions of pH 8.87, 7.37, 4.52, 3.51 and 2.38 were performed as given in the experimental method and osmolality was adjusted with 0.1 M of sucrose solution.

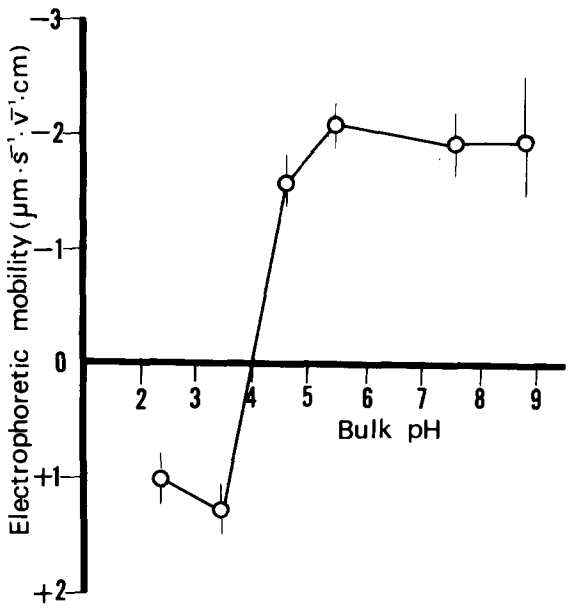

Fig. 3. Electrophoretic mobility histogram of purified synaptic vesicles as a function of bulk pH. The six observations from Fig. 2 are plotted; data for mobilities are as follows: ○, mean mobility of each section; the bar represents the standard error. The isoelectric point of the purified synaptic vesicles is as indicated, pH 4.01 ± 0.13 (S.D.).

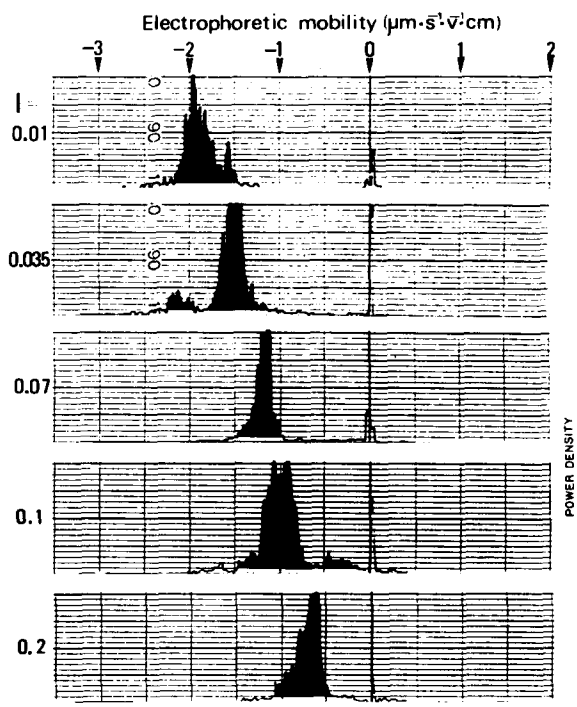

Fig. 4. Distribution and histogram of electrophoretic mobility of purified synaptic vesicles. Experimental conditions of ionic strength 0.01, 0.035, 0.07, 0.1 and 0.2 were achieved with NaCl and pH was constant at 7.37 made up with Hepes-Tris having 0.1 M sucrose. Power density, 'relative number of particles'.

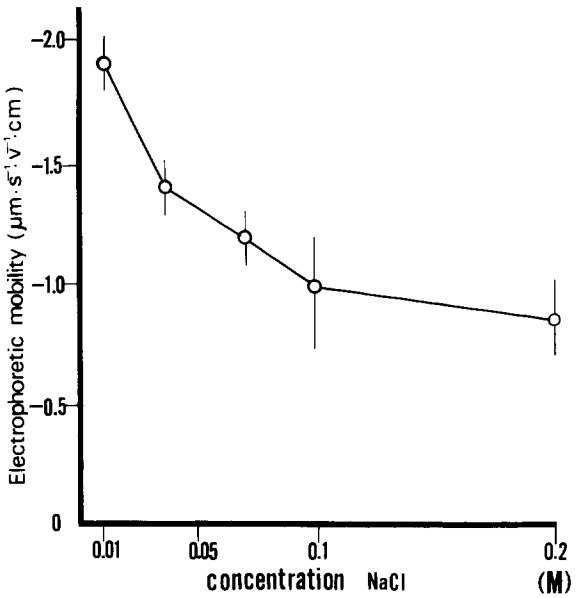

Fig. 5. Electrophoretic mobility of purified symaptic vesicles as a function of ionic strength. The five observations from Fig. 4 are plotted. ○, mean of mobility of each section; the bar represents the standard error.

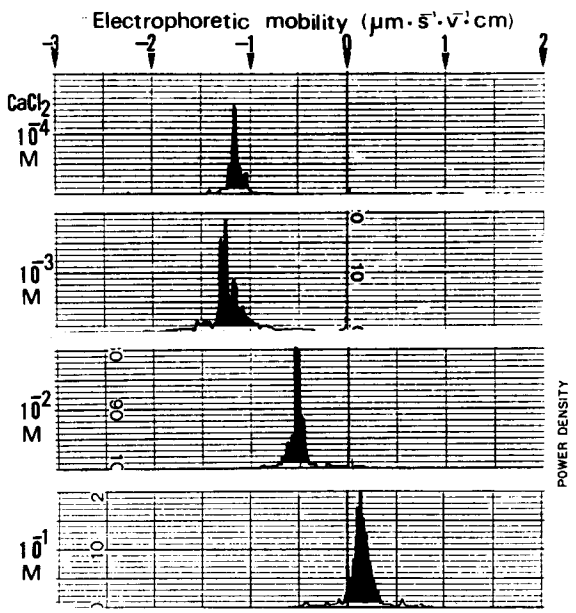

Fig. 6. Electrophoretic mobility histogram of purified synaptic vesicles as an effect of Ca^{2+}. Ionic strength adjustments were made with $CaCl_2$ for the divalent cation, using 0.1 M sucrose, and pH 7.37 was adjusted with 0.01 M Hepes-Tris solution. Power density, 'relative number of particles'.

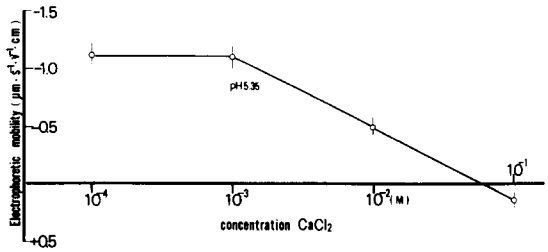

Fig. 7. Effect of $CaCl_2$ on electrophoretic mobility of purified synaptic vesicle. The four observations from Fig. 6 are plotted. ○, mean mobility; the bar represents the standard error and the pH presented is for each solution.

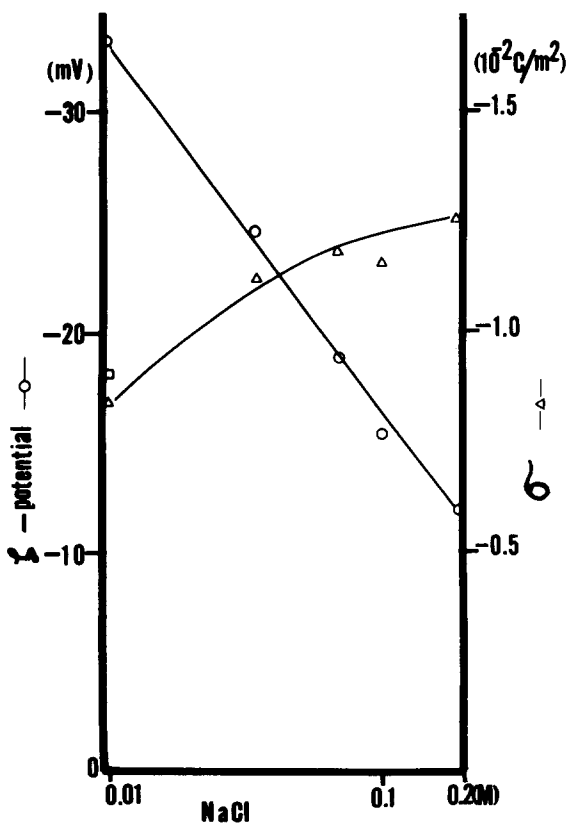

Fig. 8. ζ-potential (○) and surface charge density σ (△) of purified cerebral cortex synaptic vesicles as a function of NaCl concentration.

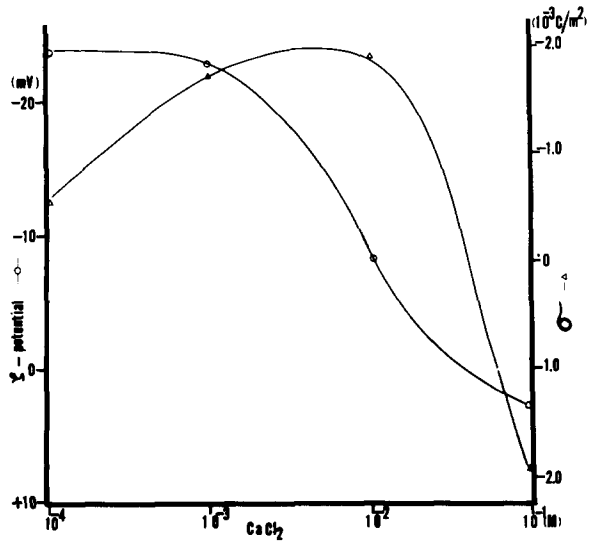

Fig. 9. ζ-potential (○) and surface charge density σ (△) of purified cerebral cortex synaptic vesicles as a function of $CaCl_2$ concentration.

Stability model of a synaptic vesicle suspension

We regard a synaptic vesicle as a sperical shell of radius r and thickness d. Imagine two synaptic vesicles with a separation of h between their surfaces immersed in an electrolyte solution, as shown in inset of Fig. 10. The SI unit system is used in the following treatment. We consider two kinds of interaction between two approaching vesicles, following the DLVO theory of colloid stability [7,8].

(A) Electrostatic interaction

Charged surfaces of vesicles cause an electrostatic interaction between two approaching vesicles. Since the surface charge arises mainly from dissociating groups, its density, σ, will remain approximately constant during the approach of vesicles. An approximate expression for the electrostatic interaction of two identical splerical particles at constant σ is given [22] by

$$V_{el}(h) = 4\pi\epsilon_r\epsilon_0\varphi_0^2 \frac{r(h+r)}{h+r} \times \ln \frac{1}{1 - \frac{r}{h+r}\exp(-\kappa h)} \quad (5)$$

with

$$\varphi_0 = \frac{\sigma}{\epsilon_r\epsilon_0\kappa} \quad (6)$$

where φ_0 is the surface potential of an isolated synaptic vesicle. Eqn. 5 ignores the influence of electrostatic fields inside vesicle and would be a good approximation for small φ_0 and large κr.

(B) Van der Waals interaction

Considering the shell structure of vesicles, we have the following expression for the interaction energy of two vesicles with a separation of h between their surfaces

$$V_v(h) = v(r, r) + v(r - d, r - d) - 2v(r, r - d) \quad (7)$$

$$v(r_1, r_2) = -\frac{A}{6}\left\{\frac{2r_1r_2}{(h+2r)^2 - (r_1+r_2)^2} + \frac{2r_1r_2}{(h+2r)^2 - (r_1-r_2)^2} + \ln\frac{(h+2r)^2 - (r_1+r_2)^2}{(h+2r)^2 - (r_1-r_2)^2}\right\} \quad (8)$$

is the interaction energy of two spheres of radii r_1 and r_2 at a separation of $h + 2r$ between their centers [23] and A is the Hamaker constant.

The total interaction energy $V(h)$ is given by the sum of $V_{el}(h)$ and $V_v(h)$

$$V(h) = V_{el}(h) + V_v(h) \quad (9)$$

The values of parameters appearing in Eqns. 5 and 7 are chosen as follows. We may assume the ζ-potential to be approximately equal to the surface potential, φ_0. The Hamaker constant, A, for biological cell membranes is reported to be of the order of 10^{-21} J [11,24,25]. Our calculations are made for $\varphi_0 = -20$ mV and $A = 0.5$, $1.2 \cdot 10^{-21}$ J. We put $r = 22.5$ nm and $d = 10$ nm. The values of other parameters are chosen as $T = 300$ K, $\kappa^{-1} = 0.8$ nm, $\epsilon_r = 80$, in accordance with physiological conditions. Results are shown in Fig. 10.

These curves have a negligibly week secondary minimum with depth smaller than 0.1 kT and rise

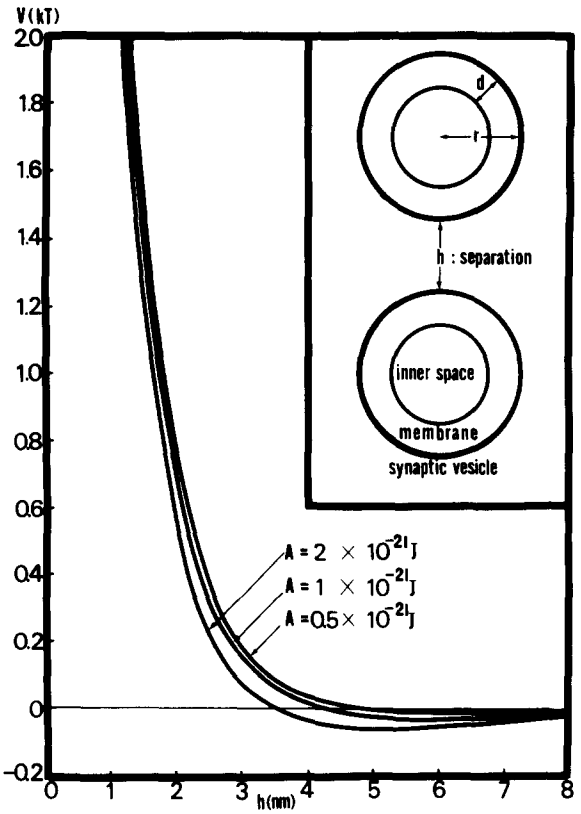

Fig. 10. Interaction of two synaptic vesicles of radius r and thickness d at a separation of h between their surfaces. Calculated with $A = 0.5,\ 1,\ 2 \cdot 10^{-21}$ J, $\varphi_0 = -20$ mV, $r = 22.5$ nm, $d = 10$ nm, $T = 300$ K, $\kappa^{-1} = 0.8$ nm, and $\epsilon_r = 80$.

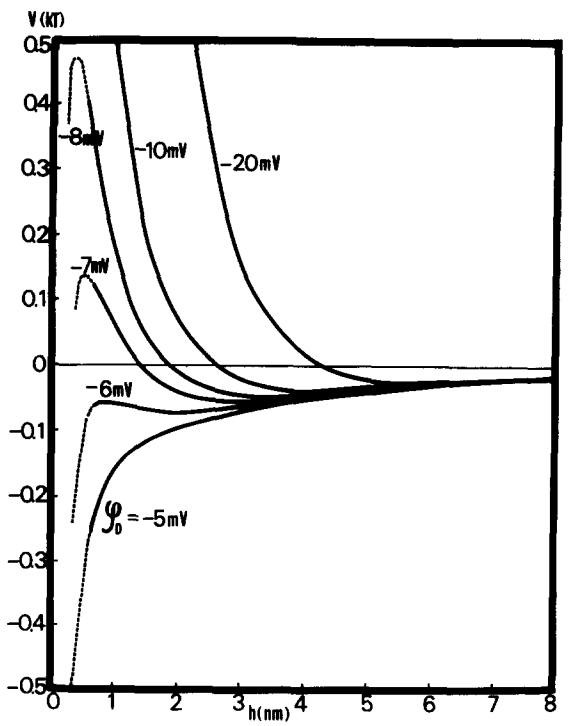

Fig. 11. Interaction energy of two synaptic vesicles. Effects of different φ_0. $A = Z \cdot 10^{-21}$ J, $r = 22.5$ nm, $d = 10$ nm, $T = 300$ K, $\kappa^{-1} = 0.8$nm, $\epsilon_r = 80$. The curves are written with dotted lines for separations less than approx 0.5 nm, where macroscopic treatment is inadequate.

rapidly with decreasing vesicle separation, leading to a potential barrier to prevent close contact of vesicles. This would be an explanation for observations by electron microscopy that a suspension of synaptic vesicles is a stable system, showing no aggregation under physiological environmental conditions.

The potential barrier, however, disappears for small φ_0, as shown in Fig. 11, in which curves are calcalculated for $A = 2 \cdot 10^{-21}$ J and several values of φ_0. Lowering the pH of vesicle solution leads to a reduction in surface charge density, σ, of vesicles, and accordingly, to a reduction in surface potential, φ_0. This may explain the observation that a decrease in pH (below pH 4) decreases the mobility of vesicles and leads to irreversible, strong aggregation of synaptic vesicles. Also, at high salt concentrations, the surface potential φ_0 decreases, as is obvious from Eqn. 6 (if σ remains constant), since the Debye-Huckel parameter, κ, is proportional to the square-root of the salt concentration. Moreover, at high salt concentrations the shielding effect reduces the electrostatic interaction V_{el}, since V_{el} involves a factor $\exp(-\kappa h)$. These salt effects may explain an observation that irreversible strong aggregation occurs at high salt concentrations above 50 mM $CaCl_2$.

Discussion

We have measured the electrophoretic mobility of the isolated cerebral-cortex synaptic vesicle. We showed that the mobility depends on pH and ionic concentration of the solution. The vesicle surface was found to be negatively charged under the physiological conditions and the isoelectric point was observed at pH 4.0 in 0.01 M NaCl solution. Our findings are essentially similar to those of the electrofocusing

method (Ohsawa, K., unpublished results) and these observations are in agreement with the results of the measurements by McLaughlin et al. [3], except in that they did not find the isoelectric point (in 0.0145 M NaCl solution) and that our measurement yielded values of the mobility rather lower than those of McLaughlin et al. [3]. We also observed that the mobility is reversed in 0.1 M $CaCl_2$ solution. This may be attributed to the adsorption of Ca^{2+} on the vesicle surface, as discussed below.

We have calculated the ζ-potential and surface charge density of the vesicle, as shown in Figs. 8–11. In NaCl solution, ζ-potential is linearly decreased with the logarithm of NaCl concentration (Fig. 8). This would be caused mainly by the shielding effect of ions. The surface charge density increases gradually in magnitude as the NaCl concentration increases (Fig. 9). A similar phenomenon was observed by Wilkins et al. [24,25] for sheep leucocytes. They suggested that the degree of surface ionization increases with increasing ionic concentration. ζ-Potential and surface charge density decrease also in $CaCl_2$ solution as the concentration increases and their sign are changed at 0.1 M concentration (Figs. 9 and 11). This may be due to the effect of adsorption of Ca^{2+} on the vesicle surface, in addition to the shielding effect of ions. Since membranes are complex structures, the idea that extrinsic proteins may be removed by altering the ionic conditions should be considered. This removal may change the net surface charge of the vesicles. Evidence supporting of this view was reported on the membrane of leucocyte [24,25].

It is generally accepted that the diffuse double layer interaction of colloid stability in a colloidal system, in which the double layer around the particle has its origin in an equilibrium distribution of potential-determining ions between the particle surface and the solution, has been considered by many investigators [9,10].

The theory of colloid stability gave a reasonable interpretation for the stability of a suspension of synaptic vesicles in a purified preparation and for the effects of pH and salt concentration in vitro. There seems a possibility that the theory of colloid stability would also be applied to the most important biological problem of the interaction between synaptic vesicles and the presynaptic membrane, which has already been discussed by Bass and Moore [1], Remler [2] and Dean [15]. But at present insufficient data on the presynaptic membrane are available, such as its surface charge density or surface potential, although there have been many electrophysiological investigations [26,27]. It seems likely that short-range interactions such as repulsion of hydration layers formed on the surfaces of vesicles and the presynaptic membrane, as are considered in Refs. 1 and 2, are involved besides the long-range interactions treated in the present study. It should be mentioned here that the above analysis is one of the possible ways to interpret the observed stability of synaptic vesicles in certain environmental solutions.

It is known that lipid vesicles composed of phosphatidylcholine, of which the geometrical size is about the same as that of the synaptic vesicle, do not aggregate in the physiological environmental solutions used in this work, in spite of having zero net surface charge and zero ζ-potential. In contrast, phosphatidylserine vesicles of the same size would aggregate in the NaCl solution, of which concentration is more than 0.5 M, even though the surface potential of the phosphatidylserine membrane is smaller (the magnitude of potential is greater) than -20 mV and the surface charge density is greater than $-0.35e/65\text{Å}^2$ in the above media [28]. These facts seem to indicate that the DLVO theory alone is not adequate to explain the aggregation phenomena even of such simple lipid membrane vesicles.

In biological cell systems, since their membranes have a more complex molecular structure, the above approach to interpretation of membrane interactions may encounter further difficulty in explaining the real causes of these cell aggregation or non-aggregation phenomena. For example, a human erythrocyte membrane processes a net negative charge in the physiological media and its ζ-potential is measured to be -14 mV [29]. The erythrocytes are stable in these suspension solutions. Although the addition of 20 mM Ca^{2+} of such erythrocyte suspension solutions reduced the ζ-potential to almost zero, the erythrocytes do not aggregate to each other at room temperature in this situation [29]. For this case, it is considered that certain macromolecules or proteins associated with the membrane or membrane surface may exert additional repulsive forces due to their steric exclusion volume on two closely approaching membranes and consequently prevent the aggregation of

two cell membranes. Similar situations may also occur for synaptic vesicles in a certain suspension medium. This type of possibility ought to be investigated in the future for stability of synaptic vesicles.

It is highly probable that in the synapse the interaction energy of the repulsive force between synaptic vesicles is present on the surface under the resting condition and a queuing vesicle with transmitter is released without aggregating to any other when localized excitation or inhibition, which invades the synaptic region, is paralleled by transient perturbations of certain fluxes through the membranes [22,24,25].

In the case of site field strength [23–25], we may infer that viscosity [16] near the sites can be expected to alter these highly axoplasmic parameters in the mobility of the synaptic vesicles.

Recently Israel and Dunant [30] reported that there is an membrane operator in presynaptic membrane and a neurochemical transmitter is not included in synaptic vesicles. If there were a channel speculation for neurotransmitter release in the presynaptic membrane, the synaptic vesicle would not be directly correlative to a neurotransmitter. However, synaptic vesicles exist in all nerve terminals of all animals and have some functions. The interaction energy between the synaptic vesicles is truly based on the physical properties of these models.

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