TENSILE STRENGTH OF THE CHROMAFFIN GRANULE MEMBRANE

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ABSTRACT Catecholamine release from chromaffin granules, suspended in sucrose solutions of various osmotic strengths, was determined at different temperatures between 2° and 44°C. Dynamic measurements showed that steady state is achieved within 15 min of incubation at all temperatures. The effect of temperature on the release was established in terms of the median granular fragility (MGF) defined as the concentration of sucrose solution causing 50% lysis. The MGF was determined as the inflection point of the Gaussian distribution of granular fragility. The MGF was found to decrease with fall in temperature implying a corresponding increase of the tensile strength of the vesicle membrane. Critical resultant forces at lysis were calculated and found to vary from 8.2 dyn/cm at 2°C to 4.2 dyn/cm at 44°C. These compare well with tensions at lysis found earlier for erythrocytes.

I. INTRODUCTION

Most neurotransmitters, hormones, and many enzymes are stored in intracellular secretory vesicles. In response to appropriate hormonal or neural stimuli, the soluble contents of the vesicles are released into the extracellular compartment. Biochemical evidence, indicating exocytosis of neurosecretory material, was first obtained by Douglas and Poisner (1) and Kirshner et al. (2). Exocytosis is considered, by many investigators, to be a process of secretion (3–6), but its precise mechanism is still unknown and has been the subject of much speculation and controversy.

A process similar to secretion is the transport of macromolecules across endothelial cell membranes and several theoretical models have been proposed for its description. Based on a statistical model, Shea et al. (7) simulated vesicular transport in terms of a Brownian motion in the presence of an absorbing barrier. Green and Casley-Smith (8) used the Fokker-Planck equation to estimate the probability of a vesicle fusing with a membrane when colliding with it. Rubin (9) used a chemical-reaction kinetics approach in which the vesicle diffusion process is coupled with the vesicular attachment/detachment process. Weinbaum and Caro (10) proposed a dynamic model by considering a constrained Brownian diffusion of vesicles subject to long-range hydrodynamic and short-range London–van der Waals force interactions with the membranes of the endothelial cell.

Because the final approach of the vesicles to the cell membrane in exocytosis and vesicular transport is dominated by a balance of physical forces, it involves a variety of mechanical and physical factors such as flow, pressure and stress distribution, temperature changes, and strength of the membrane. This subject has been ignored so far in studies of the secretory systems but has received major attention in many studies of blood circulation. In particular, investigations were extensively carried out concerning the rheology, strength and hemolysis of red blood cells (11–16) in view of the critical importance of the flexibility of these cell membranes to life (17).

In this work, the mechanical properties of the membrane of a vesicular system were studied. Chromaffin granules, the secretory vesicles of the adrenal medulla, are spherical in shape and fairly uniform in size (18). The inner volume of these membrane-bound-subcellular organelles is almost entirely occupied by a rigid chemical complex without osmotic activity (19), and the exchangeable water volume is only 10% at 0.3 M sucrose solution (20). These vesicles have provided the experimental basis for most of the current knowledge of the physiology of secretion by exocytosis (21). Recently, the adrenal medulla has been the object of intensive experimental study as a possible source of insight into the biochemical aspects of the process of exocytosis using the secretion of catecholamine as a model. For a review see Zinder and Pollard (22).

When suspensions of chromaffin granules are exposed to

This work was performed in partial fulfillment for a D.Sc. degree at the Technion-Israel Institute of Technology (Y.H.).

solutions of varying osmotic strengths, solvent penetrates into the slightly expandable vesicles until either equilibrium is achieved or lysis occurs. The inward volume flux, J, is given by the phenomenological equation for membrane transport (23).

$$J = L_p [(\pi_i - \pi_0) - (p_i - p_0)]. \quad (1)$$

L_p is a mechanical filtration coefficient and π_0, π_i, p_0, and p_i are external and internal osmotic and hydrostatic pressures, respectively.

The purpose of this work was to determine the tensile strength of the chromaffin granules membrane as a first stage for a mechanical study of exocytosis. The experimental procedure was based on exposure to osmotic pressure differences at various temperatures, thus causing lysis due to a catastrophic membrane fracture.

II. EXPERIMENTAL PROCEDURES

A. Isolation of Chromaffin Granules

Granules were prepared from fresh bovine adrenal glands following the procedure of Taugner (24) with the modifications of Hoffman et al. (25).

The procedure involves three-stage gravitational isolation of the vesicles from cell homogenates and their subsequent suspension in 0.3 M sucrose solution necessary to keep them intact. No salt buffers were used in the experimental procedures.

B. Turbidity Measurements

Turbidity measurements were performed to establish the dynamic behavior of catecholamine release and to determine the time needed to achieve a steady state. The turbidity of the granule suspensions was measured at a wavelength of 540 nm using a 635 Varian Techtron recording spectrophotometer (Varian Associates, Inc., Palo Alto, Calif.) at 23°C.

The absorbance of a freshly prepared chromaffin granule suspension in a sucrose solution, was recorded relative to a blank sample of a suspension in distilled water preincubated at 37°C. The molarities of the sucrose solutions ranged from 0 to 0.6 M. The turbidity was normalized using the value for a granule suspension in iso-osmotic 0.3 M sucrose solution as unity.

C. Chemical Assays

Catecholamines were determined by the fluorometric trihydroxyindol method as described by Von Euler and Floding (26).

D. Release of Catecholamine at Different Temperatures

An important physical factor influencing the mechanism of catecholamine release is temperature. We carried out experiments at various temperatures in order to build fragility curves under different mechanical conditions.

Chromaffin granules were incubated at several temperatures in sucrose solutions of different osmotic strengths in the range 0–2.0 M. 50 μl of the freshly prepared chromaffin granule suspension were added to test tubes containing 1 ml of sucrose solutions at the various osmotic strengths. Each set, done in duplicate, was incubated for 15 min at the desired temperature, a sufficient time for achieving a steady state (see section IIIA), and centrifuged in the cold (4°C) at 2,000 g for a similar period. The supernatant of each tube was then assayed for released catecholamine. Runs were made at 2°, 10°, 26°, 37°, 44°, and 50°C. Catecholamine release was measured for each temperature in excess of that found in the solution at the beginning of each test and normalized with respect to the maximal release value obtained by incubating the granule sample in distilled water. At the end of the granule preparation procedure, a small amount of catecholamines was occasionally present in the solution. This was most probably due to some spontaneous rupture of the isolated granules and was subtracted from the final amount of catecholamines found at the end of each experimental procedure. A sample of the sucrose solution was removed just before initiation of each experimental procedure and the amount of catecholamines found was subtracted from the final determination at the end of the incubation period.

III. RESULTS AND DISCUSSION

A. The Dynamics of Catecholamine Release

The relationship between the turbidity of chromaffin granule suspension and catecholamine release was used by Perlman (27) and by Creutz and Pollard (28). We have compared the degree of lysis found by turbidity measurements to that found by catecholamine determination in a chemical assay. Fig. 1 depicts the fragility curve of chromaffin granules for the two methods. The lysis predicted by the turbidity curve is consistently lower than that corresponding to the one found by spectrofluorometry. The difference is particularly evident in the far hypo-osmotic

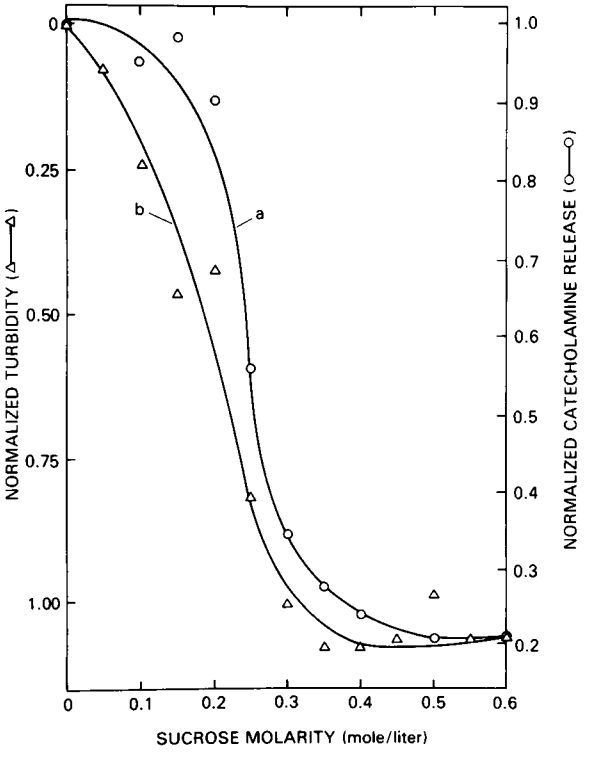

FIGURE 1 Catecholamine release determination by (*a*) chemical analysis and (*b*) turbidity measurement. Curve *a* is normalized with respect to total lysis obtained by incubation with distilled water at 37°C. Curve *b* is normalized with the maximum absorbance corresponding to that of a suspension in 0.3 M sucrose solution.

zone (0–0.15 M sucrose) where there is an abundance of membrane fragments and granule "ghosts."

The dynamic change in the turbidity of chromaffin granule suspensions in various sucrose solutions at 23°C is illustrated in Fig. 2. The curves corresponding to hyperosmotic sucrose solutions lie slightly higher than the 0.3 M curve and are not shown. This shift is probably due to shrinkage of granules, which slightly increase the total amount of light scattering.

The data of Fig. 2 were used to establish the duration of the transient state of release for each molarity. Suspensions with sucrose concentration >0.3 M reached a steady state of release at relatively short times (<3 min). The hypo-osmotic suspensions were stabilized after a time period between 6 and 12 min, being the range found between distilled water and 0.25 M sucrose solution, respectively. This demonstrates that the response time is inversely proportional to the lysis driving forces (e.g., the deviation from iso-osmotic state toward hypo-osmoticity). The response time necessary to achieve a steady state appears to be insensitive to temperature changes. Chemical assays for release at 2° and 10°C show that multiple results obtained after 15 min, 30 min, or 1 h are practically equal.

Based on these observations we established a period of 15 min as a valid incubation time for each measurement. This period ensured a reading at steady state, thus providing an adequate base for comparison between results obtained under various experimental conditions. A shorter incubation period, e.g., 10 min as was used by Perlman (27), might result in lower catecholamine release levels.

B. Temperature Sensitivity of the Fragility Curve

The effect of temperature on the osmotic fragility is presented in Fig. 3. It is assumed that the concentration

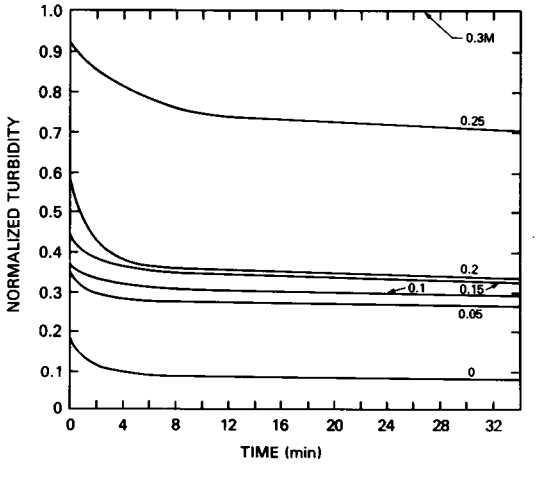

FIGURE 2 The dynamic change of turbidity of chromaffin granule suspensions in various sucrose solutions at 23°C. Unity corresponds to absorbance of a suspension in 0.3 M sucrose solution.

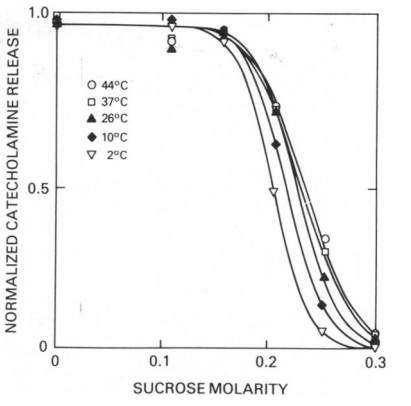

FIGURE 3 The effect of temperature on the osmotic fragility curve. Catecholamine release at each temperature, in excess to the amount found at the beginning of each test, is normalized with respect to total lysis obtained when granules were incubated in distilled water. Each curve averages between three to seven independent runs at that temperature.

within the granules is 0.3 M; therefore the data, measured in excess of the amount found initially in the solution, are normalized so that zero lysis occurs at 0.3 M sucrose solution and maximum lysis in distilled water.

Since it is commonly assumed that the granule population has a normal (Gaussian) distribution, the fragility curve can be correlated in terms of the cumulative fraction of lysis (28)

$$y = \int_{-\infty}^{x} \frac{1}{s\sqrt{2\pi}} e^{-(\xi - \xi_0)^2/2s^2} d\xi, \qquad (2)$$

where $x = 0.3$ M, (M being the sucrose solution molarity) and ξ_0 and s^2 are the distribution inflection point and variance, respectively. The latter were determined by a least-square fit to the experimental data for the various temperatures considered. It is clear that the resistance of granules to rupture increases as the temperature decreases. This behavior differs from that reported by Seeman et al. (29), who found that the osmotic fragility of erythrocytes was reduced at higher temperature. However, a comparison of these two experimental systems is not straightforward. The osmotic fragility of the erythrocytes is primarily a measure of the surface-to-volume ratio of the cell. This is due to the abililty of the erythrocyte to swell and become spherical, so that thermal changes in the fragility of the cell probably reflect an increase of this ratio with temperature. Chromaffin granules, on the other hand, are nearly spherical and the osmotic pressure difference is almost immediately supported by the granule membrane. Thus thermal changes in their osmotic fragility probably reflect changes in membrane properties.

The median granular fragility (MGF) is defined as the concentration of sucrose solution causing 50% lysis of the granules. The MGF was determined from the fragility data of Fig. 3 by considering the inflection point (median)

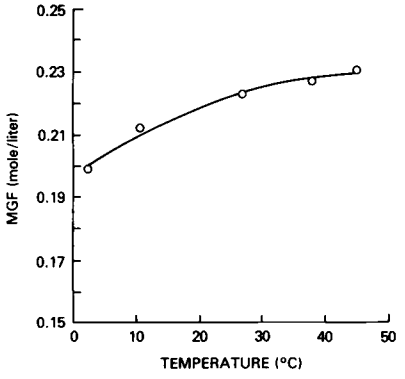

FIGURE 4 The change of the MGF with temperature. Data points represent concentration of sucrose solution causing 50% lysis at the various temperatures.

of each Gaussian curve, and is shown in Fig. 4. Our results are somewhat lower than those of Creutz and Pollard (28) who found 50% lysis at an osmolality of 0.272 and 37°C, corresponding to an MGF of 0.25, and slightly higher than those reported by Perlman (27) with an MGF of 0.21° at 30°C. This latter result is presumably due to the fact that the measurements were carried out at shorter incubation times. These three results obtained for the MGF of chromaffin granules are quite comparable, and the small differences seen could be due to slight variations in the experimental technique, leading Creutz and Pollard to find no further change in turbidity below a sucrose concentration of 0.15 M, while we show lysis continuing and reaching a maximum at 0.11 M.

Our study shows that the MGF increases slightly with temperature increase. Since this reflects a measurement of the critical yield stress of the chromaffin granule membrane, it seems to indicate a monotonic change in membrane properties, and probably structure, with temperature. The chromaffin granule membrane, as other biological membranes, is composed of lipids and proteins which confer upon the membrane a certain rigidity depending on the ratio between them. Winkler and Smith (19) have found that the chromaffin granule membrane is made up of 56.6% phospholipids, 16.6% cholesterol, 26.6% proteins, and traces of magnesium and calcium. This high lipid content could make the membrane structure very sensitive to temperature changes, and rising temperature would then accelerate the gel–to–liquid-crystalline phase transition. Changes in membrane fluidity with temperature have been demonstrated in chromaffin cell plasma membranes (30), and synthetic phospholipid bilayer vesicles (31, 32) and have been attributed to the formation of an unstable boundary between rigid and fluid domains. These changes are affected by the cholesterol-to-phospholipid ratio, especially in the artificial membrane systems where a higher cholesterol content results in lower viscosity. These studies, and others seem to support our contention of an altered physical state of the membrane with temperature change. The chromaffin granule membrane has a somewhat higher cholesterol content in contrast to that found in other biological membranes, which might be a cause for the relatively shallow slope seen in Fig. 4.

It is interesting to note that Perlman (27) observed a transition in the lysis of chromaffin granules in isotonic solutions of erythritol, arabitol and mannitol at ~16–19°C. We do not observe such a transition in lysis with hypotonic solutions nor did Perlman (27). It follows that this transition is associated with the permeability of the membrane to diffusing solutes and not with the membrane tensile strength. The transition in permeability may reflect a change in the internal structure reminiscent of the well-known glassy temperature of artificial polymers, which, being a secondary transition property, is not necessarily reflected in the membrane strength.

IV. SUMMARY

From a mechanical point of view, lysis and a subsequent release of catecholamine occurs at a critical membrane stress. Such a stress develops as a result of influx and accumulation of water due to the osmotic pressure difference.

The membrane stress, σ, can be estimated using the Laplace equation for a sphere of radius r and membrane thickness t

$$\sigma = r(p_i - p_0)/2t = r\Delta P/2t. \quad (3)$$

Using the common assumption (16, 18, 25) that, in our system, the vesicle is initially spherical and stress free and its volume change, which is due to the corresponding amount of water influx, is negligible, the concentration within the granule remains approximately constant (0.3 M equivalent to osmolality 0.332). In such a system equilibrium is attained when the increase in internal pressure balances the osmotic pressure gradient across the membrane. Eq. 1 yields for the critical pressure difference

$$\Delta P_c = (\pi_i - \pi_{MGF}) \quad (4)$$

where π_{MGF} is the osmotic pressure of the sucrose solution causing 50% granule lysis and π_i changes slightly with temperature. The following table summarizes evaluation of the critical stress, σ_c, for five temperatures, using the following average data for chromaffin granules: $r \simeq 10^{-5}$ cm and $t \simeq 0.75 \times 10^{-6}$ cm (33).

It should be noted that the deviations from these assumptions may change the calculated values of the critical tension. A deviation from sphericity will enable penetration of water into the granule, thus lowering the internal osmotic pressure. In such a case a slight increase in r is also expected. The net anticipated result though, should be lower values for the membrane force resultant, $\sigma_c t$.

It should prove interesting to compare the results of Table I to corresponding data obtained for the erythrocytes. Evans et al. (16) measured for the latter maximal tension of 10–12 dyn/cm at 25°C and 3–4 dyn/cm at

TABLE I
CRITICAL STRESS, σ_c, CALCULATED AT DIFFERENT TEMPERATURES USING THE MGF CURVE (FIG. 4)

Temperature	MGF	Osmolality	Osmolality	π_{MGF}	π_i	$\sigma_c t$
°C		mol/1,000 g	mol/liter	atm	atm	dyn/cm
2	0.199	0.213	0.228	5.14	6.769	8.23
10	0.212	0.227	0.245	5.69	6.964	6.46
26	0.223	0.241	0.259	6.35	7.360	5.10
37	0.227	0.245	0.264	6.72	7.631	4.64
44	0.230	0.249	0.268	6.97	7.803	4.21

50°C. The similarity in strength of both membranes is quite remarkable.

These results can be used in future application to mechanical analyses of processes involving vesicular membrane deformation as well as rupture and fusion. In particular they should prove important when modeling the mechanical aspects of the final approach of a granule to the cell membrane during the process of catecholamine secretion by exocytosis.

We would like to thank Avital Greenberg of the Department of Clinical Biochemistry, Rambam Medical Center for her excellent assistance with the experimental procedure.

Received for publication 30 January 1981 and in revised form 12 February 1982.

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