A BIOPHYSICAL MODEL OF THE CHROMAFFIN GRANULE

ACCURATE DESCRIPTION OF THE KINETICS OF ATP AND Cl^- DEPENDENT GRANULE LYSIS

CARL E. CREUTZ AND HARVEY B. POLLARD, *Clinical Hematology Branch, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20205 U.S.A.*

ABSTRACT A model is constructed to describe the behavior of isolated chromaffin granules (secretory vesicles of the adrenal medulla) when they are induced to release their contents by incubation with MgATP and Cl^-. The model is based on the assumption that the release event is osmotic lysis due to the ATPase dependent influx of protons and osmotically active Cl^- ions. The consequences of this influx of osmotically active particles are predicted from osmotic fragility curves determined by suspending granules in hypotonic media. Turbidity measurements on granule suspensions undergoing the ATP and Cl^- dependent release reaction are used to fit the parameters of the model. The model then successfully describes the time course, Cl^- dependence, ATP dependence, and osmotic strength suppression of the release event as monitored either by measurements of turbidity changes or of epinephrine release. The degree of suppression of release predicted in hypertonic media is also in agreement with published data on hypertonic suppression of exocytosis from several cell types: chromaffin cells, blood platelets, and parathyroid cells. Therefore, the model may also provide an accurate description of some of the events occurring during exocytosis.

INTRODUCTION

> The lowest valuation which can be put upon it is to say that the formal description of the kinetics has the same sort of use as a railway timetable, which although not the expression of a fundamental law, enables one to catch trains; the attention which has been paid to it, however, is justifiable on somewhat better grounds.... It is because the formal description of the kinetics is so highly developed that it is now impossible to make a statement about mechanism without its being immediately examined on a quantitative basis.
>
> Eric Ponder, *Hemolysis and Related Phenomena*, 1948.

Isolated chromaffin granules, the secretory vesicles of the adrenal medulla, release their contents when they are incubated at 37°C in an isotonic medium containing MgATP and Cl^- or other permeant anions (1). It has been hypothesized that this release is due to osmotic lysis of the granules resulting from the inward pumping of protons by a membrane ATPase and the concurrent influx of the permeant anion as a counterion (1–4). The importance of understanding the nature of this process is increased by the recent suggestion that the same events underly the process of exocytosis, whereby the contents of the secretory vesicle are released to the outside of the cell (5–8). The evidence suggesting that the mechanism is osmotic in nature

in both cases is primarily the ability of increased osmotic strength to suppress both processes (2–9).

This paper presents a model for the chromaffin granule release reaction that is based on the above hypothesis. A key element in the construction of the model is a relationship between the kinetics of the release reaction and the osmotic fragility curve for the granule population as determined by incubating granules in hypotonic media and measuring lysis. This approach was inspired by the pioneering work of Eric Ponder (10, 11) on red blood cell hemolysis, in which the concept of an osmotic fragility curve was developed. In the development and fitting of the model turbidity measurements were used to monitor lysis (12, 13), or, in a more abstract sense, swelling, lysis, and whatever other rearrangements occur as a consequence of an osmotic imbalance across the granule membrane. The model is found to be very successful in describing and predicting the qualitative and quantitative features of the in vitro release process. This success, we feel, provides compelling evidence for the accuracy of the osmotic lysis hypothesis of the chromaffin granule release reaction. It is suggested that the model may be useful in more detailed analyses of the chromaffin granule release reaction and of exocytosis from cells.

EXPERIMENTAL PROCEDURES

Preparation of Chromaffin Granules

Chromaffin granules were prepared from bovine adrenal medullae by homogenization genization and differential centrifugation in 0.3 M sucrose as previously described (2).

Turbidity Measurements

The turbidity of chromaffin granule suspensions was measured at 540 nm on a Gilford 250 recording spectrophotometer (Gilford Instrument Laboratories Inc., Oberland, Ohio) with a water jacketed sample chamber (13).

Assay of granule lysis in hypotonic media was conducted as follows. 200-μl aliquots of a freshly prepared chromaffin granule suspension, A540 $\simeq$3.0, were added to tubes on ice containing 1.8 ml of a sucrose solution of appropriate concentration to give the final, experimental osmolality, buffered with 1 mM MES-NaOH, pH 6. The tubes were placed in a 37°C water bath for 5 min, to replicate the temperature of the release reaction, then returned to the ice water bath to prevent further baseline lysis. Subsequently, the turbidity of the samples was read individually immediately after introduction to the spectrophotometer cuvette at room temperature. Iso-osmotic substitution of KCl for sucrose, up to levels used in the ATP, Cl^- dependent release experiments, did not significantly change the osmotic fragility of the chromaffin granules.

The release reaction induced by ATP and Cl^- was monitored by intermittent recording of the absorbance of samples incubated at 37°C in the spectrophotometer cuvettes. The standard incubation medium contained, in 1 ml, 5 mM $MgCl_2$, 5 mM Na_2ATP, 25 mM MES-NaOH (pH 6.0), and sucrose and KCl in appropriate concentrations to give the experimental Cl^- concentration and a calculated final osmolarity of 0.3. The final addition to the prewarmed incubation mixture was 0.2 ml of a chromaffin granule suspension in 0.3 M sucrose (or higher

concentrations of sucrose in some experiments) adjusted to give a final A540 in the reaction mixture of 0.3 (corresponding to ~0.13 mg/ml of protein).

The exact osmolalities of experimental solutions were determined by freezing point depression on a Precision Systems Osmette S osmometer (Precision Systems, Inc., Sudbury, Mass.)

Release of Epinephrine

Epinephrine release from granules undergoing the ATP and Cl^- dependent release reaction or hypotonic lysis was determined in supernates after centrifugation of the granules and granule membranes as previously described (2). The epinephrine was assayed by the fluorometric trihydroxyindole method at pH 2 (14).

Sampling Errors

The greatest precision in data and the fitting of the model was obtained when all the data were obtained from a single preparation of chromaffin granules over a few hours. After a few hours, changes in the osmotic fragility curve as the preparation deteriorated became significant. For the most critical data used in fitting the model measurements were made in duplicate or triplicate. The figures constructed with these data indicate the standard deviations of these measurements. However, when constrained to deal with a single preparation of granules, some of the time courses measured to test predictions of the model could be carried out only once. Accordingly, these data are presented without error estimates. Their reliability, however, should be comparable to the other data since they were always obtained under the same general experimental conditions. In these cases it is reasonable to estimate that repeated measurements would have fallen within 10% of the observed values.

Computations

Data manipulation and modeling were carried out on a DEC 10 computer through the use of MLAB, an on-line modeling program developed at the Division of Computer Research and Technology, National Institutes of Health, Bethesda, Maryland. The program and documentation of its application may be obtained by contacting Dr. Gary Knott at the aforementioned institution.

DEVELOPMENT OF THE MODEL

Determination of the Osmotic Fragility Curve

A fundamental assumption of the release model developed here is that a given imbalance in osmotic pressure on the two sides of the granule membrane, whether due to an increase in internal osmotic strength or a decrease in external osmotic strength, leads to the same change in structure (including degree of lysis) of the granule population, as reflected in the turbidity of the suspension. The first step taken in the construction of a model based on this assumption was the determination of the effect of lowering the external osmotic strength on the turbidity (A540) of a granule suspension. A monotonic decrease in turbidity was found as the molarity of the sucrose suspension was lowered from 0.3 to 0.15 M. Below this level there was no

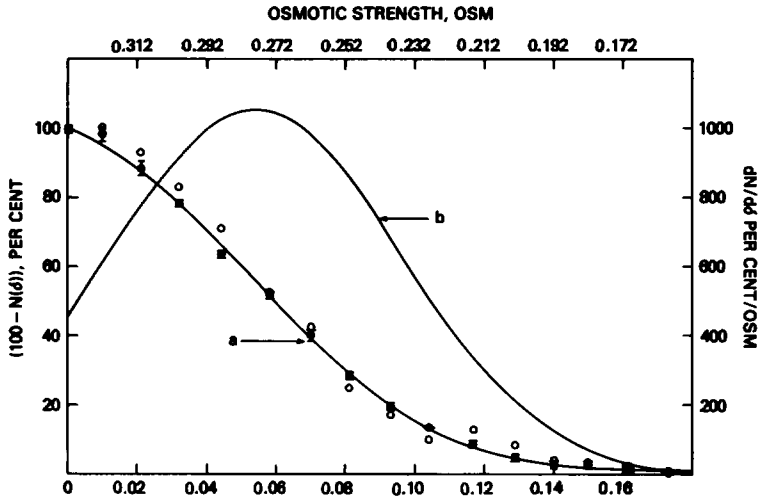


FIGURE 1 Osmotic fragility curve of chromaffin granules prepared in 0.3 M sucrose. Curve *a* is 100-$N(\delta)$, the normalized turbidity of the suspension in percent, where 100% corresponds to the turbidity in 0.332 osmolal medium (A540 ≃0.3), and 0% corresponds to the minimum turbidity seen after hypotonic lysis (A540 ≃0.09). The curve is plotted as a function of the total osmolality of the incubation medium, or of δ, the deviation from 0.332 osM. The points are measurements of turbidity in duplicate ±SD; the open circles are the percent of total epinephrine remaining in the granules; the solid curve is 100—the integral of curve *b*. Curve *b* is $dN/d\delta$, a Gaussian of the form $Ce^{-(\delta-A)^2/B}$ with A, B, and C chosen so that the integral of the curve *b* gives the best least squares fit to the turbidity data (A = 0.0540 osM; B = 3.51 × 10^{-3}osM^2; C = 1,052%/osM). This curve is referred to as the osmotic fragility curve.

further decline in the turbidity, and this minimum value was always ~30% of the turbidity at 0.3 M. Chemical measurement of the epinephrine released in hypotonic lysis demonstrated that the 70% decline in turbidity occurred when 100% of the epinephrine was released from the granules. To simplify analysis the turbidity data was normalized so that the turbidity in 0.3 M sucrose, 1 mM MES (osmolality = 0.332) was defined as 100% and the minimum turbidity (30% of the original) was defined as 0%. Data normalized in this way are presented in Fig. 1. This figure also contains data for the epinephrine released as a function of the external osmolality. The correlation with a decline in turbidity is very good except at the smaller deviations from 0.332 OsM where some swelling without granule lysis may be occurring.

The osmotic fragility curve, as used by Ponder (11), is a frequency curve giving the percentage of the population that lyses in each given reduction in the osmolality of the suspension medium. If instead of lysis we consider the decline in turbidity (which is closely correlated), then in the present context an osmotic fragility curve can be constructed using the derivative of the curve of turbidity versus osmolality in Fig. 1. That is, if 100-$N(\delta)$ is the experimental curve, where δ is the deviation from the initial osmolality (0.332 OsM) and N is the normalized turbidity decline (ranging from 0 to 100), then $dN/d\delta$ is the fragility curve. We have found that a reasonably accurate model of the fragility curve is obtained by adjusting the parameters A, B, and C, of a Gaussian curve:

$$\frac{dN}{d\delta} = Ce^{-(\delta-A)^2/B}, \tag{1}$$

so that the integral of the curve yields the best least-squares fit of $100\text{-}N(\delta)$ to the experimental points. Such a curve is shown in Fig. 1 as well as the fit of the curve to the experimental data for hypotonic lysis.

Correlation of the Kinetics of the Release Reaction with the Osmotic Fragility

The model being constructed rests on the hypothesis that the ATP dependent release reaction involves lysis due to increasing osmotic strength within the granule. To correlate this type of lysis with the hypotonic lysis analyzed above, we may regard δ as representing a positive deviation in osmolality on the inside of the granule, rather than a negative deviation on the outside of the granule. (It may be more accurate to view δ as a "virtual increase in osmolality" since changes in the granule structure and a concurrent influx of water may tend to keep the actual internal and the external osmolalities equal while the number of osmotically active particles in the granule increases.) We can then determine empirical curves for δ as a function of time during the release reaction in the manner described below.

The turbidity of a granule suspension undergoing ATP and Cl^- dependent release is measured as a function of time. It is important to note that the final extent of turbidity

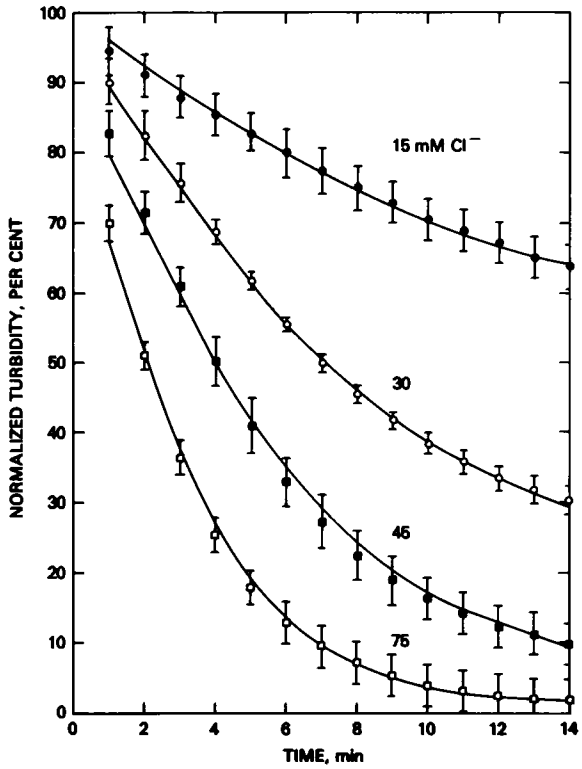


FIGURE 2 Time course of turbidity change for chromaffin granule suspensions undergoing the MgATP and Cl^- dependent release reaction, with different levels of Cl^-. Turbidity axis is in percent with 100% corresponding to the initial turbidity of A540 ≈0.3 and 0% corresponding to the turbidity at 15 min with 90 mM Cl^-. Other conditions as described in Experimental Procedures. The error bars represent SD for triplicate determinations. The solid lines represent the best simultaneous fit of the model to all of the data in the figure.

decrease under maximal release conditions (90 mM Cl^-, 5 mM Mg ATP) was found to be 70%, the same as obtained by complete hypotonic lysis, and that this decrease also corresponded to complete epinephrine release via the release reaction. Therefore the release curve under maximal release conditions could be normalized to the same scale used for hypotonic lysis: the turbidity is defined as 100% at 0 time, before the reaction begins, and 0% at infinite time. Under conditions of reduced concentrations of Cl^-, the release time courses tended to level off at intermediate levels between 0 and 100% (Fig. 2). This characteristic of the release curves has not been previously reported or anticipated, and the ability of the model to mimic this behavior, as will be shown, is an important measure of its success.

To determine $\delta(t)$, for each time point in the release curves measured at various Cl^- concentrations the value of δ is determined which makes the integral of the fragility curve from 0 to δ equal to the normalized percentage decline in the release curve. That is, if 100-$R(t)$ is the release curve (as in Fig. 2), $\delta(t)$ is determined such that

$$R(t) = \int_0^{\delta(t)} \frac{dN}{d\delta'}\, d\delta' = \int_0^{\delta(t)} Ce^{-(\delta'-A)^2/B}\, d\delta'. \tag{2}$$

This produces a set of points representing $\delta(t)$ as illustrated in Fig. 3 for a range of Cl^- concentrations. These curves constitute the fundamental correlation between the release time course and the osmotic fragility curve. The heart of the model is an equation to describe these curves and is developed in the next section.

Determination of a Model for the Curves $\delta(t)$

To construct a model that will give curves fitting the empirical data for $\delta(t)$, we must consider what activities at the granule membrane may alter the internal osmotic content of the granule. We will assume there is an active process, requiring ATP, and that there is a passive leak. Then the total change would be the sum of these two factors: $d\delta/dt_{total} = d\delta/dt_{active} + d\delta/dt_{passive}$. The hydrolysis of ATP by the membrane is known to drive protons into the granule (14) and these must be accompanied by the permeant counterion Cl^- if the inward movement of protons is not to be blocked by an increase in membrane electrical potential. The Cl^- ion enters through a specific, saturable transport site, and in the presence of excess ATP, the concentration of Cl^- is the limiting factor in the release reaction (2). Therefore we may expect that the kinetics of the active process will be governed by Michaelis-Menton kinetics with Cl^- as the substrate variable: if v is the rate of chloride entry,

$$v = \frac{V_m Cl_0}{K_m + Cl_0}, \tag{3}$$

with Cl_o the external Cl^- concentration.

Now it is not clear how many osmotically active particles are introduced in the interior of the granule by the transport of a single chloride ion. There are several possibilities: The cotransported proton may be osmotically active; the proton may be immediately buffered by the granule core constituents and therefore be osmotically "silent"; or the entry of Cl^- and H^+ may lead to solubilization of some of the small molecules trapped in the core (epinephrine, ATP, Ca, Mg) thus leading to a "recruitment" of osmotic particles. Therefore, the model incorporates a factor, f, relating $d\delta/dt$ to the rate of Cl^- influx: $d\delta/dt = v \times 1/f$. Defined this

way, $f = 1$ if the proton is osmotically silent, $f = 0.5$ if the proton is active, $f < 0.5$ if recruitment occurs. The value of f will be used as a fitting parameter, thus allowing the character of the data to determine which description is most accurate.

In modeling the passive leak, we will assume that the rate of the leak is determined by the Cl^- gradient according to Fick's law. That is, the flux of chloride across the membrane will be proportional to, and oppositely directed from, the chloride gradient. The effect this has on the number of osmotically active particles is again determined by the parameter f which relates chloride movements to changes in the internal osmolality. Thus, if v is again the chloride flux:

$$\begin{aligned} v &= K\Delta[\mathrm{Cl}] \\ &= K(\mathrm{Cl}_0 - \mathrm{Cl}_i - f\delta), \end{aligned}$$

or

$$\frac{d\delta}{dt} = \frac{K}{f}(\mathrm{Cl}_0 - \mathrm{Cl}_i - f\delta), \tag{4}$$

where K, the effective diffusion coefficient for Cl^- across the membrane, is a fitting parameter, Cl_i is the initial internal Cl^- concentration, Cl_0 is the external Cl^- concentration, and $f\delta$ represents the increase in internal Cl^- that is coupled to the change in internal osmotic strength due to both active and passive processes. The internal Cl^- concentration has been determined as 30 mM (2), and this average figure is used in the model for Cl_i.

The complete expression for the rate of change of δ combines the active and passive terms:

$$\frac{d\delta}{dt} = \frac{V_m \mathrm{Cl}_0}{f(\mathrm{Cl}_0 + K_m)} + \frac{K}{f}(\mathrm{Cl}_0 - \mathrm{Cl}_i - f\delta). \tag{5}$$

This equation may be integrated, to give an expression for $\delta(t)$:

$$\delta(t) = \frac{1}{Kf}\left(\frac{V_m \mathrm{Cl}_0}{\mathrm{Cl}_0 + K_m} + \mathrm{KCl}_0 - \mathrm{KCl}_i\right)(1 - e^{-Kt}). \tag{6}$$

Normally we anticipate $\delta(0)$, the increase in internal osmotic strength at time $t = 0$, to be zero. However, in some experiments when KCl was substituted for sucrose the osmolality of the incubation mixture was slightly less than that of the initial granule suspension. This led to a small but rapid decline in turbidity, occurring in 1 min, as the granules were exposed to the new osmotic strength. In modeling events occurring after the first minute the effect of this initial change in the osmolality of the suspension medium can be accounted for by adding a small constant term, δ_0, to the equation for $\delta(t)$.

The entire fitting process of the model thus reduces to (*a*) fitting a Gaussian expression (Eq. 1) to the osmotic fragility curve, and (*b*) fitting the above expression (Eq. 6) to the empirical data for $\delta(t)$. The only parameters that are allowed to vary in fitting $\delta(t)$ are V_m, the maximum rate for active transport of Cl^-, K_m, the affinity of the active transport system for Cl^-, f, the relation between transported (active or passive) Cl^- and the increase in osmotically active particles, and K, the diffusion constant for the passive leak of Cl^-. It is necessary to fit the model simultaneously to data from a series of Cl^- concentrations, as in Fig. 3, in order that

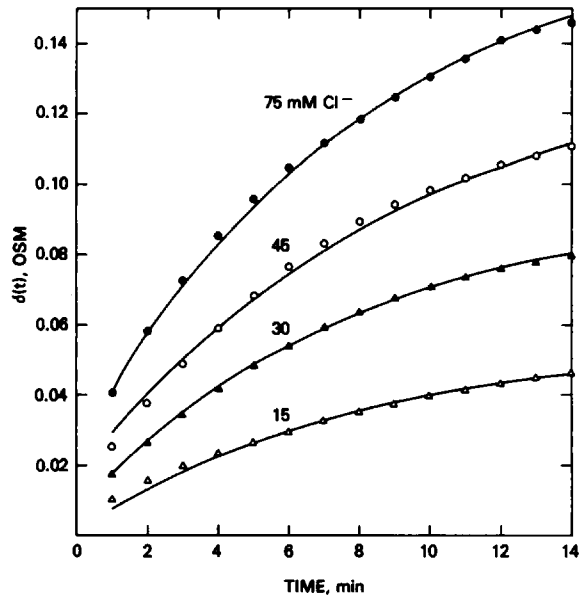


FIGURE 3 Plots of $\delta(t)$, the increase in the internal osmotic strength of the chromaffin granule preparation, at different external chloride concentrations. The solid lines were obtained by a simultaneous fit to the data shown of the expression for $\delta(t)$ incorporated in the model:

$$\delta(t) = \frac{1}{Kf}\left(\frac{V_m Cl_0}{Cl_0 + K_m} + KCl_0 - KCl_i\right)(1 - e^{-Kt}) + \delta_0.$$

The fitted values of the parameters are: $K = 0.105\ min^{-1}$; $f = 1.16$ M Cl^-/osM $V_m = 0.0177$ M/min; $K_m = 0.0145$ M.

V_m and K_m be correctly determined. After the values of the fitting parameters have been determined the model may be used to generate release time courses, as in Fig. 2, by integrating over the osmotic fragility curve.

APPLICATION OF THE MODEL

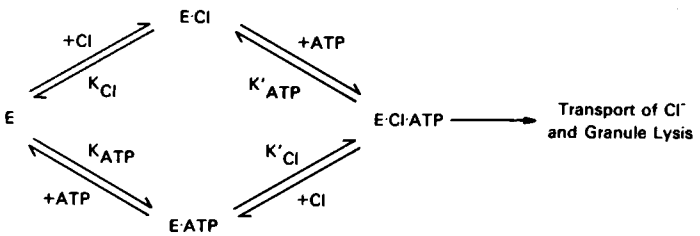
Fit of the Model to the Kinetics of Release

As seen in Fig. 2, the model successfully fits the release time courses, including the changes in rate and extent of lysis that occur at different levels of Cl^-. The release process tends to taper off at reduced Cl^- levels, rather than approaching complete release at a reduced rate, because the passive outward leak of Cl^- becomes equal in magnitude to the inward active transport of Cl^-. Although the model predicts that eventually (after the 14 min illustrated in Fig. 2) the release terminates at an intermediate level depending upon the Cl^- concentration, in fact a slow decline in turbidity is still observed at long times. This may be due to a slow permeation by sucrose since a similar decline is seen when granules are suspended in sucrose alone at 37°C.

The fitted value of f of ~1 immediately indicates that the increase in internal osmotic strength during the release reaction is due only to the Cl^- ions that have entered. This indicates that the counterion to the Cl^- is osmotically silent and is therefore also an indication

that the counterion is indeed a proton since that is the only cation for which there is a clear mechanism of buffering. The data of Njus, et al. (16) indicate that the granule core may buffer 92 μmol of protons per milliliter of intragranular volume with a pH change of one unit. This buffering capacity was reported to be fairly constant over the pH range of 4.5–7.0, encompassing the native internal pH of freshly isolated granules of 5.7 (15). A granule which requires an increase in osmolarity of 100 mosM to burst will thus be able to buffer all of the protons entering with the Cl^- while changing the internal pH by a little over 1 pH unit. Therefore, the fitted value of f is consistent with this considerable buffering capacity of the granule core.

The fitted value of the Michaelis constant, K_m, for Cl^- of the active transport system was 14.5 mM, which is lower than the previously reported values of the K_m in the release reaction (2). However, the previous data was determined at a maximum ATP concentration of 1 mM, and it was found that the K_m for Cl^- decreased as the ATP concentration was increased. If the chromaffin granule, or its proton and anion transport system, is modeled using classical kinetic theory as a bisubstrate enzyme (requiring ATP and Cl^- as substrates and with Cl^- transport and subsequent granule lysis as the product) then it can be shown that the high concentration of ATP (5 mM) used in the present experiments could have led to the low value of K_m for Cl^- that was found. Classically, a bisubstrate enzyme, E, would be modeled as shown below (16):



As shown, the reaction may follow two pathways to form the enzyme-substrate complex E-Cl^-ATP with distinct equilibrium constants depending upon which substrate attaches to E first. The enzyme substrate complex may then lead to hydrolysis of ATP, Cl^- transport, and subsequent granule lysis. In this system the K_m for chloride can be shown to be (17):

$$K_m = K_{max} \frac{K_{ATP} + 1/[ATP]}{K'_{ATP} + 1/[ATP]} \tag{7}$$

The values of K_m for Cl^- at different ATP levels, as reported previously by Pazoles and Pollard (2) for epinephrine release, and the value of K_m obtained from the present model are plotted together versus $1/[ATP]$ in Fig. 4. The above equation provides a good fit to the data, as shown in the figure, if $K_{max} = 86.79$ mM, $K_{ATP} = 0.228$ mM^{-1}, and $K'_{ATP} = 2.30$ mM^{-1}. Therefore, the value of the K_m that the model has predicted at 5 mM ATP appears consistent with the previously published values of K_m for Cl^- in the granule release reaction. The success of the classical bisubstrate model in describing this behavior suggests there is coupling between the ATPase and the Cl^- transport sites. This might be simply electrical coupling, via the granule membrane, or might reflect a more intimate association of the ATPase and a Cl^- binding site as has been seen in detergent solubilized preparations of granule membranes (18).

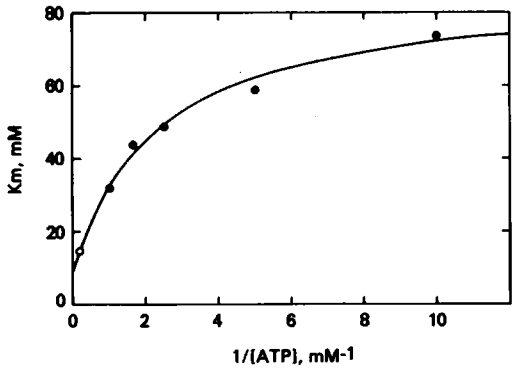


FIGURE 4 Plot of the K_m for Cl^- of the chromaffin granule release reaction as a function of 1/[ATP]. The solid points are from Table I of reference 2, the open circle is the value of K_m obtained in the present modeling experiments. The solid curve is the fitted expression $K_m = K_{max}\,(K_{ATP} + 1/[ATP])/(K'_{ATP} + 1/[ATP])$ with $K_{max} = 86.8$ mM, $K_{ATP} = 0.228$ mM^{-1} and $K'_{ATP} = 2.30$ mM^{-1}.

Predictions of the Model Concerning the ATP Dependence of the Release Reaction

A stringent test of the structure of the model may now be applied. We may eliminate the active pumping of Cl^- into the granule by removing the ATP from the incubation medium, and, in the model, setting $V_m = 0$. (Other methods of blocking the active movement of Cl^-, such as inhibiting the ATPase or shortcircuiting the proton pump with proton ionophores, could also be modeled by reducing V_m.) Then $\delta(t)$ will still increase, but only due to the passive leak of Cl^-. When incubated in the absence of ATP, the granules still release epinephrine in a Cl^- dependent manner, but at reduced rate and extent. Fig. 5 shows that the model accurately predicts the time course in turbidity change observed under these conditions. Fig. 6 illustrates a chloride titration of the extent of release occurring in a 10-min incubation period in the presence or absence of 5 mM ATP. The model has been used to generate a fitted curve to the data obtained in the presence of ATP (these data are obtained from the time courses in Fig. 2). This curve has the characteristic sigmoid appearance reported previously for the Cl^- titration of the release reaction (2). The model was also used to predict the Cl^- titration curve in the absence of ATP, and the figure illustrates that an accurate prediction is obtained. Thus the two components, active and passive, in the model for $\delta(t)$ appear to have been correctly identified and given the appropriate weights in determining the overall character of $\delta(t)$.

Predictions Concerning Suppression of the Release Reaction by Increasing External Osmotic Strength

The primary evidence that the Cl^- and ATP dependent release reaction of chromaffin granules is due to osmotic lysis is the observation that increased external osmotic strength suppresses the reaction. In the present model, an increase in the external osmotic strength due to the addition of a nonpermeant solute can be accounted for simply by moving the position of the osmotic fragility curve. Specifically, the parameter A would be increased by the increase in osmolality above 0.332 Osm. However, when the model is integrated after incorporating this change there is no agreement with experiment as the model is much more dramatically

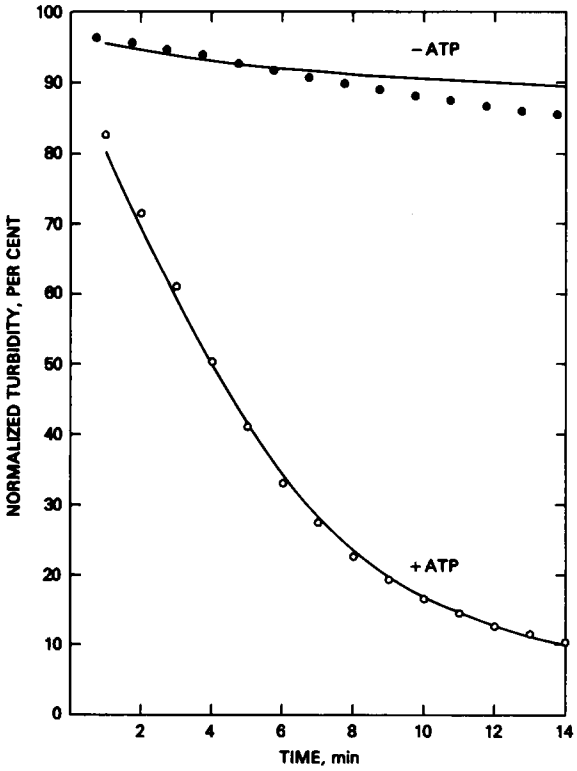


FIGURE 5 Predictions of the model for release in the absence of ATP. The lower curve is an example of the fit of the model to data for release in the presence of 5 mM ATP and 45 mM Cl^-. The top curve is the prediction of the model for release in 45 mM Cl^- but without ATP.

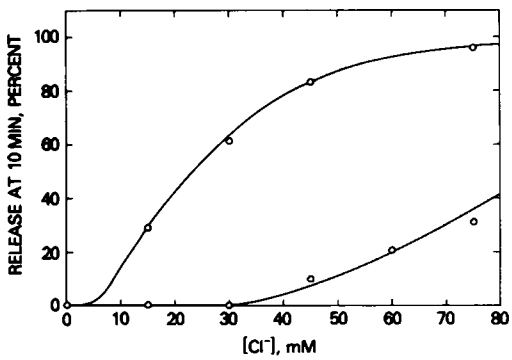


FIGURE 6 Titration of Cl^- in the release reaction. Vertical axis is the percent of the total possible turbidity change (i.e., occurring with complete release) that has occurred after a 10-min incubation at a given Cl^- concentration. The data on the top curve are measured release values in the presence of 5 mM ATP, with the solid line being the fit of the model to these data. The data on the bottom curve are measured release values in the absence of added ATP, with the solid line being the prediction of the model.

inhibited by the increase in external osmotic strength than is the experimental preparation (Figs. 7 and 8). One possible explanation of this discrepancy is that the solute (sucrose) used to increase the osmotic strength may itself have been permeant to some extent. If the leakage of sucrose into the granule under these hypertonic conditions were to occur slowly during the incubation, then the release curves should be highly suppressed at least at the earliest times. However, the release curves in hypertonic sucrose showed poor suppression even at very early times. Therefore, it seemed possible that the osmotic fragility curve had been dramatically shifted due to leakage of the sucrose before or within the first 1 or 2 min of the incubation. Measurements were, therefore, made of the osmotic fragility curves of granule populations that had been suspended and preincubated for 5 min at 37°C, in hypertonic sucrose. Examples of these curves are shown in Fig. 7, and are seen to contrast with the predicted curve for a perfectly impermeant solute. Curves were also determined for granules preincubated for 0, 1, and 10 min at 37°C (curves not shown). It was found that 80% of the shift in the fragility curve seen at 5 min had occurred before incubation, while the suspension was held on ice, and that between 5 and 10 min of incubation at 37°C there was only a few percent change in the position of the curve. This rapid shift in the fragility curve, occurring even in the cold, may have been due to physical rupture and subsequent repair of the granule membrane held in support by the core materials. The same shifts were seen when lactose was substituted for sucrose, indicating the effect was not specific to a single sugar. (Although KCl or NaCl may also be used to suppress the release of epinephrine by increasing the external osmolality, they were not used in this study because the chromaffin granules aggregate in the increased ionic strength, introducing a complicating increase in the turbidity of the suspension.) Whatever the mechanism of this shift, it was apparent that the fragility curve measured after a 5-min incubation would be a useful "average" curve to incorporate in the model for describing the release kinetics over a 15-min period.

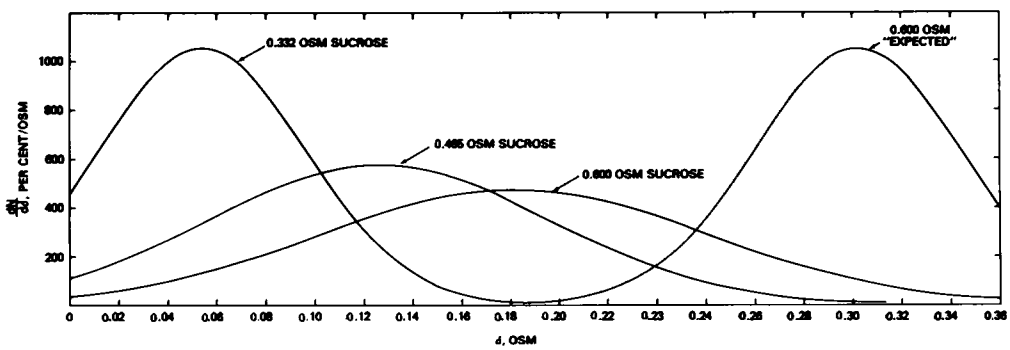


FIGURE 7 Osmotic fragility curves for chromaffin granules exposed to hypertonic conditions. The curve on the left is the fragility curve for granules prepared in 0.332 osM sucrose, reproduced from Fig. 1. The curve on the right is the "expected" fragility curve for the same preparation of granules when suspended in 0.600 osM sucrose; it is obtained from the first curve on the left simply by increasing the parameter A by 0.268 osM. The curves marked 0.465 osM and 0.600 osM are the experimental fragility curves obtained for granules preincubated in 0.465 osM and 0.6 osM sucrose, respectively. These curves are obtained from measurements of the turbidity of the suspension as the osmolality is reduced, using the type of analysis used to generate Fig. 1. In all cases, on the horizontal axis, δ represents the reduction from the initial osmolality.

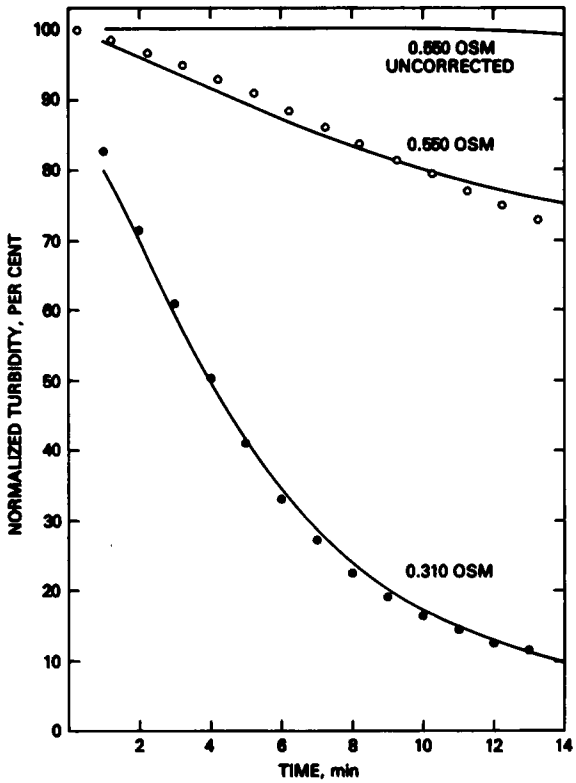


FIGURE 8 Time course of release reaction for chromaffin granules incubated in a hypertonic sucrose medium with 45 mM Cl^-, 5 mM MgATP. The bottom curve shows the fit of the model to the release (turbidity decline) occurring in a 0.332 osM medium containing 45 mM Cl^- and 5 mM MgATP. The top line shows the prediction obtained from the model for release in a 0.550 osM medium if it is assumed that the sucrose is completely impermeant and the fragility curve is shifted accordingly. The middle curve is the prediction if the experimentally determined osmotic fragility curve for granules in the hypertonic medium is used to generate the prediction. The points are the experimental observations.

Using the fragility curve measured after 5 min of incubation, the model was integrated to obtain a prediction for release occurring under hypertonic conditions. Noting that the parameters in the expression for $\delta(t)$ were not changed from the values obtained in isotonic media, the agreement of the prediction with the experimental time course in Fig. 8 seems quite good.

The suppression of epinephrine release from chromaffin granules by hypertonic sucrose was reported in 1976 by Pollard, et al. (9) in terms of the extent of release occurring in 10 min in the presence of 1 mM MgATP and 90 mM Cl^- as a function of the osmotic strength of the medium. The data appeared to be well fit by a single exponential. Data on the suppression of exocytosis from cellular systems was subsequently found to be fit by a similar exponential (5–8, footnote 1). The present model has been used in an attempt to reproduce this relationship between release and osmotic strength. The curve of Fig. 4 was used to determine a

[1]Pollard, H. B., C. J. Pazoles, and C. E. Creutz. Manuscript submitted for publication.

value for K_m to insert in the model, and Cl_o, the external chloride, was set at 90 mM. There was no need to change V_m as Pazoles and Pollard have demonstrated that the effect of ATP on the V_m of the system has saturated by the time ATP is increased to 1 mM (2).

To determine the shape of a continuous curve representing the suppression by external osmotic strength, it was necessary to obtain an estimate of the fragility curve for every value of external osmolality. Such estimates were obtained by fitting continuous functions of osmolality to the values of the parameters A, B, and C determined at several different osmolalities, as illustrated in Fig. 9 *a*. Linear models were used for the parameters A and B, as illustrated. For the parameter C, the requirement that the area under the fragility curve must be constant was used to relate C to the fitted value of B: $C = 100/\sqrt{\pi B}$. Using these fitted curves for A, B, and C, the continuous curve in Fig. 9 *b* was predicted for suppression of release by sucrose. As shown, the curve is distinct from, and yet fairly well described by a single exponential. This figure also contains the data reported by Pollard et al. for suppression of epinephrine release, and it is seen that there is a good correlation between these data and the prediction of the model. This provides a striking demonstration of the ability of the model to make predictions concerning epinephrine release as well as tubid change in the presence of ATP and Cl^- concentrations different from those used in the fitting of the model.

SUMMARY AND CONCLUSION

The model developed here for the ATP and Cl^- dependent chromaffin granule release reaction consists of the following elements: A Gaussian osmotic fragility curve, determined by measurement of lysis, or more accurately turbidity changes that are closely correlated with

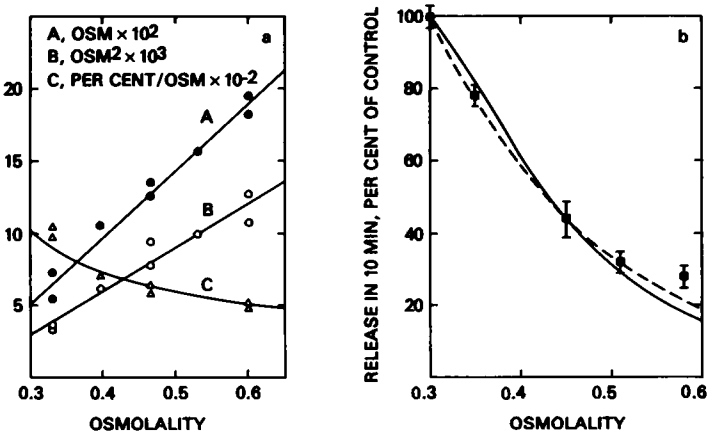


FIGURE 9 (*a*) Variation of the parameters A, B, and C in the Gaussian osmotic fragility curve $dN/d\delta = Ce^{-(\delta-A)2/B}$ as a function of initial osmolality. The data for A(●) are fit by the line $A = 0.0458 \times \text{osM} - 0.0844$. The data for B (0) are fit by $B = 0.0303 \times \text{osM} + 0.00607$. The data for C (Δ) are fit by $C = 100/\sqrt{\pi B}$, with B given by the linear expression defined above. (*b*) Suppression of the granule release reaction by increasing osmotic strength. The ordinate is the amount of release occurring after 10 min at the abscissa value of medium osmolality, expressed as a percent of the amount of release occurring in a 0.3 osM medium. [ATP] = 1 mM, [Cl^{-1}] = 90 mM. The solid line is the prediction of the model. The dashed line is the best approximation of the model's prediction by a single exponential. The data are those reported in reference 9 for epinephrine release.

lysis, induced by hypotonic media; and a model for the increase in the internal osmotic strength of the granules as a result of a membrane ATPase that pumps protons and osmotically active Cl^- ions into the granules and of a passive leak of Cl^- through the membrane. Once the three parameters of the osmotic fragility curve and the four parameters of the model for changes in the internal osmotic strength of the granules have been determined, it is possible to use the model to predict the kinetics of the release reaction under a wide variety of conditions. A single osmotic fragility curve is adequate for modeling all isotonic experiments. However, a new curve must be determined for experiments conducted at different tonicities. Although the predictions of the model are most appropriately applied to measurements of the turbidity of granule suspensions, excellent agreement is found in predicting epinephrine release, which is closely correlated with the turbidity decrease.

The ability of the model to predict accurately the characteristics of the release reaction under a variety of conditions provides compelling evidence that the underlying mechanism of the active release of epinephrine and the other contents of the chromaffin granule is osmotic lysis driven by the inward movement of Cl^- ions (or other permeant anions). The model may also become a useful tool for more detailed analysis of components of this release system. For example, it may provide a framework for studying the release induced by other anions or for determining the site of action of pharmacological agents which inhibit this process (2). Since the curve predicted by the model for the suppression of release by osmotic strength is also a good approximation to experimental data on the suppression of exocytosis from cells (5–8, footnote 1), the model may also be an accurate representation of some of the events occurring during this important cellular process.

We would like to thank Christopher Pazoles for helpful discussions and encouragement during all phases of this project, and Carol Brower and Karen Anderson for their skillful preparation of the manuscript.

Received for publication 29 August 1979 and in revised form 6 February 1980.

REFERENCES

1. POLLARD, H. B., C. J. PAZOLES, C. E. CREUTZ, and O. ZINDER. 1979. The chromaffin granule and possible mechanisms of exocytosis. *Int. Rev. Cytol.* **58**:160–198.
2. PAZOLES, C. J., and H. B. POLLARD. 1978. Evidence for stimulation of anion transport in ATP-evoked transmitter release from isolated secretory vesicles. *J. Biol. Chem.* **253**:3962–3969.
3. NJUS, D., and G. K. RADDA. 1978. Bioenergetic processes in chromaffin granules: a new persepective on some old problems. *Biochim. Biophys. Acta.* **4631E:219–244.**
4. CASEY, R. P., O. NJUS, G. K. RADDA, and P. A. SEHR. 1976. ATP-evoked catecholamines release in chromaffin granules: osmotic lysis as a consequence of proton translocation. *Biochem. J.* **158**:583–588.
5. POLLARD, H. B., K. M. TACKMAN-GOLDMAN, C. J. PAZOLES, C. E. CREUTZ, and N. R. SHULMAN. 1978. Evidence for control of serotonin secretion from human platelets by hydroxyl ion transport and osmotic lysis. *Proc. Natl. Acad. Sci. U.S.A.* **74**:5295–5299.
6. BROWN, E. M., C. J. PAZOLES, C. E. CREUTZ, G. D. AURBACH, and H. B. POLLARD. 1978. Regulation of parathyroid hormone release from dispersed bovine parathyroid cells by permeant anions. *Proc. Natl. Acad. Sci. U.S.A.* **75**:876–880.
7. POLLARD, H. B., C. J. PAZOLES, C. E. CREUTZ, A. RAMU, C. A. STROTT, P. RAY, E. BROWN, G. D. AURBACH, K. M. TACKMAN-GOLDMAN, and N. R. SHULMAN. 1977. A role for anion transport in the regulation of release from chromaffin granules and exocytosis from cells. *J. Supramol. Struct.* **7**:277–285.
8. POLLARD, H. B., C. J. PAZOLES, and C. E. CREUTZ. 1979. Evidence in support of a chemiosmotic mechanism for exocytosis from platelets, parathyroid and chromaffin cells. *In* Catecholamines: Basic and Clinical Frontiers, I. Kopin and E. Usdin, editors. Permagon Press, Oxford.
9. POLLARD, H. B., O. ZINDER, P. G. HOFFMAN, and O. NIKODEJEVIC. 1976. Regulation of the transmembrane

potential of isolated chromaffin granules by ATP, ATP analogs and external pH. *J. Biol. Chem.* **251**:4544–4550.

10. PONDER, E. 1924. The Erythrocyte and the Action of Simple Hemolysins. Oliver and Boyd, Edinburgh.
11. PONDER, E. 1948. Hemolysis and Related Phenomena. Grune and Stratton, New York.
12. TRIFARO, J. M., and A. M. POISNER. 1967. The role of ATP and ATPase in the release of catecholamines from the adrenal medulla. II. ATP-evoked fall in optical density of isolated chromaffin granules. *Mol. Pharmacol.* 3:572–580.
13. CREUTZ, C. E., C. J. PAZOLES, and H. B. POLLARD. 1978. Identification and purification of an adrenal medullary protein (synexin) that causes calcium dependent aggregation of isolated chromaffin granules. *J. Biol. Chem.* **253**:2858–2866.
14. ANTON, A. H., and D. F. SAYER. 1962. A study of the factors affecting the aluminum oxide-trihydroyindole procedure for the analysis of catecholamines. *J. Pharmacol. Exp. Ther.* **138**:360–375.
15. POLLARD, H. B., M. SHINDO, C. E. CREUTZ, C. J. PAZOLES, and J. S. COHEN. 1979. Internal pH and state of ATP in adrenergic chromaffin granules determined by ^{31}P nuclear magnetic resonance spectroscopy. *J. Biol. Chem.* **254**:1170–1177.
16. NJUS, D., P. A. SEHR, G. K. RADDA, G. A. RITCHIE, and P. J. SEELEY. 1978. Phosphorous-31 nuclear magnetic resonance studies of active proton translocation in chromaffin granules. *Biochemistry.* **17**:4337–4343.
17. LAIDLER, K. J. 1958. The Chemical Kinetics of Enzyme Action. Clarendon Press, Oxford.
18. PAZOLES, C. J., C. E. CREUTZ, A. RAMU, and H. B. POLLARD. 1980. Permeant anion activation of MgATPase activity in chromaffin granules: evidence for direct coupling of proton and anion transport. *J. Biol. Chem.* In press.