Ion Permeability of Isolated Chromaffin Granules

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ABSTRACT   The passive ion permeability, regulation of volume, and internal pH of isolated bovine chromaffin granules were studied by radiochemical, potentiometric, gravimetric, and spectrophotometric techniques. Chromaffin granules behave as perfect osmometers between 340 and 1,000 mosM in choline chloride, NaCl, and KCl as measured by changes in absorbance at 430 nm or from intragranular water measurements using ^3H_2O and [^{14}C]polydextran. By suspending chromaffin granules in iso-osmotic media of various metal ions and selectively increasing the permeability to either the cation or the anion by intrinsically permeable ions or specific ionophores, it was possible to determine by turbidity and potentiometric measurements the permeability to the counterion. These measurements indicate that the chromaffin granule is impermeable to the cations tested (Na^+, K^+, and H^+). Limited H^+ permeability across the chromaffin granule membrane was also shown by means of the time course of pH re-equilibration after pulsed pH changes in the surrounding media. The measurement of [^{14}C]methylamine distribution indicates that a significant ΔpH exists across the membrane, inside acidic, which at an external value of 6.85 has a value of 1.16. The ΔpH is relatively insensitive to changes in the composition of the external media and can be enhanced or collapsed by the addition of ionophores and uncouplers. Measurement at various values of external pH indicates an internal pH of 5.5. Use of the ionophore A23187 indicates that Ca^{++} and Mg^{++} can be accumulated against an apparent concentration gradient with calcium uptake exceeding 50 nmol/mg of protein at saturation. These measurements also show that Ca^{++} and Mg^{++} are impermeable. Measurement of catecholamine release under conditions where intravesicular calcium accumulation is maximal indicates that catecholamine release does not occur. The physiological significance of the high impermeability to ions and the existence of a large ΔpH are discussed in terms of regulation of uptake, storage, and release of catecholamines in chromaffin granules.

INTRODUCTION

The chromaffin granule of the adrenal medulla is the intracellular organelle which contains the endogenous stores of catecholamines. Since the chromaffin cells are part of the sympaticoadrenal system, the possible regulatory functions of chromaffin granules over uptake, storage, and release of catecholamines have important physiological and pharmacological implications.

However, despite significant research efforts, the mechanism of catecholamine transport at the membrane level is still unresolved. At least two mecha-

nisms have been proposed in the literature to explain the uptake of catechol-amines by chromaffin granules. According to the first, the storage-complex hypothesis, uptake occurs via the inward diffusion of amines down a concentration gradient and the high intravesicular catecholamine content is the result of the binding of amines to nondiffusable complexes (1). According to the active uptake hypothesis, the high concentration of amines in the granules is maintained by active transport across the membrane which is sufficient to counteract the loss of amines by diffusion (2). The experimental evidence fails to discriminate between the two postulated mechanisms. It has been previously noted that these mechanisms may not be mutually exclusive (3). It is possible that the actual mechanism of catecholamine uptake results from the most salient features of both, since it has been shown that: (a) an Mg^{++}-dependent ATPase exists in the chromaffin granule membrane and under appropriate conditions a net uptake of catecholamines can occur in both intact chromaffin granules (4, 5) and chromaffin granule ghosts (6, 7); (b) within the intragranular space the ATP content has been found to represent 20.5% of the dry weight of the chromaffin granules, the highest of any known organelle (8, 9), and this ATP, perhaps in conjunction with chromogranins, may form a nondiffusable storage complex with catecholamines and cations (10); and (c) in model systems catecholamines can accumulate against an apparent concentration gradient in the presence of ATP and certain soluble proteins (11, 12).

There are likewise at least two general hypotheses as to how release of catecholamines could occur: (a) intracellular release of catecholamines into the cytosol and diffusion across the cell membrane (13); and (b) fusion of the chromaffin granule membrane with the plasma membrane followed by secretion of the soluble contents directly into the extracellular space (14). While biochemical and morphological studies suggest that release occurs via the latter mechanism (15, 16), exocytosis, the sequence of biochemical events which occur in such a process is still largely unresolved. Whatever the mechanism, it is unequivocal that calcium is the necessary and sufficient stimulus-secretion coupling element (17-19).

Any mechanism relating to the regulation of catecholamine levels in the adrenal medulla must ultimately relate to control of that process at the site of the chromaffin granule membrane. Because of the known importance of the regulation of intracellular processes by cystolic ion fluxes, systematic investigations are needed to determine the permeability of the chromaffin granule membrane to various ions. A few previous reports have dealt in part with these determinations (1, 20, 21). However, comprehensive studies appear critical to the understanding of the mechanisms of catecholamine uptake and release.

In this study, ion permeability and ion transport across chromaffin granule membranes were investigated with radiochemical, potentiometric, and spectro-photometric measurements. From these studies, it was concluded that the chromaffin granule membrane is highly impermeable to H^+, Na^+, K^+, Ca^{++}, Mn^{++}, and Mg^{++}, and that a large ΔpH exists across the membrane, inside acidic. These findings are discussed in terms of physiological mechanisms for catecholamine uptake, storage, and release.

MATERIALS AND METHODS

Preparation

Chromaffin granules were isolated from bovine adrenal glands in a 0.27 M sucrose-10 mM Tris (hydroxymethyl)-aminomethane medium, pH 7.0. The chromaffin granules were purified on a D_2O-Ficoll-sucrose density gradient in order to provide isotonicity as described elsewhere (22, 23). The isolation medium was used for two subsequent washings and for the final suspension which was stored at 4°C until use. Protein was measured by the Lowry method with bovine serum albumin as the standard (24). All the experiments were performed at 24°C.

Measurement of Volume

The intragranular water content of the granules was measured by radiochemical, gravimetric, and photometric techniques. In the radiochemical technique, ^3H_2O and [^{14}C]polydextran were added to centrifuge tubes containing the desired osmolarity of KCl. The granules were added to the tubes which were centrifuged after 15 s. The supernates were carefully removed with a Pasteur pipette and the tubes gently washed with three additions of ice-cold sucrose. The pellets were denatured overnight in 0.4 ml of perchloric acid (14%). The tubes were subsequently centrifuged and 0.2 ml of the supernate was added to 5 ml of Handifluor scintillation fluid (Mallinckrodt Inc., St. Louis, Mo.) containing 0.2 ml of 3 M formic acid. The vials were then counted for ^3H and ^{14}C radioactivity. The internal water space was calculated from the total water space minus the polydextran space of the pellet. The determination of extravesicular space in mitochondria (25), chloroplasts (26), and lysosomes (27) by this technique has been well established. Lack of appreciable binding of [^{14}C]polydextran to the granules was excluded for the following reasons. First, when various concentrations of chromaffin granules were added to [^{14}C]polydextran containing medium and the granules centrifugated, the concentration of [^{14}C]polydextran measured in the supernate was found to be the same, i.e. independent of the concentration of granules added. Second, washing the granules which were initially suspended in [^{14}C]polydextran containing medium in [^{14}C]poly-dextran-free medium dramatically reduced the amount of [^{14}C]polydextran found in the pellet. Third, the same extravesicular space was calculated by doubling or halving the concentration of [^{14}C]polydextran routinely used.

The gravimetric technique utilized a similar process except that ^3H_2O was omitted. After the initial centrifugation the supernate was decanted and the inside of the tube carefully swabbed with cotton-tip applicators in order to remove the remaining water. The tubes and pellets were weighed before and after drying in an oven, the difference in weights indicating the total water space. Perchloric acid was added and the dissolved pellet prepared for scintillation counting as described previously. The dextran-accessible space was subtracted from the total water space in order to determine the intravesicular water content.

Turbidity was measured with a Hitachi model 124 Double Beam Spectrophotometer at 430 nm in order to minimize unwanted contributions by adrenochrome absorbance. Granules were added to a cuvette containing the desired osmolarity of KCl, NaCl, or choline chloride and the instantaneous absorbance changes followed. The osmolarity of the solutions used was measured with an Osmette Precision Osmometer model 2007 (Osmonics, Inc., Hopkins, Minn.).

Measurements of Ion Movement

Net ion fluxes were measured with ion-specific electrodes, metallochromic indicators,

and atomic absorption. K^+ and H^+ fluxes were simultaneously measured with a Beckman 39137 cationic electrode (Beckman Instruments, Inc., Fullerton, Calif.) connected with a Corning 100 pH meter (Corning Scientific Instruments Medford, Mass.), and a Thomas 73030 pH electrode (Arthur H. Thomas Co., Philadelphia, Pa.) connected with a Corning 109 pH meter, and a dual channel recorder.

Ca^{++} uptake was measured by dual wavelength spectrophotometry with the indicator Arsenazo III (28). This dye measures ionized calcium concentrations and, with respect to other metallochromic indicators, has the advantage of: (a) high sensitivity and selectivity to Ca^{++}; (b) measurable absorbance changes at around 700 nm, far from absorbing pigments and adrenochrome; and (c) low sensitivity to pH changes. The measurements were obtained with a dual wavelength spectrophotometer designed and built at the Johnson Foundation, University of Pennsylvania, by use of the wavelength pair 675-685 nm.

Mg^{++} uptake was measured by atomic absorption spectroscopy in the supernate, after sampling and centrifugation of aliquots of the reaction mixture. The assay was performed with a Varian Techtron AA-5 atomic absorption spectrophotometer (Varian Associates, Palo Alto, Calif.) using the resonant line 2,851 Å.

Measurement of Catecholamines

Catecholamines were measured by three methods. In the first, granules were rapidly spun down and the catecholamines assayed spectrophotometrically from the supernate after oxidation by ferricyanide (29). Adrenochrome formation was measured at 480-575 nm with a Johnson Foundation dual wavelength spectrophotometer. In the second method, ferricyanide (0.25%) was added directly to the sample cuvette and catecholamine release measured as adrenochrome formation at 520-540 nm. In the third method, the endogenous fluorescence of the catecholamines was used to monitor release. It has been shown that the fluorescence yield and fluorescence lifetime of catecholamines increase in disrupted granules (30). This observation was employed to measure the kinetics of release of catecholamines. In this method, granules were added to a cuvette containing 0.27 M sucrose-10 mM Tris (hydroxymethyl)aminomethane maleate (pH 6.9) and the fluorescence increase was measured in a Perkin-Elmer MPF2A Fluorescence Spectrophotometer with excitation at 280 nm and emission at 320 nm (Perkin-Elmer Corp., Norwalk, Conn.).

Measurements of ΔpH

$[^{14}C]$Methylamine distribution was used to determine the ΔpH across the membrane of the granules. The method is based on the fact that amines freely permeate biological membranes in their neutral form (and only in this form) with equilibrium being reached when $RNH_{2(inside)} = RNH_{2(outside)}$. It readily follows, then, for amines of high pK_a that $R\text{-}NH_3^+_{(inside)}/R\text{-}NH_3^+_{(outside)} = H^+_{(inside)}/H^+_{(outside)}$ and, since amines would be expected to concentrate inside acidic vesicles, the ratio of the intravesicular concentration to that in the medium should give a measure of the ΔpH. The $[^{14}C]$methylamine distribution ratio was then corrected for the extravesicular H_2O space which was determined by the distribution of $[^{14}C]$polydextran. The experiment was performed by adding 0.2 ml of granules to microcentrifuge tubes containing 1 ml of the reaction mixture and the desired tracers (^3H_2O and $[^{14}C]$polydextran or $[^{14}C]$methylamine). After 4 min, the tubes were centrifuged in a Coleman 8-611 desk microcentrifuge (Coleman Instruments Div., Perkin Elmer Corp., Oak Brook, Ill.). From each tube, a 100-μl sample was withdrawn from the supernate and mixed with 0.2 ml of 14% perchloric acid. A slice was then made across the bottom of the tube so that approximately half of the pellet was cut and this portion of the pellet also was mixed with 0.2 ml of perchloric acid. The supernates and the pellets remained in the acid overnight. A sample from each tube was withdrawn, mixed with the

scintillation fluid, and counted for ^3H and ^{14}C as described elsewhere in this section. Water content ^3H_2O, $[^{14}C]$polydextran, and $[^{14}C]$methylamine distributions were calculated by the relative activities in the pellet and in the supernate by a method similar to that described previously (31). The ΔpH was calculated by the equation listed in Table I and on the assumption that the concentration of methylamine in the external water space was similar to that in the supernate.

Materials

Tris(hydroxymethyl)aminomethane, Mes (2-[N-morpholino]-ethanesulfonic acid), PIPES (piperazine-N-N'-bis[2-ethane-sulfonic acid]), and EGTA (ethyleneglycol-bis-[β-amino-ethyl ether]N,N'-tetracetic acid) were purchased from Sigma Chemical Co., St. Louis, Mo. Valinomycin was obtained from Calbiochem, Los Angeles, Calif. FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) was a gift from Dr. P. G. Heytler of E. I. DuPont de Nemours, Inc., Wilmington, Del. Nigericin and X537A were kindly supplied by Dr. J. Berger of Hoffman-La Roche, Nutley, N. J., and A23187 was from Dr. R.

TABLE I
EFFECT OF MEDIA ON ΔpH

Medium*	$[^{14}C]$Polydextran space / ^3H_2O Space	$[^{14}C]$Methylamine / ^3H_2O Space	$[^{14}C]$Methylamine (IN)‡ / $[^{14}C]$Methylamine (OUT)	$\Delta pH = \log \dfrac{C_{in}}{C_{out}}$
185 mM Choline chloride-10 mM Tris-maleate	0.57	6.8	14.6	1.16
185 mM NaCl-10 mM Tris-maleate	0.37	6.4	9.6	0.98
185 mM KCl-10 mM Tris-maleate	0.43	6.6	10.8	1.03
270 mM Sucrose-10 mM Tris-maleate	0.45	7.6	13.1	1.18

* Parallel experiments were performed under the same conditions in the presence of (a) $[^{14}C]$polydextran and H_2O and (b) $[^{14}C]$methylamine and H_2O. Granules were added to the incubation medium (pH 6.85) so that the content was 11.5 mg protein/ml. After 4 min the granules were rapidly centrifuged. The distributions of the traces were measured as described in Materials and Methods.

$$\ddagger \; \frac{C_{in}}{C_{out}} = R + (R - 1)\left(\frac{X}{1-X}\right) \; \text{where} \quad R = \frac{[^{14}C]\text{methylamine}}{^3H_2O \text{ space}}; \quad X = \frac{[^{14}C]\text{polydextran}}{^3H_2O \text{ space}}.$$

Hamill of Eli Lilly and Co., Indianapolis, Ind. All the ionophores were dissolved in ethanol and the volume of ethanol added for each experiment did not exceed 5 $\mu l/ml$ of the reaction mixture. $[^{14}C]$Methylamine (10.6 mCi/mmol), $[^{14}C]$polydextran (1.12 mCi/g), and ^3H_2O were purchased from New England Nuclear, Boston, Mass.

RESULTS

Osmotic Properties of Isolated Chromaffin Vesicles

The measurement of volume changes induced by the presence of anions and cations provides insight into both the permeability properties of the membrane and the capacity of the organelle to respond to changes in its osmotic environment. Chromaffin granules were found to swell when placed in a medium containing metal ions, with the extent of the swelling depending upon the external osmolarity (Fig. 1) as has been reported previously (32). However, swelling did not relate to the external osmolarity in a simple linear manner but rather showed a triphasic behavior. As the osmolarity was reduced from 1,000

mosM to 350 mosM, a small swelling was observed which is attributed to the re-equilibration of the external water space induced by suspending the chromaffin granules in a medium of impermeant ions. From 350 mosM to 200 mosM there was a large magnitude and rapid swelling several orders of magnitude larger than that of the first phase and accompanied by catecholamine release (Fig. 1). At osmolarities below 200 mosM, no further swelling or catecholamine release was observed.

Thus, only a limited region of the osmotic spectrum (greater than 350 mosM) could be utilized for studies of volume regulation since when granules suspended in KCl of this osmotic region were resuspended in isotonic sucrose it was
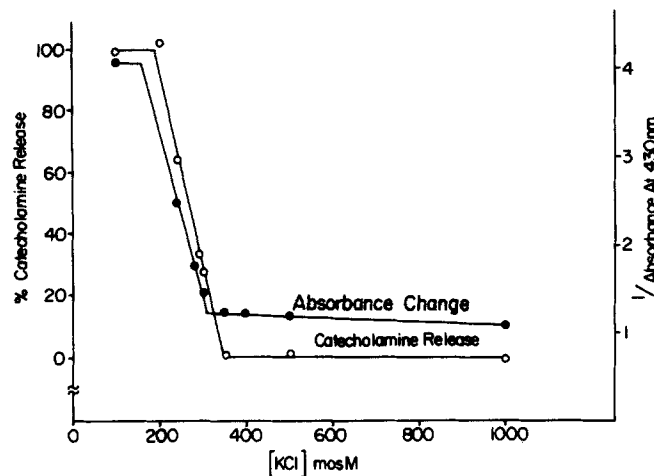
FIGURE 1. Percent catecholamine release and swelling of chromaffin vesicles as a function of osmolarity. The reaction mixture contained 10 mM Tris maleate (pH 6.95) and the various concentrations of KCl indicated in the abscissa. The chromaffin granule concentrations were 0.695 mg of protein/ml for swelling and 2.78 mg of protein/ml for catecholamine release measurements. The catecholamines were assayed from the supernate after centrifugation as adrenochrome formation by using 0.25% ferricyanide as the oxidizing agent. Adrenochrome was measured at 485–575 nm on a Johnson Foundation double beam spectrophotometer.

found that the swelling was reversible and they could again behave as perfect osmometers. Moreover, granules washed in buffered 0.8 M sucrose and/or 0.4 M sucrose also behaved as perfect osmometers; however, the swelling commensurate with catecholamine release was irreversible (data not shown). The integrity of the catecholamine stores can be maintained at these high osmolarities for a long period of time (several hours). It must be noted that conclusions derived from studies of organelle function in reaction mixtures composed of metal ions of low tonicity should be reevaluated in light of these effects (33–35).

At an osmolarity of 380 mosM, it was possible to study rapidly the membrane permeability to ions. If the ions are impermeable, the vesicles should behave as osmometers obeying the relationship

$$V_c = \frac{k}{c} + V_\infty,$$

where V_c is the internal volume at an external concentration of solute c, k is a constant, and V_∞ is the volume at infinite concentration of external solute. Fig. 2 illustrates that the relationships holds if the inverse absorbance is substituted for volume. Quantitatively similar results were obtained in KCl, NaCl, and choline chloride media. Measurements of the actual internal water space via radiochemical and gravimetric techniques showed that a linear relationship exists over this range as well with the internal water space being 1.98 μl/mg of protein at 400 mosM and the osmotic dead space 1.04 μl water/mg of protein. That the chromaffin granules behave as perfect osmometers in KCl, NaCl, and choline chloride implies that the chromaffin granule membrane is impermeable to either of these small ions, or to both.
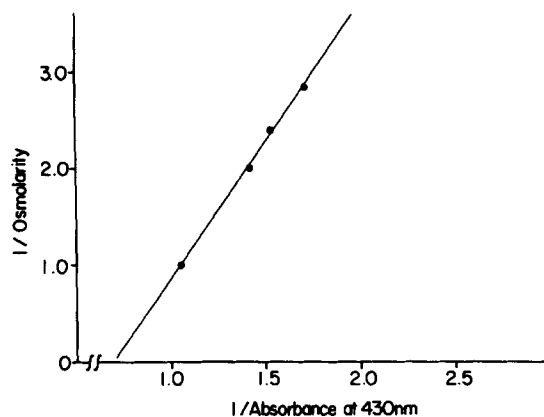

FIGURE 2.   Light scattering of chromaffin granules as a function of osmolarity. The reaction mixture contained 10 mM Tris maleate (pH 6.95), the various concentrations of KCl indicated, and chromaffin granules (0.80 mg of protein/ml).

Monovalent Cation Permeability

In order to investigate rapidly the permeability of the membrane to individual anions and cations, swelling of the chromaffin granules was monitored in iso-osmotic solutions. Since swelling in such a system can only occur when both anion and cation can penetrate the membrane, the rate of permeation of an individual ion was studied under conditions where the counterion was freely permeable. The latter was obtained with intrinsically permeable ions or in the presence of specific ionophores. In the case of ammonium salts, it is thought that the uncharged species NH_3 readily passes through the chromaffin granule membrane, down a concentration gradient. Once within the intravesicular space, NH_3 associates with a proton and the gradient equilibration will be dictated by the permeability to the counterion. The net penetration is NH_4 anion and, since the internal osmotic pressure has now been increased, the granule swells. In the case of a weak acid, the undissociated form rapidly enters the granule moving down its electrochemical gradient. Once inside, the acid dissociates and its entrance will be limited by the permeability of the cation present in the medium. Fig. 3 shows the results of such a system wherein the granules were suspended in iso-osmotic solutions and swelling was monitored. Maximal

swelling would be expected to occur whenever both the anion and cation are considered to be freely permeable, as in the case of ammonium acetate. As evidenced in Fig. 3 A, swelling induced by ammonium acetate is rapid and complete since neither the anion nor the cation is limiting the influx of the other.
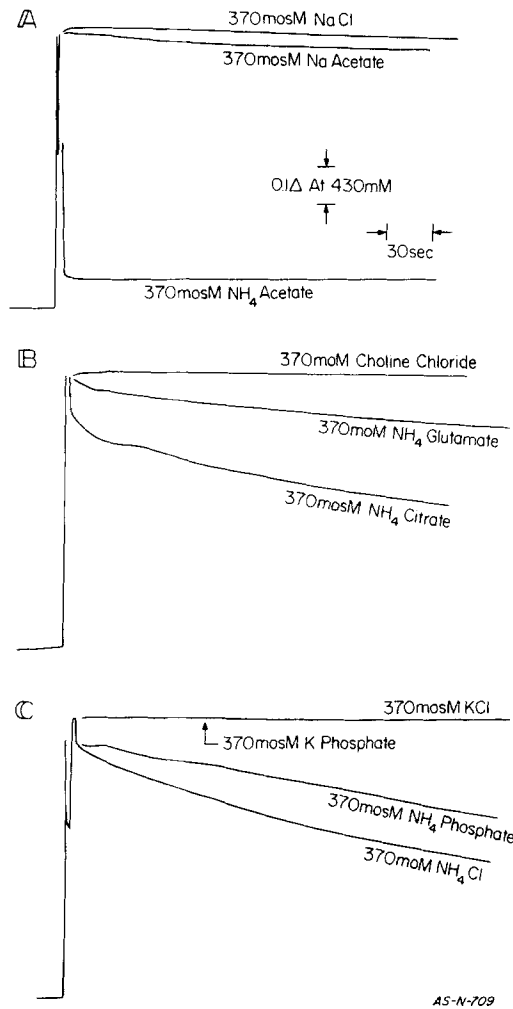

FIGURE 3 A-C.   Light scattering of chromaffin vesicles (1.80 mg of protein/ml) suspended in iso-osmotic solutions of various inorganic salts. 10 mM Tris maleate (pH 6.85) was present in each sample.

Chromaffin granules suspended in NaCl do not swell, indicating that the membrane may be impermeable to Na^+ or Cl^-, or to both. Suspension in sodium acetate allows for the determination of Na permeability since the penetration of the cation is now the rate-limiting step. It can be seen that only a very limited swelling occurs in the presence of sodium acetate, indicating that the membrane is impermeable to Na^+. Fig. 3 C shows that neither KCl nor K phosphate pro-

duces a significant swelling. It was also observed that the granules are slightly permeable to glutamate and more permeable to citrate and chloride (Fig. 3 B).

Ionophores and uncouplers which increase the permeability of the membrane specifically to one ionic species also permit the study of the natural passive permeability of the membrane to the counterion, which will tend to distribute according to its electrochemical gradient. Fig. 4B shows that the addition of valinomycin, an ionophore which transports K^+ electrogenically across artificial
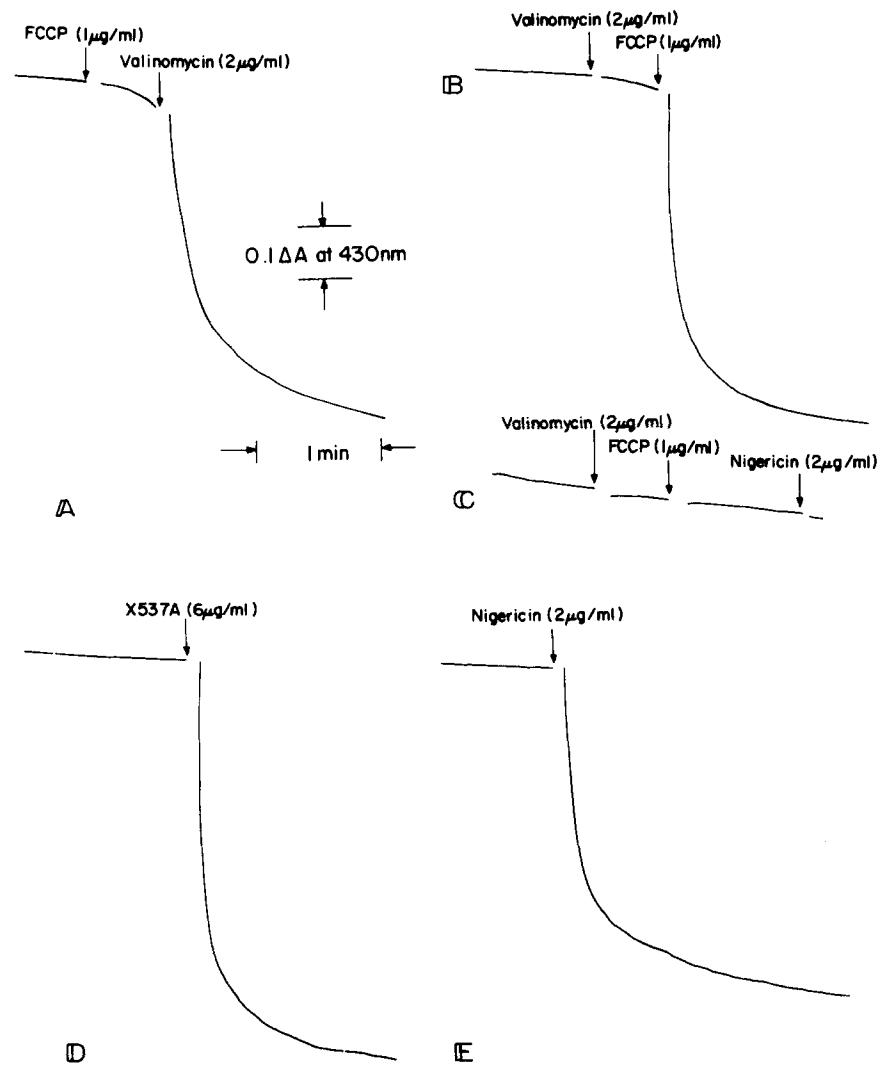

FIGURE 4 A–E. Swelling of chromaffin vesicles induced by various ionophores. Chromaffin vesicles (1.98 mg of protein/ml) were added to a reaction mixture of 185 mM KCl-10 mM Tris maleate (pH 6.85) in A, B, D, and E. 185 mM choline chloride-10 mM Tris maleate (pH 6.85) was used in C. The reactions were initiated by ionophore addition. Absorbance decrease (swelling) is indicated as a downward deflection.

and biological membranes, produces only a limited swelling of the chromaffin granules suspended in a KCl medium. This is because a significant and rapid movement of K^+ into the vesicle cannot occur unless a similar charge moves in the opposite direction. Thus, although valinomycin causes the membrane to become freely permeable to K^+, the movement of the charged ion establishes a diffusion potential which limits the further influx of K^+. The subsequent addition of FCCP, an uncoupler which makes the membrane permeable to protons, provides an exchangeable charge. Thus, in the presence of both valinomycin and FCCP, electroneutral K^+ influx and H^+ efflux can occur which results in a large and rapid swelling. A similar effect was observed when the order of addition was reversed (Fig. 4 A). Nigericin, a monocarboxylic acid ionophore known to catalyze an electroneutral K^+:H^+ exchange, produces a swelling similar in rate and extent to that induced in the presence of both valinomycin and FCCP (Fig. 4 E). X537A, also a monocarboxylic acid ionophore capable of transporting K^+ and H^+ but possessing the additional properties of being able to transport Ca^{++} and catecholamines through lypophilic phases (36–39), produces an effect similar to that of nigericin (Fig. 4 D). Fig. 4 C serves as a control wherein choline chloride was used as the suspending medium. No swelling was observed upon addition of valinomycin, FCCP, or nigericin, since none of the ionophores forms a complex with and transports choline. X537A, however, is capable of producing a large dose-dependent swelling of the granules when suspended in choline chloride (data not shown). This effect is not likely to be due to influx of ions since X537A cannot directly facilitate choline or chloride movement through the membrane. The effect is probably attributable to the ability of X537A of facilitating transport of catecholamines through lipophilic phases. Since catecholamines are probably stored in large aggregate complexes intravesicularly and X537A is capable of releasing at least 95% of the endogenous stores (23), one likely explanation would be that swelling is due to the dissociation of the intravesicular storage complex mediated by the efflux of catecholamines catalyzed by X537A.

Potentiometric measurements utilizing K^+- and H^+-sensitive electrodes and dual channel recording provide a way to measure simultaneously and quantitatively ionophore-induced K^+ and H^+ fluxes. In these experiments, granules were suspended in choline chloride and K^+ and H^+ activities outside the chromaffin vesicles were recorded. The addition of FCCP produced only a minute release of K^+ (Fig. 5 A) and an external acidification. This H^+ release was larger than expected but can be explained by the acidity of the intravesicular space (see following section). The subsequent addition of valinomycin facilitated the movement of a counterion and K^+ efflux and proton influx could occur. Valinomycin addition (Fig. 5 B) caused a K^+ release which was rapidly accelerated by FCCP addition. The initial acidification upon administration of valinomycin gave some variability depending upon the experimental conditions.

Nigericin addition resulted in a K^+ efflux and H^+ influx in a 1:1 stoichiometry (Fig. 5 D). The simultaneous addition of valinomycin and FCCP, however, failed to produce 1:1 stoichiometry. This may be attributed to the concerted action of two ionophores which need not maintain obligate coupling of ion movements, especially in the presence of different electrochemical gradients. X537A, which

is capable of complexing K^+ as well as Ca^{++}, H^+, and catecholamines, acts similarly to nigericin.

H^+ Permeability and Conductance

The preceding observations obtained with measurements of osmotic space and with turbidimetric and potentiometric measurements, all indicate that the chromaffin granule has a low permeability to K^+ and H^+. The permeability of the membrane to H^+ can be tested in another way: if the chromaffin granule
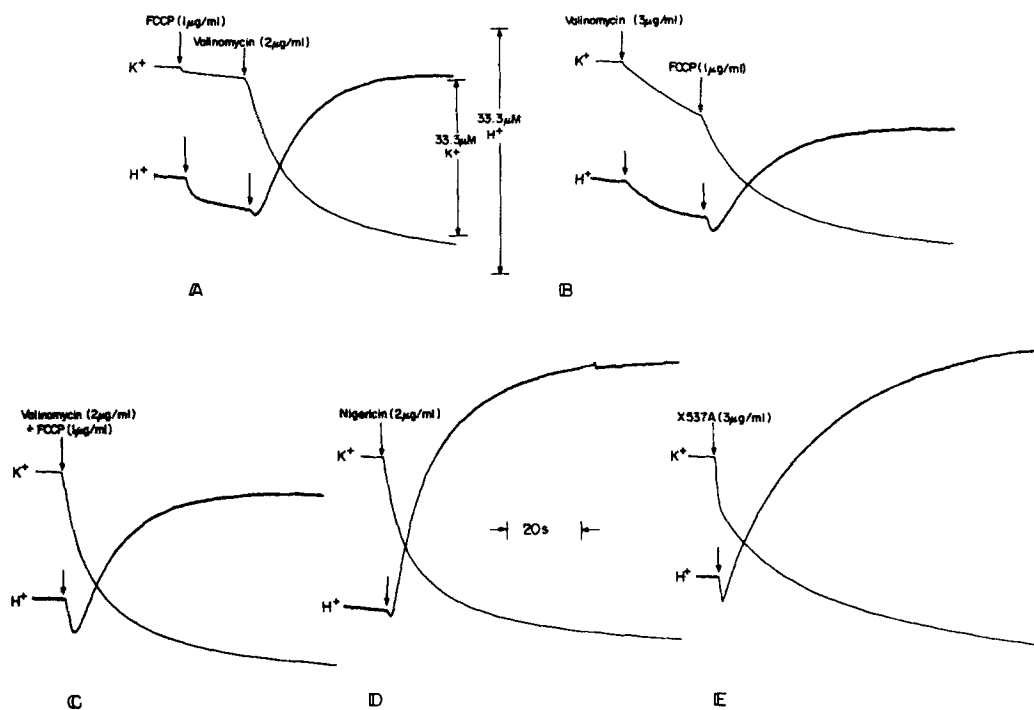

FIGURE 5 A–E. Simultaneous recordings of K^+ release and H^+ uptake induced by FCCP and valinomycin (A–C), nigericin (D), and X537A (E). The reaction mixture consisted of 185 mM choline chloride–1 mM Tris maleate (pH 6.85) and chromaffin granules (1.91 mg of protein/ml).

membrane has a low permeability to H^+ then there should be permitted only a slow acid-base equilibration between the external medium and the intravesicular space. This requirement can be tested by adding a pulse of H^+ or OH^- to a lightly buffered chromaffin granule suspension and observing the progress of the titration of the intravesicular buffering capacity with a sensitive pH recording system. Pulsed additions of acid or base to a suspension of chromaffin granules should result in: (*a*) the titration of the outer aqueous phase and of the part of the granules exposed to the suspension medium; and (*b*) the titration of the intravesicular space separated from the medium by the chromaffin granule membrane. The conductance of the membrane to H^+ or OH^- can be calculated by the time course of (*b*) according to the equation (40):

$$C_M = \frac{B}{t^{1/2}} \ln 2$$

where C_M is the conductance expressed as micrograms ions H^+ or OH^-/second pH unit gram of protein, B is the buffering power differential, and $t^{1/2}$ the time for half-equilibration of the slow phase. When the suspension is pulsed with OH^- (Fig. 6 A), it is seen that a re-equilibration does not occur and the change in the external pH persists. Similar results are also obtained for an H^+ pulse (data not shown). K^+ addition produced a more rapid equilibration which was increased in the presence of valinomycin. When FCCP is added an extremely rapid re-equilibration occurrs. The detergent Triton X-100 abolishes the impermeability feature of the membrane and causes the membrane to become freely permeable to protons so that rapid equilibration can occur. The value of the ΔpH in the presence of Triton is taken as the measure of the total internal and external buffering capacity. If the pH on both sides of the chromaffin vesicle membrane was the same before the OH^- pulse then the equilibration value approached during re-equilibration after the OH^- pulse would be that denoted by the Triton X-100 ΔpH value. The data show that this is not the case and suggest that the intravesicular space is acidic. The external K^+, which is present in a concentration sufficient to cause a net inward K^+ gradient, allows for the optimal observation of H^+ efflux which can be coupled to K^+ influx. When the external pH is lowered to 5.55 (Fig. 6 B) and therefore the pH on both sides of the membrane before the titration approaches equality, the dramatic effects of valinomycin and FCCP upon the re-equilibration are abolished.

Because this experiment indicated that the internal space of the vesicles is acidic, methylamine distribution was utilized in order to measure the magnitude of the ΔpH across the chromaffin granule membrane. This technique, which is based upon the premise than an amine permeates only in the neutral form, has been applied with great success to various other organelles such as chloroplasts (41, 42), chromatophores (43), lysosomes (44, 45), and bacteria (46). Table I lists the effects of various media on ΔpH. It was found that in a choline chloride-10 mM Tris maleate medium a ΔpH existed of 1.16. In order to determine if this ΔpH was media dependent, the distribution experiments were undertaken in various external media (NaCl, KCl, and sucrose). The observation that the observed ΔpH did not vary significantly with the external medium is taken as evidence that the ΔpH did not arise secondarily from the establishment of a Donnan equilibrium. Moreover, the fact that the chromaffin granule membrane is largely impermeant to cations reduces the possibility of the establishment of a Donnan effect. Table II lists the effects of various ionophore and uncoupler additions and external pH on the ΔpH. Nigericin, which has been seen to catalyze a 1:1 K^+:H^+ exchange of endogenous K^+ for an external H^+, was found to cause a further acidification of the intravesicular space in accordance with its mechanism of action. Conversely, the addition of nigericin to chromaffin granules suspended in a medium containing 10 mM KCl and thus facilitating an extravesicular K^+ for an internal H^+ collapsed the ΔpH to 0.79. The largest decrease in ΔpH occurred upon addition of A23187, a dicarboxylic acid capable of transporting calcium ions in exchange for protons in a 1:2 ratio (47). Significantly, subsequent addition of nigericin resulted in a partial re-establishment of

the ΔpH. The external pH was varied over the range 4.50–7.85 in order to determine the effect on the ΔpH. It was found that a change in the external pH produced a corresponding and equal change in the ΔpH between pH 5.85 and 7.85 from 0.3 to 1.9. Below pH 5.50 the calculated ΔpH was very small (0.1 pH)

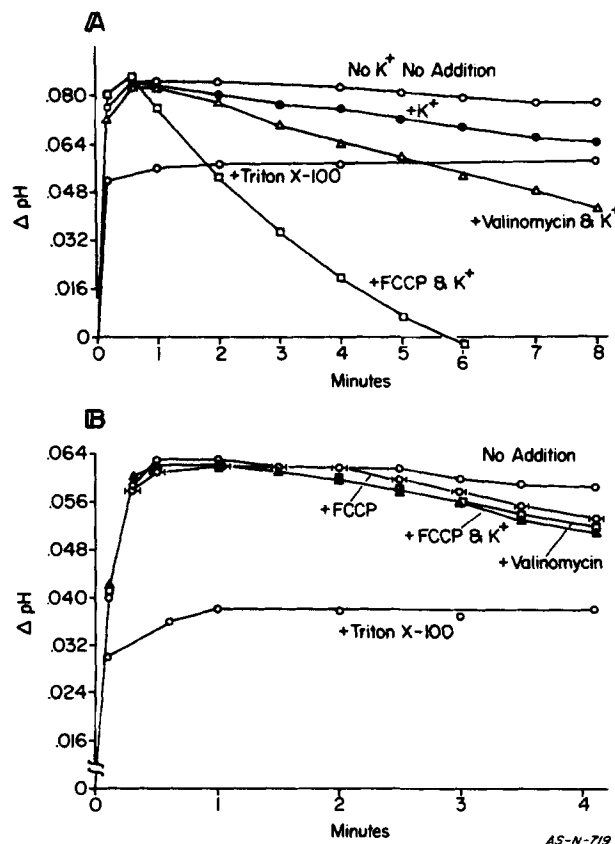FIGURE 6 A, B.   Time course of the pH changes of the external solution during pulsed base titrations of chromaffin vesicles (4.4 mg of protein/ml). The reaction mixtures contained, in addition to chromaffin vesicles, 185 mM choline chloride-1 mM Tris maleate at pH 6.95 (A) and 185 mM choline chloride-2 mM Pipes-Tris at pH 5.55 (B). At time zero 5 μl of 50 mM NaOH was injected. Triton X-100 (final concentration 0.1%) was added 5 minutes before the base pulse. The final concentrations of the additions were: K^+ (8.3 mM), valinomycin (1.49 μg/ml), and FCCP (1.49 μg/ml).

and was independent of the external pH. Extrapolation of the values obtained from pH 5.85–7.85 to zero ΔpH yielded a pH value of 5.50 which is taken as the value of the intravesicular pH.

Ca^{++} and Mg^{++} Permeability

When simultaneous K^+ and H^+ movements were monitored in the presence of A23187, it was found that there was no K^+ movement (Fig. 7) but a rapid acidification of the medium was found in the presence of external Mg^{++} or Ca^{++}.

This H$^+$ release could be blocked in the presence of the calcium chelator EGTA (Fig. 7 C). However, it rapidly appeared upon subsequent addition of 25 μM Mg^{++}. The effect of nigericin upon K$^+$ and H$^+$ gradients (Fig. 7 D) is shown for comparison.

In order to determine if Mg^{++} was indeed being transported into the granule via the ionophore A23187, the concentration of Mg^{++} in the external medium was measured by atomic absorption spectrophotometry. When Mg^{++} was added to the external medium in the absence of the ionophore, it was found that it

TABLE II

EFFECT OF IONOPHORES AND EXTERNAL pH ON ΔpH

Medium*	[¹⁴C]Polydextran space / ³H₂O Space	[¹⁴C]Methylamine / ³H₂O Space	[¹⁴C]Methylamine (IN) / [¹⁴C]Methylamine (OUT)	$\Delta pH = \log \frac{C_{in}}{C_{out}}$
Experiment I				
Control (185 mM choline chloride-10 mM Tris maleate)	0.57	6.84	14.6	1.16
+ Nigericin (10 μg/ml)	0.51	11.5	22.2	1.34
+ 10 mM KCl + nigericin (10 μg/ml)	0.52	3.46	6.11	0.79
+ 1 mM CaCl₂ + A23187 (10 μg/ml)	0.55	1.58	2.31	0.37
+ 1 mM CaCl₂ + A23187 (10 μg/ml) + nigericin (10 μg/ml)	0.48	1.98	2.90	0.46
Experiment II				
0.27 M Sucrose-20 mM Tris Mes				
pH 4.50	0.46	1.16	1.29	0.11
pH 5.35	0.46	1.13	1.30	0.11
pH 5.70	0.36	1.43	1.67	0.23
pH 6.05	0.36	2.40	3.20	0.51
pH 6.55	0.36	5.65	8.26	0.91
pH 7.50	0.32	30.3	44.1	1.64
pH 7.85	0.35	47.4	86.1	1.93

* Experiment I: Parallel experiments were performed under the same conditions in the presence of (a) [¹⁴C]polydextran and ³H₂O, and (b) [¹⁴C]methylamine and ³H₂O. Granules were added to the indicated medium (pH 6.85) so that the content was 11.5 mg of protein/ml. Ionophores were added at the indicated concentration and after 4 min the granules were rapidly centrifugated. 10 mM KCl and 1 mM CaCl₂ were added as indicated, before incubation.
Experiment II: Conditions were identical to those of Experiment I except that no ionophores were added and pH was varied as indicated.

could all be recovered in the supernate (Fig. 8). However, addition of A23187 resulted in a dramatic disappearance of Mg^{++} from the medium.

When calcium uptake was measured by the calcium indicator dye Arsenazo III, it was found that the Ca^{++} added was not taken up by the granules until the addition of A23187, which produced a rapid Ca^{++} uptake (Fig. 9 A). This uptake could be partially or totally inhibited by the appropriate addition of Mg^{++} (Fig. 9 B). The steady-state uptake of Ca^{++} at varying external calcium concentrations was progressively decreased upon increased external magnesium concentrations, with complete inhibition of Ca^{++} uptake at all external calcium concentrations studied occurring at 100 μM Mg^{++}. When the kinetics of calcium uptake and H$^+$ release were measured at various A23187 concentrations, calcium uptake and H$^+$ release were found to follow the same time course with an average ratio

of 1 Ca^{++} accumulated per 2 H^+ released (Fig. 10). Thus, it can be concluded that: (*a*) the addition of A23187 to chromaffin granules suspended in a medium containing external calcium causes calcium uptake in exchange for H^+ in a ratio of 1:2; (*b*) the uptake of calcium can be inhibited by Mg^{++} which is also transported by the ionophore; and (*c*) the calcium and magnesium found intravesicularly are predominantly bound.
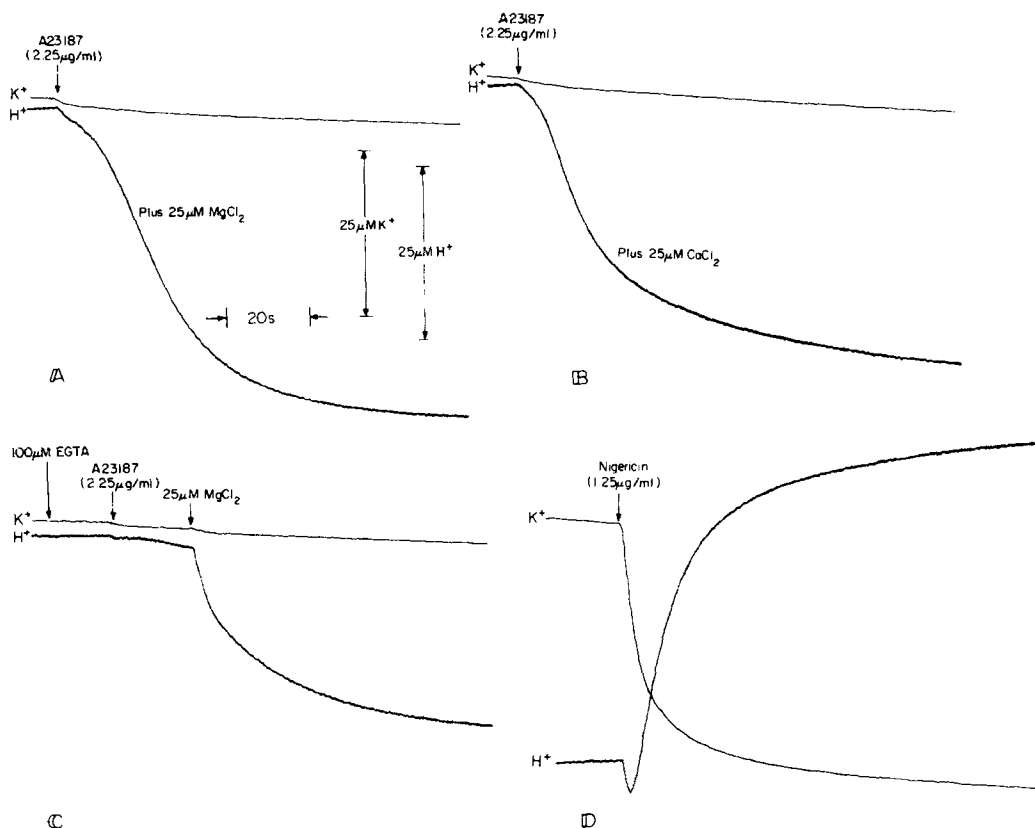

FIGURE 7 A-D. Simultaneous recordings of K^+ release and H^+ uptake in the presence of A23187 (A-C) and nigericin (D). The reaction mixture consisted of 185 mM choline chloride-1 mM Tris maleate (pH 6.85) and chromaffin granules (1.95 mg of protein/ml).

While it is clear that calcium does not penetrate the chromaffin granule membrane before the addition of A23187, it is nonetheless possible that calcium is freely permeable but that uptake cannot occur due to the absence of an exchangeable cation or anion. Fig. 11 A shows that no Ca^{++} uptake was detected after addition of valinomycin which facilitates K^+ diffusion across the membrane. The fact that externally added Ca^{++} is unable to penetrate the granules driven by endogenous K^+ gradients is taken as further evidence that the membrane of the granules is impermeable to Ca^{++}. The addition of FCCP also failed

to produce Ca^{++} uptake (data not shown). The subsequent addition of FCCP, after A23187 administration, had no effect upon the steady state calcium levels. The addition of nigericin, after calcium uptake via A23187 (Fig. 11 B) or before A23187 addition (Fig. 11 C) produced an increased uptake of calcium. However, in the presence of external K$^+$ this effect was abolished (Fig. 11 D). The increased Ca^{++} uptake upon addition of nigericin can be explained on the basis of the H$^+$-releasing mechanism of A23187 wherein a significant uptake of calcium is accompanied by a large H$^+$ efflux. The increased internal pH may limit either the binding of Ca^{++} to an intravesicular site or the exchange of Ca^{++} for H$^+$ by A23187. The addition of nigericin, which in a K$^+$-free medium would tend to
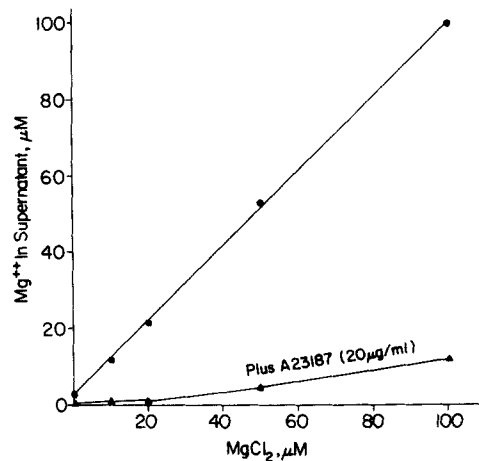

FIGURE 8.   The effect of A23187 on Mg^{++} uptake. The reaction mixture contained 0.27 M sucrose-10 mM Tris maleate (pH 6.85), chromaffin granules (2.55 mg of protein/ml), and the concentration of MgCl$_2$ as indicated in the abscissa. The final volume was 2.0 ml. A23187 (20 μg/ml) was added to one half of the tubes while the same amount of ethanol was added to those serving as controls. After 5 min, the tubes were centrifuged in a Sorvall RC-2B centrifuge at 8,500 rpm for 30 min and the supernate was removed and assayed for magnesium content as described in Materials and Methods.

increase the intravesicular concentration of H$^+$, may relieve this pH restriction and further uptake can occur. This claim is further supported by the observation that nigericin addition during the initial phase of A23187-induced calcium uptake fails to increase the rate although it does increase the extent of calcium uptake (data not shown).

X537A, the carboxylic acid ionophore which is capable of translocating K$^+$, H$^+$, calcium, and catecholamines in various natural and artificial membranes, failed to produce an uptake of calcium at low concentrations (Fig. 12). Even at a high concentration of X537A, the amount of calcium uptake is far less than that induced by A23187. This observation provides supplementary support to the conclusion, which was previously reported, that X537A is capable of direct facilitation of catecholamine gradient equilibration (23). The fact that X537A is able to release catecholamines without perturbing the calcium gradient provides

a method to determine if the nature of the internal calcium binding sites is directly related to catecholamine stores. When X537A is added to the chromaffin granule suspension after calcium uptake by A23187 has reached a steady state, an additional calcium uptake is seen (Fig. 13 A). However, when external K^+ (5
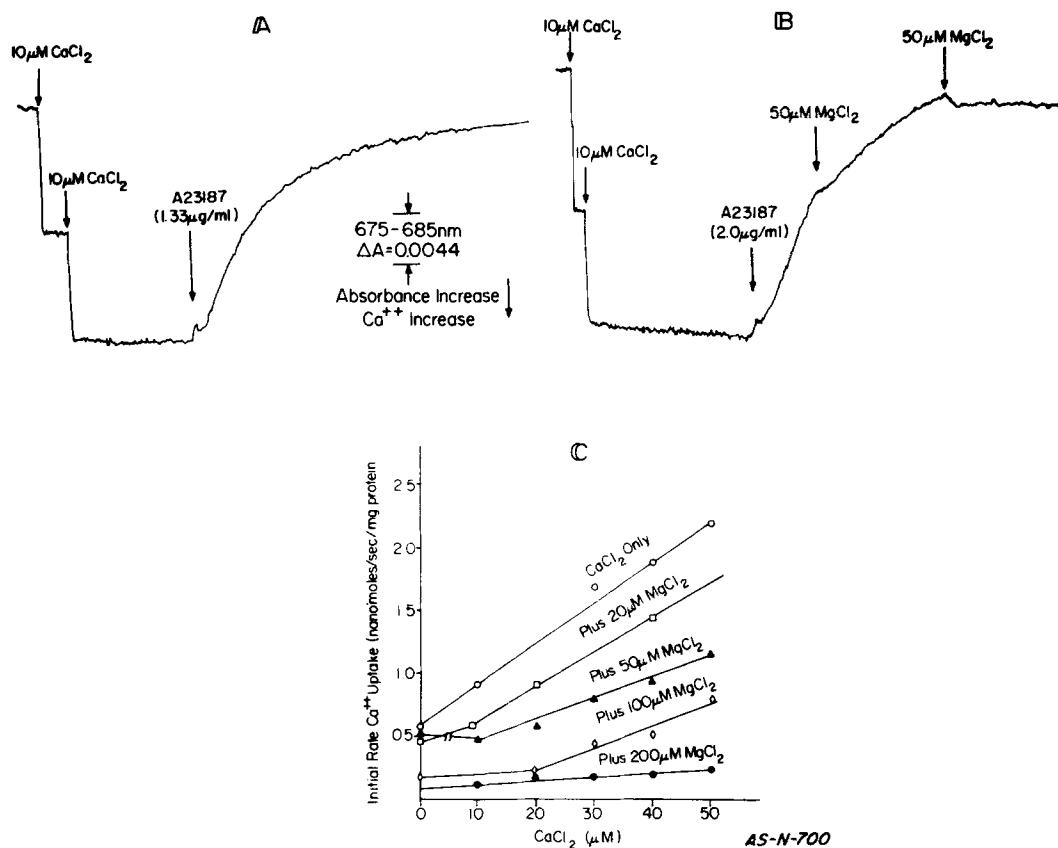

FIGURE 9 A-C.   Time course of calcium uptake mediated by A23187 in isolated chromaffin vesicles (A), the effect of Mg^{++} upon the Ca^{++} uptake (B), and a plot of the initial rate of Ca^{++} uptake at various Mg^{++} concentrations (C). The reaction mixture contained 0.270 M sucrose-10 mM Tris maleate (pH 6.95), 30 μM Arsenazo III, chromaffin vesicles (0.45 mg of protein/ml), and the concentrations of $MgCl_2$ and $CaCl_2$ indicated in the figure.

mM) is present this effect of X537A is abolished (Fig. 13 B). It appears likely that X537A is acting by the same mechanism as nigericin in this case, i.e. $K^+:H^+$ exchange resulting in reacidification of the intravesicular space. In the presence of external K^+ this exchange occurs in the opposite direction and the effect is abolished. It has already been shown that X537A is capable of such a $K^+:H^+$ exchange (Fig. 4 C). It was also observed that significant calcium uptake by A23187 occurred even when over 95% of the endogenous catecholamines were released by previous addition of X537A (Fig. 13 C). This calcium uptake was
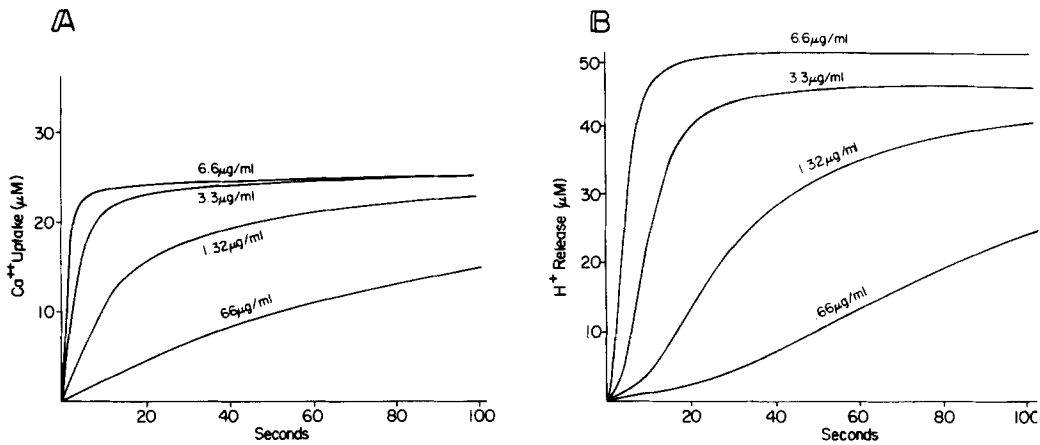
FIGURE 10 A, B.   Kinetics of Ca^{++} uptake (A) and H^+ release (B) as a function of A23187 concentration. The reaction mixture contained 0.27 M sucrose-10 mM Tris maleate (pH 6.95), 30 μM Arsenazo III, and chromaffin granules (1.39 mg of protein/ml). Tris maleate was decreased to 2 mM in B. Reaction volume was 3.0 ml.
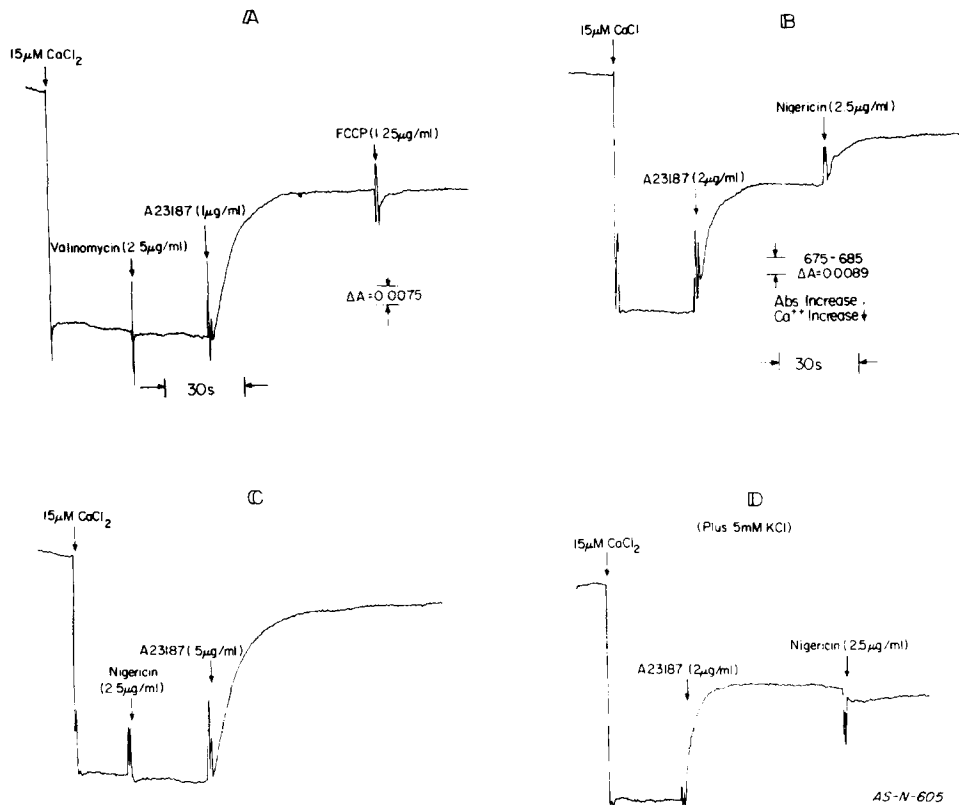

FIGURE 11 A–D.   The effect of valinomycin, FCCP, and nigericin upon A23187-mediated calcium uptake. The reaction mixture was identical to that in Fig. 9 except for the concentration of chromaffin vesicles which was 0.50 mg of protein/ml.

decreased if the reaction proceeded in the presence of external K$^+$ (Fig. 13 D). These results indicate that calcium can be bound intravesicularly in the absence of catecholamines and supports the previous observation that the magnitude of the uptake is dependent upon the internal pH.

Fig. 14 shows that steady-state calcium uptake via A23187 in isolated chromaffin granules as a function of the Ca^{++} concentration results in a curve possessing a linear portion from 0 to 40 μM external calcium and a saturable region above 40 μM. The linear nature is indicative of the presence of high affinity noninteracting binding sites for Ca^{++}. At the point where the saturation effect beings, the steady-state Ca^{++} uptake amounts to 50 nmol/mg protein.

Cation Movements and Catecholamine Release

Of profound physiological significance is whether calcium uptake via A23187 is capable of producing catecholamine release. Catecholamine release can be measured directly by monitoring the formation of adrenochrome and noradren-
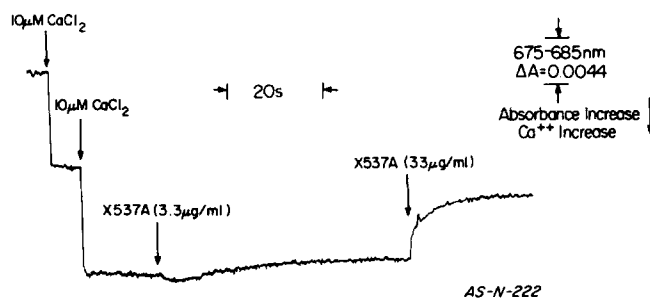

FIGURE 12.   The kinetics of calcium uptake in isolated chromaffin vesicles induced by X537A. The reaction mixture was similar to that of Fig. 9.

ochrome, the oxidation products of epinephrine and norepinephrine, which are formed by using ferricyanide as the oxidant and measured in a double-beam spectrophotometer. X537A which has been shown to release at least 95% of the endogenous catecholamines (23) can be utilized to determine the maximum catecholamine release. The results of such an experiment are presented in Fig. 15. The addition of X537A (in the absence of externally added calcium) produced a rapid efflux of catecholamines. However, when A23187 was added in the presence of varying amounts of external calcium, no significant catecholamine release occurred until the external calcium concentration approached 1 mM and even at this concentration, catecholamine efflux was a slow process. It was determined by separate measurements that the maximum steady-state calcium uptake with A23187 occurred at 30 μM external calcium and thus catecholamine release seen at an external calcium concentration of 1 mM was not occurring via a process related to intravesicular levels of calcium. Similar results were obtained when a 185 mM KCl-10 mM Tris maleate medium was substituted for the sucrose medium used in the experiment presented in Fig. 15 (data not shown), i.e. no catecholamine release was seen upon the addition of calcium or the subsequent addition of A23187 until the Ca^{++} levels reached millimolar values. Moreover, the rate and extent of calcium uptake with A23187 was found
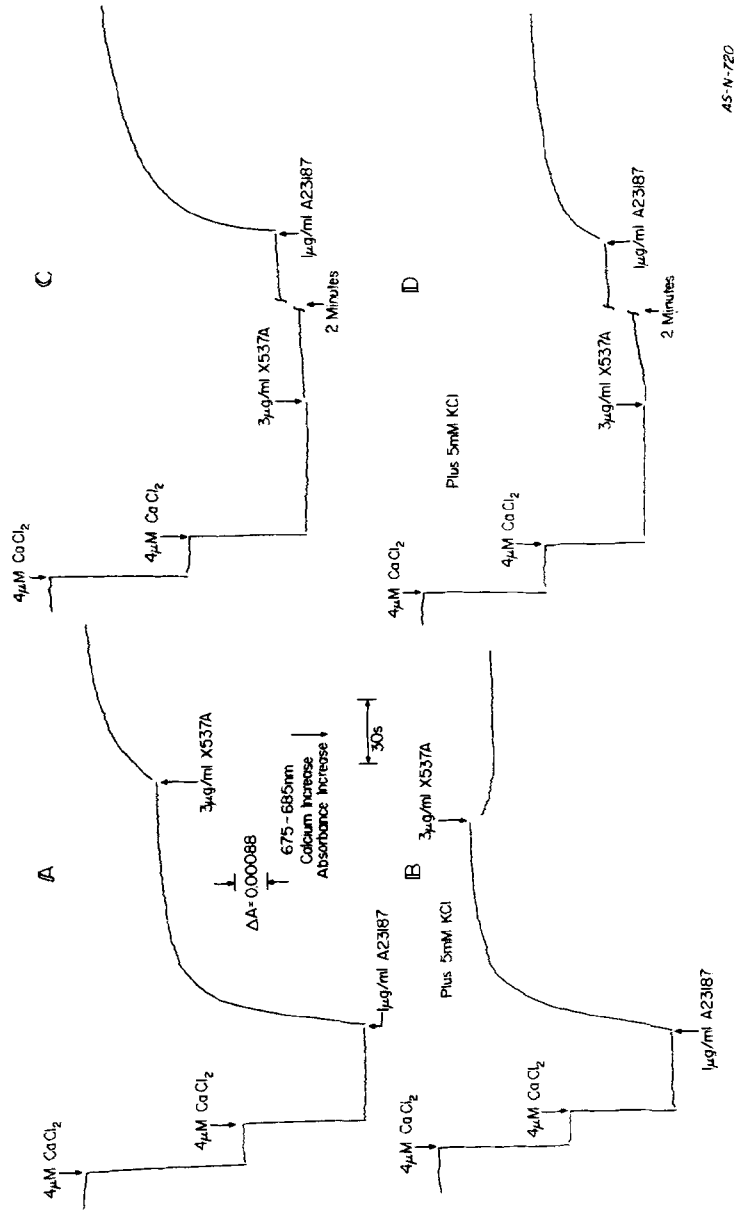
FIGURE 13 A–D. The effect of subsequent additions of A23187 and X537A on calcium uptake in isolated chromaffin vesicles. The reaction mixture was similar to that in Fig. 9 except for the concentration of chromaffin vesicles which was 0.135 mg of protein/ml.
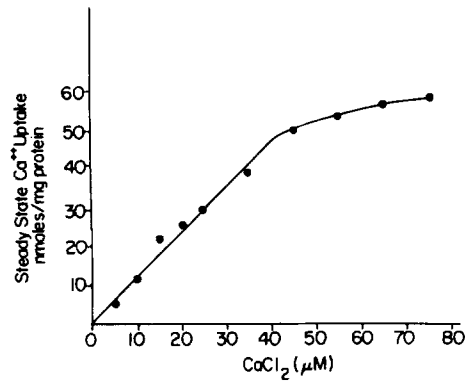
FIGURE 14. Steady-state values of calcium uptake mediated by A23187 as a function of the external Ca^{++} concentration. Experimental conditions were as follows: 0.27 M sucrose-10 mM Tris-maleate (pH 6.95), 30 μM Arsenazo III, chromaffin granules (0.34 mg of protein/ml), and calcium as indicated. A23187 (5 μg/ml) was added to initiate the reaction and the values of Ca^{++} uptake represent the values after the achievement of steady-state levels (approximately 1 min).
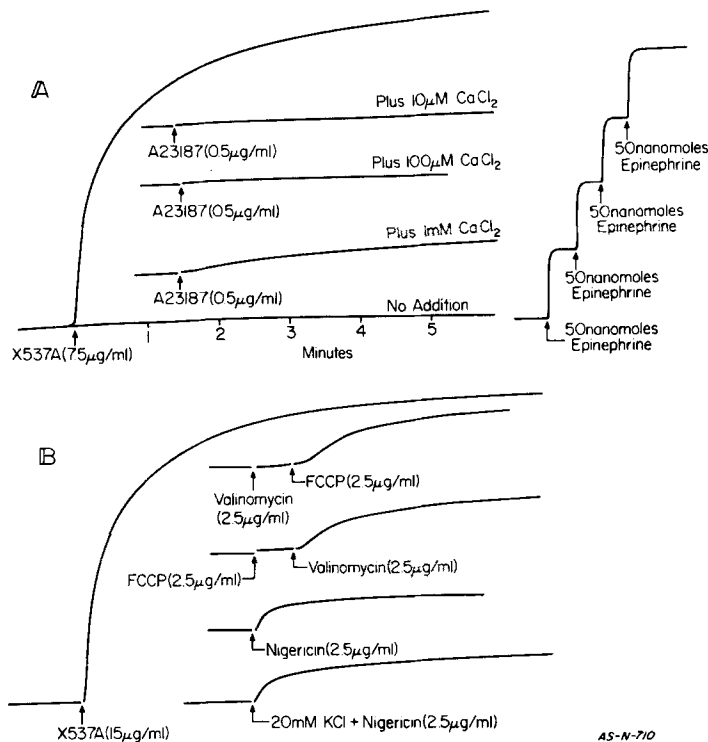

FIGURE 15 A, B. The effect of various ionophores upon catecholamine release in isolated chromaffin vesicles. The reaction medium consisted of 0.27 M sucrose-10 mM Tris maleate (pH 6.95), 0.25% ferricyanide, and chromaffin vesicles (0.18 mg of protein/ml). Catecholamine release was monitored as adrenochrome formation in a double beam spectrophotometer at 540-520 nm.

to be similar in both KCl and sucrose media. These experiments determine that an increase in the intravesicular calcium concentration is incapable of releasing significant intragranular stores of catecholamines (greater than 1%).

Also shown are the effects of valinomycin, FCCP, and nigericin upon the release of catecholamines (Fig. 15B). Valinomycin and FCCP can release catecholamines only when both exist simultaneously with neither alone producing a significant release. Nigericin is seen to cause a small release in the presence or absence of external K^+. That in the former case nigericin would tend to decrease the ΔpH and in the latter to increase the ΔpH is taken as evidence that the release is not dependent upon the ΔpH. Moreover, the observation that A23187, which also causes a severe collapse of the ΔpH, does not cause release, supports the claim. Thus, release of catecholamines by nigericin or FCCP plus valinomycin may occur via redistribution of K^+ gradients. This observation may relate to the necessity for the chromaffin granule's maintaining a low permeability to K^+.

DISCUSSION

The understanding of mechanisms of catecholamine storage and distribution rests upon the knowledge of the physicochemical properties of the chromaffin granule membrane. In this investigation a variety of techniques were employed to obtain unequivocal measurements of certain properties of the chromaffin granule membrane, relating particularly to ionic permeability and volume regulation. The results provide some insight into the mechanisms of catecholamine release and raises intriguing possibilities as to the fundamental processes underlying the mechanism of uptake of catecholamines and maintenance of catecholamine stores.

While chromaffin granules were first isolated in 1953 (48), the initial studies were hampered by the questionable purity of the fractionated material. Later, preparations were performed on sucrose gradients which dramatically increased the purity of the preparation (49, 50). However, since isolation was undertaken in 1.6 M sucrose, osmolarity was not preserved and rapid lysis occurred when the hypertonic sucrose was replaced by isotonic media. Relatively recently, a preparation has been reported based upon the utilization of a D_2O-Ficoll-sucrose gradient (22). This procedure provides a relatively pure fraction of chromaffin granules and, most importantly, maintains isotonicity. It has been shown in this study that chromaffin granules exhibit a high degree of fragility when placed in a medium which is hypotonic. However, it has also been shown that chromaffin granules isolated by the Ficoll-sucrose-D_2O technique and suspended in a medium of metal ions of sufficiently high osmolarity (greater than 340 mosM) are capable of regulation of volume and maintenance of catecholamine stores for an extended period.

Because earlier studies have utilized chromaffin granule preparations which possessed a high degree of contamination by other subcellular organelles, particularly mitochondria and lysosomes, or failed to maintain isotonicity throughout the isolation procedure, the conclusions reached from these studies, particularly with regard to the fragility, the passive permeability, and the ability of the

chromaffin granule to maintain internal catecholamine stores, must be re-evaluated.

Impermeability to Ions

Studies with photometric, potentiometric, and radiochemical techniques indicate that even when one ion is rendered freely permeable to the chromaffin granule membrane there is no corresponding movement of the counterion in spite of an exceedingly high gradient. These results, which indicate that the chromaffin granules are impermeable to Na^+, K^+, H^+, Mg^{++}, and Ca^{++}, suggest that the chromaffin granule membrane may possess one of the lowest permeabilities to cations of any known subcellular organelle. While the net anion fluxes were not quantitated as they were for the cations, it is clear from turbidity measurements using a freely permeable cation that even in the presence of an exceedingly large downhill gradient for anions, the rate of permeation of several anions was relatively low. This may have a tremendous significance for the mechanism of uptake and release and the role which the chromaffin granule membrane must play in maintenance of catecholamine stores. The chromaffin granule is distinguished from other intracellular organelles by its high ratio of cholesterol to lipid and its extremely high content of lysophosphatidylcholine which may represent up to 20% of the total lipid-phosphorus. The high content of cholesterol and lysophosphatides may result in the existence of a unique lipid milieu in the chromaffin granule membrane which may account for its permeability properties.

H^+ Conductance

Experiments wherein the permeability of the membrane to an ion was made quite high by the addition of the appropriate ionophore allowed determination of the passive permeability of the counterion. These experiments showed that even in the presence of a significant H^+ gradient the net flux of H^+ was quite low (Fig. 5). When an acid-base titration was undertaken at a pH at which the H^+ gradient was minimal (pH 5.55), only a very slow re-equilibration was observed (Fig. 6 B). Due to technical difficulties resulting from measurements at low pH and the resolution of the measuring equipment, a precise measurement of conductance could not be undertaken. However, a value for conductance representing the upper limit for proton conductance could be calculated and was found to be 0.088 μg ion H^+/s pH unit g of chromaffin granules. This value compares with the values obtained for those organelles which maintain the lowest measured conductance to protons (51); for mitochondria, 0.075 μg ion H^+/s pH unit g. and for *Micrococcus denitrificans* of 0.071 μg ion H^+/s pH unit g. Since this calculation sets the maximum level for proton conductance for the chromaffin granules it is likely that the actual conductance is lower than that given above and may prove to be the lowest of any known organelle.

ΔpH

The existence of a ΔpH across the chromaffin granule membrane was indicated by the observation of H^+ release in the presence of valinomycin plus K^+ and by

the "overshoot" on the acid-base titration in the presence of FCCP. Moreover, these effects could be elicited from granules which were several days old. Because the volume of the granule does not permit a direct measurement of the internal pH with a microelectrode, methylamine distribution was used in order to obtain a measure of the ΔpH across the chromaffin granule membrane. The values obtained indicate that the internal pH is maintained at pH 5.50 and is not affected by changes in the external pH.

Under steady-state conditions, the margin of error in this technique involves mainly the magnitude and extent of binding of methylamine and this was excluded for the following reasons. First, when disrupted membranes were prepared by lysis of chromaffin granules in distilled water and overnight dialysis, the measurement of the methylamine distribution ratio yielded a ΔpH value corresponding to 0.1. When lysis was initiated by resuspension of the chromaffin granules in 20 mM Tris maleate a distribution ratio corresponding to a value of 0.3 pH units was obtained which could be due to binding and/or incomplete lysis and set an upper limit for the contribution of binding to the calculations. Second, doubling the methylamine concentration produced no effect on ΔpH measurements. Third, the addition of ionophores and uncouplers known to perturb proton gradients was able to alter the ΔpH in a predictable way in the chromaffin granules.

The physiological role of the ΔpH is speculative at this time, although the possibility exists that it may serve at least two functions. First, because catecholamines are rapidly oxidized at alkaline pH (52), the low intravesicular pH may serve to maintain the stores in the unoxidized form. Second, if catecholamine permeation of the chromaffin granule membrane occurs via the uncharged species (as does methylamine permeation) then the possibility exists that catecholamines can distribute across the membrane according to the ΔpH. If the intravesicular space is maintained at pH 5.5 and the cytosolic pH is in the region 7.2-7.4 (based upon measurements in cells from other tissues [53-55]), then a pH difference of 1.7-1.9 exists. If the catecholamines are capable of distributing according to the ΔpH, then a distribution gradient could occur of 50-80:1 based purely upon the existence of a ΔpH and the passive permeability of the chromaffin granule to uncharged catecholamines. The high degree of passive ion impermeability may play a role in the maintenance of the ΔpH and of the intragranular catecholamine stores which are both sensitive to changes in their ionic and osmotic environment. It is likely, of course, that most of the intragranular catecholamine stores exist in a bound form. Currently under investigation is the process by which the H^+ gradient is generated and maintained. The primary candidate is a proton-translocating ATPase similar at least conceptually to that observed in submitochondrial particles and chloroplasts. An additional possible physiological role for the acidic intravesicular pH deals with the enzymatic activity of dopamine-β-hydroxylase. The enzyme has been reported to have a pH optimum at 5.5 and to be virtually inactive at pH 7.0 (56).

Calcium and Magnesium Permeability

It was conclusively shown that the chromaffin granule membrane maintains a

low permeability to calcium. Even when a downhill gradient of endogenous K^+ or H^+ was made available for exchange with calcium, no net inward flux of calcium was observed (Fig. 11). Only when the permeability was induced by a calcium ionophore was calcium accumulated against an apparent concentration gradient. However, since the calcium ionophore can only facilitate the equilibration of calcium across the membrane, the magnitude of the uptake is a result of the accessibility of Ca^{++} (or Mg^{++}) to endogenous binding sites.

It has been reported that the intravesicular calcium content of isolated chromaffin granules is 42 nmol/mg of protein (57). If this is the case, then the steady-state calcium uptake data (Fig. 14) indicate that the intravesicular calcium content can at least be doubled since in the presence of A23187 an uptake of 50 nmol/mg of protein was measured at an external calcium concentration of 40 μM. It is not known whether the sites which calcium occupies were occupied previously. However, it is known that appreciable Ca^{++} can be bound inside the granules in the absence of catecholamines (Fig. 13 C). A potentially powerful technique exists for probing the nature of this site. By utilizing Arsenazo III and a wavelength pair specific for Mn^{++} (655–640) it was found that the chromaffin granule could accumulate Mn^{++} in the presence of A23187 (data not shown). Due to manganese's paramagnetic properties, it may be possible to explore the nature of the internal binding site by EPR techniques.

The impermeability of the chromaffin granule membrane to calcium makes it unlikely that transient changes in cytosolic calcium concentration are capable of causing release of intravesicular catecholamine stores. That even an increase in intravesicular calcium is incapable of causing catecholamine release was unequivocally shown through the use of the calcium ionophore A23187 which was able effectively to double the intravesicular content of calcium without initiating catecholamine release. Since the obligatory nature of calcium as the stimulus-secretion coupling element is well characterized, any theory which proposes release from chromaffin granules far removed from the chromaffin cell membrane must effectively deal with the obtained results. Previous reports which noted that external calcium was capable of releasing catecholamines used calcium concentrations two to three orders of magnitude higher than cystolic levels (58, 59). Since the internal binding sites for calcium are saturated in the micromolar range, the releasing effect of calcium at high concentrations probably is due to its effect on the external membrane phase. Recent evidence shows that calcium can mediate the fusion of lipid vesicles (60). While it is clear that such a process could not occur deep within the chromaffin cell, a similar process has been proposed to occur at the cell membrane where the transient local calcium concentration may approach large values (20, 61, 62). Inherent in discussions of this type, however, is the caution which must be exercised in attempting to extrapolate results obtained from isolated granules to the process of stimulus-secretion coupling in the intact cell.

The role which the chromaffin granule plays in calcium homeostasis is speculative at this time. While it is clear from this study that chromaffin granules can bind significant quantities of calcium intravesicularly and there have been reports that the calcium content of chromaffin granules isolated from rapidly

stimulated adrenal glands is increased (63), the mechanisms of calcium translo-cation and regulation are unknown. There have been several reports of a calcium-stimulated ATPase activity in the chromaffin granule membrane (1, 64). However, an uptake of calcium by an Mg^{++}-ATPase-stimulated reaction in intact granules has not been reported.

Measurement of Catecholamines

Until now, kinetic measurements of catecholamine release and uptake have been limited by the absence of techniques which allow for the continuous measure-ment of catecholamine fluxes. Certain previous studies (65, 66) utilized light-scattering techniques to investigate release but until now samples had to be taken from incubation mixtures and pelleted before catecholamine content could be measured. However, two techniques are presented (Fig. 16) which can be used to monitor continuously catecholamine uptake or release. In the first, catecholamine release can be monitored by observation of the formation of the oxidation products of the catecholamines using ferricyanide as the oxidant and double beam spectroscopy. This was the method used in Fig. 15. This method is limited only by the rate of oxidation of the released catecholamines. It can be seen from the calibration curve (Fig. 16 A) that the addition of exogenous catecholamines results in an instantaneous measurement of the oxidized catecholamine on the time scale upon which the observation was made. Release by the continuous monitoring of oxidation products is compared with data obtained by sampling a similar mixture, centrifugating the suspension, and determining the catecholamine content of the supernate (Fig. 16 B).

It has recently been reported that the potential exists for monitoring catechol-amine release and uptake based upon the endogenous fluorescence properties of the catecholamines, in particular the property that release of bound catechol-amines results in a fluorescent enhancement (30). This prediction is confirmed by monitoring the fluorescence of the endogenous catecholamines in the pres-ence of various concentrations of X537A (Fig. 16 C). It is readily observed that qualitatively similar curves were generated by this method when compared to the two previous methods. Quantitatively, the curves are shifted due to the use of different volumes and chromaffin granule content. While release via X537A is readily shown in this case, the possibility exists, at least theoretically, that Mg^{++}-ATP-stimulated uptake of catecholamines could be monitored by fluorescent quenching. However, the practical application is subject to severe technical problems and good qualitative data are difficult to obtain. The combination of these two techniques provides a still more potentially powerful method for catecholamine measurement. Since oxidized catecholamines do not fluoresce, the possibility exists of simultaneously monitoring the release of catecholamines by their oxidation products as well as the intravesicular amount and state (bound or unbound) of the remaining catecholamines by fluorescence and polarization techniques.
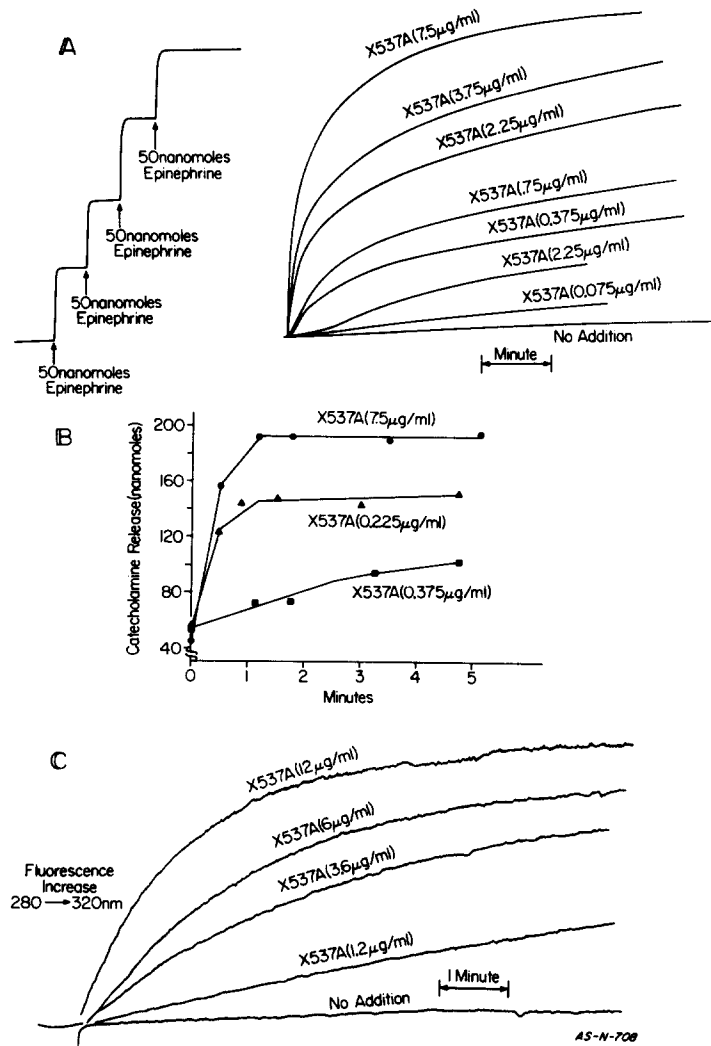
FIGURE 16 A-C. The effect of X537A upon catecholamine release as measured by direct monitoring of adrenochrome formation in the suspension (A), assay of the supernate for catecholamines after pelleting of the chromaffin vesicles (B), and direct monitoring of endogenous catecholamine release in the suspension medium by fluorescence (C). The reaction mixture for A was similar to that in Fig. 15. In B, the reaction mixture was 0.27 M-sucrose-10 mM Tris maleate (pH 6.95) and chromaffin vesicles (0.18 mg of protein/ml). After X537A addition, at the times indicated, samples were taken from the mixture and pelleted with an Eppendorf desk centrifuge. The supernate was removed and catecholamine oxidation prevented by HCl addition. Catecholamines were assayed as adrenochromes after ferricyanide addition in a dual wavelength spectrophotometer at 485–575 nm. In C, the reaction mixture contained 0.27 M sucrose-10 mM Tris maleate (pH 6.95) and chromaffin vesicles (0.240 mg of protein/ml).

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