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MECHANISMS OF ACCUMULATION OF TYRAMINE, METARAMINOL, AND ISOPROTERENOL IN ISOLATED CHROMAFFIN GRANULES AND GHOSTS

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Abstract—The effects of the transmembrane pH gradient (ΔpH) and the transmembrane potential gradient (ΔΨ) on the uptake of several sympathomimetic amines were investigated, using bovine adrenal chromaffin granules isolated in isotonic sucrose. As previously described [R. Johnson and A. Scarpa, *J. biol. Chem.* **254**, 3750 (1979)], freshly isolated chromaffin granules maintain an intragranular pH of 5.5 as measured by [^{14}C]methylamine distribution and, in the presence of ATP, generate a ΔΨ of 80 mV, positive inside, as measured by [^{14}C]thiocyanate distribution. When tryamine, metaraminol, and isoproterenol (1–50 mM) were added to well-buffered suspensions of granules at pH 7.0, a dose-related alkalinization of the granule interior was observed. Study of the time-resolved influx of the same amines labeled radiochemically (5–21 μM) revealed that all the amines were accumulated against an apparent concentration gradient. However, while accumulation of [^{14}C]serotonin and [^{3}H]isoproterenol was totally inhibited by reserpine, [^{14}C]tryramine accumulation was inhibited by only 60% and [^{14}C]metaraminol uptake was unaffected. The ATP-dependent generation of a ΔΨ produced a stimulation of amine uptake in the order: serotonin > isoproterenol > tyramine; metaraminol accumulation was not enhanced by ATP addition. The relationship between the electrochemical proton gradient ($\Delta\bar{\mu}_{H^+}$) and the electrochemical gradient for each of the sympathomimetic amines ($\Delta\bar{\mu}_A$) was investigated utilizing chromaffin ghosts devoid of endogenous matrix gradients or components. All amines were accumulated in the presence of ΔpH alone. In the presence of ΔΨ alone, [^{14}C]serotonin, [^{14}C]tyramine, and [^{3}H]isoproterenol were accumulated, but no [^{3}H]metaraminol uptake was demonstrable. The results indicate that serotonin and isoproterenol accumulated in isolated chromaffin granules and ghosts via a reserpine-sensitive mechanism, driven by the magnitude of the electrochemical proton gradient. Conversely, metaraminol permeated the membrane of the chromaffin granule through the apolar lipid phase and distributed according to the ΔpH alone. Tyramine uptake proceeded by both mechanisms. The implications of the mechanism of accumulation of these potent physiologic and pharmacologic agents for their *in vivo* action are discussed.

The subcellular amine-containing organelles of the sympathico-adrenal system accumulate endogenously synthesized catecholamines and store these biogenic amines prior to their release into the synaptic cleft or the blood [1–7]. *In vitro* investigations utilizing isolated chromaffin granules and synaptic vesicles suggest that the mechanism of uptake is dependent upon a reserpine-sensitive carrier-mediated transport event which is coupled to the electrochemical proton gradient ($\Delta\bar{\mu}_{H^+}$)† across the membrane of the organelle [8–13]. In addition to catecholamines, adrenergic granules accumulate a wide variety of catecholamine derivatives which are found either endogenously or as a consequence of administration of a pharmacologic agent [1, 4, 14–18].

Tyramine is an example of a sympathomimetic amine found within chromaffin granules and in sympathetic nervous tissue in small amounts [19–23]. Despite long-standing investigation, its physiologic role and implication in pathologic states remain speculative [24]. Metaraminol, on the other hand, is a pharmacologic amine known to exert a profound effect upon many physiological variables even at low concentrations [25–27]. Previous investigations of the accumulation of tyramine and metaraminol in nerve and adrenal medullary storage granules have revealed that, in contrast to the catecholamines, these amines were accumulated into the granules by an ATP and reserpine-insensitive mechanism which was not further elucidated [28–30].

In light of the recent advances in the understanding of the stoichiometric coupling of the catecholamine accumulation to the electrochemical proton gradient, and due to the probable importance of these sympathomimetic amines in overall amine homeostasis, a reinvestigation of the uptake mechanism of other amines of physiologic and pharmacologic relevance, such as tryamine and metaraminol, was undertaken. Isoproterenol, an important pharmacologic sympathomimetic amine which binds to terminal β-

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† Abbreviations: $\Delta\bar{\mu}_{H^+}$, electrochemical gradient for protons; $\Delta\bar{\mu}_A$, electrochemical gradient for amines; DMSO, dimethylsulfoxide; ΔpH, transmembrane proton gradient; ΔΨ, transmembrane electrical gradient; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; isoproterenol, *N*-isopropylnorepinephrine (Iso); metaraminol, *m*-hydroxyphenylpropanolamine (Me); SCN^-, thiocyanate ion; serotonin, 5-hydroxytryptamine; tyramine, *p*-hydroxyphenylethylamine (Try); and Hepes, H-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

receptors [31–33], was also examined for its ability to accumulate in chromaffin granules and ghosts.

The results indicate that sympathomimetic amines are transported into isolated chromaffin granules via two mechanisms: (1) reserpine-sensitive, carrier-mediated transport driven by the $\Delta\bar{\mu}_{H^+}$, and (2) permeation of the apolar phase of the membrane in the uncharged form, driven by the ΔpH. The physiological and pathological implications of these results are discussed below.

MATERIALS AND METHODS

Preparation of chromaffin granules. Intact bovine adrenal chromaffin granules were isolated and purified by a previously published method [34] involving differential centrifugation and a continuous isotonic density gradient of Percoll. The isolation medium contained 0.27 M sucrose and 30 mM Tris–maleate (pH 7.00). The purification procedure consistently resulted in an extremely pure granule population as shown by high ATP and catecholamine content per mg protein, and minimal subcellular organelle contamination in the granule fraction compared with the other gradient fractions [34]. All experiments were done within 16 hr after animal slaughter.

Preparation of chromaffin ghosts. Chromaffin ghosts were prepared by a method published previously [11]. Briefly, freshly isolated chromaffin granules were lysed in hypotonic medium, pelleted, and resuspended in one of two media: (1) 185 mM KCl, 10 mM ascorbic acid, 5 mM Tris–maleate, pH 7.00; or (2) 185 mM sodium isethionate, 20 mM ascorbic acid, 5 mM Tris–maleate, pH 7.00. The suspension was then dialyzed overnight at 4°. After 15 hr, the ghosts were washed in fresh medium and resuspended in the same medium containing 30 mM buffer plus 10 μM iproniazid phosphate.

Measurement of ΔpH. [^{14}C]Methylamine distribution was used to determine the ΔpH across the membrane of the granules as described previously [35, 36]. The concentration of methylamine added was 8.7 μM. This is a highly reproducible method routinely used in many laboratories with a variety of subcellular organelles (for review see Ref. 37). The method is based on the observation that amines freely permeate biological membranes in the uncharged form (and only in this form), with equilibrium being reached when $[RNH_2]_{inside} = [RNH_2]_{outside}$. For amines of high p$K_a$, $[RNH_3^+]_{(inside)}/[RNH_3^+]_{(outside)} = [H^+]_{(inside)}/[H^+]_{(outside)}$. Because amines concentrate inside acidic vesicles, the logarithm of the ratio of the intragranular [^{14}C]methylamine concentration to that in the external medium (log C_{in}/C_{out}) gives a measure of the ΔpH. Chromaffin granules were incubated in [^{14}C]methylamine and ^3H_2O, or [^{14}C]polydextran and ^3H_2O (to correct for extragranular water space), as described previously [38]. Water content (^3H_2O), [^{14}C]polydextran, and [^{14}C]methylamine distribution were calculated by the relative activities in the pellet and supernatant fractions, using the equation: $C_{in}/C_{out} = R + (R - 1)\,[x/(1 - x)]$, where R = [^{14}C]-methylamine/^3H_2O space, and x = [^{14}C]polydextran/^3H_2O space.

Measurement of ΔΨ. [^{14}C]SCN^- distribution across the chromaffin granule membrane was utilized to measure the transmembrane potential. This lipophilic anion has been shown previously to permeate biological membranes and distribute in accordance with a positive, inwardly directed potential in a highly reproducible manner [37]. The distribution of [^{14}C]SCN^- (3.5 μM) was measured as discussed above for [^{14}C]methylamine distribution, and the membrane potential was calculated according to the Nernst equation: $\Delta\Psi = 58.8 \log [^{14}C]SCN^-_{in}/[^{14}C]SCN^-_{out}$.

Measurement of [^{14}C] *and* [^{3}H]*amine distribution.* The distributions of [^{14}C]-5-hydroxytryptamine (serotonin) (3.5 μM) and [^{14}C]tyramine (4.2 μM) were determined by the method described for the measurement of [^{14}C]methylamine distribution. Metaraminol and isoproterenol distributions were determined using [^{3}H]metaraminol (0.3 μM) and [^{3}H]isoproterenol (0.4 μM), plus [^{14}C]polydextran (1 mg/ml). These methods allow a rapid and highly reproducible measurement of the distribution of a labeled species across a biological membrane when expressed as a ratio of the internal to external concentration [10, 11]. Specific membrane binding was determined by two methodologies. In the first, the distribution of the labeled compound was determined in intact granules under conditions wherein the electrochemical proton gradient had been completely eliminated by the addition of FCCP (6.7 g/ml) and ammonia (50 mM). In the second, the distribution was measured in a suspension of scattered membranes formed by hypo-osmotic lysis of the chromaffin granules. Binding values from the two methods, which were closely comparable, were averaged, subtracted from the distribution values obtained under experimental conditions, and did not exceed 2% (for serotonin), 15% (for tyramine), 10% (for metaraminol), and 6% (for isoproterenol) of the observed accumulations.

Potentiometric recording of sympathomimetic amine uptake. The experimental procedure for on-line kinetic accumulation of isoproterenol and epinephrine into isolated chromaffin ghosts is described elsewhere.* In brief, amines were measured as their reversible oxidation products detected by a glassy carbon-working electrode set at a potential of 0.5 V vs an Ag/AgCl reference electrode with an auxiliary steel electrode. The glassy carbon electrode was maintained at that fixed potential with a model CV-1A amperometric controller (Bioanalytical Systems, Inc., Indianapolis, IN). The output from the controller was connected to a standard single pen recorder. The electrodes and amperometric controller were housed in a Faraday cage to minimize extraneous interference and to optimize the signal-to-noise ratio.

Calculations. The ΔpH is defined as $pH_{in} - pH_{out}$. Therefore, the ΔpH is always negative in these experiments (indicating an acidic intragranular space). Curve fitting was determined by linear regression using the method of least squares, and all data discussed exhibited a coefficient of determination (r^2) not less than 0.95.

* R. G. Johnson, S. Hayflick, S. E. Carty and A. Scarpa, unpublished data.

Materials. All standard reagents, including tyramine hydrochloride, 5-hydroxytryptamine creatinine sulfate, isoproterenol bitartrate, and reserpine (dissolved in DMSO) were purchased from the Sigma Chemical Co., St. Louis, MO. Metaraminol bitartrate was the gift of Dr. Clement A. Stone, Merck, Sharp & Dohme, West Point, PA. FCCP, obtained from the Pierce Chemical Co., Rockford, IL, was dissolved in ethanol, and the volume added did not exceed 5 μl/ml of the reaction mixture. [^{14}C]Methylamine (48.1 mCi/mmole), [$^{3}H_2O$] (1 mCi/g), [^{3}H]metaraminol (12.2 Ci/mmole), and [^{14}C]polydextran (0.503 mCi/g) were purchased from the New England Nuclear Corp., Boston, MA. [^{14}C]SCN^- (62.0 mCi/mmole), [^{14}C]serotonin (60 mCi/mmole), and [^{14}C]tyramine (50 mCi/mmole) were obtained from the Amersham Corp. (Arlington Heights, IL).

RESULTS

Amine permeation in response to the ΔpH across the chromaffin granule membrane. Isolated chromaffin granules maintain an acidic intragranular matrix space of pH 5.5 as measured by [^{14}C]methylamine and ^{31}P nuclear magnetic resonance [36, 39–43]. Due to the low endogenous permeability of the membrane to protons and to the large internal buffering capacity, this acidic intragranular pH is independent of the pH of the extragranular medium and persists for several days when isolated granules are stored at 4° [38, 39]. The effect of large external concentrations of the amines under study upon the existing ΔpH was studied to determine the intrinsic amine permeability of the membrane as described previously [11, 38]. In brief, if a weak base permeates the membrane as the neutral, lipophilic species, then reprotonation of that species within the intragranular space should result in an internal alkalinization dependent upon the magnitude of the internal buffering capacity, which is constant and has been measured previously [38]. Primary and secondary amines such as tyramine, metaraminol, and isoproterenol would thus accumulate in acidic matrix spaces based upon their pK_a values by permeating the membrane in the uncharged form. This, in fact, is the principle by which [^{14}C]methylamine distribution has proven to be efficacious in the measurement of ΔpH.

In the experiment illustrated in Fig. 1, various

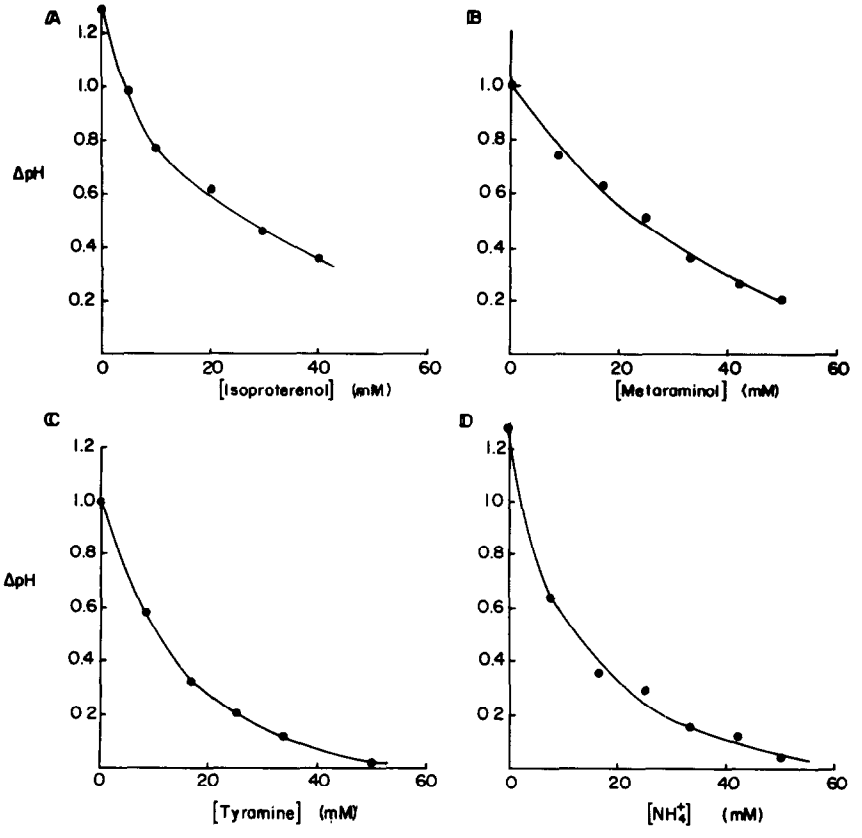

Fig. 1. Dose-dependent effect of isoproterenol, metaraminol, tyramine, and ammonia on the ΔpH in isolated chromaffin granules. Each reaction mixture contained 0.27 M sucrose, 30 mM Tris–maleate (pH 7.0), 1 mM ascorbate, 10 μM iproniazid phosphate, chromaffin granules (4.4 mg protein/ml), [^{14}C]methylamine, and $^{3}H_2O$, and either (A) isoproterenol bitartrate, (B) metaraminol bitartrate, (C) tyramine hydrochloride, or (D) ammonium sulfate at the concentrations indicated. The sample volume in each case was 1.2 ml. After 20 min the samples were centrifuged for 4 min in a model 4200 Eppendorf desk microcentrifuge. The supernatant fraction and pellet were assayed for radioactivity as described under Materials and Methods. Temperature was 24°.

concentrations of tyramine, metaraminol, isoproterenol, and ammonia were added to well-buffered suspensions of isolated chromaffin granules. For each amine, the additions resulted in a dose-dependent alkalinization of the intragranular space. That strikingly similar quantitative results were achieved for the sympathomimetic amines as for ammonia indicates that these three amines can permeate the chromaffin granule membrane in a manner qualitatively and quantitatively similar to the uptake of catecholamines, which have been found to accumulate according to the magnitude of the ΔpH [9, 11]. The observation that these curves are not precisely superimposable probably relates to: (1) the differential permeability of the associated anions (chloride ≫ bitartrate); (2) absence of steady-state conditions due to the wide range of permeabilities of the amines under investigation; and (3) various effects of the added amine and its anion upon the integrity of the intragranular storage complex.

The qualitative kinetic influx of these sympathomimetic amines into isolated chromaffin granules was then studied using radiochemically labeled amines present at concentrations too low to perturb the ΔpH. 5-Hydroxytryptamine (serotonin), a biogenic amine which maintains a high specificity for the amine transporter with excellent rates of uptake and minimal intragranular binding [1, 11, 15, 19, 44], was utilized as a control. All the amines tested (Fig. 2) exhibited a time-dependent influx into the chromaffin granules. [^{14}C]Tyramine and [^{3}H]metaraminol accumulation was similar in rate and extent to that of [^{14}C]serotonin. However, [^{14}C]isoproterenol uptake was about one order of magnitude less in rate and extent when compared to the other amines. Each of the amines was released from the granules when the ΔpH was collapsed upon the addition of large concentrations of ammonia (arrows); the lack of complete efflux is consistent with some intragranular binding of the amines [15].

Effect of reserpine upon amine accumulation. The results observed in Fig. 2 could be explained either by amine permeation across the apolar membrane phase or by specific carrier-mediated transport. To discriminate between these two mechanisms, the effect of reserpine upon amine accumulation was studied (Fig. 3). Reserpine is an extremely apolar analogue of serotonin which is an irreversible inhibitor of amine transport and is postulated to bind to a putative catecholamine carrier molecule (for review see Ref. 45). The addition of reserpine has no effect upon the ΔpH or ΔΨ across the chromaffin granule membrane [9, 10, 38]. When the uptake of sympathomimetic amines was measured as a function of reserpine concentration (Fig. 3), several conclusions could be made: (1) serotonin and isoproterenol accumulations were totally inhibited by reserpine; (2) tyramine accumulation was inhibited by 60%; and (3) metaraminol uptake was very insensitive to reserpine addition. These results suggest that serotonin and isoproterenol uptake proceeds by a carrier-mediated reserpine-sensitive process, metaraminol uptake by a process similar to that of methylamine and ammonia (via permeation of the uncharged species across a lipid moiety), and tyramine accumulation by both pathways.

Effect of ATP addition upon accumulation of sympathomimetic amines into isolated chromaffin granules. The addition of ATP to chromaffin granules suspended in a medium of impermeant anions results in the generation of a transmembrane potential (ΔΨ), inside positive, of 80 mV without a measured change in the ΔpH; the presence of FCCP abolishes

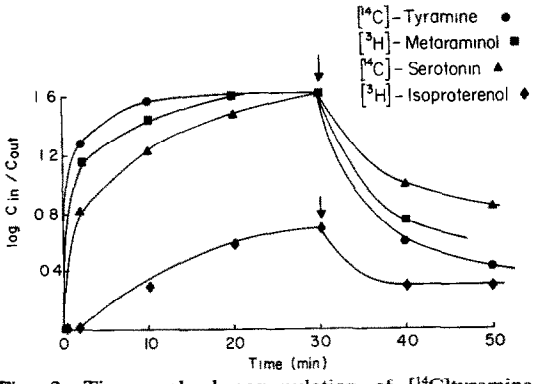

Fig. 2. Time-resolved accumulation of [^{14}C]tyramine, [^{3}H]metaraminol, [^{14}C]serotonin, and [^{3}H]isoproterenol in isolated chromaffin granules, expressed as the logarithm of the ratio of the internal to external concentrations. The reaction mixtures contained 0.27 M sucrose, 20 mM Tris–maleate (pH 7.00), chromaffin granules (1.7 mg protein/ml), 1 mM ascorbate, 10 μM iproniazid phosphate, radioisotope-labeled amine, and either $^{3}H_2O$ or [^{14}C]polydextran. $(NH_4)_2SO_4$ (30 mM) and FCCP (5.00 μg/ml) were added after 30 min had elapsed (arrows). The total initial volume was 10 ml. At the times indicated, 1.2 ml samples were centrifuged and assayed as described in Fig. 1. Temperature was maintained at 37° by use of a water bath.

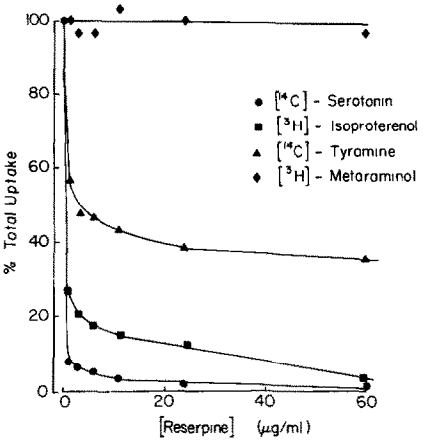

Fig. 3. Inhibition of amine accumulation by reserpine. In addition to chromaffin granules (5.5 mg protein/ml), the reaction mixtures contained 0.27 M sucrose, 30 mM Tris–maleate (pH 7.00), 1 mM ascorbate, 10 μM iproniazid phosphate, radioisotope-labeled amine, $^{3}H_2O$ or [^{14}C]polydextran, and reserpine dissolved in DMSO at the concentrations indicated. The total amount of DMSO added did not exceed 5 μl/ml. The volume of each reaction mixture was 1.3 ml. After 15 min, the samples were centrifuged and the procedure outlined in Fig. 1 was followed. Temperature was 24°. Percent total uptake was determined relative to the observed total uptake with no reserpine in the experimental medium.

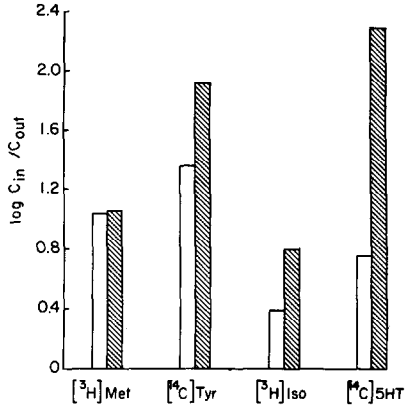

Fig. 4. Effect of $\Delta\Psi$ upon the accumulation of amines in isolated chromaffin granules. In a total volume of 3 ml, chromaffin granules (4.6 mg protein/ml) were added to 0.27 M sucrose, 20 mM Tris–maleate (pH 7.00), 1 mM ascorbate, 10 μM iproniazid phosphate, radioisotope-labeled amine, and ^3H_2O or [^{14}C]polydextran. After dividing each incubation volume in half, 8 mM MgATP was added to one portion (shaded bar), and FCCP (6.7 μg/ml) plus 8 mM MgATP was added to the other portion (clear bar). Aliquots (1.2 ml) of each mixture were centrifuged after 30 min and processed as described in Fig. 1. The temperature was 24°.

the $\Delta\Psi$ but leaves the ΔpH intact [9, 11]. When the effect of the $\Delta\Psi$ upon the sympathomimetic amine accumulation was investigated (Fig. 4), the steady-state electrochemical gradient for amines ($\Delta\tilde{\mu}_A$, expressed in mV) for [^{14}C]serotonin and [^{3}H]isoproterenol was increased by 200 and 100%, respectively, over the uptake observed when FCCP (and hence no $\Delta\Psi$) was present. [^{14}C]Tyramine accumulation was increased by 40%, but [^{3}H]metaraminol by less than 3%. The results, together with those of the previous experiment utilizing reserpine (Fig. 3), are consistent with the two mechanisms of accumulation outlined above.

Quantitation of the effect of the ΔpH and $\Delta\Psi$ upon amine accumulation in isolated chromaffin ghosts. The conclusions from experiments employing intact chromaffin granules suggest that two mechanisms exist for accumulation of biogenic amines, a carrier-mediated reserpine-sensitive mechanism (mechanism 1), and permeation of the amine through the apolar lipid membrane (as with methylamine) (mechanism 2). However, results from experiments utilizing chromaffin granules can be equivocal, due to the high content of endogenous amines which can exchange for the accumulating amine, the presence of intragranular binding sites which prevent the measurement of the amine distribution under steady-state conditions, and the existence of endogenous proton concentration, proton electrical, and other ion gradients. For these reasons, further experiments were carried out with chromaffin ghosts formed by hypo-osmotic lysis of the chromaffin granules (to remove the endogenous soluble components), extensive dialysis, and reformation in isotonic media.

When ghosts are suspended in a solution containing a permeable anion, the addition of ATP results in the inward movement of protons [9, 11]. The transmembrane potential thus formed, positive inside, allows chloride to permeate the chromaffin granule membrane as it moves down its electrochemical gradient, dissipating the potential and permitting further H^+ influx to proceed. A large ΔpH and negligible $\Delta\Psi$ result [9, 11]. Conversely, when

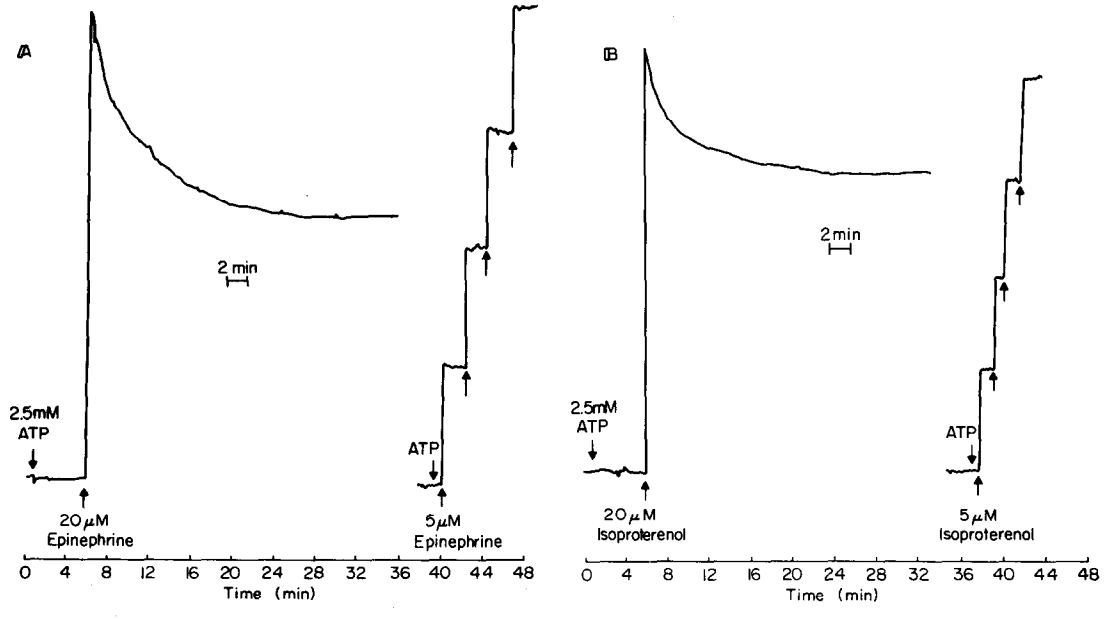

Fig. 5. Kinetic measurement of epinephrine and isoproterenol accumulation in isolated chromaffin ghosts utilizing potentiometric measurements. The reaction mixture contained 185 mM Na^+ isethionate, 30 mM Hepes (pH 6.80), and chromaffin ghosts (1.3 mg/ml). The reaction volume was 2.0 ml. The disappearance of amines from the medium was measured with a glassy carbon voltammetry electrode; more details are given under Materials and Methods. The temperature was 24°. The calibration curve was done in a separate experiment.

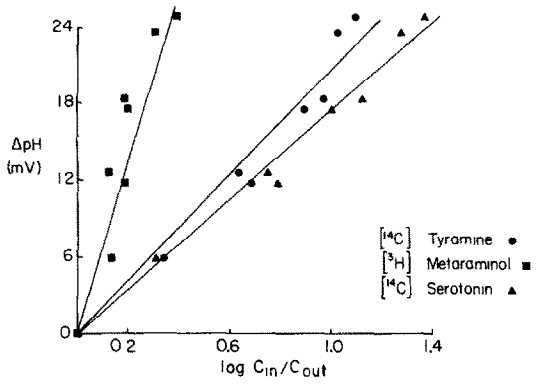

Fig. 6. Effect of ΔpH on amine accumulation into chromaffin ghosts. Ghosts were formed in 185 mM KCl, 20 mM ascorbate, and 5 mM Tris–maleate buffer at pH 7.0, as described under Materials and Methods. The reaction mixture contained 185 mM KCl, 20 mM ascorbate, 30 mM Tris–maleate buffer (pH 7.0), 10 μM iproniazid phosphate, radiolabeled amine, and either [^{14}C]polydextran or ^3H_2O, as well as 8 mM MgATP and chromaffin ghosts (0.7 mg protein/ml). The incubation volume was 1.2 ml. The range of ΔpH values was generated by addition of 0–35 mM $(NH_4)_2SO_4$ to the incubation mixture. After 20 min had elapsed, the samples were centrifuged for 7 min and processed as in Fig. 1. The experiment was performed at 24°.

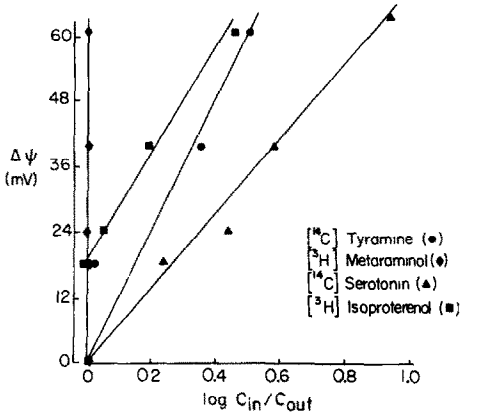

Fig. 7. Effect of the ΔΨ on amine accumulation in chromaffin ghosts. As described under Materials and Methods, chromaffin ghosts were formed in 185 mM sodium isethionate, 20 mM ascorbic acid, and 5 mM Tris–maleate buffer (pH 7.0). The reaction mixture contained 185 mM sodium isethionate, 20 mM ascorbic acid, 30 mM Tris–maleate buffer (pH 7.0), 10 μM iproniazid phosphate, and radiolabeled amine and either [^{14}C]polydextran or ^3H_2O, as well as 8 mM MgATP and chromaffin ghosts (0.7 mg protein/ml). Total incubation volume was 1.2 ml, with a range of ΔΨ values generated by the addition of 0–60 mM NaSCN to the incubation mixture. After 20 min, the samples were centrifuged for 7 min and processed as in Fig. 1. The experiment was performed at 24°.

ghosts are formed in a highly buffered medium containing an impermeant anion (isethionate), the ATP-dependent inward movement of H^+ generates a large potential which, in the absence of corresponding cation efflux or anion influx, is rapidly limited. A large ΔΨ and negligible ΔpH result [9, 11].

The availability and unique properties of the ghost preparation permitted a re-evaluation of the transport of isoproterenol by utilizing a potentiometric electrode which, under well-defined conditions, is selective for biogenic amines [46]. In these experiments, ghosts were formed in both chloride and isethionate media, and ATP-dependent accumulation of isoproterenol (Fig. 5B) was compared with that of epinephrine (Fig. 5A). In every case, no uptake was measured in the absence of ATP, and the ATP-dependent uptake was totally inhibited by reserpine (data not shown). The kinetic measurement of the accumulation of isoproterenol provides further evidence that uptake can proceed against a net concentration gradient and substantiates that the rate and extent of its accumulation are significantly less than that of the physiological substrate epinephrine. Tyramine and metaraminol were not tested in this system, as they cannot be detected by the electrode.*

When ghosts were formed and suspended in KCl-containing medium, a ΔpH approaching 0.5 pH units was generated (Fig. 6). All of the amines tested showed a linear, dose-dependent relationship between the magnitude of accumulation and the magnitude of the ΔpH. However, while metaraminol distribution revealed a 1:1 correspondence between accumulation and the magnitude of the ΔpH, the stoichiometry (proton:amine ratio; see Ref. 11) for serotonin was nearly 2. The tyramine stoichiometry was intermediate but close to that of serotonin. Isoproterenol distribution was not included since, unlike the other amines which clearly reached equilibrium with the proton gradient during the time of the experiment, isoproterenol distribution was still far from equilibrium during the length of the experimental incubation.

In the presence of ΔΨ alone, a wide range of relationships was observed between the magnitude of accumulation and the magnitude of the ΔΨ (Fig. 7). [^{3}H]Metaraminol did not accumulate in the presence of a ΔΨ; even in the presence of a 60 mV potential, no [^{3}H]metaraminol uptake was measured. Conversely, a linear and corresponding relationship (1:1) was observed between the ΔΨ and serotonin accumulation. The values for [^{3}H]isoproterenol and [^{14}C]tyramine were intermediate between those of [^{3}H]metaraminol and [^{14}C]tyramine. Other experiments (not shown) demonstrate that as incubation times were increased, the tyramine and isoproterenol distribution relationships approached that for serotonin (1:1), suggesting that, under the experimental conditions chosen as optimal for maintaining a constant potential, the equilibrium gradient for tyramine and metaraminol was not entirely attained.

DISCUSSION

Data from several laboratories [11–13, 38, 47–54] interested in the measurement of proton gradients and distribution of biogenic amines have led to a model for biogenic amine accumulation into isolated storage granules which is generally accepted [1, 11, 13]. The essence of the model is that a H^+-translocating ATPase exists within the membrane

* R. G. Johnson, S. Hayflick, S. E. Carty and A. Scarpa, unpublished data.

and is responsible for the inward movement of protons, thus generating a ΔpH, inside acidic, and a ΔΨ, inside positive. These proton electrical and concentration gradients form the electrochemical gradient for protons ($\Delta\bar{\mu}_{H^+}$), defined as [55–58]: $\Delta\bar{\mu}_{H^+} = \Delta\Psi - Z\Delta pH$, where $Z = (2.3\,RT/F)$.

When suspended at pH 7.4, chromaffin granules can maintain a ΔpH of 1.9 units, acidic inside, and a ΔΨ of 80 mV, positive inside. Inward catecholamine movement occurs against a net concentration gradient by a reserpine-sensitive carrier; the driving force is the electrochemical proton gradient ($\Delta\bar{\mu}_{H^+}$). Recent results obtained using purified ghost preparations have provided unequivocal evidence for the role of the $\Delta\bar{\mu}_{H^+}$ in the accumulation of biogenic amines and indicate that the magnitude of the driving force is equal to $\Delta\Psi - 2Z\Delta pH$ [11, 13]. The proportional relationship between the electrochemical gradient for amines ($\Delta\bar{\mu}_A$) and the $\Delta\bar{\mu}_{H^+}$ does not permit discrimination of the species of amine which is transported (i.e. cation, anion, neutral, or zwitterion; see Ref. 11 for complete discussion). At this time, however, the evidence is most consistent with binding of a neutral amine to a negatively charged molecule [9].

The present study utilized isolated chromaffin granules due to their ready isolation in high yield and purity and advantageous ability to form chromaffin ghosts. The evidence to date, however, is consistent with the notion that the mechanism of catecholamine accumulation outlined above may be universal, with ready extrapolation to synaptic vesicles [8, 50, 53], as well as to subcellular organelles which contain other biogenic amines such as serotonin (dense granules of platelets) [59, 60] and histamine (large core granules of mast cells) [61].

An amine which is accumulated via the catecholamine carrier in equilibrium with the $\Delta\bar{\mu}_{H^+}$ (mechanism 1) would be expected to exhibit the following properties: (1) reserpine sensitivity; (2) structural specificity; and (3) distribution at equilibrium according to the relationship: $\log C_{in}/C_{out} = \Delta\Psi - 2Z\Delta pH$. On the other hand, those amines which permeate the apolar lipid phase of the membrane in the uncharged form (mechanism 2) would be expected to demonstrate (1) reserpine insensitivity; (2) a rate of permeation proportional to the lipophilicity of that amine; and (3) distribution at equilibrium such that $\log C_{in}/C_{out} = \Delta pH$. The most salient aspects of these models are illustrated in Fig. 8.

The observations that the addition of high doses of tyramine, metaraminol, and isoproterenol all produced an alkalinization of the intragranular space (Fig. 1) and that radiochemically labeled sympathomimetic amines were taken up and released in response to the magnitude of the driving force (Fig. 2) demonstrated the net accumulation of these amines but did not discriminate between the two mechanisms (Fig. 8). However, the effects of reserpine (Fig. 3), ATP addition (Fig. 4), ΔpH alone (Fig. 6), and ΔΨ alone (Fig. 7) allow the following conclusions to be made: (1) metaraminol accumulates in isolated chromaffin granules by a reserpine-insensitive mechanism with distribution at equilibrium according to the magnitude of the ΔpH; the ΔΨ does not affect metaraminol uptake; (2) serotonin and isoproterenol uptake, like that previously described for catecholamines, occurs by a reserpine-sensitive, carrier-mediated mechanism driven by both the ΔΨ and ΔpH; and (3) tyramine can accumulate by either pathway (Fig. 8).

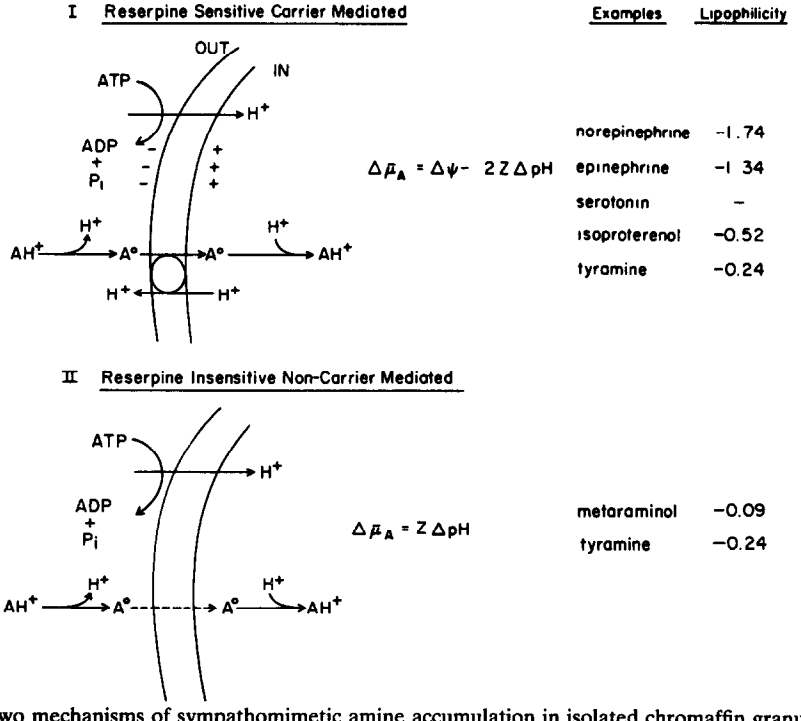

Fig. 8. Two mechanisms of sympathomimetic amine accumulation in isolated chromaffin granules and ghosts. The lipophilicities of the amines listed are expressed as logarithm of the *n*-octanol/water partition coefficients, as measured previously [62].

Previous studies have dealt with tyramine and metaraminol uptake into isolated chromaffin granules [30, 38, 63]. In each report it was concluded that uptake of these two sympathomimetic amines is characterized by reserpine and ATP insensitivity. However, the mechanism by which apparent net accumulation of the amines occurred was not investigated, nor was it hypothesized. Several years later it was advanced by Stjärne [3] that the uptake of these amines may be dependent upon their lipid solubility, although no rationale was given to explain their uptake against a concentration gradient. Recently, a systematic and elegant study of the lipophilicity of the catecholamines and related compounds was reported [62]. The lipophilicity of the amines studied is listed in Fig. 8 (the more positive the value, the greater the lipophilicity). As can be seen, the amines possessing the highest lipophilicity, metaraminol (−0.27) and tyramine (−0.34), are the amines which have been shown here to accumulate by a non-reserpine-sensitive mechanism. This conclusion does not, however, exclude binding of the amines to the amine transporter. In fact, tyramine and metaraminol are excellent competitive inhibitors of epinephrine uptake [15, 29], indicating that the affinity for binding of the amine to a membrane carrier and the rate of translocation of the amine carrier complex are not equivalent.

The results of this study have several potential physiologic and pharmacologic implications. First, while manifesting a rate of accumulation slower than that of the other amines studied, isoproterenol clearly accumulated against a net concentration gradient (Figs. 2 and 4), an effect which would be expected to occur *in vivo*. This finding is consistent with a previous report that isoproterenol accumulates in sympathetic nerve granules [64]. The absence of significant accumulation of isoproterenol by perfused organs and tissue slices has led several investigators to hypothesize that this sympathomimetic amine, in contradistinction to the circulating catecholamines, is not transported at the level of the axonal membrane [65–67]. Utilization of the potentiometric technique illustrated in Fig. 5 and elsewhere, coupled with well-defined cell systems such as that of the cultured rat pheochromocytoma, will permit a rapid and sensitive method with which to fully document the interaction of the amine with an axonal-like membrane.

Second, the mode of action of tyramine and metaraminol when adminstered systemically has been postulated to include (1) release of norepinephrine from extraneural sites [68–71]; (2) competitive inhibition of norepinephrine uptake into the storage granules [15, 29]; (3) direct displacement of endogenous amines within the intragranular matrix space by accumulated tyramine and metaraminol [72]; (4) formation of octopamine by β-hydroxylation of tyramine [73, 74]; and (5) direct interaction of the sympathomimetic amines with α and β receptors [68, 75, 76]. The results reported here suggest that, in addition to their pharmacological action, the effects of tyramine and metaraminol may relate to (1) perturbation of the existing electrochemical proton gradient with redistribution of endogenous stored catecholamines, and (2) uptake proceeding independently of the presence of normal physiologic regulators of the catecholamine transporter molecule.

The conclusions may help to explain several previous observations. The high concentrations of tyramine required to release catecholamines from perfused adrenal glands [77] may reflect the large intragranular buffering capacity of the chromaffin granules [38]; a large influx of tyramine through the apolar phase would be required before alkalinization of the intragranular matrix space and redistribution of the endogenous catecholamines would be observed. The observed absence of a strict stoichiometric release of norepinephrine by tyramine in sympathetic nerves [78] may also be dependent upon perturbation of the ΔpH. Because the driving force for catecholamine accumulation is $\Delta\Psi - 2Z\Delta pH$ [11, 13], and the ratio of the internal to external catecholamine concentrations varies as the square of the proton gradient, a small change in the ΔpH results in a significant perturbation of the electrochemical equilibrium gradient for catecholamines. And finally, a single sympathomimetic amine may exert multiple actions; in this respect tyramine can perturb the magnitude of the ΔpH by permeation through the apolar phase of the membrane, causing a redistribution of endogenous catecholamines, as well as by competitively blocking catecholamine uptake through the reserpine-sensitive carrier site.

With the application of sensitive techniques, such as high pressure liquid chromatography and mass spectroscopy, to the identification and quantitation of the endogenous biogenic monoamines in the central nervous and peripheral sympathetic systems, it has become apparent that many analogs of the catecholamines are present and may exist in substantial quantities. Because of the paramount role of the storage organelles in the overall regulation of amine homeostasis, interaction of the sympathomimetic amines with these organelles has important implications. In this study two mechanisms for accumulation of sympathomimetic amines have been elucidated. The chromaffin granule and ghost preparations, coupled with the radiochemical and potentiometric techniques outlined in this report, provide an excellent model for the investigation of the transport of these as well as other sympathomimetic amines, and for the study of the storage and release of those monoamines found endogenously.

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