Biochemical Pharmacology, Vol. 30, No. 7, pp. 763–769, 1981.
Printed in Great Britain.

0006-2952/81/070763-07 $02.00/0

ACCUMULATION OF AMANTADINE BY ISOLATED CHROMAFFIN GRANULES

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(*Received* 17 *May* 1980; *accepted* 29 *August* 1980)

Abstract—The accumulation of amantadine and the effect of this pharmacologic amine upon the magnitude of the transmembrane proton gradient (ΔpH) and of the transmembrane potential gradient ($\Delta\Psi$) were investigated in bovine adrenal chromaffin granules isolated in isotonic sucrose. Freshly isolated chromaffin granules have an intragranular pH of 5.5, as measured by [^{14}C]methylamine distribution [R. G. Johnson and A. Scarpa, *J. gen. Physiol.* **68**, 601 (1976)]. The addition of amantadine (1–50 mM) to well-buffered suspensions of granules at pH 7.0 resulted in a dose-related alkalinization of the granule interior. Similar results were obtained with equivalent external concentrations of ammonia. When the time-resolved influx of labeled amines into the granules was studied radiochemically, using low external amine concentrations, the accumulation of [^{3}H]amantadine was quite similar to that of [^{14}C]methylamine with regard to rate and extent over a wide range of magnitudes of the transmembrane proton gradient. However, unlike biogenic amine accumulation into the chromaffin granule, which is driven by both transmembrane proton and potential gradients, the accumulation of [^{3}H] amantadine was not stimulated by the existence of a transmembrane potential, nor was it inhibited by reserpine. Moreover, low concentrations of amantadine did not competitively inhibit biogenic amine accumulation in the isolated granules. These results indicate that amantadine can distribute across the membrane of chromaffin granules according to the magnitude of the endogenous ΔpH, and suggest that *in vivo* amantadine may be concentrated and stored as a pharmacologic agent in amine containing granules.

The uses of amantadine hydrochloride (1-adamantanamine, Symmetrel) as a therapeutic agent in the clinical treatment of Parkinson's disease and as an antiviral prophylactic agent have been extensively investigated and reviewed [1, 2]. Amantadine has recently been shown to also inhibit thrombin-stimulated platelet aggregation *in vitro* [3]. However marked the success of this drug in clinical treatment, its mechanism(s) of action remain(s) unelucidated and the subject of some controversy. Although there is basic agreement that amantadine permeates all cell membranes [1, 4, 5], its anti-Parkinsonian action is variously attributed to (a) a dopaminergic stimulation of post-synaptic receptors [6]; (b) an inhibition, at high concentration, of catecholamine reuptake by blockage of ionic channels [7, 8], or by an unknown non-carrier-specific mechanism [1, 9, 10]; or (c) an enhanced release, at low concentrations, of dopamine and norepinephrine within critical regions of the brain [1, 11]. The antiviral activity of amantadine has been postulated to lie in an alteration of the host cell membrane so as to decrease the penetration of sensitive viral strains [1], and amantadine has been found to inhibit the uptake of 5-hydroxytryptamine (serotonin) into intact platelets [1, 12]. To our knowledge, however, no investigation to date has endeavored to elucidate mechanisms operating at a level of interaction between amantadine and the specific subcellular organelles in which biogenic amines are stored.

Amine containing subcellular organelles have been found to accumulate, against an apparent concentration gradient, a wide variety of structurally related amine compounds. Among these are 5-hydroxytryptamine, stored in the dense granules of platelets, and the catecholamines dopamine, epinephrine, and norepinephrine, stored in the chromaffin granules of the adrenal medulla. The common features of these compounds are: (a) they each consist of a closed ring moiety, (b) each has a primary amino grouping, and (c) carboxyl groups are absent. Examination of the amantadine molecule reveals that, as a primary amine, it incorporates all three features. Given the known dopaminergic actions of amantadines *in vivo*, we attempted to determine whether amantadine accumulates inside chromaffin granules and if the same molecular uptake mechanisms and regulation exist for this compound as for the biogenic amines.

MATERIALS AND METHODS

Preparation of chromaffin granules. Intact chromaffin granules were isolated from bovine adrenal glands that were obtained at a local slaughterhouse and transported to the laboratory on ice. The adrenal medullae were dissected free and subjected to homogenization and differential centrifugation, as described previously [12, 13], in an isolation medium of 0.27 M sucrose, 10 mM Tris–maleate buffer, pH 7.0. Chromaffin granules were purified from other subcellular organelles by centrifugation through a

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Ficoll-D_2O-sucrose density gradient, which has the advantage of preserving the isotonicity of the granule suspension, as described elsewhere [12]. The isolation medium was utilized for two subsequent washings and for the final suspension, which was stored at 4° until use. Total protein was measured by the method of Lowry *et al.* [14], using bovine serum albumin as the standard.

Preparation of erythrocytes. Human venous blood was collected from a volunteer into a solution of sodium citrate (2.5%). The erythrocytes were allowed to settle for 20 min at 24°, after which time the platelet-rich plasma was removed and the cells were washed three times in a solution of 350 mM sucrose, 10 mM Tris–maleate, pH 7.30. The erythrocytes were then resuspended in this solution and stored at 4° until use.

Measurement of ΔpH. [^{14}C]Methylamine distribution across the membrane of the chromaffin granule was used to measure the ΔpH. This is a highly reproducible method used routinely by many laboratories, with a variety of subcellular organelles (for review see Ref. 15). The technique is based on the observation that amines freely permeate biological membranes only in the uncharged form, reaching equilibrium when $[R—NH_2]_{inside} = [R—NH_2]_{outside}$. Because methylamine has a high pK_A, $[R—NH_3^+]_{inside}/[R—NH_3^+]_{outside} = [H^+]_{outside}/[H^+]_{inside}$ for steady-state distribution. Therefore, the logarithm of the ratio of the [^{14}C]methylamine intragranular concentration to that in the external medium ($\log C_{in}/C_{out}$) gives a measure of the ΔpH. Under equilibrium conditions, the margin of error in this technique involves mainly the magnitude and extent of binding of methylamine. The contribution of aspecific binding effects to the calculations is thought to approximate 0.15 pH units [16]. Due to variations in viability of the isolated granules from preparation to preparation, slight deviations are observed in the measurement of the ΔpH under various conditions.

Chromaffin granules were incubated with [^{14}C]methylamine (8.2 μM) and ^3H_2O, or with [^{14}C]polydextran and ^3H_2O (to correct for extragranular water space); incubations were carried out in the dark. Aliquots of this suspension were centrifuged in an Eppendorf model 3200 desk centrifuge for 4 min and processed for radioassay as described previously [13]. Distributions of [^{14}C]methylamine and ^3H_2O were calculated by their relative activities in the pellet and supernatant fraction, using the equation (for complete explanation see Ref. 15):

$$C_{in}/C_{out} = R + (R - 1)\,[x/(1 - x)]$$

$$R = \frac{[^{14}C]\text{methylamine}}{^3H_2O\text{ space}};\ \text{and}\ x = \frac{[^{14}C]\text{polydextran}}{^3H_2O\text{ space}}$$

Measurement of ΔΨ. [^{14}C]SCN^- distribution across the chromaffin granule membrane was utilized to measure the transmembrane potential. This lipophilic anion has been previously shown to permeate biological membranes and distribute in accordance with a positive inwardly directed potential in a highly reproducible manner [13]. The distribution of [^{14}C]SCN^- (3.5 μM) was measured as discussed above for methylamine distribution and the membrane potential was calculated according to the Nernst equation:

$$\Delta\Psi = 58 \log \frac{[^{14}C]SCN^-_{in}}{[^{14}C]SCN^-_{out}}$$

Measurement of [^{14}C]*catecholamine and* [^{3}H]*amantadine distribution.* The distributions of [^{14}C]-5-hydroxytryptamine (serotonin) (10.1 μM) and [^{14}C]epinephrine (10.1 μM) were determined by the method described for the measurement of [^{14}C]methylamine distribution. Although 5-hydroxytryptamine is not found endogenously within the adrenal medulla, it has the highest specificity for uptake *in vitro* and the lowest affinity for intragranular binding sites [17]. Therefore, it has been used with great success previously to measure biogenic amine accumulation [18]. Amantadine distribution was determined using [^{3}H]amantadine (1.2–2.4 μM) plus [^{14}C]polydextran, and ^3H_2O plus [^{14}C]polydextran (to correct for extragranular water space). This method allows a rapid and highly reproducible measurement of the distribution of a labeled species across a biological membrane when expressed as the ratio of the internal to external concentration. In the case of [^{14}C]methylamine and [^{14}C]thiocyanate distribution, significant binding was excluded [13]. For the biogenic amines and amantadine, a 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 protonmotive force had been completely eliminated by the addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and a large concentration of ammonia. In the second, the distribution was measured in a suspension of scattered chromaffin membranes formed by hypo-osmotic lysis of the chromaffin granules. Binding values from the two methods, which were closely comparable, were averaged and subtracted from the distribution values obtained under experimental conditions, and did not exceed 9 per cent for the amines and 12 per cent for amantadine.

Materials. Trizma base, maleic acid, reserpine, and 5-hydroxytryptamine were purchased from the Sigma Co., St. Louis, MO. Amantadine hydrochloride was the gift of Dr. Charles C. Whitney, DuPont du Nemours, Inc., Wilmington, DE. [^{14}C]Methylamine (50.1 mCi/mmole), [^{14}C]epinephrine (59.4 mCi/mmole), and ^3H_2O (1 mCi/g) were purchased from the New England Nuclear Corp., Boston, MA. [^{14}C]KSCN (59 mCi/mmole) and [^{3}H]amantadine (350 mCi/mmole) were purchased from Amersham/Searle, Arlington Heights, IL. All other chemicals (reagent grade) were obtained from the Arthur H. Thomas Co., Philadelphia, PA.

RESULTS

Reduction of the ΔpH *across the membrane of the chromaffin granule by amantadine.* It is well established that the isolated chromaffin granule, when suspended in a well-buffered medium at pH 7.0, exhibits an endogenous ΔpH (inside acidic)

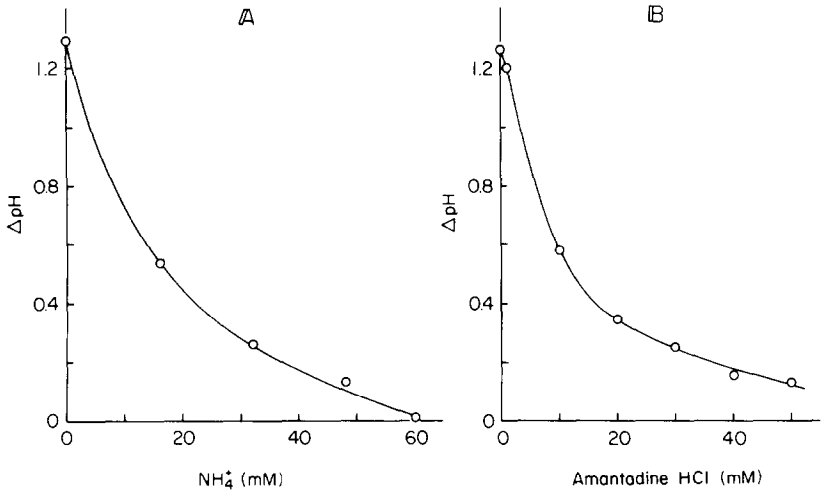

Fig. 1. Effect of amine addition upon the ΔpH of isolated chromaffin granules. Panel A: Dose-dependent effect of NH_4^+ upon the ΔpH. The reaction contained 0.27 M sucrose, 10 mM Tris–maleate (pH 7.0), chromaffin granules (6.1 mg/ml), [^{14}C]methylamine, and ^3H_2O, in an incubation volume of 1.2 ml. $(NH_4)_2SO_4$ was added at the indicated concentrations and, after 20 min of incubation in the dark, samples were centrifuged in an Eppendorf desk microcentrifuge for 4 min. The supernatant fraction and pellet were assayed for radioactivity as described under Materials and Methods. Temperature was 24°. Panel B: Dose-dependent effect of amantadine upon the ΔpH. Experimental conditions were as described in panel A, except that amantadine–HCl was added instead of $(NH_4)_2SO_4$ at the concentrations indicated.

approaching 1.5 pH units; that is, the intragranular space is maintained at pH 5.5 [13, 16]. The effect of high concentrations of ammonia and amantadine on the magnitude of the ΔpH across the chromaffin granule membrane is illustrated in Fig. 1. One way in which to test whether a molecule can permeate a biological membrane in the uncharged form is to measure the ΔpH at various external concentrations of that compound. If a weak base permeates the membrane in the unprotonated, uncharged form, then the reprotonation of the species in the intragranular space should result in an alkalinization of that space, dependent in magnitude upon the internal buffering capacity (for review, see Ref. 19). The buffering capacity in isolated chromaffin granules has been measured previously [20]. As reported by this laboratory, the addition of biogenic amines at high concentrations to isolated chromaffin granules

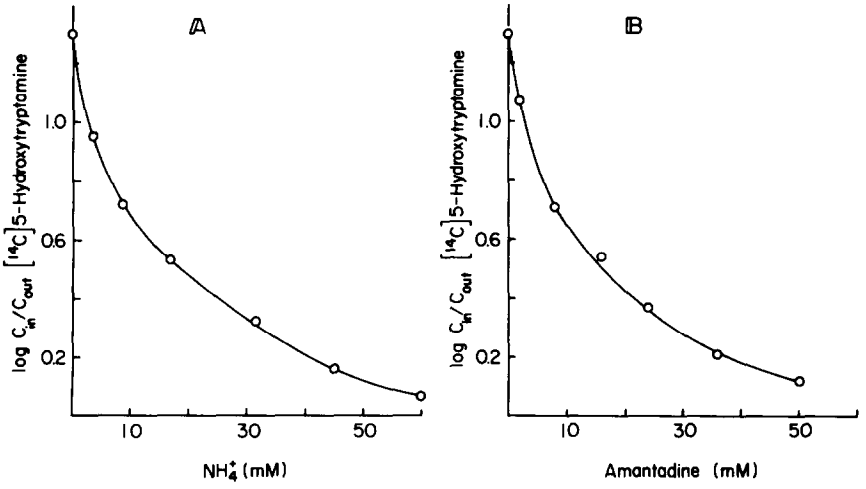

Fig. 2. Effect of amine addition upon ^{14}C-labeled biogenic amine uptake into chromaffin granules. Panel A: Dose-dependent effect of NH_4^+ upon [^{14}C]-5-hydroxytryptamine uptake. Chromaffin granules (6.8 mg/ml) were suspended in a reaction mixture of 0.27 M sucrose, 10 mM Tris–maleate (pH 7), containing [^{14}C]-5-hydroxytryptamine and ^3H_2O, in an incubation volume of 1.2 ml; $(NH_4)_2SO_4$ was added at the concentrations illustrated. After incubation for 20 min in the dark, the reaction was stopped and samples were treated as described in the legend for Fig. 1A. Binding of [^{14}C]serotonin, determined as described in Materials and Methods, accounted for 9 per cent of the maximum observed accumulation and was subtracted. Temperature was 24°. Panel B: Dose-dependent effect of amantadine upon [^{14}C]-5-hydroxytryptamine uptake. Samples were treated exactly as in panel A, except that amantadine–HCl was added instead of $(NH_4)SO_4$ at the concentrations shown. Binding of [^{14}C]serotonin was 6 per cent of the maximum observed accumulation and was subtracted.

produces a dose-dependent decrease in the ΔpH through alkalinization of the intragranular space, suggesting that these compounds also permeate the membrane in the uncharged form [13]. In Fig. 1A ammonia, a small amine, was added to a well-buffered suspension of chromaffin granules, and a dose-dependent decrease in the ΔpH resulted. Likewise, the addition of amantadine at various concentrations to suspensions of chromaffin granules also produced a dose-dependent decrease in the ΔpH (Fig. 1B). The fact that these two curves are not precisely superimposable probably relates to (1) differential effects on the integrity of the intragranular storage complex, (2) methodological difficulties in accurately measuring the internal water space under conditions of large magnitude swelling, and (3) the fact that the amantadine salt included chloride, which is the most permeant anion documented to enter isolated chromaffin granules [16], whereas the ammonium salt was sulfate, a much less permeant anion [16]. This result suggests that, at least at high concentrations, amantadine also permeates the membrane of the chromaffin granule in the uncharged form, and thus can accumulate within an acidic intragranular space.

To investigate this phenomenon further, the effect of large concentrations of amantadine upon the distribution of a micromolar concentration of [^{14}C]-5-hydroxytryptamine was studied (Fig. 2B). In this experiment, the concentration of labeled 5-hydroxytryptamine was sufficiently small so that, when added to a suspension of chromaffin granules, it did not result in perturbation of the ΔpH. As a control (Fig. 2A). the effect of ammonia addition upon [^{14}C]-5-hydroxytryptamine distribution was determined. The addition of NH_4^+ at various concentrations, which was associated with a dose-dependent decrease in the ΔpH (Fig. 1A), resulted in a dose-dependent decrease in the accumulation of [^{14}C]-5-hydroxytryptamine. This observation illustrated that 5-hydroxytryptamine accumulation was dependent upon the magnitude of the ΔpH across the granule membrane (see also Ref. 13). The results shown in Fig. 2B suggest that the addition of amantadine in high concentrations also produced a dose-dependent decrease in the accumulation of biogenic amine into the chromaffin granule.

Time-resolved influx of [^{3}H]*amantadine into isolated chromaffin granules.* To explore the kinetic influx of amantadine into isolated chromaffin granules, [^{3}H]amantadine was added at low concentrations that had been found not to perturb the intragranular pH. The addition of [^{14}C]methylamine and [^{3}H]amantadine to suspensions of chromaffin granules at room temperature produced almost instantaneous equilibrium distributions (data not shown). Therefore, in an effort to resolve kinetic influx and efflux of these amines, the same experiment was undertaken at 4° (Fig. 3). The addition of [^{14}C]methylamine to a suspension of granules resulted in a rapid equilibrium distribution of this compound (Fig. 3A), with the magnitude of the accumulation taken as a measure of the endogenous ΔpH. When the ΔpH was reduced by ammonia addition, there was an efflux of the [^{14}C]methylamine until a new equilibrium distribution was reached. Like [^{14}C]methylamine, [^{3}H]amantadine accumulation, when expressed as the logarithm of the internal

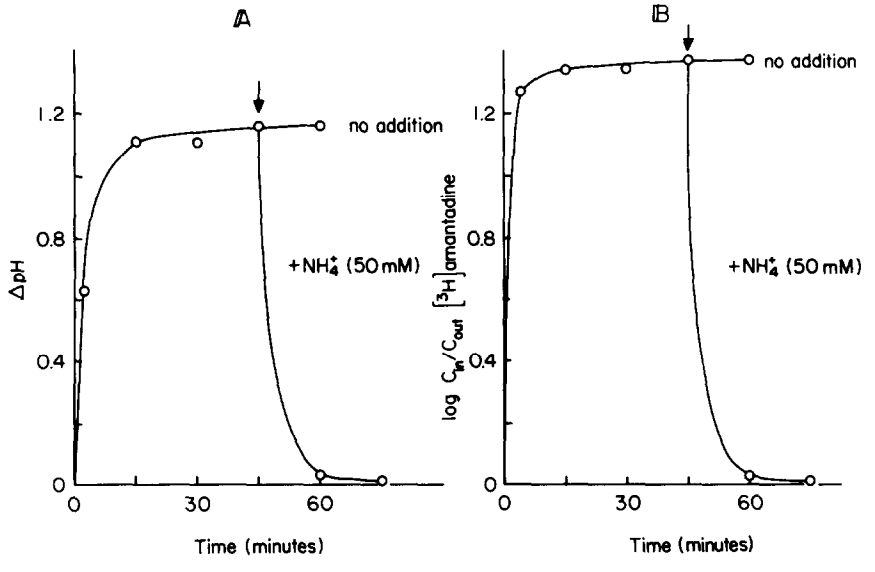

Fig. 3. Time-resolved measurements of ΔpH (A) and [^{3}H]amantadine accumulation (B) under identical conditions. The reaction mixture consisted of chromaffin granules (6.7 mg/ml), 0.27 M sucrose, 10 mM Tris–maleate (pH 7.0), and either [^{14}C]methylamine and $^{3}H_2O$ (A) or [^{14}C]dextran and [^{3}H]amantadine (B). The total incubation volume was 10 ml. The reaction mixture was incubated in the dark and at the indicated times aliquots were removed, centrifuged, and assayed as described in Fig. 1A. $(NH_4)_2SO_4$ (30 mM) was added to half of the remaining reaction mixture at the time indicated. Binding of [^{3}H]amantadine, determined as described in Materials and Methods, was 12 per cent of the maximum accumulation observed and was subtracted. Temperature was maintained at 4° using a water bath.

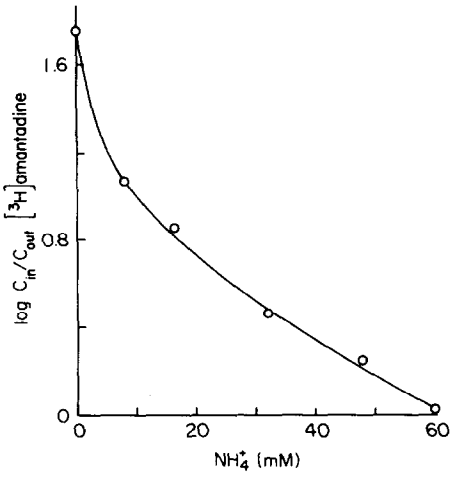

Fig. 4. Dose-dependent effect of NH_4^+ upon [^{3}H]amantadine uptake. Chromaffin granules (6.5 mg/ml) were suspended in reaction mixtures of 0.27 M sucrose, 10 mM Tris–maleate (pH 7.0), [^{3}H]amantadine, and [^{14}C]dextran, to a total volume of 1.2 ml. $(NH_4)_2SO_4$ was added at the concentrations shown. After a 20-min incubation in the dark, samples were centrifuged and assayed as described in Fig. 1A. Binding of [^{3}H]amantadine, determined as described in Materials and Methods, was 10 per cent of the maximum observed accumulation and was subtracted. Temperature was 24°.

to external concentration, approached the magnitude of the existing ΔpH. Moreover, as with the distribution of [^{14}C]methylamine, the addition of ammonia resulted in an efflux of [^{3}H]amantadine to a new equilibrium distribution value. The distribution ratio of [^{3}H]amantadine was consistently 0.1–0.2 units higher than that of [^{14}C]methylamine. Previous independent experiments, however, have suggested that methylamine may slightly underestimate the ΔpH by approximately 0.15 pH units [21].

To determine whether these observations were specific only for the chromaffin granule or whether accumulation of [^{3}H]amantadine in the presence of a ΔpH is a more generalized property pertaining to any biological system, the distribution of [^{3}H]amantadine was studied in the human red blood cell by establishment of a Donnan equilibrium for chloride [22] (data not shown). The results were again consistent with the notion that [^{3}H]amantadine can accumulate inside the acidic intragranular space by permeating the membrane in the uncharged form such that at equilibrium its distribution approaches the magnitude of the ΔpH.

The magnitude of [^{3}H]amantadine accumulation by the chromaffin granule was closely correlated with the magnitude of the ΔpH in a dose-dependent fashion (Fig. 4). In this experiment, the ΔpH was reduced by adding increasing concentrations of ammonia (as in Fig. 1A) and, in consequence, the accumulation of [^{3}H]amantadine also decreased in a dose-dependent manner.

Effect of a transmembrane potential upon [^{3}H]*amantadine accumulation.* That the chromaffin granule possesses an endogenous acidic intragranular pH upon isolation has been illustrated in the above figures and elsewhere [13, 16]. Moreover, it was shown previously that when MgATP is added to a suspension of chromaffin granules, a transmembrane potential, positive inside and approaching 100 mV [16], can be measured by the distribution of [^{14}C]SCN^-, a lipophilic anion. This is presumably due to activation of a H^+ translocating ATPase within the chromaffin granule membrane [23]. As determined previously, amine accumulation by the chromaffin granule can be driven not only by the magnitude of the pH gradient, but also by the magnitude of the transmembrane potential, independently of the ΔpH [13]. The ΔΨ across the chromaffin granule membrane was measured by [^{14}C]SCN^- in the presence and absence of ATP (Fig. 5A); a large concentration of NH_4^+ was added to the suspension of chromaffin granules so that the

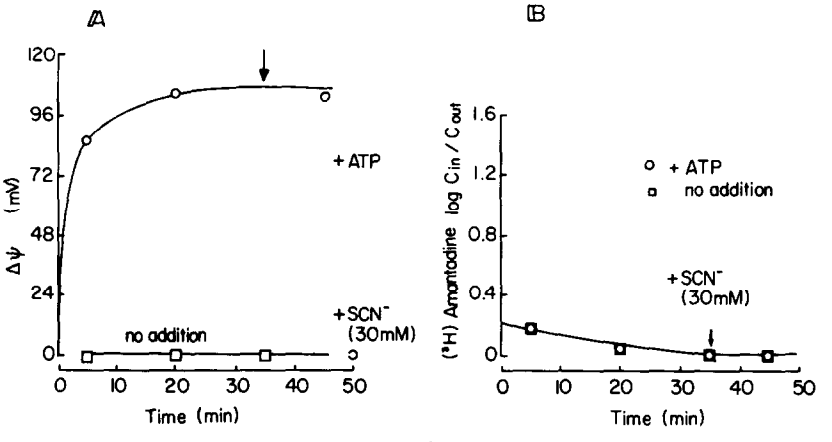

Fig. 5. Time-resolved measurements of ΔΨ and [^{3}H]amantadine uptake under identical conditions. Chromaffin granules (6.9 mg/ml) were incubated in quadruplicate reaction mixtures containing 0.27 M sucrose, 10 mM Tris–maleate (pH 7.0), 25 mM $(NH_4)_2SO_4$, and either [^{14}C]SCN^- and ^3H_2O (A), or [^{14}C]dextran and [^{3}H]amantadine (B) (total volumes 5 ml). MgATP (8 mM) was added to one-half of the samples, and the suspensions were incubated in the dark. NaSCN (30 mM) was added to all suspensions at the time illustrated (arrow). Samples of 1.2 ml were removed at the times shown and were treated as discussed in Fig. 1A. [^{3}H]Amantadine binding was determined as described in Materials and Methods and was equal to that observed in Fig. 3. Temperature was 24°.

endogenous ΔpH was reduced to 0.2 pH units. As evidenced, in the absence of ATP no ΔΨ was generated. When ATP was present, however, the ΔΨ approached 100 mV, and could then be reduced by the addition of the electrogenic proton translocator FCCP.

When the effect of a transmembrane potential upon the rate and extent of [^{3}H]amantadine accumulation was investigated under the same conditions (Fig. 5B), no significant potential-stimulated accumulation occurred [the small accumulation of [^{3}H]amantadine was found to be due to the small ΔpH (0.2 units) which existed]. In contrast, in a control experiment (data not shown), 5-hydroxytryptamine accumulation under these conditions approached a steady-state value of 1.2 when expressed as the logarithm of the ratio of the internal to external concentrations after a 15-min incubation. These observations suggest that, while the accumulation of amantadine occurred according to the endogenous ΔpH as with other biogenic amines, unlike the biogenic amines, no net accumulation of amantadine can be stimulated by the presence of a ΔΨ.

Effect of reserpine upon [^{3}H]*amantadine accumulation.* Analysis of the experiments presented suggests that perhaps amantadine was not permeating the membrane of the chromaffin granule via the putative amine carrier. One test of this hypothesis is to study the effect of reserpine upon the distribution of amantadine. Reserpine has been found to be a highly selective inhibitor of biogenic amine accumulation in chromaffin granules [24], presumably by binding irreversibly to a carrier molecule, and to have no effect upon either the ΔpH or an ATP-generated ΔΨ. In Fig. 6, the effect of reserpine upon the accumulation of [^{14}C]epinephrine and [^{3}H]amantadine was studied. As can be seen, the addition of reserpine resulted in a dose-dependent decrease in the magnitude of [^{14}C]epinephrine uptake, in agreement with previous results from this and other laboratories. But even at concentrations that are known to completely inhibit biogenic amine accumulation, no significant effect was observed on the accumulation of [^{3}H]amantadine. This suggests that amantadine can accumulate against an apparent concentration gradient by permeating through the apolar lipid phase of the granule membrane, without translocation through a catecholamine carrier site.

Effect of amantadine upon biogenic amine accumulation. Although the evidence in Fig. 5 is consistent with the notion that net translocation of amantadine does not occur via an amine transport site, the possibility that low concentrations of amantadine could inhibit catecholamine accumulation by binding to the putative carrier or inhibiting catecholamine translocation could not be ruled out. For this reason, the effect of various small concentrations of amantadine (1–250 μM) upon the accumulation of [^{14}C]dopamine and [^{14}C]hydroxytryptamine was investigated (data not shown). The results indicated that, even at concentrations well over one order of magnitude greater than that of the biogenic amines, the presence of amantadine had no effect upon uptake of labeled dopamine and only a small effect (less than 5 per cent inhibition) upon uptake of labeled 5-hydroxytryptamine; thus, amantadine was not found to inhibit biogenic amine accumulation by the chromaffin granule.

DISCUSSION

Current thinking as to the mechanism of biogenic amine accumulation has been reviewed recently [18].

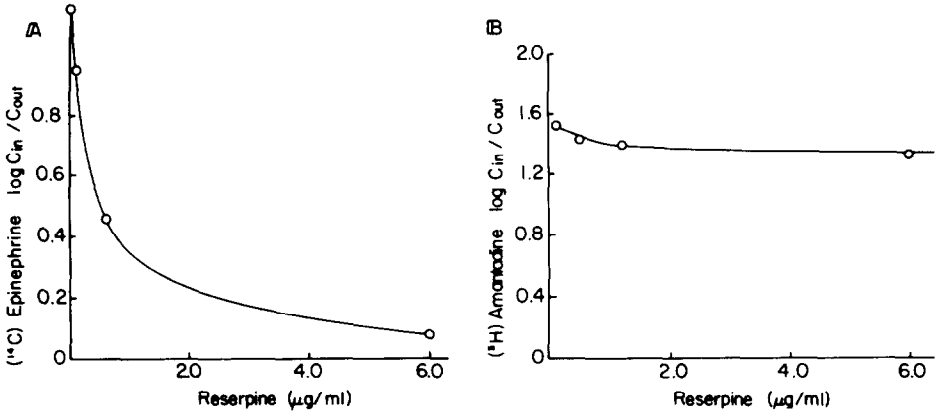

Fig. 6. Dose-dependent effect of reserpine on [^{14}C]epinephrine and [^{3}H]amantadine uptake. The reaction mixture consisted of chromaffin granules (7.0 mg/ml) suspended in 0.27 M sucrose, 10 mM Tris–maleate (pH 7.0), containing either [^{14}C]epinephrine and ^3H_2O (A), or [^{14}C]dextran and [^{3}H]amantadine (B). Sample volume was 1.2 ml. Reserpine dissolved in dimethylsulfoxide (DMSO) was added at the concentrations indicated and the samples were incubated for 20 min. Content of DMSO in the reaction mixture was 10 μl/1.2 ml; the addition of DMSO alone was found to have no effect upon [^{14}C]epinephrine accumulation. Centrifugation of the samples and assay of radioactivity proceeded as described in Fig. 1A. Binding of [^{14}C]epinephrine and [^{3}H]amantadine accounted for 10 and 11 per cent, respectively, of the total accumulation observed in this experiment.

In brief, the model suggests that amine accumulation occurs via a specific reserpine-sensitive catecholamine translocase, present in the granule membrane, which is dependent upon the magnitude of the ΔpH and $\Delta\Psi$, i.e. upon the protonmotive force maintained by a H^+ translocating ATPase. It has been shown that, in the absence of ATP, amines can distribute according to the magnitude of the ΔpH [18, 20]. For this to occur, catecholamines, like methylamine, may permeate the membrane in the uncharged form. When amantadine was added to the chromaffin granules at high concentrations (Fig. 1), there was a dose-responsive decrease in the ΔpH, suggesting that at least at high concentrations permeation occurred in the uncharged form in response to the acidic granule interior. Measurement of [^{3}H]amantadine accumulation at concentrations that did not affect the ΔpH reveals that the distribution closely parallels that of the ΔpH (Fig. 3). Moreover, unlike accumulated catecholamines, amantadine was not tightly bound within the intragranular storage complex, since reduction of the proton gradient resulted in rapid re-equilibration of the molecule. In contradistinction to biogenic amines, the uptake of [^{3}H]amantadine was extremely rapid, with a time course closely following that of [^{14}C]methylamine, even at low temperatures (Fig. 3). In addition, [^{3}H]-accumulation decreased in proportion to the ΔpH (Fig. 4). The excellent correlation with [^{14}C]methylamine distribution suggests that amantadine may prove to be an indicator of the ΔpH.

The addition of ATP to intact chromaffin granules resulted in the establishment of a membrane potential without an effect on the magnitude of the ΔpH. Catecholamine accumulation in isolated chromaffin granules, however, is stimulated by more than one order of magnitude [13]. That the addition of ATP had no effect upon amantadine accumulation (Fig. 5) is good evidence that amantadine was not being taken up into the chromaffin granules by a carrier-mediated process. Moreover, since its accumulation was not reserpine-sensitive (Fig. 6), and since there was no evidence that amantadine competed with catecholamines for accumulation into the granule, interaction of amantadine with the carrier in any fashion is not likely. Rather, amantadine, like methylamine and ammonia, appeared to permeate the chromaffin granule membrane through the apolar lipid phase.

The internal pH of the chromaffin granule approaches 5.5 and that of the cytosol 7.4; therefore, the ΔpH *in vivo* approaches 2.0 pH units. This means that, if amantadine distributed according to the ΔpH *in vivo*, the concentration of amantadine would be 100 times greater in the intragranular space than in the cytosol. With a reported clinical plasma level of 0.5 mM [3], and the high lipophilicity of the compound, it would be expected that extremely high levels of amantadine would be found to exist in the CNS and, particularly, within amine containing storage vesicles.

Synaptic vesicles have been shown to possess many of the properties of chromaffin granules with regard to their structure, composition, mechanism of release, and the role of the protonmotive force in the mechanism of amine accumulation [25]. The results presented here suggest that if a ΔpH exists across the membrane of synaptic membranes (which the evidence to date supports), then amantadine would accumulate in synaptic vesicles within the substantia nigra of the brain as well. In addition to a large net accumulation, the interaction of amantadine might perturb the magnitude of the ΔpH across the synaptic vesicles with implications for amine accumulation and storage, and it certainly would result in the concomitant release of amantadine upon release of stored biogenic amines. The finding that amantadine was concentrated within an amine containing subcellular storage organelle may help to contribute to the elucidation of the mechanism of amantadine action *in vivo*.

Acknowledgements—Many thanks are due to Mr. Dan Brannen and Ms. Tina Davidson for preparation of the manuscript and to Dr. Charles C. Whitney (E. I. DuPont de Nemours & Co.) for his gifts of amantadine–HCl and of [^{3}H]amantadine used in the preliminary experiments. We are also indebted to Mr. Mike Beers and Ms. Susan Brannen for their expert technical assistance. This work was supported by Grant 77-675 from the National Institutes of Health. R. G. Johnson is a recipient of Medical Scientist Training Program Grant GM-20246.

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