Solubilization and Reconstitution of the Catecholamine Transporter from Bovine Chromaffin Granules†

Ron Maron, Hana Fishkes, Baruch I. Kanner, and Shimon Schuldiner*

ABSTRACT: The catecholamine transporter from bovine chromaffin granules has been solubilized by using low concentrations of sodium cholate in the presence of phospholipids. The functional solubilized protein has been incorporated into liposomes after removal of the detergent either by gel filtration or by dialysis. Reserpine-sensitive accumulation against a concentration gradient is achieved by artifically imposing a pH gradient across the membrane. In the reconstituted system adenosine 5′-triphosphate (ATP) serves as an energy source only at higher detergent concentrations. The proton-translocating adenosine triphosphatase (ATPase) is solubilized in parallel with the increasing efficiency of ATP as an energy source. Several criteria are proposed to distinguish between carrier-mediated (reserpine sensitive) and unmediated transport in the reconstituted system. The reserpine-sensitive process shows affinity and specificity toward biogenic amines similar to that of the native system. The results presented in this communication provide further support for the contention that concentrative uptake in biogenic amine storage vesicles is driven by a transmembrane pH gradient, which, in the native system, is generated by a proton-translocating ATPase. Moreover, the assays described provide a tool for the isolation and purification of the transport protein.

The chromaffin granules are involved in the transport, storage, and secretion of catecholamines in the adrenal medulla (Smith, 1968; Stjärne, 1972; Kirshner, 1974; Winkler & Smith, 1975). The isolated granules catalyze the uptake of large amounts of adrenaline in a process which is dependent on ATP (Kirshner, 1962; Carlsson et al., 1963) and is inhibited by reserpine and by proton ionophores (Kirshner, 1962; Bashford et al., 1975a). The granules have been found to contain a membrane-bound ATPase (Banks, 1965; Kirshner et al., 1966; Hasselbach & Taugner, 1970; Bashford et al., 1975b) which is stimulated by the proton conductors (Bashford et al., 1975b).

Uncoupler-sensitive pH gradients (ΔpH, interior acid) (Casey et al., 1977; Johnson & Scarpa, 1976; Pollard et al., 1976; Johnson et al., 1978) and ATP-dependent changes in the fluorescence of 1-anilinonaphthalene-8-sulfonate (Bashford et al., 1975b) have been detected in intact granules. Moreover, it has been demonstrated that the addition of high concentrations of adrenaline resulted in a decrease of the preexisting proton gradient. In addition, by manipulating the external pH (internal pH was constant at 5.6), a relationship between adrenaline transport and the H^+ gradients was observed (Johnson et al., 1978). According to these observations, a possible mechanism of concentrative uptake might be the generation of a pH gradient (acidic interior) by the proton-translocating ATPase, followed by a distribution of the catecholamine dictated by the above-mentioned pH gradient. From such a mechanism, it follows that if the uncharged species of the amine is the one translocated, the final concentration gradient will be determined by the transmembrane pH gradient. By using chromaffin granule membrane vesicles derived from the intact granules by osmotic shock (Philips, 1974; Schuldiner et al., 1978), it has been possible to show that the imposition of a ΔpH (interior acid) across the membrane of these vesicles induces the ATP-independent reserpine-sensitive accumulation of both adrenaline (Schuldiner et al., 1978) and 5-hydroxytryptamine (5-HT)[1] (Phillips, 1978). The accumulation of the various biogenic amines appears to be mediated by one carrier and is competitively and reversibly inhibited by reserpine (Kanner et al., 1979).

In order to further understanding of the translocation process at a molecular level, it is obviously helpful to identify and isolate the proteins involved in the process. In the present communication we describe conditions for the solubilization and reconstitution of functional catecholamine transporter. The techniques described provide a rapid and convenient assay for the purification of the transport protein.

Experimental Procedure

Preparation of Chromaffin Granule Membrane Vesicles. Chromaffin granules were prepared from bovine adrenal glands essentially as described by Kirshner (1962). Membrane vesicles were obtained by osmotic shock, frozen, and stored in liquid air (Phillips, 1974; Schuldiner et al., 1978).

Solubilization and Reconstitution of the Catecholamine Transporter. For solubilization of the catecholamine transporter, membrane vesicles were rapidly thawed, diluted in 20 volumes of a solution containing 0.15 M KCl and 0.01 M K-Hepes (pH 7.4) (buffer K), collected by centrifugation (39000*g*, 30 min), and resuspended in the same buffer to a protein concentration of 22 mg/mL. Sodium cholate was added dropwise to a final concentration of 0.9%, the suspension (300 μL) was transferred to a tube containing 2.8 μmol of partially purified (Kagawa & Racker, 1971) dried soybean phospholipids (asolectin; Associated Concentrates, Woodside, NY) and sonicated for a few seconds in a bath-type sonicator (Bransonic, B-12). After incubation for 10 min at 0 °C, the preparation was centrifuged at 130000*g* for 90 min. The supernatant was passed through a Sephadex G-50 column (grade fine, 1 × 18 cm) which was previously equilibrated with buffer K. The turbid fractions eluting at the void volume and

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[1] Abbreviations used: 5-HT, 5-hydroxytryptamine; buffer K, solution containing KCl (0.15 M) and K-Hepes (0.01), pH 7.4.

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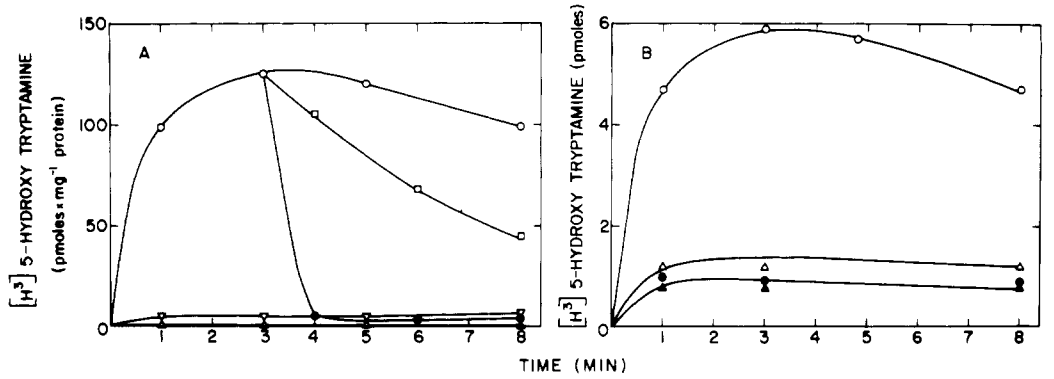

FIGURE 1: Reconstitution of the catecholamine transporter from bovine chromaffin granules. The catecholamine transporter was solubilized and reconstituted as described under Experimental Procedure. The final protein concentration of the reconstituted proteoliposomes was 15.7 mg/mL. Uptake was measured by diluting 3 μL of the above suspension into 300 μL of a mixture containing 0.3 M sucrose, 0.01 M Tris–Hepes, pH 8.5, 0.09 μM 5-hydroxy[G-^{3}H]tryptamine (27.8 Ci/mmol), and 5 μM nigericin. (A) Complete system (○); nigericin omitted (▽); 5-hydroxytryptamine (□) and sodium cholate (●) added to final concentrations of 50 μM and 0.8%, respectively, 3 min after the reaction was initiated; and (△) membranes assayed before the Sephadex step (see Experimental Procedure). (B) Complete system in the absence (○) or presence (△) of 2 μM reserpine; reconstituted mixture prepared in the absence of protein assayed in the presence (▲) or absence (●) of 2 μM reserpine.

which contained 60–80% of the protein loaded were collected and centrifuged at 130000g for 30 min. The pellet was resuspended in a minimal volume of buffer K to yield a protein concentration of ~10 mg/mL.

In some experiments the cholate-soluble supernatant was dialyzed overnight against 100 volumes of buffer K. Before the experiment the proteoliposomes were collected by centrifugation and resuspended in a minimal volume of buffer K to yield a protein concentration of ~10 mg/mL. The specific activity of the proteoliposomes obtained by this technique was similar to that of the proteoliposomes obtained by Sephadex filtration. The results of the experiments described in the present communication were obtained by the latter technique.

In a recent study it has been shown that liposomes obtained by this technique are bigger and more homogeneous than those obtained by most other methods (Brunner et al., 1976). This method of detergent removal is superior because of its rapidity and its reproducibility. For processing of very small amounts of protein we have recently utilized small columns, as described by Penefsky (1977). The protein recovery was over 95%, and since it is not diluted after the passage through the column, in many cases there is no need for concentrating the proteoliposomes by centrifugation.

Transport Assays. ATP-dependent transport was assayed at 30 °C in 0.3 mL of a medium containing (in final concentration) 0.3 M sucrose, 10 mM Tris–Hepes (pH 8.5), 2.5 mM $MgSO_4$, 5 mM Na-ATP, and 0.09 μM 5-hydroxy[G-^{3}H]tryptamine (27.8 Ci/mmol). The reaction was initiated by addition of a small aliquot (3 μL) of proteoliposomes containing 20–40 μg of protein. At a given time, uptake was terminated by rapid addition of 2 mL of a cold solution of 0.3 M sucrose containing 10 mM Tris–Hepes (pH 8.5) and immediate filtration through membrane filters (Schleicher and Schuell, 0.45-μm pore size, or Millipore, 0.22 μM). The filters were then rapidly washed with another 2 mL of the latter solution, removed from the suction apparatus, dried, and assayed for radioactivity by liquid scintillation spectrometry.

ΔpH-dependent transport was assayed at 30 °C in 0.3 mL of a medium containing (in final concentration) 0.3 M sucrose, 10 mM Tris–Hepes (pH 8.5), 2.5 mM $MgSO_4$, 5 μM nigericin, and 0.09 μM 5-hydroxy[G-^{3}H]tryptamine (27.8 Ci/mmol). The reaction was initiated by addition of a small aliquot (3 μL) of proteoliposomes containing 20–40 μg of protein. Termination of the reaction and counting were performed as described above. Activity was always completely dependent on the presence of nigericin and on a K^+ gradient ($K_{in} > K_{out}$).

ATPase Assays. ATPase activity was assayed at 37 °C in 1 mL of a medium containing (in final concentrations) 0.3 M sucrose, 20 mM K-Hepes (pH 7.4), 2.5 mM ATP, 2.5 mM $MgSO_4$, 5 mM PEP, and 10 units of pyruvate kinase (625 units/mg). The reaction was initiated by the addition of a small aliquot (25 μL) of membrane vesicles containing ~100 μg of protein. After 20 min, the reaction was stopped by the addition of 0.1 mL of 40% trichloroacetic acid. Inorganic phosphate released was assayed as described by Lohmann & Jendrassik (1926) in the presence of 1% sodium dodecyl sulfate.

Chemicals. 5-Hydroxy[G-^{3}H]tryptamine (27.8 Ci/mmol) was purchased from Amersham. Asolectin was from Associated Concentrates, Woodside, L.I., NY. Nigericin was the generous gift of Dr. R. J. Hosley, Eli Lilly. All other chemicals were from commercial sources and of the highest purity available.

Results and Discussion

The data presented in Figure 1 describe the results of a typical reconstitution experiment. Thus, membranes treated with 0.9% sodium cholate showed no ability whatsoever to accumulate 5-hydroxytryptamine. When the 130000g supernatant (which contained ~30% of the protein) is passed through a Sephadex column, the proteoliposomes formed do accumulate 5-HT against its concentration gradient. In the experiment described, K^+-containing membranes are diluted 100-fold into a medium devoid of K^+. Addition of nigericin, an ionophore which catalyzes the exchange of potassium and hydrogen ions, induces a fast uptake of 5-HT which after ~2 min reaches a steady-state level of 110 pmol/mg of protein (Figure 1A). If nigericin is omitted from the medium (Figure 1A) or in the presence of external potassium (not shown), no uptake is observed. This accumulation requires the integrity of the membrane, since addition of sodium cholate to a final concentration of 0.8% causes an efflux of the previously transported solute. Addition of an excess of unlabeled solute induces an efflux of the preaccumulated material. This finding supports the contention that the accumulated material is not metabolized and also that a saturable component (i.e., the transporter) is involved in the process. Accumulation is observed only under conditions in which a ΔpH is artificially generated across the membrane. ATP, which is presumably

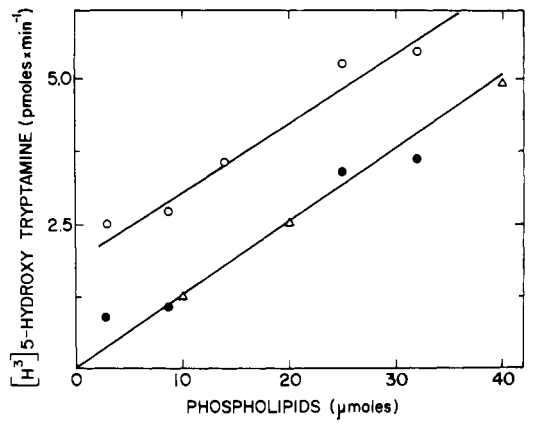

FIGURE 2: 5-Hydroxytryptamine uptake in proteoliposomes reconstituted with different amounts of phospholipids. The catecholamine transporter was solubilized and reconstituted as described under Experimental Procedure except that the lipids were added at the indicated amounts to aliquots containing 6.6 mg of protein each. The assay was performed as indicated in Figure 1 in the presence (●) or absence (○) of 2 μM reserpine; (△) liposomes to which no protein was added.

the physiological energy source for accumulation in the native system, is almost completely ineffective under the experimental conditions described.

Reserpine, a well-known inhibitor of catecholamine accumulation in intact granules and in chromaffin granule membrane vesicles, inhibits uptake in the reconstituted system as well. However, the inhibition in the reconstituted system is never complete (Figure 1B). The levels of uptake achieved in the presence of reserpine are very similar to the uptake of a system to which no protein was added (Figure 1B).

The correlation between the uptake levels in the absence of any protein and the uptake in the presence of reserpine is maintained under all the experimental conditions tested. Thus, for example, when the amount of lipids present during reconstitution is increased from 2.8 to 40 μmol, the uptake in the absence of any added protein increases linearly as a function of the increase in the concentration of lipids (Figure 2). In parallel, the fraction of the uptake which is sensitive to reserpine decreases as a function of the increasing amount of lipids during reconstitution (Figure 2). Thus, reserpine-sensitive transport constitutes more than 65% of the measured activity up to ~10 μmol of added lipids. When more than 30 μmol is present during reconstitution, the sensitive fraction decreases to less than 25%. These findings can be explained on the basis of the observation that catecholamines are accumulated in liposomes in response to a pH gradient even in the absence of any added protein [Nichols & Deamer (1977) and Figure 3B]. However, the carrier-mediated transport shows different properties than those detected in the absence of protein (Figure 3). (a) Reserpine sensitivity: carrier-mediated transport is sensitive to reserpine while the unmediated transport is not. (b) Competition: accumulation of 5-HT is competitively inhibited by other biogenic amines only when the transport is carrier mediated. Thus, uptake of 5-HT into proteoliposomes, but not into liposomes, is inhibited by epinephrine and norepinephrine with apparent K_I values comparable to those observed in the native system. (c) Temperature sensitivity: boiling of proteoliposomes (but not of liposomes) destroys transport activity. (d) Saturation: when an excess of unlabeled 5-HT is added after radioactive substrate is accumulated, the previously accumulated solute is released from proteoliposomes (Figure 1A) but not from liposomes (data not shown). Moreover, when the initial rate of 5-HT transport is measured in proteoliposomes as a function

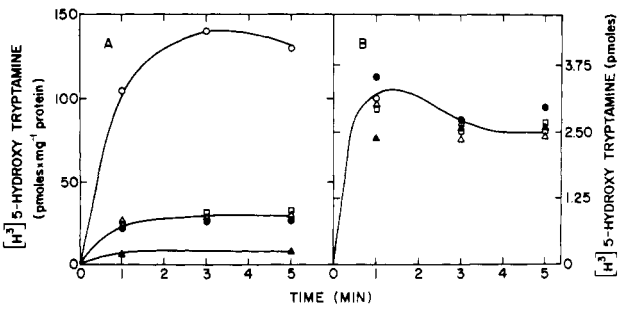

FIGURE 3: Carrier-mediated 5-HT accumulation as compared to unmediated transport. (A) The catecholamine transporter was solubilized and reconstituted as described under Experimental Procedure. The final protein concentration of the reconstituted proteoliposomes was 14.4 mg/mL. (B) In parallel, phospholipids to which no protein was added were treated in the same way except that the phospholipid concentrations were manipulated so that the final amount in the assay was 5 times higher than that of the reconstituted proteoliposomes. Under these conditions the amounts of 5-HT accumulated by liposomes are comparable to those accumulated by proteoliposomes. The assay was performed as described in Figure 1 (○) except that the following compounds were added to yield the indicated final concentrations: epinephrine, 50 μM (△); norepinephrine, 50 μM (□); and reserpine, 2 μM (●). In one case (▲) the membrane suspensions were boiled during 3 min before dilution into the reaction medium. In both cases (A and B) no uptake was observed when nigericin was omitted from the assay medium.

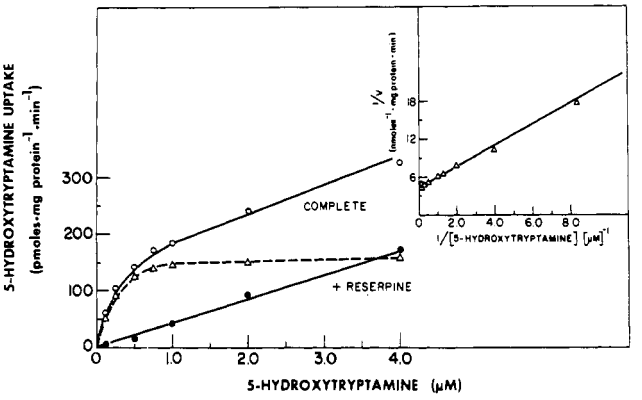

FIGURE 4: Kinetics of 5-HT uptake in the reconstituted system. Reconstitution was performed as described under Experimental Procedure. The final protein concentration of the reconstituted proteoliposomes was 17.6 mg/mL. 5-HT uptake was measured in duplicate at the indicated concentrations in the presence (○) or absence (●) of 2 μM reserpine. After 1 min of reaction, uptake was terminated and assayed as described under Experimental Procedure.

of 5-HT concentration in the medium, two components are detected, a saturable one, reserpine sensitive, with an apparent K_m of 0.4 μM (which is in the same range of the K_m of the native system) and a nonsaturable one which is reserpine insensitive and does not saturate in the range of 5-HT concentrations tested (up to 20 μM). Accumulation into liposomes shows no saturation (Figure 4). In these experiments the assay was performed by diluting reconstituted proteoliposomes into media containing increasing 5-HT concentrations (up to 20 μM). The uptake into liposomes (not shown) or into proteoliposomes in the presence of reserpine shows a linear dependence on the external 5-HT concentration. Uptake into proteoliposomes on the other hand shows a biphasic dependence. When the reserpine-sensitive fraction of this uptake is plotted as a function of the external 5-HT concentration, saturation is observed. As already stated, the apparent K_m of this fraction (as calculated from Lineweaver–Burk plots) is 0.4 μM, which is somewhat lower than the apparent K_m of the native system, 4 μM (Kanner et al., 1979). It is not clear what the significance of this difference is. The V_{max} of the

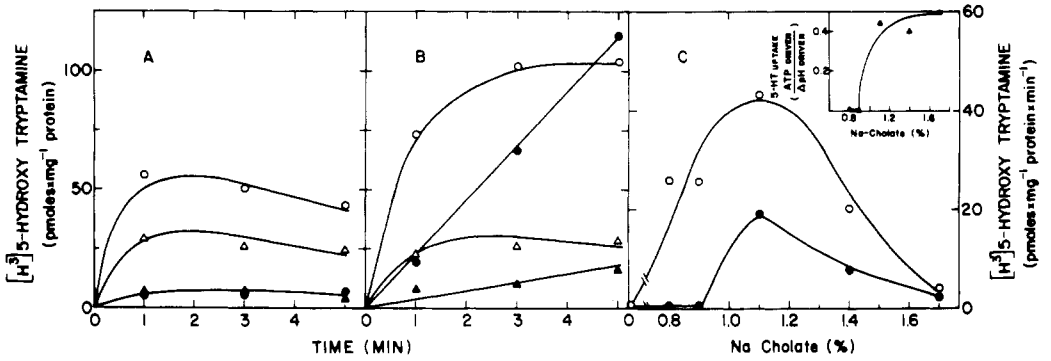

FIGURE 5: Differential solubilization of the catecholamine transporter and the H^+-translocating ATPase at different cholate concentrations. Solubilization and reconstitution were performed as described under Experimental Procedure except that the cholate concentrations were as indicated. The final protein concentration of the reconstituted proteoliposomes was between 6.4 and 7.4 mg/mL. Uptake was measured by diluting 3 μL of the above suspension into 300 μL of a mixture containing 0.3 M sucrose, 0.01 M Tris–Hepes, pH 8.5, 0.09 μM 5-hydroxy[G-^{3}H]tryptamine (27.8 Ci/mmol), and either 5 μM nigericin (open symbols) or 5 mM ATP and 2.5 mM $MgSO_4$ (closed symbols). In every case a control containing 2 μM reserpine was assayed (triangles). (C) The activity plotted is the reserpine-sensitive fraction; (A) 0.8% cholate during extraction; and (B) 1.1% cholate.

reserpine-sensitive fraction was 252 pmol min^{-1} (mg of protein)$^{-1}$. Taken as a whole, the described findings are best explained by assuming that the reconstituted preparation consists of a mixture of proteoliposomes containing the catecholamine transporter, proteoliposomes in which the carrier is absent, and liposomes with no protein incorporated. The last two classes of structures show accumulation which is not saturable and which is not reserpine sensitive.

The liposomes may be partially fractionated from the proteoliposomes by centrifuging through a 0.35 M sucrose cushion. The uptake by the lipid-enriched fraction which did not penetrate the cushion was inhibited by reserpine only slightly (17%) while the uptake of the protein-rich liposomes was inhibited more than 70%. Proteoliposomes that were not subjected to this treatment showed a 34% inhibition by reserpine. In these experiments, the amount of phopholipid added per 6.6 mg of protein was 25 μmol.

The lipid requirement of the system cannot be easily demonstrated because of the instability of the transporter in the absence of externally added lipids. When phospholipids are added only after the centrifugation step, 91% of the activity is lost compared to the control in which phospholipids were added 5 min after the detergent.

The maximal amount of functional transporter solubilized in the presence of sodium cholate is obtained at a detergent concentration of 1.1%. Increasing the concentration above 1.1% brings about a decrease in the activity of the solubilized protein (Figure 5C). As previously stated, at low detergent concentration ATP is a poor source of energy (Figure 5A). However, ATP becomes more and more effective as the cholate concentration is increased (parts B and C of Figure 5). At the same time one can demonstrate that the fraction of Mg^{2+}–ATPase catalytic activity that is released to the supernatant increases with an increase in the detergent concentration (not shown). It should be noted that although the catalytic activity of the Mg^{2+}–ATPase in the supernatant continues to increase even above 1.7% cholate (data not shown), the ATP-dependent transport shows an optimum at 1.1% and thereafter starts decreasing. This decrease is rather parallel to the one shown by the ΔpH-dependent transport, suggesting again that above 1.1% cholate the transporter's functionality is impaired. On the basis of the data presented, it is suggested that there is a sequential solubilization of the two membrane components tested. When both components are solubilized, reconstitution may render a fraction of the proteoliposomes with both activities and then ATP-dependent transport, which requires the presence both of the Mg^{2+}–ATPase and the catecholamine transporter, may be detected. If one assumes that the transport rates observed are proportional to the amount of functional proteoliposomes, it can be calculated that at the optimal concentration of detergent ~40% of the transporter-containing population also contains a functional proton-translocating ATPase.

The differential effects described tend to strengthen the contention that the protein which does not precipitate during centrifugation is indeed solubilized. Further support for the above is provided by the finding that almost 90% of the solubilized protein is included in a Sepharose 6B column (not shown).

The reconstitution of the biogenic amine carrier has a special problem of its own. The carrier is rather labile and requires the presence of phospholipids for activity. On the other hand, increasing amounts of lipids result in noncarrier-mediated transport (Figures 2 and 3). Thus, a suitable compromise had to be found between these opposing requirements and this has been described in this communication.

The results presented in this communication strengthen the contention that concentrative uptake of biogenic amines in storage organelles is driven by a transmembrane pH gradient generated by the ATPase. Thus, it has been possible to solubilize and reconstitute each of the two components directly involved in the accumulation: the proton-translocating ATPase on the one hand and the catecholamine transporter on the other. The transporter catalyzes accumulation of the amines, even in the absence of the ATPase molecule, provided a pH gradient is artificially imposed across the membrane. It should be noted in this context that we have been unable to show accumulation when electrical potentials are artificially imposed across the membrane. Although these negative results do not rule out the possibility that the translocation process is electrogenic (e.g., by an exchange with a proton), they tend to support the idea that the species translocated is the neutral amine. Effects of electrical potential on the movement of adrenaline have been detected only in the intact granule (Johnson et al., 1978; Njus & Radda, 1978; Holz, 1978). Since in this preparation the amine does not remain free in solution after its translocation, the effect of the electrical potential could be secondary and due to inhibition of other systems active in the intact organelle. These effects of membrane potential are not detected either in membrane vesicles (Phillips, 1978; Schuldiner et al., 1978) or in proteoliposomes. Hopefully, it will be possible to answer this

question as well as others when a functional purified transporter is available. The techniques described in this communication provide a fast and useful assay for the above described purification.

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A Proton and Carbon-13 Nuclear Magnetic Resonance Spectroscopy Study of the Conformation of a Protonated 11-*cis*-Retinal Schiff Base†

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ABSTRACT: Model visual pigment chromophores, *N*-(11-*cis*-retinylidene)propylimine (I) and the trifluoroacetate and chloride salts of I, have been prepared in highly purified form and their proton (^{1}H) and carbon-13 (^{13}C) nuclear magnetic resonance (NMR) spectra analyzed. The ^{1}H NMR chemical shifts and coupling constants for *N*-(11-*cis*-retinylidene)-propyliminium trifluoroacetate indicate that the polyene chain is planar from C(7) to C(12) and twisted about the C(12)–C(13) bond, forming a mixture of distorted 12-s-cis and distorted 12-s-trans conformations. Therefore, a protonated 11-*cis*-retinal Schiff base relieves steric strain due to interactions of 10-H with 13-CH_3 and 14-H in a manner similar to 11-*cis*-retinal. Significant differences are seen between the behavior of the respective ^{13}C chemical shifts of the 11-cis and all-trans isomers upon going from retinal to the imine to the protonated imine. A comparative analysis of the ^{13}C NMR chemical shifts of the 11-cis and all-trans isomers of these chromophores, using the steric interaction hypothesis to explain the γ shift, indicates that protonation of I in $CDCl_3$ at −65 °C results in a change of the more stable conformation from distorted 12-s-cis to distorted 12-s-trans. Determination of the ^{1}H nuclear Overhauser enhancement of 10-H upon irradiating 13-CH_3 of the protonated retinylidenimine in acetone-d_6 also supports this result. A consideration of the effects of solvent and temperature on the solution conformation of the retinylidene chain in a protonated Schiff base leads to conclusions in agreement with the NMR studies. It is proposed from these results and resonance Raman experiments that the conformation of the chromophore of rhodopsin is distorted 11-cis-12-s-trans.

The chromophore of the vertebrate visual pigment rhodopsin is postulated to be a specifically perturbed 11-*cis*-retinylidene group that is bound through a protonated Schiff base linkage to an amino group of the apoprotein opsin (Morton & Pitt, 1955; Kropf & Hubbard, 1958; Hubbard, 1969; Rimai et al., 1970; Lewis et al., 1973; Oseroff & Callender, 1974; Abrahamson & Fager, 1973; Mathies & Stryer, 1976; Callender et al., 1976). The hypothesis of a protonated Schiff base was stated by Morton & Pitt (1955) to explain the properties of "indicator yellow" and by Kropf & Hubbard (1958) to explain the large difference between the visible absorptions of retinal and rhodopsin. Further evidence was obtained by Bownds (1967) and Fager et al. (1972), who demonstrated that the chromophore could be covalently attached to opsin through an amino linkage by reacting the pigment with $NaBH_4$ or

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