Topography of Rhodopsin in Rod Outer Segment Disk Membranes. Photochemical Labeling with *N*-(4-Azido-2-nitrophenyl)-2-aminoethanesulfonate†

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ABSTRACT: Rod cell disk membranes have been photochemically reacted with the water-soluble, membrane-impermeable nitrene precursor *N*-(4-azido-2-nitrophenyl)-2-aminoethanesulfonate [NAP-taurine, NAPT]. Rhodopsin, minor membrane proteins, and lipids all incorporate the (nitrophenyl)[^{35}S]taurine (NPT) label. We also find that rhodopsin may be labeled in the dark using prephotolyzed NAPT. This reaction is presumably due to long-lived photoproducts of NAPT. NAPT modifies rhodopsin in the membrane in a selective manner; some cyanogen bromide peptides of NPT-rhodopsin contain appreciable NPT label and some contain essentially none. Precise specific radioactivities could not be determined for the [^{35}S]NPT-peptides since loss of label from some of the peptides was observed during purification procedures. Rhodopsin's carboxyl-terminal cyanogen bromide peptides are well labeled when the protein is modified in disk membranes but the amino-terminal peptide is poorly labeled. When rhodopsin is labeled in reconstituted membranes in which both surfaces of rhodopsin are accessible to reagent, labeling of rhodopsin's amino-terminal peptide is enhanced. These results are consistent with a model in which rhodopsin's carboxyl-terminal region is located at the cytoplasmic (external) surface of disk membranes and its amino terminus is located at the intradiskal membrane surface.

Rhodopsin is the chromoprotein of rod cells in the retina which is responsible for black and white vision. The outer segments of rod cells contain stacks of disk-shaped membranes which contain the photoreceptor protein rhodopsin. Approximately 95% of the intrinsic membrane protein of disk membranes is rhodopsin (Smith et al., 1975; Krebs & Kühn, 1977).

It now appears clear that rhodopsin is a transmembrane protein. The cytoplasmic (external) disk membrane surface of membrane-bound rhodopsin is susceptible to attack by proteases (Saari, 1974), and it has been shown that the carboxyl-terminal region of rhodopsin is located at this cytoplasmic membrane surface (Hargrave & Fong, 1977). The amino-terminal region of rhodopsin contains rhodopsin's sites of carbohydrate attachment (Hargrave, 1977). Histochemical evidence places carbohydrate internally in the disk membrane (Röhlich, 1976), and lectin-binding studies support localization of rhodopsin's carbohydrate at the internal surface of the disk membrane (Adams et al., 1978). The transmembrane nature of rhodopsin is convincingly demonstrated by iodination studies on disk membranes and reconstituted vesicles (Fung & Hubbell, 1978).

The mechanism of action of rhodopsin as a photoreceptor is presumably related to its mode of insertion in the disk membrane. Rhodopsin has been postulated to act by regulating the release of a transmitter substance, Ca^{2+}, from the internal compartment of the disk membrane (Hagins, 1972). It has also been hypothesized that rhodopsin acts via its surface to affect rod cell biochemical events which lead to transduction of the photon absorption event to that of neural transmission (Hubbell & Bownds, 1979). Whatever we learn concerning the membrane orientation of rhodopsin is likely to be of value in understanding its function and mechanism of action.

Membrane-bound rhodopsin has been studied by a number of surface probes. Different proteolytic enzymes cleave rhodopsin internally in one region (Pober & Stryer, 1975) or two regions (Sale et al., 1977) as well as at the carboxyl terminus (Hargrave & Fong, 1977). One of the regions where proteolytic cleavage occurs is also subject to modification by transglutaminase (Pober et al., 1978). The enzyme lactoperoxidase can also modify rhodopsin in the membrane. A small amount of lactoperoxidase-catalyzed introduction of iodine into tyrosyl residues can be obtained, which is considerably increased by introduction of an iodination-enhancement label into rhodopsin (Fung & Hubbell, 1978).

Chemical modification of rhodopsin's surface has been reported. Isethionyl acetimidate, an imido ester which was designed to be membrane impermeable (Whiteley & Berg, 1974), has been used to modify the amino groups of disk membranes (Raubach et al., 1974). Vectorial labeling with isethionyl acetimidate and choline acetimidate indicates that rhodopsin spans the membrane although these reagents have been demonstrated to be slightly permeable to the disk membrane (Nemes et al., 1980).

All of the above labeling techniques are rather specific in the sites which can be labeled. The enzymatic techniques will only allow labeling of sites based upon the limited substrate specificity of the enzyme and upon the conformation of the surface-exposed region of the protein. Thus, transglutaminase can only label "highly exposed" glutaminyl residues (Iwanij, 1977). Isethionyl acetimidate and choline acetimidate would be restricted to chemical modification of lysyl residues in proteins. If it is desired to label *any* surface region of a protein, a probe of broad specificity would be required. Reagents which generate nitrenes or carbenes offer such an opportunity (Knowles, 1972).

NAPT[1] was designed to be a membrane-impermeable reagent capable of labeling any type of molecule at a mem-

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[1] Abbreviations used: buffer A, 67 mM sodium phosphate (pH 7.0) and 1 mM each in $MgCl_2$, Na_2EDTA, and DTT; DTT, dithiothreitol; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; NAPT, *N*-(4-azido-2-nitrophenyl)-2-aminoethanesulfonate or NAP-taurine; NPT, (nitrophenyl)taurine; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; Cl_3AcOH, trichloroacetic acid; TrTAB, tridecyltrimethylammonium bromide.

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brane surface (Staros, 1974). Its application to the study of erythrocyte membranes showed that it was capable of labeling all of the proteins which had previously been ascribed to the external surface of the erythrocyte and several minor ones which had not been previously labeled on the external surface by other reagents (Staros & Richards, 1974). Subsequent studies have shown that NAPT is capable of reacting with side chains of any amino acid but that it does exhibit some preference (Matheson et al., 1977). There appears to be some preference in the labeling of the erythrocyte band 3 protein compared to some of the other proteins labeled (Staros & Richards, 1974). This is presumably explained not by particular amino acid content but by the preferential interaction of NAPT with the protein due to band 3's function as an anion-transporting protein (Knauf et al., 1978). Although Staros & Richards (1974) demonstrated the membrane impermeability of NAPT at 0 °C, NAPT has been demonstrated to cross the membrane at 37 °C (Staros et al., 1975). The anion-transporting protein, band 3, appears to be responsible for the membrane permeability which can be demonstrated for NAPT in the erythrocyte (Knauf et al., 1978). There is no reason to believe that the reagent should be membrane permeable in the absence of such a transport system.

Experimental Section

Materials

Analytical reagent grade chemicals were used without further purification unless otherwise specified. The following materials were purchased from Sigma Chemical Co.: D-α-tocopherol, methyl α-D-glucopyranoside, methyl α-glucoside, Hepes, L-α-phosphatidylethanolamine (egg yolk, type III), L-α-phosphatidylcholine (egg yolk, type III-E), hyaluronidase (type V, ovine testes), chondroitinase ABC (*Proteus vulgaris*), concanavalin A–Sepharose 4B, Sephadex G-50 (20–80 μm), and Triton X-114. Thermolysin and octyl β-D-glucopyranoside (octyl glucoside) were obtained from Calbiochem-Behring. Hydroxylapatite (DNA grade Bio-Gel HTP) and protein standard mixtures for calibration of $NaDodSO_4$ gels were purchased from Bio-Rad Laboratories. Teflon filters (LSWP 025, 5.0 μm) were obtained from Millipore Inc. We synthesized tridecyltrimethylammonium bromide (TrTAB) from tridecyl bromide (Columbia Organics, Columbia, SC) and trimethylamine (Eastman) according to the procedure of Hong & Hubbell (1972). Sodium dodecyl sulfate (Sipon WD from Alcolac, Inc., Baltimore, MD) was recrystallized from 80% ethanol. Acrylamide (Eastman) was recrystallized from chloroform. We decolorized cholic acid (Sigma) and recrystallized it as described by Fung & Hubbell (1978). [^{3}H]Formaldehyde (New England Nuclear) was diluted with nonradioactive formaldehyde to a final specific radioactivity of 3.4×10^4 dpm/mmol. Sodium cyanoborohydride (Aldrich) was recrystallized from acetonitrile–methylene chloride (Jentoft & Dearborn, 1979). The white powder was stored in a desiccator over P_2O_5. 11-*cis*-Retinal was the gift of Hoffmann-La Roche Inc. The solid was dissolved in ethanol and its concentration was determined spectrophotometrically with $E_{380nm}^{1\%,1cm} = 878$ (Hubbard et al., 1971).

Eyes were collected at a local slaughter house and kept on ice in the dark prior to dissection. Retinas were removed under dim red light and quick frozen by dropping into liquid nitrogen. Retinas were stored in tightly wrapped containers at −80 °C.

$NaDodSO_4$–polyacrylamide gel electrophoresis and gel staining were performed according to Fairbanks et al. (1971). Protein molecular weights were determined from a plot of log M_r vs. mobility by using standard proteins for calibration. Coomassie blue stained gels were scanned by using a Varian Techtron 635 spectrophotometer equipped with a gel scanning attachment. Gels for radioactive counting were sliced into 1-mm slices by using a Mickel gel slicer (Brinkmann Instruments, Inc.). Gel slices were dissolved by warming with 30% H_2O_2 (0.5 mL/1-mm gel). Radioactive samples were mixed with a xylene–Triton X-114 cocktail (Anderson & McClure, 1973) containing Scinti-Prep (Fisher). Radioactive counting was performed with a Searle Mark III liquid scintillation counter.

Acid hydrolysis of peptides, proteins, and aliquots from column fractions was performed in an evacuated glass chamber containing constant-boiling HCl (Dreyer & Bynum, 1967). Amino acid analysis was performed either on a Beckman 119CL amino acid analyzer equipped with a Model 126 data system or on a Durrum D500 amino acid analyzer.

Rod outer segment disk membranes were prepared according to the method of Smith et al. (1975). They were used immediately or stored at 4 °C in the dark suspended in 5% sucrose with α-tocopherol and under an argon atmosphere. When rod outer segments, rather than disk membranes, were prepared, we used the method of Papermaster & Dreyer (1974) as adapted by McDowell & Kühn (1977). Rhodopsin was purified by chromatography on hydroxylapatite using the detergent TrTAB (Hong & Hubbell, 1973). Reconstituted membrane vesicles containing rhodopsin were prepared by using purified rhodopsin, sodium cholate, and egg phospholipids as described by Fung & Hubbell (1978). Rhodopsin content was measured with $E_{498nm} = 40\,600$ (Wald & Brown, 1953). Thermolysin digestion of membranes was performed as described previously (Hargrave & Fong, 1977).

[^{35}S]NAPT was synthesized following the procedure of Staros et al. (1975) and purified by using the ion-exchange procedure of Staros & Richards (1974). NAPT was identified by chromatography on silica gel GF (Merck) thin-layer plates by using 2-propanol–formic acid–water (20:1:15) (Matheson et al., 1977). The concentration of NAPT was determined spectrophotometrically by using $E_{471nm} = 4730$ (Staros & Richards, 1974). The specific radioactivity was determined to be 64.8 Ci/mol when first synthesized. Specific radioactivity was recalculated for each experiment. NAPT was stored in aqueous solution (~4.4 mM) in small aliquots in the dark at −20 °C.

Methods

Photolysis of membrane samples was performed by using an apparatus based upon the design of Staros (1974). Disk membranes were suspended at 2 to 3 mg/mL in buffer A. In a typical experiment 100 μL of NAPT solution was added to 500-μL membranes. Samples were contained in a Corex test tube (Corning No. 8441) which was inserted into a machined Teflon holder fitted with 2 O rings. This was fitted to a brass shaft which was turned at 90 rpm by a flash evaporator motor (Buchler No. PFE-1BN). Temperature was maintained at 20 °C by a circulating water bath through a double-walled cooling jacket. Illumination was provided from below by a Sylvania photoflood lamp (R32, 375 W) at a distance of 15 cm.

In early experiments designed to determine reaction conditions, NAPT incorporation into disk membranes was evaluated by use of a filter assay. Measured aliquots (20–50 μL) of the reaction mixture were withdrawn at timed intervals and mixed with 1 mL of ice-cold 10% Cl_3AcOH (1 mM in DTT). Samples were transferred to a Millipore filter by using six additional 1-mL portions of the Cl_3AcOH solution, and the filter was washed with three 10-mL portions of the Cl_3AcOH

solution. Radioactivity retained by the filters was determined by liquid scintillation counting. Values for "blank" were subtracted from the experimental values. Blank values were determined by mixing membrane samples with reagent in the dark, immediately quenching with Cl_3AcOH solution, and filtering as described above. Such values were typically equivalent to 10% of the total counts incorporated into the membranes during the first 5 min of labeling.

After experimental conditions had been established as described above, the time course of incorporation of NAPT into *rhodopsin* was determined. Disk membranes which had been reductively methylated with [^{3}H]formaldehyde were employed for these experiments. Measured aliquots (20–50 μL) of the NAPT reaction mixture were withdrawn at timed intervals and mixed by a vortex mixer with 1 mL of ice-cold buffer A. Samples were centrifuged 15 min at 37000*g*. The pellet was resuspended, and the centrifugation and wash procedure was repeated three times. Membrane pellets were dissolved in $NaDodSO_4$-dissociation medium to a final concentration of 1 mg/mL. Aliquots (20–50 μL) were taken for $NaDodSO_4$–polyacrylamide gel electrophoresis. Gels were fixed and stained, and the stained rhodopsin band was excised for radioactive counting. In some experiments duplicate gels were run, and a region of the unstained gel (corresponding to the rhodopsin band in the stained gel) was excised for determination of the ^{35}S and ^{3}H content.

Disk membranes or rod outer segments were suspended in buffer A and labeled with NAPT as previously described to test for the presence of labeled glycosaminoglycans. Labeled membranes were suspended in and pelleted from small volumes of the buffer recommended for the enzymes to be employed: 150 mM Tris hydrochloride–acetate (pH 8.0) for chondroitinase and 300 mM sodium acetate–chloride (pH 5.4) for hyaluronidase (Saito et al., 1968).

Membrane samples containing ~200 μg of rhodopsin were then suspended in 500 μL of buffer, and 5 μL 1 M PMSF in dioxane was added to inhibit any proteolytic activity which might be present in the carbohydrases (Gaw, 1977). One unit of chondroitinase or 1 unit of hyaluronidase was then added, and incubation was performed for 1 h at 37 °C.

Purification of NPT-rhodopsin. Membrane samples which had been photolyzed in the presence of NAPT for 5 min were diluted to 10 mL with ice-cold buffer A and centrifuged at 37000*g* for 30 min. The pellet was resuspended, and the washing and centrifugation steps were repeated 3 times. The pellet was resuspended in buffer A at 1 mg/mL, and 11-*cis*-retinal was added in threefold molar excess to the rhodopsin originally present. Rhodopsin regeneration was allowed to proceed in the dark at room temperature for 3 h. The membranes were pelleted and resuspended in 50 mM Tris–acetate (pH 6.9); the procedure was repeated 2 times. Solid octyl glucoside was added to the membrane suspension to a final concentration of 50 mM. Following centrifugation the supernatant material was made 1 mM in $MgCl_2$, $MnCl_2$, and $CaCl_2$ prior to chromatography on concanavalin A–Sepharose (Steineman & Stryer, 1973). After unbound material had been collected, rhodopsin was eluted from the column with the same buffer made 0.5 M in methyl α-glucoside.

Reductive Methylation. Disk membranes were reductively methylated based on the procedure of Jentoft & Dearborn (1979). Membranes were suspended in 100 mM Hepes buffer (pH 7.5). Twenty-two micromoles of [^{3}H]formaldehyde was added per mg of rhodopsin. The reaction mixture was made 20 mM in $NaCNBH_3$ and allowed to react at 4 °C for 24 h. Membranes were pelleted by centrifugation (15 min at 37000*g*) and resuspended in buffer A, and the washing procedure was repeated 5 times.

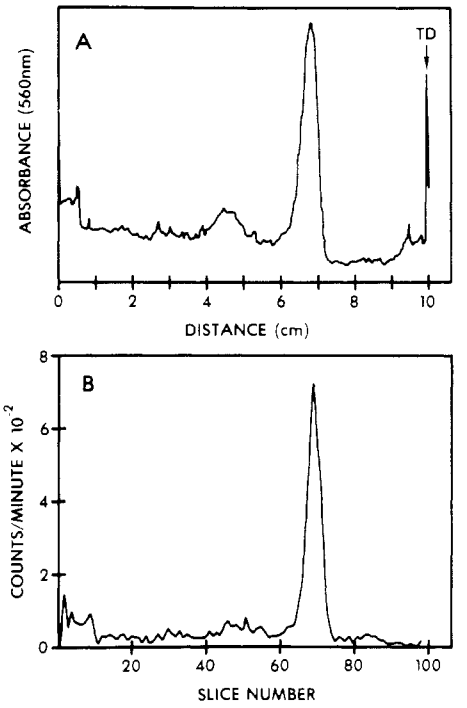


FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis profiles of disk membranes labeled with [^{35}S]NAPT. (A) Coomassie blue profile. 34 μg of protein was applied to the gel. Following electrophoresis, a nichrome wire was inserted in the gel to mark the center of the tracking dye (TD). The gel was fixed with acetic acid–2-propanol and stained with Coomassie blue. (B) ^{35}S profile. The gel in panel A was sliced and counted as described under Experimental Section. A line connects individual data points.

Preparation of Cyanogen Bromide Peptides of NPT-rhodopsin. NPT-rhodopsin, prepared by chromatography on concanavalin A–Sepharose, was dialyzed to remove the detergent. Reduction and aminoethylation of the protein were performed as previously described (Hargrave, 1977). Aminoethylated rhodopsin was submitted to cyanogen bromide cleavage in 70% formic acid at a protein concentration of less than 10 mg/mL and with 3 times its weight in CNBr. After 24-h reaction at room temperature, the reaction mixture was carefully degassed under vacuum and then loaded directly on a column of Sephadex G-50 equilibrated in 20% formic acid.

Samples for manual ninhydrin analysis were evaporated and acid hydrolyzed. Ninhydrin analysis was performed essentially as described by Hirs (1967).

Results

On the basis of preliminary experiments, standard conditions of labeling were chosen which yielded good incorporation of label into the membranes but minimized time of photolysis. The profile of incorporation of [^{35}S]NAPT into disk membrane proteins is shown in Figure 1. The bulk of the radioactivity is seen to be associated with the dominant Coomassie blue staining polypeptide (M_r 35000) which has been demonstrated to be the protein opsin (Daemen, 1973; Papermaster & Dreyer, 1974). Incorporation of the NPT label by rhodopsin provides evidence that rhodopsin has a portion of its polypeptide chain in contact with the external aqueous environment of the disk membrane.

The time course of the reaction was studied as a function of incorporation of [^{35}S]NAPT into rhodopsin. Precision in these measurements was obtained by incorporating ^{3}H into rhodopsin's amino groups by reductive methylation, thus al-

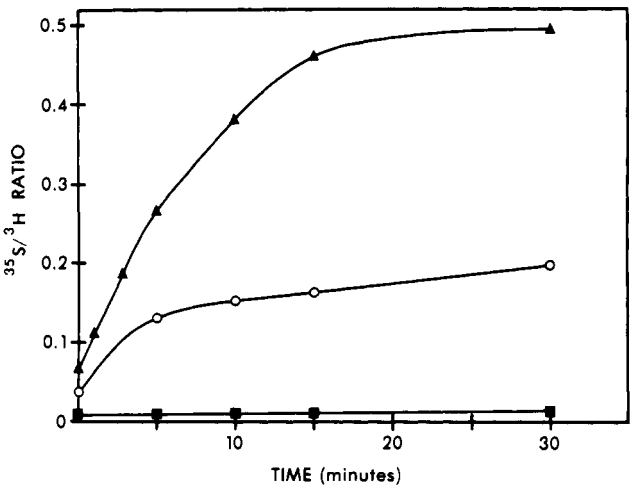


FIGURE 2: Time course of incorporation of [^{35}S]NAPT into reductively methylated [^{3}H]rhodopsin in disk membranes. [^{35}S]NAPT was mixed with reductively methylated ^{3}H-labeled disk membranes and treated as described below and under Experimental Section. Washed membranes were solubilized and submitted to NaDodSO$_4$–polyacrylamide gel electrophoresis. Each rhodopsin band was excised and its ^{35}S and ^{3}H content was determined. (▲) [^{35}S]NAPT was mixed with ^{3}H-labeled disk membranes and the mixture was photolyzed. Samples were removed at the time indicated. (○) [^{35}S]NAPT was photolyzed for 5 min and then mixed with nonphotolyzed ^{3}H-labeled disk membranes in the dark. Incubation was performed for the times indicated, in the dark. (■) ^{3}H-Labeled disk membranes and [^{35}S]NAPT were incubated in the dark. No photolysis was performed.

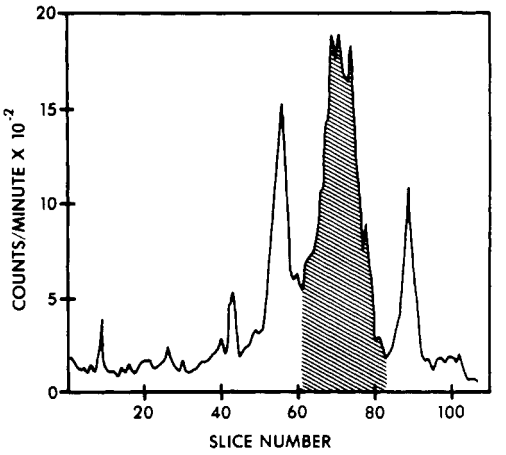


FIGURE 3: NaDodSO$_4$–polyacrylamide gel electrophoresis radioactivity profile of [^{35}S]NPT rod cell outer segment membranes. [^{35}S]-NAPT-labeled outer segments were submitted to NaDodSO$_4$–polyacrylamide gel electrophoresis and the unfixed gels were sliced. Rhodopsin is located in slices 50–60. The crosshatched area represents radioactive material which becomes depleted by washing the membrane and is removed by fixing the gel. Label in the small molecules peak in the tracking dye region (slices ~85–93) is removed by fixing the gel.

lowing [^{35}S]NAPT incorporation to be measured in relation to the ^{3}H internal standard. The results of such time course of reaction studies are presented in Figure 2. Incorporation of ^{35}S into rhodopsin appears to approach a maximum at 20–30-min photolysis time with more than half of the reaction occurring in the first 5 min. Negligible incorporation of ^{35}S into rhodopsin occurs in the absence of photolysis. [However, a measurable but small amount of incorporation does occur upon overnight incubation in the dark (M. Mas, unpublished observations).] We decided to investigate whether ^{35}S incorporation might also occur *in the dark following photolysis.* That such a reaction does indeed occur is shown in Figure 2. This reaction, occurring in minutes rather than milliseconds, cannot involve nitrene insertion (see Discussion). We chose to limit our study to the ^{35}S incorporation which occurs during the period of photolysis; thus, the washing procedure following photolysis, described under Experimental Section, was always performed immediately. As a result of these and other experiments, a 5-min photolysis time was selected for all of our studies.

As demonstrated above (Figure 1), when disk membranes labeled with NAPT were analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis, the fixed and stained gels showed rhodopsin to be the dominant labeled component. Duplicate gels which had been neither fixed nor stained showed the presence of a broad and heterogeneous labeled component migrating in the region between the rhodopsin band and tracking dye (Mas, Wang, and Hargrave, unpublished experiments). Since this material is absent in the stained gel (Figure 1), it must have been extracted from gels by the acetic acid–2-propanol fixing solution. For several disk membrane preparations, we found the amount and apparent molecular weight distribution of this component to be variable compared to that of rhodopsin. This heterogeneous ^{35}S material was also found when rod outer segment membrane preparations were reacted with NAPT (Figure 3). Washing of the labeled membranes removed some of this material, and it was completely solubilized by incubation of the labeled membranes with various buffers for 1 h at 37 °C. We subsequently determined that both [^{35}S]NAPT and photolyzed [^{35}S]NAPT alone will produce apparently the same broad ^{35}S band upon NaDodSO$_4$–polyacrylamide gel electrophoresis. It would therefore appear that variable amounts of the reagent and its photolysis products remain with the disk membranes under our experimental conditions and account for the extractable ^{35}S material shown in the crosshatched area in Figure 3. However, because of the possibility that the reagent may have reacted with membrane-associated macromolecules (see Discussion), several tests were performed. Labeled membrane preparations were incubated separately with chondroitinase, hyaluronidase, and trypsin. Such incubations failed to alter this ^{35}S material; thus, it does not appear to contain protein or the glycosaminoglycans chondroitin sulfate or hyaluronic acid. It therefore appears likely that this ^{35}S material which can be removed by washing of the membrane does not reflect the labeling of membrane surface materials and is due only to the NAPT.

The labeling of minor membrane protein components is seen clearly in Figure 3. For example, the M_r 225 000 protein described by Papermaster et al. (1976) has incorporated ^{35}S (gel slices 8 and 9, Figure 3). In Figure 3 we also observe ^{35}S migrating at the dye front where small molecules including lipids are found. To test for label incorporation into lipids, we measured the ^{35}S incorporated into Cl_3AcOH-washed disk membranes (by the filter assay) and extracted the filters with chloroform–methanol. In one experiment we observed approximately 70% of the counts to be extractable. Since the reagent and its hydrolysis products should be water soluble and the reaction of NAPT with other membrane components should not lead to chloroform–methanol soluble products, it appears that lipids have been reacted.

[^{35}S]NAPT-labeled membranes were submitted to limited proteolytic digestion by thermolysin in order to further characterize the nature of [^{35}S]NAPT incorporation into rhodopsin. The membrane-bound digestion products F1 and F2 were formed along with a small amount of an intermediate, O′, as observed previously (Hargrave & Fong, 1977). These rhodopsin digestion products also contain the [^{35}S]NAPT label (Figure 4B). This suggests that all membrane-bound digestion products of rhodopsin still retain regions which are available

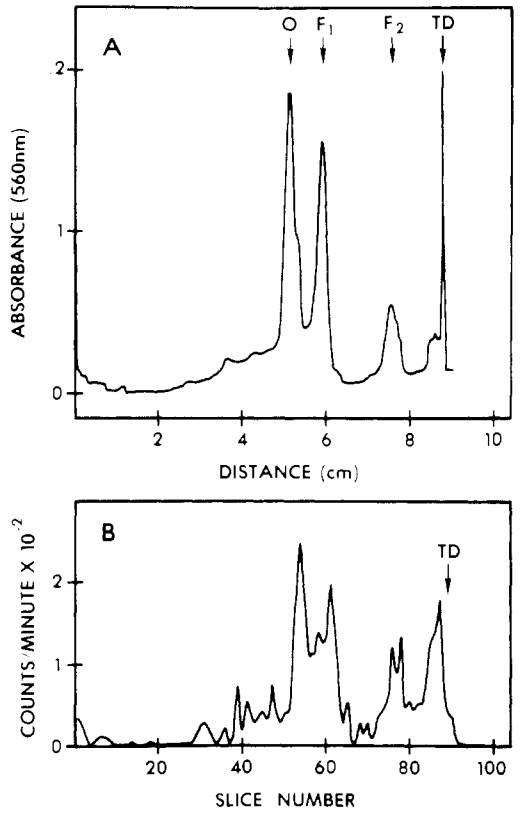
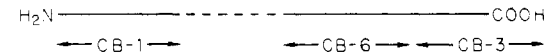


FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis profile of thermolysin-digested disk membranes labeled with [^{35}S]NAPT. (A) 22 μg of protein was loaded on the gel, and the gel was stained with Coomassie blue. (B) A duplicate of the gel in panel A, but 44 μg of protein was used. The fixed gel was sliced and its radioactivity was determined.

Scheme I



for labeling by NAPT at the disk membrane cytoplasmic surface but which are resistant to proteolysis by thermolysin.

We wished to determine which peptides of rhodopsin were well labeled in disk membranes as a way of exploring rhodopsin's membrane topography. Since in other studies we have determined the location of three of rhodopsin's cyanogen bromide peptides in the covalent sequence of the protein, information about their exposure will aid in constructing a model for rhodopsin's mode of membrane association. This information can be further extended by comparison of labeling of rhodopsin in artifical reconstituted membranes in which both surfaces of rhodopsin are accessible to reagent (Fung & Hubbell, 1978). The location of these peptides is presented in Scheme I.

The sequence of the 38 amino acid amino-terminal cyanogen bromide peptide (CB-1) has been reported (Hargrave et al., 1980). The 31 amino acid carboxyl-terminal cyanogen bromide peptide (CB-3) has been isolated and its structure has been determined (Fong, 1978; Hargrave et al., 1979). Adjacent to it in the sequence is a cyanogen bromide peptide 8 amino acids in length (CB-6) (McDowell & Griffith, 1978). These three peptides can be located easily when a cyanogen bromide digest of rhodopsin is submitted to gel filtration.

NPT-rhodopsin was purified from labeled disk membranes in order to investigate what peptides in the rhodopsin molecule are available to NAPT in the native membrane. First, rhodopsin was regenerated in photolyzed disk membranes by incubation with 11-*cis*-retinal in the dark prior to solubilization

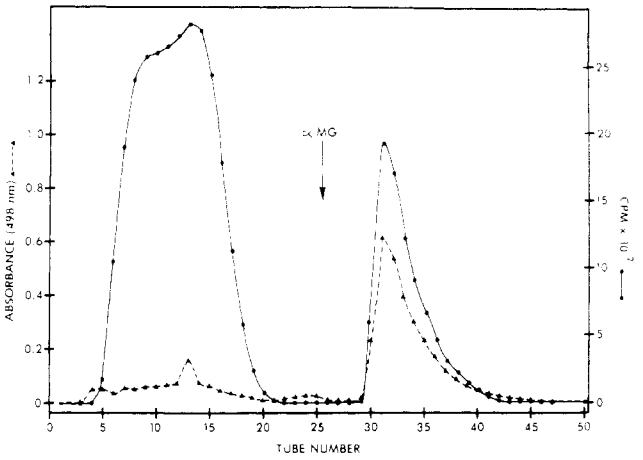


FIGURE 5: Concanavalin A–Sepharose chromatography of detergent-solubilized [^{35}S]NAPT-labeled disk membranes. Disk membranes containing 5 mg of rhodopsin were photolyzed with [^{35}S]NAPT. Membranes were washed, incubated with 11-*cis*-retinal in the dark, and solubilized with octyl glucoside. Chromatography was performed in the dark at 12 mL/h on a 0.9 × 4.5 cm column of concanavalin A–Sepharose equilibrated in buffer containing octyl glucoside. At the point shown by the arrow, buffer containing 0.5 M methyl α-glucoside (αMG) was introduced at a flow rate of 4 mL/h. 1-mL fractions were collected and 10-μL aliquots were removed for radioactive counting. Tubes 30–40 were pooled and found to contain 2.3 mg of rhodopsin (based on A_{498}).

in detergent. [^{35}S]NPT-rhodopsin was purified from other components of the reaction mixture by chromatography on concanavalin A–Sepharose (Figure 5). Lipids and other materials not bound to the column emerge unretarded in the first 20 fractions. These fractions were not pink, and their absorbance at 498 nm is due to light scattering. Rhodopsin was eluted with methyl α-glucoside. [^{35}S]NPT-rhodopsin prepared in this manner was mixed with unlabeled rhodopsin, aminoethylated, and reacted with cyanogen bromide. The cyanogen bromide peptides were chromatographed on Sephadex G-50 in order to partially separate them (Figure 6A). Ninhydrin analysis and the ^{35}S profile show that only five of the eight ninhydrin peaks eluting from the column contain appreciable amounts of label. Thus, some peptides (e.g., pool 6) are well labeled and others (e.g., pool 5) are poorly labeled. Pool 1 contains several high molecular weight peptides which remain to be characterized (Hargrave, McDowell, and Curtis, unpublished experiments). Pools 2 and 5 contain peptides which are essentially devoid of label. Pool 8 represents the salt region of the column: amino acid analysis shows a great deal of ammonia present but virtually no amino acids. Radioactivity in the peak probably arises from breakdown of some NPT-protein label during the CNBr cleavage step in 70% formic acid. Each of the three remaining pools contains a peptide whose position in the rhodopsin sequence is known. (1) Rhodopsin's 31 amino acid carboxyl-terminal peptide (CB-3) is found in pool 4, which contains appreciable label. The ninhydrin profile of pool 4 has been found to coincide with ^{32}P of this peptide which contains the phosphorylation sites of rhodopsin (Fong, 1978). (2) The 8 amino acid peptide adjoining peptide CB-3 in the sequence (CB-6) is found in pool 6. CB-6 contains a cysteine which is reactive toward *N*-ethylmaleimide (McDowell & Griffith, 1978). The identity of the [^{35}S]NPT peptide of pool 6 with peptide CB-6 has been determined by the demonstration of cochromatography of the [^{35}S]NPT pool with [^{3}H]NEM CB-6 (Wang, McDowell, and Hargrave, unpublished experiments). (3) Pool 3, which contains only a small amount of label, contains rhodopsin's amino-terminal glycopeptide (CB-1). Verification of its elution

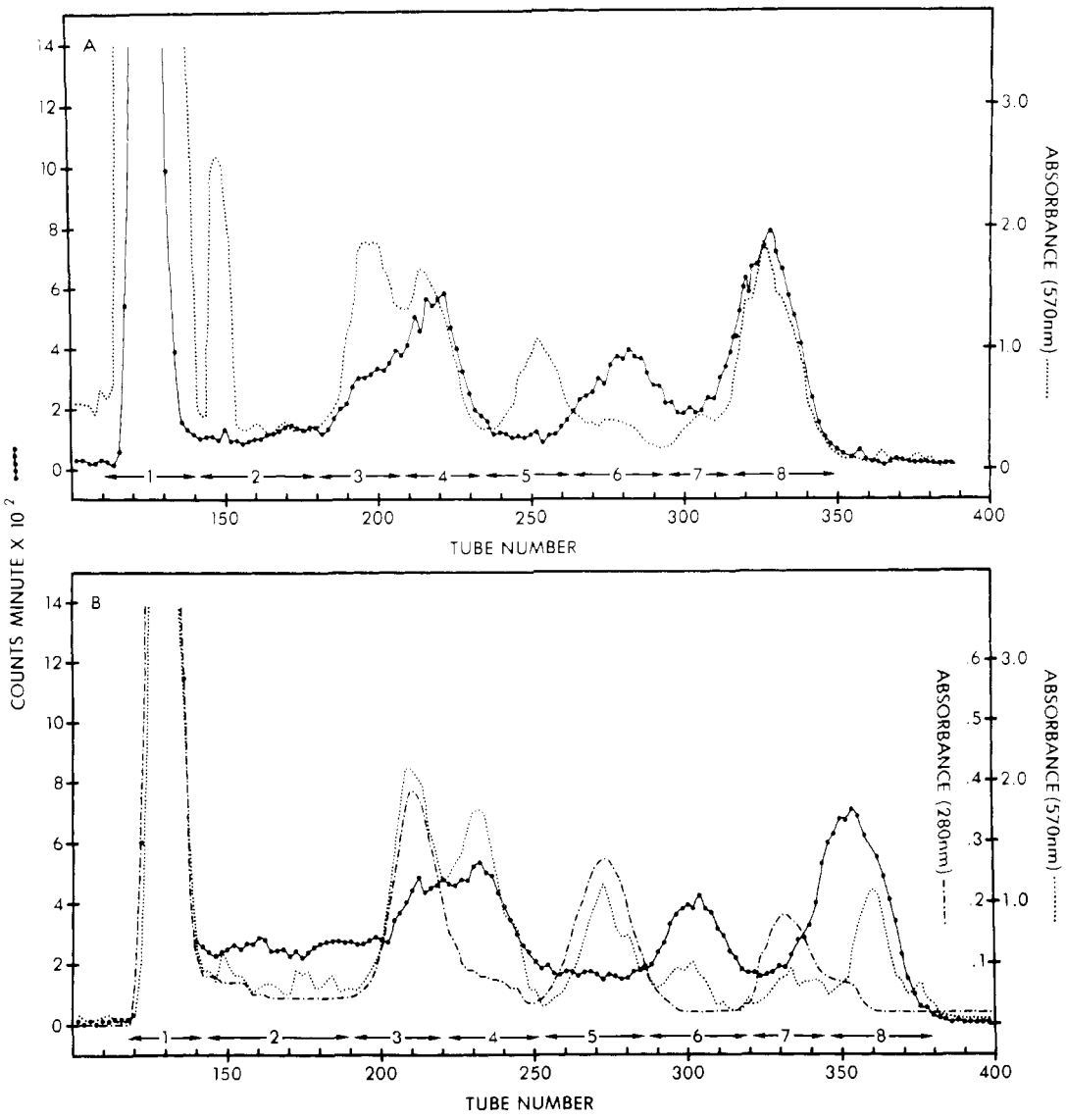


FIGURE 6: Chromatography of cyanogen bromide peptides of [^{35}S]NPT-rhodopsin from disk membranes and from reconstituted membranes. Each peptide mixture in ~10 mL was applied to a 2.5 × 200 cm column of Sephadex G-50 and eluted with 20% formic acid. Approximately 3-mL fractions were collected by drop counting. 500-μL aliquots of alternate tubes were taken for radioactivity determination, and 50-μL aliquots were taken for acid hydrolysis and ninhydrin analysis. (A) Disk membranes: 10 mg of NPT-rhodopsin purified from disk membranes was mixed with 76 mg of rhodopsin. The mixture was aminoethylated and cyanogen bromide cleaved. The peptides, containing 7.1 × 10^5 cpm, were chromatographed as shown above. Eight column fraction pools were made as shown in the figure. The peptide shown by ninhydrin analysis in pool 2 is not consistently observed in different experiments. (B) Reconstituted membranes: 12 mg of NPT-rhodopsin purified from reconstituted membranes was mixed with 70 mg of rhodopsin. The mixture was aminoethylated and cyanogen bromide cleaved. The digest, containing 9.9 × 10^5 cpm, was subjected to chromatographic analysis. The data points for radioactivity for the fractions in pool 1 are not connected with a solid line in order to increase the clarity of the figure.

position is obtained from the A_{280nm} due to its tyrosines and tryptophan (see Figure 6B) and by glucosamine analysis of the column fractions (Wang and Hargrave, unpublished experiments). Of these three peptides, the two which contain the bulk of the label are the carboxyl-terminal peptides in pool 4 (CB-3) and in pool 6 (CB-6).

In order to simultaneously label *both* membrane-exposed surfaces of rhodopsin, the [^{35}S]NAPT labeling was performed on rhodopsin in reconstituted membranes. NPT-rhodopsin from these reconstituted membranes was purified and cyanogen bromide cleaved, and its peptides were chromatographed (Figure 6B). All pools which contain label in the previous experiment (Figure 6A) are found to be labeled, as would be expected. However, there is now enhanced label incorporation in pool 3 which contains the amino-terminal peptide CB-1. Peptide CB-1 contains three tyrosyl residues and a tryptophanyl residue (Hargrave et al., 1980), and its elution position is shown by the 280-nm profile across pool 3. In an attempt to express the labeling on a quantitative basis, the pooled fractions were analyzed for radioactivity and amino acid content (Table I). The two carboxyl-terminal peptides (in pools 4 and 6) are labeled to approximately the same extent

Table I

	disk membranes[a]		reconstituted membranes[b]	
pool	cpm/μg[c]	ratio to pool 4	cpm/μg	ratio to pool 4
3	5.49	0.48	5.93	0.70
4	11.4	1	8.51	1
6	19.9	1.74	15.8	1.86

[a] Pools from the experiment shown in Figure 6A. [b] Pools from the experiment shown in Figure 6B. [c] Micrograms of amino acids determined by acid hydrolysis of pool aliquots, followed by quantitative amino acid analysis.

in each preparation; 1:1.74 in disks and 1:1.86 in reconstituted membranes. The extent of label incorporation into the N-terminal peptide (pool 3) rises from 0.48 in disks to 0.70 in reconstituted membranes, an increase of 46%. Similar results were obtained in a duplicate experiment. This enhancement of the extent of labeling of the amino-terminal peptide in reconstituted membranes suggests that the amino terminus of more molecules is accessible to the reagent in the reconstituted membrane preparation than in the disk membrane preparation.

To enable even better quantitation of the labeling reaction and comparison of the two membrane systems, we desired to completely purify the peptides CB-1 and CB-3 and determine their specific radioactivities. Both NPT-peptides were subsequently isolated in pure form, but our radioactivity profile for gel filtration steps showed that ^{35}S label was being lost from CB-3. This prevented us from obtaining valid specific radioactivity measurements for these peptides. The lack of stability of at least some of the NPT-peptide bonds during extended purification procedures precludes gathering of totally quantitative comparative labeling information with this reagent and possibly with nitrenes generally.

Discussion

We have determined that the photoreactive reagent NAPT can successfully label rhodopsin and other components of rod cell disk membranes. This nitrene label was selected due to its high reactivity, low specificity, and membrane impermeability which should allow it to label any molecular species on a membrane surface. We conclude that the labeling of rhodopsin by NAPT supports the model which places rhodopsin's carboxyl terminus on the external surface of the disk membrane and its amino terminus at the internal surface of the disk membrane.

Disk membranes were carefully prepared and stored in order to preserve the integrity of their sidedness. Isosmotic buffers were employed and mild reaction conditions were chosen. In our preliminary studies to determine the conditions for membrane photolysis, we observed photolytic damage to rhodopsin. High intensity illumination of rhodopsin for periods of 30 min–1 h in the absence of NAPT caused fuzziness and broadening of the rhodopsin band as observed by NaDodSO$_4$–polyacrylamide gel electrophoresis (Mas and Hargrave, unpublished experiments). We suspect that these results may be explained by retinal-sensitized oxidation which leads to lipid peroxidation and subsequent modification of rhodopsin, possibly by malonaldehyde (Delmelle, 1978; Farnsworth & Dratz, 1976). Thereafter, we flushed our reaction vessel with argon and restricted photolysis times to 5 min, conditions which caused no discernible photolytic damage to rhodopsin yet allowed good incorportion of reagent.

We observed that the incorporation of radioactivity from the reagent into rhodopsin did not stop following cessation of illumination. This phenomenon was explored by measuring incorporation of label into rhodopsin in the dark in a reaction in which reagent alone was photolyzed (Figure 2). Since the lifetime for aryl nitrenes is on the order of 10^{-4} s (Reiser et al., 1968), this light-initiated reaction which continues in the dark after 30 min involves a long-lived nitrene photoproduct. Undoubtedly such a reaction also occurs during the period of illumination. Therefore, it appears from the data in Figure 2 that this reaction may account for as much as 40% of the labeling of rhodopsin observed during our usual 5-min photolysis experiments.

It is well-known that nitrenes enter into a variety of reactions in addition to insertion into C–H bonds (Gilchrist & Rees, 1969). Ring enlargement can occur (Doering & Odum, 1966), forming a reactive species, reportedly an azepine (Huisgen et al., 1958) or a ketimine (Chapman & LeRoux, 1978). The ring enlargement product is subject to nucleophilic attack, e.g., forming a substituted azepine with an amine. The rearrangement product(s) of NAPT would thus be subject to attack by nucleophilic groups at the membrane surface, yielding a variety of products in addition to the secondary amides formed by C–H insertion. It is even possible that nonnitrene photolysis products may be involved in the labeling [the photolysis of (*p*-nitrophenyl)alanine has been reported to covalently label a protein (Escher & Schwyzer, 1974)]. Such side reactions may account for the long-lived labeling reactions observed in the dark and which are undoubtedly also occurring during the 5-min photolysis reaction. Some of the products formed may be less stable than a secondary aryl amine and may explain the lack of stability of the [^{35}S]-NPT–peptide linkage observed during the purification of peptide CB-3. Loss of label appears to have occurred during purification of peptides from photolytic aryl azide labeling in a previous investigation (Galardy et al., 1974).

Of interest is the observation that when disk membranes and rod outer segments are reacted with NAPT, heterogeneous labeled material is found which is removed from the NaDodSO$_4$–polyacrylamide gels during fixing and staining (see Figure 3). The reagent alone and the photolyzed reagent alone appear to yield the same material on NaDodSO$_4$ gels, but the possibility that the reagent had in fact reacted with some component of the membrane preparation could not be ignored. The polydisperse nature of the ^{35}S material, its failure to precipitate in the gel, and its lack of sensitivity to trypsin indicate that the material does not contain protein. Since it is partially removed by buffer washing and does not migrate at the dye front where lipids migrate, it would not appear to be lipid. The possibility that the reagent may have reacted with glycosaminoglycans was investigated due to reports of the presence of chondroitin sulfate and hyaluronic acid as components of the interphotoreceptor matrix material (Ocumpaugh & Young, 1966; Bach & Berman, 1971) and their probable presence in rod outer segments (Godfrey, 1973). In addition, failure of the membrane-bound rhodopsin to react with fluorescein isothiocyanate prior to water washing of the membranes has been ascribed to shielding effects of complex carbohydrates (Dratz & Schwartz, 1973). However, we were not able to digest this material by incubation of labeled membranes with chondroitinase or hyaluronidase. It therefore appears that the heterogeneous ^{35}S material may not contain membrane-surface components and may be composed entirely of NAPT photolysis side products. Noncovalently attached photoproducts have been observed in the reaction of NAPT with other membranes (J. Staros, personal communication). These side products could be removed from the membrane by reisolation of the labeled membranes by sucrose gradient centrifugation (J. Staros, personal communication). Such a membrane reisolation step was included in subsequent NAPT-labeling studies (Staros & Richards, 1974).

Rhodopsin is the principal protein of disk membranes and is the principal protein labeled by NAPT. In contrast to the relatively high degree of specificity shown by other chemical and enzymatic probes, NAPT should be sufficiently nonspecific so that any portion of the polypeptide chain which is available to the reagent should be capable of incorporating label. The exhaustive labeling performed by Matheson et al. (1977) showed that all of the 16 different kinds of amino acids located on the surface of ribonuclease could be modified by NAPT (although some selectivity was observed). We therefore ex-

pected (and observed) that, when thermolysin digestion was performed on NPT-labeled membranes, the membrane-bound fragments of rhodopsin retained some label. Action of the macromolecular probe thermolysin will be restricted by its size, proteolytic specificity, and the conformation of the surface regions of rhodopsin. Only two regions of the membrane-bound molecule are susceptible to digestion by thermolysin: the carboxyl-terminal and a single internal region (Hargrave & Fong, 1977).

The labeling of CNBr peptides was compared for rhodopsin labeled in disk membranes and rhodopsin labeled in reconstituted membranes in order to obtain topographic data for rhodopsin. Labeling specificity appears to have been obtained, inasmuch as some peptides become labeled and some do not. Both carboxyl-terminal peptides CB-3 and CB-6 are well labeled. Peptide CB-3 was known to be surface exposed, and our present data now shows that the extended carboxyl-terminal sequence represented by peptide CB-6 is also available at the membrane surface. The amino-terminal peptide CB-1 shows some label incorporation when disks are modified, and the labeling extent increases by nearly half when reconstituted membranes are labeled. This is consistent with the current model for rhodopsin in which the amino terminus is located on the intradiskal membrane surface (Fung & Hubbell, 1978; Hargrave et al., 1980). We might have expected to observe no labeling at all for CB-1 in disk membranes. Several explanations may be suggested. Some of the label eluting in the position of CB-1 (as shown by the A_{280} profile over pool 3 in Figure 6B) may not be due to CB-1. Incomplete CNBr cleavage produces at least 7% CB-3 linked to CB-6, and this larger peptide coelutes with CB-1 (Fong, 1978). Also, the disk membrane preparation may contain a small fraction of inside-out disks. Treatment of disk membrane preparations with concanavalin A–Sepharose (Adams et al., 1978) will remove any inverted disks, but such a procedure was not applied to our membrane preparations. The small amount of chemical modification could also reflect penetration of some reagent across the membrane during the 5-min time of the reaction. Finally, the reagent may have modified part of the carboxyl-terminal region of peptide CB-1, which could traverse the membrane (although we consider this unlikely).

To our knowledge this is the first study which has obtained information concerning topographic details for the primary structure of a membrane protein using a water-soluble nitrene precursor. The study has been useful not only in demonstrating what parts of the protein rhodopsin are surface exposed but also in demonstrating limitations in the use of nitrenes for such studies.

We plan to extend these studies using reagents which may prove to be superior to NAPT. In spite of the high degree of water solubility of NAPT and its ability to be completely removed from membranes by washing, its structure is amphipathic and it is possible that the nitrene end of the reagent could insert several angstroms into the membrane prior to reaction. A multiply charged reagent in which the azido group was located closer to the sulfonic acid moiety might be more tightly restricted to external surface labeling (Dockter, 1979). Finally, the formation of side products following NAPT photolysis appears to be substantial. New reagents should be designed and evaluated with this factor in mind.

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Modulation of Neurotransmitter Transport by the Activity of the Action Potential Sodium Ion Channel in Membrane Vesicles from Rat Brain†

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ABSTRACT: (1) Transport of the neurotransmitters γ-aminobutyric acid (GABA) and L-glutamic acid was measured in isolated membrane vesicles derived from rat brain, with an artificially imposed electrochemical gradient of sodium ions (out > in) as a major driving force. Both transport processes were strongly inhibited by the alkaloid neurotoxin veratridine. Tetrodotoxin, which by itself had a slight stimulatory effect, completely reversed the inhibition by veratridine. (2) The degree of inhibition of neurotransmitter transport by veratridine was strongly dependent on the nature of the internal cation. With internal Tris or lithium ions inhibition by the neurotoxin was relatively poor as compared to the case when internal potassium was used. The parameter was not dependent on the nature of either the internal or the external anion. (3) The membrane vesicles catalyzed the uptake of $^{22}Na^+$ which was 3-10-fold enhanced by veratridine. This effect was completely reversed by tetrodotoxin. The veratridine-stimulated sodium ion uptake obeyed Michaelis-Menten kinetics [apparent K_m = 11 mM, V_{max} = 150 nmol/(min mg of protein)]. Vesicles which had been allowed to accumulate $^{22}Na^+$ rapidly lost their radioactivity upon dilution into sodium-containing media, provided nigericin was present during dilution. (4) Veratridine-dependent sodium ion accumulation was also highly dependent on the nature of the internal cation, Tris and lithium being relatively poor in comparison with potassium. The nature of either the internal or the external anion was not important. (5) The concentration dependence of the inhibiting effect of veratridine on GABA transport paralleled that of its stimulatory effect on $^{22}Na^+$ uptake (the half-maximal effects ranged from 20 to 30 μM). A similar parallel was found between the reversal of the effects of veratridine on both processes by tetrodotoxin (half-maximal effects between 10 and 20 nM). (6) It is concluded that in the isolated membrane vesicles functionality of the action potential Na^+ channels is preserved and that, in rat brain, the sodium-coupled neurotransmitter high-affinity uptake systems for GABA and L-glutamic acid in the vesicles originate predominantly from the presynaptic plasma membrane.

High-affinity sodium-dependent uptake systems for a wide range of neurotransmitters have been implicated in termination of transmitter action on postsynaptic receptors (Iversen, 1971) as well as in maintaining constant levels of transmitters in the neurons (Hedgvist & Stjärne, 1969). These uptake systems have been identified in several types of brain preparations such as synaptosomes (Iversen, 1971, 1973; Kuhar, 1973; Bennett et al., 1974). Various observations have led to the proposal that ion gradients, generated primarily by devices such as the $(Na^+ + K^+)$-ATPase, are the immediate driving force for neurotransmitter accumulation (Bogdanski et al., 1968; Martin & Smith, 1972; Holtz & Coyle, 1974). Recently, this idea has been supported more directly by using membrane vesicles isolated after osmotic shock of synaptosomal preparations

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