Photolabile Protecting Groups for an Acetylcholine Receptor Ligand. Synthesis and Photochemistry of a New Class of *o*-Nitrobenzyl Derivatives and Their Effects on Receptor Function†

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ABSTRACT: Two compounds have been synthesized that feature a photosensitive *o*-nitrobenzyl moiety attached directly to the carbamate nitrogen of carbamoylcholine. The well-characterized acetylcholine analogue, carbamoylcholine, was released from these derivatives in response to laser light pulses at wavelengths between 300 and 355 nm. Photolysis products were isolated by high-performance liquid chromatography and identified by chemical and spectroscopic analysis. The yield of carbamoylcholine molecules per photon absorbed was 0.25. A short-lived photochromic intermediate in the photolysis reaction was detected by laser flash photolysis. A single laser flash induced an instantaneous increase in absorbance at 406 nm, followed by a first-order decay to products, with a half-time of 0.07 ms for one of the compounds [*N*-[1-(2-nitrophenyl)ethyl]carbamoylcholine iodide] in aqueous buffers at pH 7 and 23 °C. Decay rates and quantum yields depended on the nature of the substituent on the protecting group. Evidence is presented in support of the conclusion that the transient species is an *aci*-nitro intermediate that decays directly to carbamoylcholine and therefore determines its rate of release. The photosensitive carbamoylcholine derivatives activated the nicotinic acetylcholine receptor only after photolysis, as determined by $^{86}Rb^+$ flux measurements with membrane vesicles prepared from *Torpedo californica* and *Electrophorus electricus*. Before photolysis, the compounds interacted weakly with the acetylcholine-binding sites as shown by competitive inhibition of acetylcholine-stimulated flux at high concentrations. The compounds did not induce receptor desensitization at a significant rate. The new compounds afford several major advantages over other photoactivatable acetylcholine analogues. The novel linkage of the *o*-nitrobenzyl group to nitrogen suggests that this technology may be used to prepare photoactivatable derivatives of other neurotransmitters that contain an amino group.

The application of fast-reaction techniques to the investigation of transmembrane processes permits one to investigate the effects of compounds that affect these processes over a wide concentration range. In kinetic measurements of the ion translocation controlled by the acetylcholine receptor, it has been possible to make measurements before the receptor is inactivated (desensitized) and after the inactivation has gone to completion, and the rate coefficients for receptor inactivation and reactivation could be measured independently. Much new information, and reconciliation of seemingly contradictory results obtained in electrophysiological and biochemical studies, has been achieved by this technique [reviewed by Hess et al. (1983)].

So far this approach has only been applicable to the investigation of acetylcholine receptor function in membrane vesicles prepared from the electric organs of *Electrophorus electricus* and *Torpedo* species. In principle, chemical kinetic investigations of receptors can be carried out on cell surfaces, making it possible to vary the concentration of ligands over a wide range without partially desensitizing the receptor before the measurements are made. The photoisomerizable agonist 3,3′-bis[(trimethylammonio)methylene]azobenzene bromide (Bis-Q), introduced by Bartels et al. (1971), appeared to be ideal for such investigations. The photoinduced isomerization of *cis*-Bis-Q to the trans form produces an active receptor–ligand [for a review see Lester & Nerbonne (1982)]. After separation of *cis*- and *trans*-Bis-Q by high-performance liquid chromatography (HPLC) (Delcour et al., 1982), it was shown (Delcour & Hess, 1986) in *E. electricus* electroplax vesicles that *cis*-Bis-Q itself inactivates (desensitizes) the receptor and that the trans form becomes an inhibitor of receptor function at progressively lower concentrations as the transmembrane voltage is decreased to more negative values. *trans*-Bis-Q is not an agonist for the *T. californica* receptor (Delcour & Hess, 1986).

Here we report the synthesis of a photolabile protecting group that may be useful with all neurotransmitters that contain an amino group (carbamoylcholine, γ-aminobutyric acid, dopamine, and so forth) because the chemistry of the photolysis reaction is expected to depend mainly on the protecting groups. The protecting groups that we have used are (2-nitrobenzyl)amine and [1-(2-nitrophenyl)ethyl]amine. The *o*-nitrobenzyl moiety has been used extensively as a photosensitive protecting group for carboxylate, phosphate, hydroxyl, and amine residues in organic synthesis (Morrison, 1969; Pillai, 1980). Recently, it has been used to derivatize biological substrates that can be released rapidly by photolytic irradiation in situ. For instance, the development and application of *o*-nitrobenzyl photochemistry to achieve rapid changes in concentration of ATP (Kaplan et al., 1978; McCray et al.,

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1980; Goldman et al., 1982, 1984) and cyclic nucleotides (Engels & Schlaeger, 1977; Nerbonne et al., 1984) are well documented.

In the investigations described, carbamoylcholine derivatives were chosen because carbamoylcholine is a stable analogue of acetylcholine with known pharmacological properties and has been particularly well characterized in electroplax preparations from *E. electricus* (Hess et al., 1979; Cash & Hess, 1980; Aoshima et al., 1981) and *Torpedo* species (Neubig & Cohen, 1980; Walker et al., 1981b, 1982). We report here the synthesis and properties of two photosensitive *N*-*o*-nitrobenzyl derivatives that released carbamoylcholine in response to photolytic irradiation at wavelengths between 300 and 355 nm. Photolysis proceeded in aqueous solutions at neutral pH, and quantum yields and photolysis rates were determined for the compounds. The effects of parent compounds and of their photolysis products on the nicotinic acetylcholine receptor were investigated in detail.

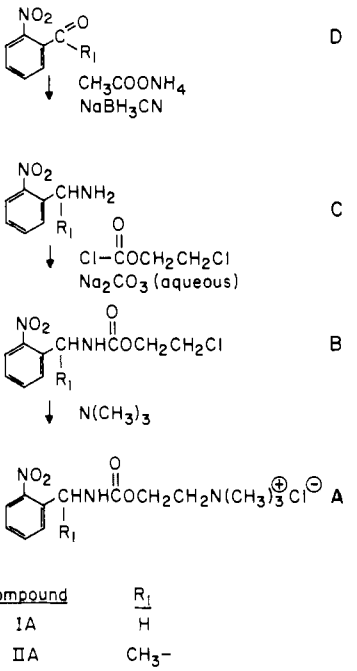
Experimental Procedures

Visible and ultraviolet absorption spectra were obtained on a Cary 219 recording spectrophotometer (Varian Instruments). Proton NMR spectra were recorded on a Varian CFT-20; chemical shifts are given in parts per million (ppm) relative to tetramethylsilane (internal standard). Melting points were determined with a Fisher-Johns melting point apparatus and are uncorrected. Elemental analysis was done by Schwartzkopf Microanalytical Laboratories (Syracuse, NY).

Acetylcholine Receptor Assays. Compounds were evaluated by a standard tracer ion ($^{86}Rb^+$) flux procedure (Walker et al., 1981b) using acetylcholine receptor rich membrane vesicles prepared from the electric organ of *E. electricus* or *Torpedo californica*. Receptor binding activity was evaluated by a competition assay using ^{125}I-labeled α-bungarotoxin as described (Walker et al., 1981a). Ion-flux measurements in the millisecond time region were performed with a quench–flow technique (Cash & Hess, 1981).

High-Performance Liquid Chromatography. A Waters 6000A injector and 440 ultraviolet absorbance detector at 254 nm were used. System 1: For separations of cationic compounds, a Bio-Sil TSK IEX-530 CM ion-exchange column was equilibrated with aqueous sodium chloride (10–200 mM) containing 10% ethanol. System 2: For separations of uncharged hydrophobic compounds a μBondapak C_{18} reversed-phase column (Waters Associates, Inc.) was used in 60% aqueous acetonitrile.

Photolysis. Samples (0.5–2.5 mL) were photolyzed in a 1 cm × 1 cm quartz curvette under N_2 with stirring in front of a Lambda Physik excimer laser (50–100 mJ per 5-ns pulse at 308 nm). Smaller samples (200 μL) were photolyzed without stirring in a specially designed cylindrical quartz cuvette (0.2 cm in diameter, 1.5 cm in length) in front of a neodymium:YAG laser (15–25 mJ per 30-ns pulse at 355 nm). For flash photolysis, a water-cooled Holobeam ruby laser with a typical 3.5-J output was used. The laser was Q-switched with a Pockel cell, resulting in a single pulse of 50 ns. Output was frequency-doubled with an angle-tuned KDP (potassium dihydrogen phosphate) crystal. The 347-nm secondary beam with output energies up to 70 mJ was separated from the primary beam with a Schott 5181 glass filter. Output of the primary beam was measured with a ballistic thermopile and a Keithley nanovoltameter and that of the secondary beam with a Scientec Disc Calorimeter energy meter. For absorption measurements, the secondary beam was allowed to fall on a 2-mm quartz cell set at 45° so that the path length was 2.8 mm. Detecting light from a tungsten iodide lamp was orthogonal to the laser beam and at 45° to the reaction cell, resulting in a 2.8-mm path length. The volume irradiated was determined by a 6-mm-diameter aperture. Spectroscopic signals were monitored by use of an EMI 9824B photomultiplier. A CS_2 solution with a 1-cm light path was placed between the quartz cell containing the sample and the photomultiplier.



Compound	R_1
IA	H
IIA	CH_3-

FIGURE 1: Structure and synthesis of N-substituted carbamoylcholines.

Synthesis. The general approach for synthesizing N-substituted choline carbamates is outlined in Figure 1 as first described by Sprinson (1941). An aqueous solution of (2-nitrobenzyl)amine hydrochloride (C, Figure 1) is condensed with 2-chloroethyl chloroformate to give N- and O-substituted carbamates (B). Halogen ethyl carbamates (B) are then converted to choline derivatives (A) by treatment with anhydrous trimethylamine. [1-(2-Nitrophenyl)ethyl]amine was prepared by reductive amination of carbonyl compounds (Borch et al., 1970).

2-Chloroethyl N-(2-Nitrobenzyl)carbamate (IB). (2-Nitrobenzyl)amine hydrochloride (6.0 g, 0.032 mol; Overlook Industries, Inc.) was added to 36 mL of 10% aqueous sodium carbonate (0.034 mol) and cooled on an ice bath. 2-Chloroethyl chloroformate (4.6 g, 0.032 mL; Pfaltz and Bauer, Inc.) was added dropwise with stirring and the mixture chilled on ice for 2 h. After being warmed to room temperature the reaction was neutralized with aqueous sodium carbonate and extracted 4 times with 50 mL of methylene chloride, and the extract was dried over anhydrous sodium sulfate. The solvent was evaporated in vacuo and the product crystallized from hot hexane: yield, 7.3 g (87%); mp 105–106 °C (uncor); NMR ($CDCl_3$) δ 7.5–7.8 (m, 4 H, aromatic), 5.6 (br s, 1 H, amide), 4.5 (d, 2 H, benzylic), 4.2 (t, 2 H, methylene), and 3.5 (t, 2 H, methylene).

The chloroethyl carbamate was converted to the iodoethyl carbamate to improve the yield of the trimethylamine reaction. Chloroethyl carbamate (7.3 g) was dissolved in 40 mL of methyl ethyl ketone, sodium iodide (20.9 g, 0.3 mol) was added, and the mixture was refluxed for 12 h. The reaction mixture was cooled and filtered over Whatman No. 1 filter paper and the solvent evaporated. 2-Iodoethyl *N*-(2-nitrobenzyl)carbamate was crystallized from hot hexane: yield,

8.8 g (89%); mp 86–87 °C. NMR in $CDCl_3$ revealed a spectrum identical with that of chloroethyl carbamate, except that the most upfield triplet shifted to δ 3.15.

N-(2-Nitrobenzyl)carbamoylcholine Iodide (IA). Iodoethyl carbamate (5 g, 0.016 mol) was dissolved in anhydrous methyl ethyl ketone, and the mixture was cooled on ice. A stoichiometric amount of anhydrous trimethylamine was added (3.8 mL of 25% trimethylamine in methyl ethyl ketone; Eastman Organic Chemicals). The reaction vessel was sealed and allowed to stand at room temperature overnight. The solvent was evaporated, the residue extracted with distilled water, and the water phase lyophilized. Compound IA was crystallized from the lyophilized powder in methyl ethyl ketone: yield, 4.2 g (65%); mp 118–119 °C; NMR (D_2O) δ 7.5–7.9 (m, 4 H, aromatic), 4.5 (s, 2 H, benzylic), 4.4 (t, 2 H, methylene), 3.6 (t, 2 H, methylene), and 3.15 (s, 9 H, *N*-methyl). IR 1700 (C=O), 3000–3500 (N–H) cm^{-1}. Anal. Calcd for $C_{13}H_{20}O_4N_3I$: C, 38.10; H, 4.90; O, 15.60; N, 10.30; I, 31.0. Found: C, 38.29; H, 5.05; 0, 15.23; N, 10.17; I, 30.99.

[1-(2-Nitrophenyl)ethyl]amine (IIC). 2-Nitroacetophenone (8.25 g, 0.05 mol; Aldrich Chemical Co.) was added to 100 mL of anhydrous methanol. With stirring, ammonium acetate (38.5 g, 0.5 mol) was added, followed by sodium cyanoborohydride (2.2 g, 0.035 mol; Aldrich Chemical Co.). The reaction vessel was sealed and stirred in the dark for 72 h at room temperature. The suspension was filtered on Whatman No. 1 filter paper and the filtrate evaporated to dryness. The residue was dissolved in approximately 200 mL of 1 N HCl and then extracted with ether (4 × 50 mL). The ether phase was discarded. While mixing on ice, the pH of the aqueous phase was raised to greater than 11 by addition of potassium hydroxide pellets. A second ether extraction (4 × 50 mL) removed the amine product. The ether solution was dried over anhydrous sodium sulfate and evaporated to give a yellow oil: yield, 1.28 g (15.5%); NMR ($CDCl_3$) δ 7.2–7.9 (in, 4 H, aromatic), 4.7 (g, 1 H, benzylic), 1.9 (s, 2 H, amine), 1.5 (d, 3 H, methyl). TLC on silica gel in chloroform gave a single spot (R_f 0.3).

N-[1-(2-Nitrophenyl)ethyl]carbamoylcholine Iodide (IIA). With compound IIC as starting material, compound IIA was synthesized by following the same procedure as described for compound IA. Yields were similar to those reported for compound I; mp 126–127 °C. NMR spectra (in $CDCl_3$ or D_2O) were also similar except for methyl protons at δ 1.6 (d, 3 H) and benzylic protons at δ 5.3 (q, 1 H). Anal. Calcd for $C_{14}H_{22}O_4N_3I$: C, 39.73; H, 5.24; N, 9.93. Found: C, 39.25; H, 5.48; N, 9.89.

Results

Two photosensitive derivatives of carbamoylcholine were synthesized by the scheme shown in Figure 1. The final compounds, IA and IIA, were obtained in highly pure form by crystallization, as shown by melting point determinations, thin-layer chromatography, and high-performance liquid chromatography (HPLC). HPLC of compound IA and of compound IIA on a cation-exchange column revealed a single symmetrical peak. The elution volume (V_e) depended on the concentration of salt in the eluting solvent; as expected, V_e increased as the salt concentration decreased. The proposed structures for compounds IA and IIA were consistent with elemental analysis and spectroscopic analysis by UV absorption, proton NMR, and IR (see Table I and Experimental Procedures).

Photolysis and Identification of Products. Figure 2A illustrates the marked changes in UV absorption spectra that were observed when aqueous solutions of compound IIA were photolyzed by repeat light pulses, from an excimer laser at 308 nm. The maximum at 262 nm, characteristic of the 2-nitrobenzyl moiety, decreased while two new spectral features appeared, a maximum at 280 nm and a shoulder at 315 nm. For each compound, spectral changes were identical with changes observed when the analogous 2-nitrobenzyl alcohol was photolyzed. Carbamoylcholine and the major photolytic side products were isolated from photolyzed solutions by HPLC and identified by chemical and spectroscopic analysis (Figure 2B). Peaks labeled 1 and 5 were present before

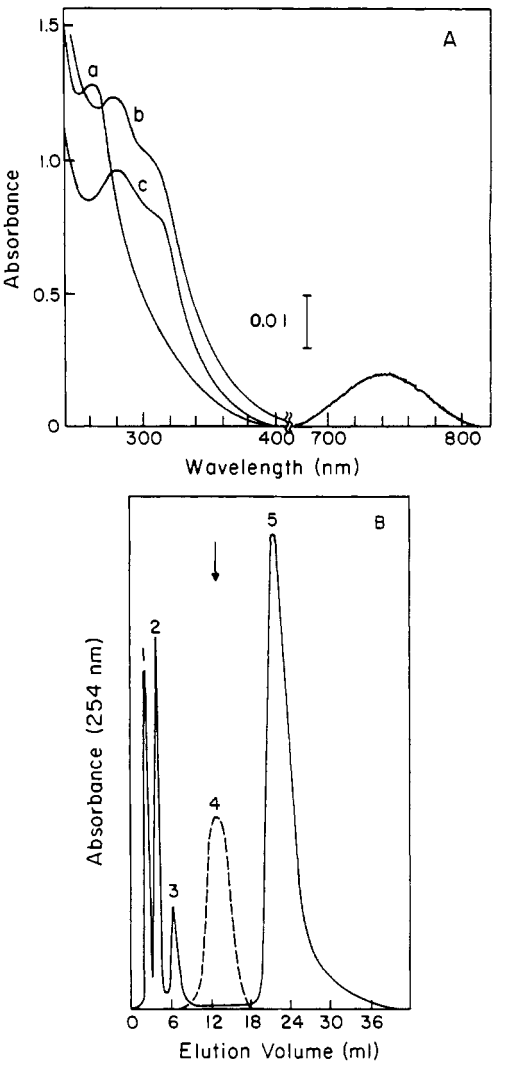


FIGURE 2: (A) Effect of laser photolysis on ultraviolet and visible absorption spectra of compound IIA. An aqueous solution of 1 mM compound IIA in 0.1 M 4-morpholinepropanesulfonic acid (MOPS), pH 7.0, was exposed to an excimer laser beam at 308 nm for 10 s (80 mJ per pulse; 10 Hz). Aliquots were removed before (a) and after (b) photolysis and diluted to 250 μM, and spectra were recorded on a Cary 219. Another aliquot was chromatographed by HPLC (see panel B, legend), and the absorption spectrum of the major photoproduct is shown (c). The bar shows the increase in sensitivity required to detect the absorbance maximum at 740 nm. (B) High-performance liquid chromatography of photolyzed mixtures of compound IIA. A cation-exchange column (Bio-Sil TSK IEX-530 CM) was equilibrated with 50 mM NaCl and 10% ethanol. A 100-μL aliquot of 1 mM photolyzed compound IIA was injected at a flow rate of 1.0 mL/min with the detector set at 2.0 absorbance units for full-scale deflection. Peaks were collected and analyzed by absorption spectroscopy: 1, λ_{max} = 220 nm; 2, λ_{max} = 280, 310 nm (see panel A); 3, λ_{max} = 275, 305 nm; 4, none; 5, λ_{max} = 262 nm. The arrow represents the elution volume for authentic carbamoylcholine run separately in the same system. Peak 4 was detected by a ninhydrin assay for ammonia (see Experimental Procedures).

photolysis and represent the iodide counterion (λ_{max} = 220 nm) and unphotolyzed compound IIA (λ_{max} = 262 nm), respectively. Peaks labeled 2–4 appeared after photolysis and increased with increasing exposure to the photolyzing beam.

The ultraviolet/visible spectrum of one photoproduct isolated by the HPLC procedure (peak 2, Figure 2B) is shown in Figure 2A. The spectrum is identical with that of 2-nitrosoacetophenone generated by photolysis of 1-(2-nitrophenyl)ethanol and features a broad absorbance maximum at 740 nm, which is characteristic of aromatic nitroso compounds (Tapuhi & Grushka, 1982). This material, whether generated by photolysis of compound IIA or 1-(2-nitrophenyl)ethanol, was extracted from aqueous photolysis solutions into chloroform and ran as a single major peak when chromatographed on a reversed-phase HPLC column in 40% or 60% aqueous acetonitrile. Another apparent photoproduct in the HPLC profile (peak 3, Figure 2B) is believed to be a dimer of the nitroso compound.

The photoproduct of primary interest was carbamoylcholine. Standard samples of authentic carbamoylcholine chloride (Sigma Chemical Co.) eluted at 13 mL in the HPLC profile shown (arrow, Figure 2B), as detected by spectrophotometric chemical assays specific for carbamates (Doulakas, 1975) or amino nitrogen after acid hydrolysis (Schiffman et al., 1964). After photolysis of the N-substituted carbamoylcholine derivatives, free carbamoylcholine was detected in the expected HPLC fractions (peak 4). Furthermore, when HPLC fractions were scanned for biological activity by using receptor-rich vesicles and a $^{86}Rb^+$ flux assay (see Experimental Procedures), the material in peak 4 both *activated* and *desensitized* the nicotinic acetylcholine receptor as expected for carbamoylcholine. No other HPLC fractions showed activity. Release of carbamoylcholine increased with increasing exposure to the photolyzing beam and coincided with the disappearance of the parent compound. The number of carbamoylcholine molecules produced per absorbed photon (product quantum yield) was estimated by quantifying the amount of carbamoylcholine released after various exposure times. The concentration of the parent compound was adjusted so that 96% of the incident light was absorbed. The number of photons reaching the sample was calculated by knowing the energy output from the laser, or more accurately by use of a ferric oxalate chemical actinometer with a known quantum yield as recommended by Calvert and Pitts (1966). By this approach, quantum yield values for production of carbamoylcholine ranged from 0.2 to 0.3, suggesting a reasonably efficient photochemical process.

Flash Photolysis. Analysis of compounds IA and IIA in a flash photolysis apparatus revealed a spectral transient, characterized by an instantaneous (<10 μs) increase in absorbance, followed by a first-order decay back to nearly the initial absorbance (Figure 3A). The short-lived species was detected when photolysis was monitored in the near UV (above 350 nm) and showed an absorbance maximum at 406 nm (Figure 3B). The *magnitude* of the absorbance increase was proportional to the concentration of compound in the cuvette and to the energy output from the laser (15–70 mJ). The *rate* of decay depended on the structure of the photosensitive protecting group (Table I) and on the pH (Figure 3C). The carbamate derivatives tested displayed a pH profile with a minimum rate at pH 8.5, while at both higher and lower pH the rate increased. The fastest photolysis rate observed was 5×10^4 s^{-1} at pH 6 for compound IIA. The photolysis rate for compound IA was less by a factor of about 20.

Caged ATP was analyzed in these flash photolysis experiments because caged ATP has the same photosensitive *o*-nitrobenzyl protecting group and a similar spectral transient

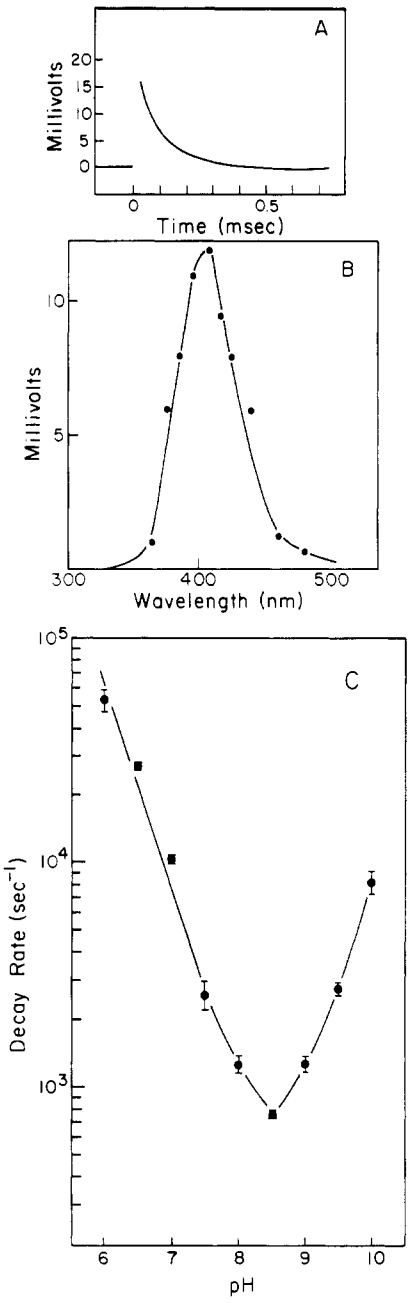


FIGURE 3: Flash photolysis of compound IIA. (A) In a quartz cuvette (path length = 2 mm) set at 45° in respect to the laser direction, 2 mM compound IIA in 0.1 M MOPs, pH 7.0, 23 °C, was exposed to a single 40-mJ pulse from a frequency-doubled ruby laser at 347 nm. Spectral changes were observed at 90° from the laser direction after a brief delay (<10 μs) and displayed on an oscilloscope. The monochromator was set at 406 nm where the absorbance of the unphotolyzed solution was low. The millivolt scale was set to zero by using the initial absorbance of the solution (100% transmission), and full scale was 250 mV (0% transmission). (B) Ultraviolet and visible spectrum of the observed absorbance change. Compound IIA (2 mM) in 0.1 M Tris, pH 8.0, 22 °C, was exposed to a 30-mJ pulse from the laser at 347 nm. At each wavelength the unphotolyzed solution was set to 0 mV (100% transmission), and 250 mV represented full scale. (C) pH dependence of *aci*-nitro decay rate. Solutions of compound IIA (1 mM) were prepared in the following buffers, and the *aci*-nitro decay rate was determined by flash photolysis: 0.1 M 4-morpholineethanesulfonic acid (MES), pH 6.0 and 6.5; 0.1 M MOPs, pH 7.0 and 7.5; 0.1 M 2-[[tris(hydroxymethyl)methyl]-amino]ethanesulfonic acid (TES), pH 7.0 and 7.5; 0.1 M Tris, pH 8.0, 8.5, and 9.0; 0.1 M borate, pH 9.0, 9.5, and 10.0. The decay rate did not depend on the nature of the buffer. Error bars represent standard deviations of three to six determinations.

that was examined in detail previously (McCray et al., 1980). Flash photolysis of caged ATP under our experimental con-

Table I: Spectral Properties and Photolysis Rates of Parent Compounds[a]

compound	λ_{max} (nm)	ϵ_M (M^{-1} cm^{-1})	*aci*-nitro decay,[b] $t_{1/2}$ (ms)
IA	262	5200	1.7 ± 0.1
IIA	262	5200	0.067 ± 0.002
caged ATP	260	19600	4.6 ± 0.3

[a]Structures of compounds are given in Figure 1. Caged ATP used here was the α-methyl 2-nitrobenzyl derivative (Kaplan et al., 1978; McCray et al., 1980). Absorption properties were determined in aqueous solutions and were independent of pH above pH 6.0. [b]Spectral transients observed in flash photolysis were analyzed at pH 7.0 and room temperature. Values represent the average of three determinations ± the standard deviation.

ditions revealed a spectral transient at 406 nm. A rate constant of 1.8×10^9 M^{-1} s^{-1} was calculated from a semilogarithmic plot of the decay rate as a function of pH, consistent with the previous study (McCray et al., 1980). The photosensitive carbamates investigated here were similar to caged ATP in the range pH 6–8 where a decrease of 1 pH unit increased the decay rate by a factor of approximately 8. However, above pH 8.5 the decay rate *increased* for compounds IA and IIA (Figure 3C) but continued to decrease for caged ATP. Overall, photolysis was faster for the carbamoylcholine derivatives than for caged ATP (Table I).

Finally, the comparison between caged ATP and compound IIA in the flash photolysis experiments provided a second measure of the quantum yield. Previously, the yield of ATP from caged ATP was shown to be a linear function of laser energy. A quantum yield between 0.5 and 0.6 was calculated from the data, in agreement with published results (Kaplan et al., 1978; McCray et al., 1980). Similarly, the amounts of intermediate species generated during flash photolysis were found to be linear functions of laser energy for both caged ATP and compound IIA. The slope of a plot of absorbance change vs. laser energy was greater by a factor of 2 for caged ATP than for compound IIA under the same conditions [2.5 mM, 0.1 M tris(hydroxymethyl)aminomethane (Tris), pH 8.0, 23 °C]. By comparison then, compound IIA has a quantum yield of 0.25. The value for compound IA was estimated to be the same.

Effects on Nicotinic Acetylcholine Receptor. In contrast to the potent receptor agonist carbamoylcholine, the N-substituted carbamoylcholine derivatives were inactive when receptor-mediated ion flux was measured with $^{86}Rb^+$ and native membrane vesicles prepared from *E. electricus* or *T. californica*. Compounds IA and IIA did, however, interact significantly with the receptor, presumably at the acetylcholine recognition sites. Figure 4A illustrates the inhibition of acetylcholine-stimulated $^{86}Rb^+$ flux by compound IA which was observed in the range 0.2–2 mM. The extent of inhibition depended on the concentration of both the inhibitor and acetylcholine, suggesting a competitive interaction. Furthermore, the compounds decreased the rate of ^{125}I-labeled α-bungarotoxin binding to *Torpedo* receptor in a concentration-dependent manner (data not shown).

Preincubation of receptor-rich membrane vesicles revealed an additional inhibitory effect of compounds IA and IIA. Preincubation with agonists (carbamoylcholine, acetylcholine) is known to cause a rapid inactivation (desensitization) of receptor-mediated ion flux (Hess et al., 1979), but with the N-substituted derivatives, inactivation was very slow. Figure 4B compares directly the rates of inactivation measured for carbamoylcholine (0.5 mM, $t_{1/2}$ = 0.6 s) and compound IA (0.5 mM, $t_{1/2}$ = 10 min). Inactivation by compound IA was

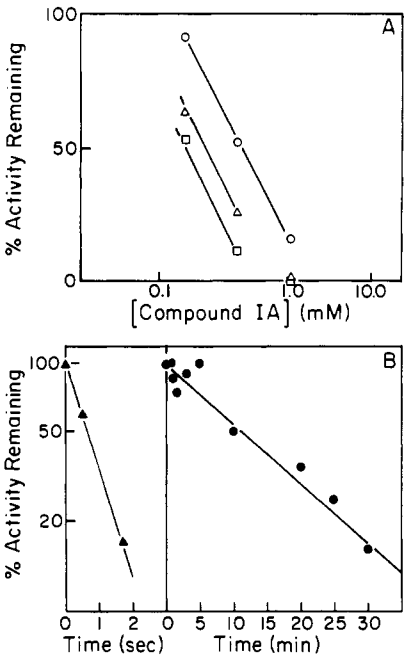


FIGURE 4: Effects of parent compounds on acetylcholine-stimulated $^{86}Rb^+$ flux in *E. electricus* membrane vesicles. (A) In a quench–flow device, vesicles were mixed with an equal volume of $^{86}Rb^+$ solution containing acetylcholine and compound IA, and flux was measured for 200 ms before quenching with *d*-tubocurarine. Three acetylcholine concentrations were used: (□) 0.1, (△) 0.3, and (○) 1 mM; the concentration of compound IA is given on the abscissa. Percent activity remaining was calculated from $\ln [(M_\infty - M_{200})(M_\infty)^{-1}]_{\text{no inhibitor}} / \ln [M_\infty - M_{200})(M_\infty)^{-1}]_{+\text{inhibitor}}$, where M_∞ represents the maximum uptake of $^{86}Rb^+$ into the vesicles and M_{200} is $^{86}Rb^+$ content after 200 ms of flux (Aoshima et al., 1981). The solid lines are drawn to help the reader differentiate between experiments done at different acetylcholine concentrations. (B) Time-dependent inhibition of acetylcholine-stimulated flux. Membrane vesicles were preincubated with inhibitor for the period of time given. Flux then proceeded for 200 ms in the presence of 2 mM acetylcholine before quenching with *d*-tubocurarine. The rate coefficient for the inactivation of the receptor by carbamoylcholine (▲, 0.5 mM, α = 1.1 s^{-1}) served as a control. The two curves were normalized to 100% at zero preincubation time. Compound IA (●, α = 0.07 min^{-1}) inhibited acetylcholine-stimulated flux by 80% at this concentration (0.5 mM) without preincubation.

faster than that by compound IIA and occurred at about $^1/_{1000}$th the rate of that with the same concentration of carbamoylcholine (Figure 4B).

The main photolytic side product isolated by HPLC (Figure 2B), and identified as 2-nitrosoacetophenone, was tested for possible toxic effects on the receptor preparation. 2-Nitrosoacetophenone had no effect on acetylcholine-stimulated $^{86}Rb^+$ flux at concentrations approaching 1 mM. At concentrations above 1 mM, receptor-mediated flux was irreversibly inactivated but could be protected from inactivation by the addition of stoichiometric amounts of sodium bisulfite. This problem of inhibition by 2-nitrosoacetophenone was first observed with purified Na^+,K^+-ATPase and solved by addition of glutathione or sodium bisulfite (Kaplan et al., 1978). Inclusion of these compounds in the experiments also prevented observable toxic effects when the *o*-nitrobenzyl group was removed from ATP in situ (Kaplan et al., 1978; Goldman et al., 1982).

Discussion

The synthesis and characterization of two photosensitive *o*-nitrobenzyl derivatives of carbamoylcholine has been presented. The *o*-nitrobenzyl moiety has been used extensively as a photosensitive protecting group in organic synthesis, and

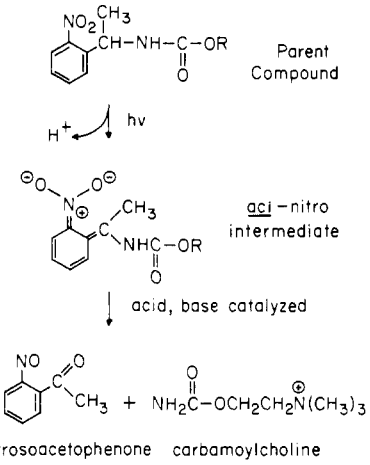


FIGURE 5: Proposed mechanism for release of carbamoylcholine.

the photochemical mechanism is fairly well understood (Morrison, 1969). We have synthesized analogues with this protecting group linked directly to the carbamate nitrogen of carbamoylcholine and then demonstrated that it could be photolytically removed. This represents the first demonstration that protection and deprotection of carbamate groups can be achieved with this photosensitive group. Carbamoylcholine and the major photolytic side products were isolated from photolyzed solutions of the *N*-2-nitrobenzyl derivatives by HPLC and identified by chemical and spectroscopic analysis. The main photolytic side product was an aromatic nitroso carbonyl compound. Thus photoinduced removal of the protecting group involves an intramolecular oxidation–reduction reaction between the nitro group and the adjacent benzylic carbon (Figure 5).

Rates of carbamoylcholine release and details of the photochemical mechanism were analyzed by flash photolysis. When spectral changes in the near UV were monitored, an intermediate species was detected with absorption properties different from those of the parent compound. The intermediate was similar in absorption spectrum (λ_{max} = 406 nm) to the *aci*-nitro intermediate originally identified in flash photolysis studies with 2-nitrotoluene (Wettermark, 1962) and more recently with caged ATP (McCray et al., 1980). The presumed *aci*-nitro species (McCray et al., 1980) observed with compounds IA and IIA decayed exponentially to products in the millisecond time region. The biphasic dependence of the decay rate on pH was characteristic of all carbamates tested. In the pH range 6–8, the pH dependence was similar to that observed with caged ATP. At neutral pH caged ATP and compound IIA (which have the same α-methyl 2-nitrobenzyl protecting group) most likely follow a similar photochemical reaction pathway, which requires protons and releases 2-nitrosoacetophenone besides the biological substrate (Figure 5). For caged ATP, it has been demonstrated that release of ATP occurs at a rate determined by the decay of the *aci*-nitro species (McCray et al., 1980).

The unique effects observed at high pH (>8.5) (Figure 3C) with the carbamate derivatives undoubtedly reflect differences in the chemistry of the carbamate leaving group. It is well documented that amine, amide, and carbamate groups can form adducts with carbonyl compounds (Challis & Challis, 1970). Some features of this addition reaction are as follows: (i) the reaction is readily reversible, (ii) the rate-determining step is carbon–nitrogen bond formation and cleavage, and (iii) the reaction is both acid- and base-catalyzed. The isolation and identification of carbamoylcholine and 2-nitrosoacetophenone as major products of the photolysis reaction initially suggested that the release mechanism was analogous to the reverse of this addition reaction. The properties of the *aci*-nitro decay reaction clearly demonstrated here, in particular, the acid and base catalysis, further suggested a direct correlation between the *aci*-nitro decay reaction and carbamoylcholine release. Most importantly, the data indicate that decay of the *aci*-nitro species reflects the rate-determining cleavage of the carbon–nitrogen bond between carbamoylcholine and the photosensitive *o*-nitrobenzyl moiety. Details of the photolytic mechanism are not yet clear although considerable evidence from investigations of other 2-nitrobenzyl compounds suggests that a nitro-group oxygen initiates release by attacking the benzyl carbon [reviewed in Morrison (1969)].

The new compounds afford several advantages over another photoactivable acetylcholine analogue, Bis-Q. Compounds IA and IIA are thermally and chemically stable and are not sensitive to subdued room light. Laser photolysis generates an acetylcholine analogue, carbamoylcholine, with known pharmacological properties in a variety of systems. Before photolysis, the compounds exert minimal inhibitory effects on nicotinic receptor preparations. The new compounds provide a way of rapidly and accurately varying carbamoylcholine concentrations over a wide range in organized biological systems that are not suited to rapid-mixing techniques. In conjunction with electrophysiological measurements, photolytic release of carbamoylcholine will allow a chemical kinetic approach to be applied to a wide variety of receptor preparations with improved time resolution. The results presented here suggest that it may be possible that a variety of neurotransmitters, including glycine, γ-aminobutyric acid, and serotonin, can be protected with the *o*-nitrobenzyl group on an amino nitrogen and then photolytically released at specific sites.

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Registry No. IA, 100311-50-0; IB, 100311-52-2; IC·HCl, 24835-08-3; IIA, 100311-51-1; IIB, 100311-53-3; IIC, 100311-54-4; $ClCO_2(CH_2)_2Cl$, 627-11-2; NMe_3, 75-50-3; $O_2NC_6H_4CH_2NHCO_2(CH_2)_2Cl$-*o*, 100311-55-5; $O_2NC_6H_4Ac$-*o*, 614-21-1; *o*-ONC_6H_4Ac, 25798-61-2; $NH_2CO_2(CH_2)_2N^+Me_3$, 462-58-8; *o*-$O_2NC_6H_4CH(Me)NHCO_2(CH_2)_2Cl$, 100311-56-6; acetylcholine, 51-84-3.

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Anisotropy Decay Associated Fluorescence Spectra and Analysis of Rotational Heterogeneity. 1. Theory and Applications†

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ABSTRACT: Individual fluorescence spectra for species in a heterogeneous system can be determined by using differences between the rotational correlation times of those components. Each spectrum derived is associated with a particular fluorescence anisotropy decay function; hence, they are anisotropy decay associated spectra (ADAS). We have previously shown [Knutson, J. R., Walbridge, D. G., & Brand, L. (1982) *Biochemistry 21*, 4671–4679] that a system containing different decay functions for total intensity can be resolved into constituent decay-associated spectra. ADAS extends the technique into the realm of fluorescence polarization, making use of the often disparate Brownian rotations found in heterogeneous biochemical systems. In this paper, we present the basic theory for ADAS in various heterogeneous systems and then present an example of ADAS resolving a binary mixture of macromolecules into "fast-rotor" (smaller or more mobile) and "slow-rotor" (larger or less mobile) components. They correctly superimpose spectra taken for the unmixed components. In the companion paper [Davenport, L., Knutson, J. R., & Brand, L. (1986) *Biochemistry* (following paper in this issue)], a specific application to a problem of importance of lipid biochemistry—e.g., the origin of the membrane probe order parameter in lipid bilayers—is presented, demonstrating the role rotational heterogeneity may play in biochemical fluorescence.

The Brownian rotations of both small molecules and macromolecules are of key importance in biochemistry. A number of biophysical techniques have been used to provide data about these motions; each, in turn, helps characterize either the size and shape of rotors or the local angular constraints of the molecular environment.

Among the biophysical techniques used to study macromolecules, fluorescence polarization has been especially useful. The historic work of Perrin (1934, 1936) framed a relationship between the lifetime-averaged depolarization process and hydrodynamic (Stokes–Einstein) parameters. These relationships between size, shape, viscosity, temperature, and lifetime have been widely used in biochemistry. The use of extrinsic labels to characterize proteins was spearheaded by Weber and co-workers (Weber, 1952, 1953a,b, 1973). In order

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