# Radiochemical Synthesis and Photochemical Properties of the Uncoupler 2-Azido-4-nitrophenol, a Versatile Photoaffinity Labeling Reagent†

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ABSTRACT: 2-Amino-4-nitrophenol was tritiated in an acid-catalyzed hydrogen exchange reaction. Radioactive 2-azido-4-nitrophenol with a specific radioactivity up to 21 mCi/mmol was synthesized from 2-amino-4-nitrophenol by diazotization and azide coupling. The photochemical properties of the uncoupler, 2-azido-4-nitrophenol, were studied as free solute and as ligand bound to uncoupler binding sites in bovine serum albumin and mitochondria. Based on product analyses, irradiation of free or bound 2-azido-4-nitrophenolate with visible light results in the formation of nitrene intermediates with a singlet to triplet ratio of 6:1 to 9:1. 2-Azido-4-nitrophenolate and bovine serum albumin form a strong 1:1 complex ($K_D$ = 0.7 $\mu$M) which can be converted into a photoproduct with a covalent bond between the label and the protein. The acid dissociation constant of the protein-bound 2-amino-4-nitrophenol moiety is strongly pH dependent. Photoaffinity labeling of mitochondria by 2-azido-4-nitrophenolate follows a pattern expected from equilibrium binding studies using normal and lipid-depleted particles: polypeptides were found to bear 90–95% of the radioactive label, and 5–10% of the latter was bound to phospholipids. Two polypeptides (approximately 56 000 and 31 000 daltons) were associated with 60% of the label, indicating a high degree of specific photochemical labeling.

Over the past 30 years, experiments with uncouplers have helped to elucidate many important aspects of the energy metabolism in mitochondria, chloroplasts, and bacteria. Because of the unique ability of uncouplers to dissipate chemical and electrochemical energy rather than inhibit energy transfer reactions, they are now a standard tool in the study of the energy metabolism in biological membranes.

Molecular interactions between uncouplers, soluble proteins, and membrane components have been investigated by a variety of techniques including equilibrium binding studies (Cantley & Hammes, 1973; Hanstein & Hatefi, 1974), experiments with fluorescent (Kraayenhof, 1971) and spin-labeled uncouplers (Hsia et al., 1972; Chen & Hsia, 1974), studies of titer and pH dependence of uncoupler effectiveness (Kaplay et al., 1970; Wilson et al., 1971), and affinity labeling with alkylating (Wang et al., 1973) and photolyzable (Hanstein & Hatefi, 1974; Katre & Wilson, 1977) uncouplers. Photoaffinity labeling with aromatic azides is a recent and promising technique which offers a number of advantages over conventional affinity labeling (Knowles, 1972). Most importantly, because of the stability of the labeling molecules in the dark, it is possible to study both equilibrium interactions and photoaffinity labeling in the same system and under a wide variety of conditions. The uncoupler 2-azido-4-nitrophenol (NPA), a photosensitive analogue of the classical uncoupler 2,4-dinitrophenol, has been synthesized in this laboratory. Equilibrium binding of NPA by mitochondria from beef heart (Hanstein & Hatefi, 1974), rat liver, yeast (unpublished), and brown adipose tissue (Cyboron & Dryer, 1977) has been studied in detail, and the relation between binding affinity and uncoupling potency has been discussed (Hanstein, 1976).

Table I: Thin-Layer Chromatography of Phenol Derivatives on Silica Gel

| compound | $R_f$ | solvent |
|---|---|---|
| 2,4-dinitrophenol | 0.80 | A |
| 2-azido-4-nitrophenol | 0.78 | A |
| 2-azido-4-nitrophenol | 0.27 | B |
| 4-amino-2-nitrophenol | 0.44 | A |
| 2-amino-4-nitrophenol | 0.25 | A |
| 2,4-diaminophenol | 0.0 | A |

The present paper reports a radiochemical synthesis of 2-azido-4-nitrophenol and explores some photochemical properties of this uncoupler as it interacts with water, a water-soluble protein, and a biomembranous system. A detailed account of photoaffinity labeling of mitochondria, submitochondrial preparations, and soluble mitochondrial ATPase ($F_1$) will be presented in a separate communication.

## Experimental Procedures

Thin-layer chromatography of nitrophenol derivatives was performed on silica gel sheets (polygram SIL N-HR, Brinkmann) in methylene chloride–acetic acid (9:1, v/v; solvent A), or benzene–acetic acid (75:11, v/v; solvent B). The $R_f$ values listed in Table I were obtained in solvent saturated chambers at room temperature (23 °C). The spots were visualized with ammonia vapor.

*Tritiated 2-Azido-4-nitrophenol.* In order to obtain the best yield in the tritiation of 2-amino-4-nitrophenol, it is necessary to use a very pure starting material. 2-Amino-4-nitrophenol is most easily recrystallized as the fluoborate in fluoboric acid (48–50%). The pure starting material can be regenerated from the fluoborate by adjusting the pH of an aqueous solution with sodium hydroxide to 4.8–5.0.

Purified 2-amino-4-nitrophenol (200 mg), water (1 mL), containing the desired amount of tritium oxide, and concentrated hydrochloric acid (1 mL) are placed into a 10-mL glass ampule (Bellco) and kept at 115–120 °C for 48–60 h. The dark reaction mixture is transferred to a 50-mL round-bottomed flask and lyophilized in a closed system. After removal of easily exchangeable tritium, the dark residue is taken up in 0.5 N HCl and filtered through a small amount

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of cotton wool. The filtrate is brought to pH 4.8–5.0 with sodium hydroxide, and the resulting crystals of 2-amino-4-nitrophenol are filtered off. In typical experiments, tritiated 2-amino-4-nitrophenol was obtained in 70–80% yield with a specific radioactivity of 3.1 and 21 mCi/mmol from 1 Ci (48 h) and 5 Ci (60 h) of tritium oxide, respectively. The radiochemical purity as determined by thin-layer chromatography (Table I) was ≥97%.

Essentially the same procedure can be used to obtain tritiated ethidium bromide. Thus, by heating 100 mg of ethidium bromide with 2 mL of 6 N hydrochloric acid containing 150 mCi of tritium oxide, a pure product containing 0.67 mCi/mmol was obtained.

Tritiated 2-amino-4-nitrophenol (247 mg) in fluoboric acid (48–50%) (4.1 mL) is cooled with an ice bath. A solution of sodium nitrite (166 mg in 0.6 mL of cold water) is added dropwise to the briskly stirred reaction mixture at a rate slow enough to keep the temperature below 5 °C. After 30 min, the vessel is transferred to a cooling bath containing an ice–rock salt mixture. Sodium azide (234 mg in 0.7 mL of water) is added slowly under continuous stirring. The reaction mixture is kept below −10 °C for 30 min and then transferred back into the ice bath where it is kept for another 30 min. After addition of 5 mL of water, the mixture is twice extracted with 10 mL of ethyl ether each. The combined extracts are washed with 5 mL of water and dried with anhydrous magnesium sulfate for about an hour. The ether solution of 2-azido-4-nitrophenol is evaporated, and the residue dissolved in a minimal amount of ether is applied to a column (0.9-cm inner diameter) packed 40-cm high with silica gel (0.05–0.2 mm, Merck) in ether. With ether as the eluant, 2-azido-4-nitrophenol is the first colored material to appear from the column. The fraction containing 2-azido-4-nitrophenol is evaporated to dryness, redissolved in a minimal amount of ether, diluted with *n*-pentane until turbid, and allowed to stand at −20 °C for crystallization. The yield is 60–70%, and the radiochemical purity determined by thin-layer chromatography is ≥99.5%. Small amounts may be conveniently purified by sublimation at 75 °C in a vacuum of about 0.3 mmHg. Crystalline 2-azido-4-nitrophenol is light yellow. It darkens upon prolonged exposure to light, but is indefinitely stable in the freezer when protected from light.

2-Amino-4-nitrophenol Fluoborate. Anal. Calcd for $C_6H_7N_2O_3BF_4$ (mol wt 241.94): C, 29.79; H, 2.92; N, 11.58; F, 31.41. Found: C, 29.79; H, 3.02; N, 11.53; F, 31.15. Melting point: 183–206 °C (dec). UV–Vis absorption in N/1000 NaOH: 274 ($\epsilon_{mM}$ 6.7), 443 nm ($\epsilon_{mM}$ 13.8) (lit. 275 nm, $\epsilon_{mM}$ 6.9; 446 nm, $\epsilon_{mM}$ 13.4; Doub & Vandenbelt, 1955). NMR (1 N DCl/$D_2O$): 7.84 ppm (d, 3-H), 7.78 (dd, 5-H), 6.75 ppm (d, 6-H); $J_{5,6}$ = 9 Hz, $J_{3,5}$ = 3 Hz, $J_{3,6} \sim 0$.

2-Azido-4-nitrophenol. Anal. Calcd for $C_6H_4N_4O_3$ (mol wt 180.13): C, 40.01; H, 2.24; N, 31.11. Found: C, 40.03; H, 2.31; N, 31.05. Melting point: 94–95 °C (dec). IR: 4.69 μm ($CHCl_3$) $RN_3$. NMR ($Me_2SO$/1 N DCl = 80/1): 7.78 (d, 3-H), 7.96 (dd, 5-H), 6.75 ppm (d, 6-H); $J_{5,6}$ = 9 Hz, $J_{3,5}$ = 3 Hz, $J_{3,6} \sim 0$.

*Selective Hydrolysis.* The material extracted from NPA-labeled mitochondria (0.43 nmol of NPA/mg of protein) by treatment with 96% and 90% acetone (Lester, 1967) was freed from solvent and lyophilized. Two hundred milligrams of this powder was reextracted with 12 mL of chloroform:methanol (2:1) containing 2,6-di-*tert*-butyl-4-methylphenol (1 mM). After evaporation and dissolution in a small volume of chloroform–methanol, the material containing about 0.12 nmol of NPA/μmol of phosphorus was applied to a column (38 × 1.2 cm) packed with 8 g of silica gel and developed with 30 mL each of chloroform, chloroform:methanol (4:1), chloroform:methanol (3:2), and methanol (Ansell & Hawthorne, 1964). Fractions of 3.1 mL were collected and assayed for radioactivity. Two main radioactive peaks eluted with chloroform:methanol (4:1) and methanol, respectively. The first radioactive peak containing phosphatidylethanolamine and 50–70% of the radioactive material was subjected to repeated selective hydrolyses at 37 and 60 °C (Duncan et al., 1971). The aqueous phase of the first hydrolysis at 37 °C was concentrated, passed through Dowex 50W-X4 as described, and analyzed for radioactivity and phosphorus content (Chen et al., 1956). The organic phase containing fatty acids and uncleaved phospholipids was evaporated, taken up in a small amount of chloroform:methanol (2:1), and applied to a small column packed with silica gel. Fatty acids were eluted with hexane:diethyl ether:acetic acid (90:10:1) (Dittmer & Wells, 1969). Thereafter, phospholipids were eluted with chloroform:methanol:water (70:30:5), analyzed for radioactivity and phosphorus, and subjected to selective hydrolysis at 60 °C in an otherwise identical procedure.

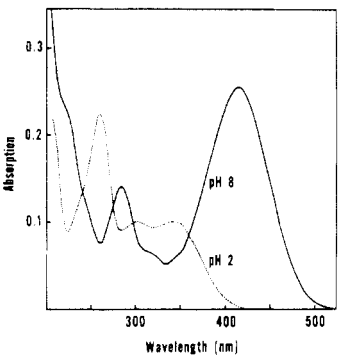


FIGURE 1: Absorption spectra of the neutral and anionic forms of 2-azido-4-nitrophenol (NPA). NPA (17 μM) in 10 mM sodium phosphate buffer, pH 8 (—), and in 10 mN hydrochloric acid (···).

## Results

*Radiochemical Synthesis of 2-Azido-4-nitrophenol.* The synthesis of radioactive NPA involves tritiation of the precursor 2-amino-4-nitrophenol at 120 °C for 48–60 h in 6 N hydrochloric acid containing tritium oxide. The optimal reaction conditions were found in deuterated media, using NMR spectroscopy to monitor the disappearance of aromatic protons. A detailed NMR analysis of partially deuterated 2-amino-4-nitrophenol (not shown) suggested that the proton adjacent to both the nitro and the amino group is by far the most reactive, followed by the proton which is ortho to the hydroxyl group, and by the proton adjacent to the nitro group and in para position to the amino group. These results suggest a neighboring effect of the amino group. It appeared possible therefore that ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide) might be tritiated under similar conditions. This proved to be the case, and the specific radioactivity of tritiated ethidium bromide obtained by this method (0.67 mCi/mmol from exchange in 150 mCi of tritium oxide) is similar to the degree of tritiation of 2-amino-4-nitrophenol (3.1 and 21 mCi/mmol from exchange in 1 and 5 Ci of tritium oxide, respectively).

*Photochemical Properties.* Figure 1 shows the spectra of 2-azido-4-nitrophenol at pH 8 and 2. It is seen that both the anionic and the neutral forms have fairly complex spectra with maxima at 416, 320 (shoulder), and 285 nm for the former and 343, 301, and 260 nm for the latter. The spectrum of undissociated 2-azido-4-nitrophenol is similar to the spectrum

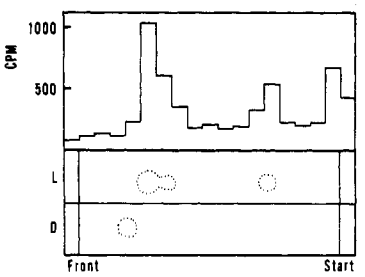


FIGURE 2: Photolysis of NPA in phosphate buffer. NPA (0.2 mM) in phosphate buffer (50 mM, pH 8.0) was irradiated in an argon atmosphere for 60 min at room temperature in an arrangement described earlier (Hanstein & Hatefi, 1974). Twenty microliters each of this solution and the original, nonirradiated solution were applied to a thin-layer chromatography sheet and developed in solvent A (see Experimental Procedures). The spots were visualized with ammonia vapor as indicated in strip L (photolyzed material) and strip D (starting material). In strip L, the space between the solvent front and the starting line was divided into small areas which were separately scratched off, collected, and extracted with methanol. The extracts (2 mL each) were acidified with 100 $\mu$L of glacial acetic acid and counted in a dioxane-based cocktail. The results are shown in the upper part of the figure.

of 2,4-dinitrophenol in 0.1 N hydrochloric acid (not shown), except for the absorption at 343 nm which may therefore be attributed to the azido group (Reiser & Wagner, 1971). These spectra indicate that visible light in the range of 400–500 nm will photolyze the anionic form exclusively, since the energy in this wavelength region (239–299 kJ/mol) is sufficient to break the RN–$N_2$ bond (Reiser & Wagner, 1971). It is therefore necessary to know the p$K_a$ of 2-azido-4-nitrophenol. The pH dependence of the absorption at 416 nm was measured and a p$K_a$ value of 5.62 ± 0.05 (room temperature, 0.1 M phosphate) was determined. Thus, 2-azido-4-nitrophenol is a significantly weaker acid than 2,4-dinitrophenol with a p$K_a$ of 4.0 (Cowles & Klotz, 1948).

The primary photolysis products of azides are nitrenes which are known to exist in two nonresonant electronic structures, i.e., as diradicals (triplet) and electron sextets (singlet). Interaction of singlet nitrenes with other molecules usually results in covalent linkages. In contrast, the main reaction of triplet nitrenes is hydrogen abstraction. Therefore, only singlet nitrenes are generally useful for photoaffinity labeling. Because of the short lifetime of singlet nitrenes (in the order of 100 ns, Reiser & Wagner, 1971), the relative contributions of the singlet and triplet form can be determined only by product analysis based on the differences in reactivity mentioned above (Lwowski, 1967; Reiser & Leishon, 1971). An estimate of the singlet/triplet ratio in NPA can be obtained as follows. Exhaustive photolysis of 0.2 mM NPA in 0.05 M phosphate buffer with light in the visible region resulted in a product mixture which can be resolved by thin-layer chromatography into three main spots (Figure 2). The slowly migrating spot below $R_f$ 0.3 was identified as 2-amino-4-nitrophenol on the basis of spectral properties, p$K_a$ measurements, and the fact that in thin-layer chromatography it comigrates with an authentic sample of 2-amino-4-nitrophenol under a variety of conditions. The instability of the two other products prevented further characterization. Based on the radioactivities recovered, 2-amino-4-nitrophenol comprised 15% of the photolysis products. In contrast to the two other main products, 2-amino-4-nitrophenol itself is completely stable under the photolyzing conditions used (not shown). Therefore, and with the generally accepted assumption that primary amines are produced exclusively from triplet nitrene through two consecutive hydrogen abstractions, the fraction of triplet nitrene in the primary photolysis product can be estimated to

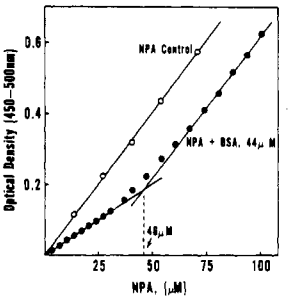


FIGURE 3: High-affinity binding of 2-azido-4-nitrophenol by bovine serum albumin. Absorption spectra of increasing concentrations of NPA in 25 mM phosphate (pH 8.0) were recorded in the presence (44 $\mu$M) and absence of BSA, and the difference in optical density at 450 nm was plotted as a function of the concentration of NPA. Temperature: 30 °C.

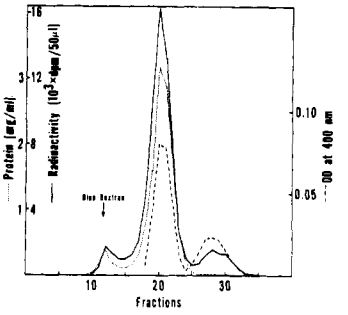


FIGURE 4: Gel filtration of the photoproducts of 2-azido-4-nitrophenol in the presence of bovine serum albumin. BSA (1 $\mu$mol) and NPA (0.31 $\mu$mol) in 10 mL of a buffer containing sucrose (0.25 M) and Tris-HCl (10 mM, pH 7.5) were irradiated for 60 min at 17–23 °C. The reaction mixture was lyophilized, taken up in 4.9 mL of sodium phosphate buffer (0.1 M, pH 7.0), and applied, together with Blue Dextran 2000, to a column packed with Ultrogel ACA 34 (1.5 × 47 cm) in the same buffer. The fractions (2.95 mL) were eluted at room temperature at a rate of about 1 mL/min. The appearance of Blue Dextran (arrow) was monitored at 630 nm. Protein was determined according to Lowry et al. (1951).

be ≥15%. Thus, possibly up to 85% of the nitrenes formed from 2-azido-4-nitrophenol may have been in the singlet state, i.e., competent for photoaffinity labeling.

*Interaction with Bovine Serum Albumin.* The properties of protein-bound NPA are best studied with a water-soluble protein such as bovine serum albumin which has a high affinity for uncouplers and which does not absorb in the visible region. Similar to other uncouplers (Weinbach & Garbus, 1966a,b; Tabachnik et al., 1970) NPA interacts strongly with bovine serum albumin. Upon binding to the protein, the spectrum of NPA undergoes a blue shift (not shown) which can be used to determine stoichiometry and affinity of the binding equilibrium. Figure 3 shows that only 1 mol of NPA/mol of bovine serum albumin binds with concomitant blue shift in the spectrum of the uncoupler anion. The data points near the intersection of the two limiting straight lines can be used to estimate the dissociation constant, $K_D = 0.7 \pm 0.3$ $\mu$M. This value is close to the value of 1.1 $\mu$M obtained for 2,4-dinitrophenol (Tabachnik et al., 1970).

Photochemical labeling of bovine serum albumin under conditions where essentially all of the NPA is protein bound allows a comparison between the fractions of singlet nitrene derived from protein bound and free uncoupler. Figure 4 shows that, after exhaustive irradiation of NPA in the presence of excess bovine serum albumin, most of the radioactivity remained associated with the protein during the gel filtration experiment. Only about 11% of the radioactivity eluted after serum albumin. The electrophoretic behavior under denaturing and dissociating conditions (Weber & Osborn, 1969) indicated

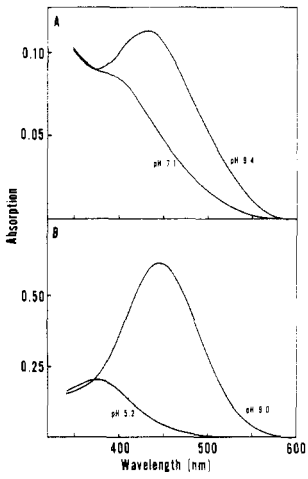


FIGURE 5: Absorption spectra of NPA-labeled bovine serum albumin and 2-amino-4-nitrophenol. (A) Spectra of fraction 21 (see Figure 4) containing NPA-labeling BSA. (Upper curve) pH 9.39; (lower curve) pH 7.08. Buffer: 0.1 M sodium phosphate. Isosbestic point at about 374 nm. (B) 2-Amino-4-nitrophenol fluoborate (approximately 63 $\mu$M). (Upper curve) pH 9.00; (lower curve) pH 5.15. Buffer: 0.1 M sodium phosphate. Isosbestic point at 372 nm.

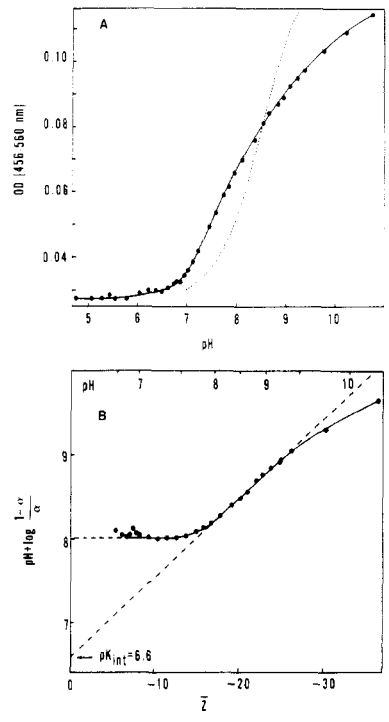


FIGURE 6: Light absorption of NPA-labeled bovine serum albumin as a function of pH. Spectra of fraction 21 in Figure 4 were recorded, and the difference in optical density at 456 and 560 nm was plotted as a function of increasing alkalinity. The pH was increased by the addition of small amounts of 2 N NaOH and measured directly in the spectral cell with the use of a microelectrode (Brinkmann EA 147) and a five-digit pH meter (Orion 801 A). The pH values obtained before and after the recording of the spectra agreed within 0.002 unit below pH 11. The spectral changes were completely reversible between pH 6.9 and 9.4. The absorption values shown are corrected for dilution. The dotted line is a theoretical curve calculated for a $pK_a$ value of 8.5, with 0.13 and 0.027 as the maximal and minimal optical densities, respectively. (B) Apparent acid dissociation constant of the chromophore in NPA–BSA as a function of the net charge on the protein. The ordinate values are $pK_a$ values calculated from the data shown in A, using 0.13 and 0.027 as the maximal and minimal optical densities, respectively. The values of the net average charge $\bar{Z}$ of BSA were derived from data published by Tanford et al. (1955) as described by Klotz & Fiess (1960). The slope between $\bar{Z} = -18$ and $-26$ is 0.094, corresponding to $w = 0.104$ in eq 1.

that the radioactive label is indeed covalently bound to the albumin molecule. Thus, approximately 90% of the protein-bound NPA engaged in photoaffinity labeling, presumably through a singlet state as the reactive intermediate. This number is close to the estimate of maximally 85% singlet nitrene obtained in the absence of protein (Figure 2).

The products of photoaffinity labeling with NPA are expected to be derivatives of 2-amino-4-nitrophenol. Thus, NPA labeled proteins and 2-amino-4-nitrophenol should have similar absorption spectra. As seen in Figure 5 the spectra of NPA–BSA (panel A) and 2-amino-4-nitrophenol (panel B) are similar at pH 9 and exhibit maxima at about 432 and 446 nm. There are also similarities between the spectra of protonated NPA–BSA and 2-amino-4-nitrophenol. The latter has a maximum at about 373 nm (panel B), while in the spectrum of the former a shoulder at about 400 nm is observed together with the onset of the protein absorption.

In order to obtain an estimate of the dissociation constant of the chromophore in NPA–BSA, the absorption at 456 nm was determined as a function of the pH. The resulting curve (Figure 6A) is flatter and less symmetric than the usual sigmoidal titration curves (dotted line). Such distortions are commonly observed in titration curves of proteins and have been explained by electrostatic interactions of charges on the macromolecule, among other things. According to Linderstrøm-Lang (1924) and Scatchard (1949), the effect of the average net charge $\bar{Z}$ on the $pK_a$ and the fractional degree of ionization $\alpha$ can be approximately described by eq 1, where $K_{int}$ is the hypothetical intrinsic dissociation constant,

$$pK_a = pH + \log \frac{1-\alpha}{\alpha} = pK_{int} - 0.868w\bar{Z} \qquad (1)$$

and $w$ is an empirical parameter related to the electrostatic work required to remove a proton from the protein. Thus, a value of $pK_{int}$ can be obtained from a plot of the left terms in eq 1 as a function of the net average charge (Tanford et al., 1955). Such a plot is shown in Figure 6B. Above pH 8 the $pK_a$ is a function of $\bar{Z}$ as expected from eq 1, and the value of the "intrinsic" dissociation constant obtained from data between pH 8 and 9.5 is 6.6. Below pH 7.6 the $pK_a$ is close to 8.0 and independent of $\bar{Z}$. The $pK_a$ of free 2-amino-4-nitrophenol was determined spectroscopically and found to be 6.9 under the conditions used for NPA (lit. 6.6, Bader, 1890; 7.0, Cowles & Klotz, 1948). These data and the spectral comparisons described above suggest that photochemical labeling of BSA with NPA results in a protein derivative of 2-amino-4-nitrophenol containing a covalent bond between the nitrogen and the protein.

*Photoaffinity Labeling of the Mitochondrial Membrane.* In mitochondria, both proteins and phospholipids appear to be able to bind uncouplers reversibly. This is shown in experiments in which uncoupler binding by normal and lipid-depleted particles is compared. Uncoupler binding by mitochondria and submitochondrial particles as a function of the uncoupler concentration can be resolved into (a) unspecific binding characterized by a partition coefficient, and (b) specific binding defined by the number of binding sites and a dissociation constant (Hanstein & Hatefi, 1974). These contributions have been separated by a least-squares method in Figure 7, and it is seen that removal of 80% of the phospholipids decreased unspecific binding by about 50% without strongly altering specific binding.

For photoaffinity labeling studies with NPA it is important to know whether the uncoupler anion or the neutral species are engaged in specific and/or unspecific binding, because only the anion is photoreactive under the conditions used. Precision absorption spectroscopy indicated that specifically bound NPA

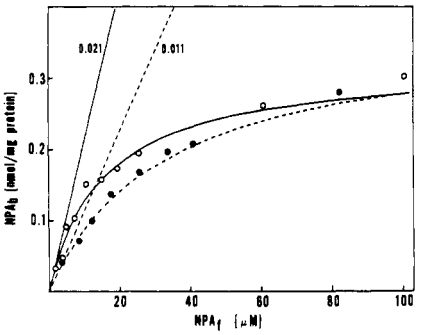


FIGURE 7: Effect of phospholipid content of submitochondrial particles on specific and unspecific uncoupler binding. Solid lines: specific (open circles) and unspecific (straight line) binding by submitochondrial particles (Löw & Vallin, 1963). Dashed lines: specific (closed circles) and unspecific (straight line) binding by lipid-depleted submitochondrial particles. The binding experiments were performed and analyzed as described (Hanstein & Hatefi, 1974), except that higher centrifugal forces (100000$g$ for 40 min) were used to sediment the particles. Lipid-depleted particles were obtained by consecutive extractions of submitochondrial particles with 96% and 90% acetone (Lester, 1967). The phosphorus contents of control and lipid-depleted particles were 0.46 and 0.09 $\mu$mol/mg of protein, corresponding to 0.43 and 0.08 mg of phospholipid/mg of protein, respectively.

Table II: Effect of pH on the Dissociation Constant of NPA Bound to the Specific Uncoupler Binding Site of Mitochondria[a]

| pH | $K_D$ ($\mu$M) |
|---|---|
| 7.00 | 5.4 |
| 7.51 | 2.9 |
| 8.06 | 5.4 |
| 8.52 | 8.1 |
| 9.00 | 5.9 |
| 9.42 | 5.5 |
| | av 5.5 ± 1.7 |

[a] For conditions, see Figure 8.

is predominantly in anionic form (K. L. Poff & W. G. Hanstein, unpublished). This conclusion is supported by a study of uncoupler binding as a function of the pH of the medium (Table II). The dissociation constant (5.5 ± 1.7 $\mu$M) is found to be independent of the pH in the range of pH 7.0–9.4, i.e., 1.5–3.9 pH units above the p$K_a$. These data indicate that the uncoupler anion is indeed the ligand interacting with the uncoupler binding site. The pH dependence of unspecific binding is shown in Figure 8, and it is seen that, above pH 8.5, this type of binding is constant, while below pH 8 it strongly increases with decreasing pH. Thus, it appears that unspecific binding involves anionic uncoupler and, below pH 8, increasing amounts of the neutral form as well. As to the type of binding, it is likely that uncoupler anions are capable of interacting with phospholipid bilayers similar to other lipophilic anions such as 1-anilino-8-naphthalenesulfonate (Lesslauer et al., 1971, 1972) in a way in which the negatively charged part of the molecule stays in the layer of polar groups of the phospholipids, while the more lipophilic moiety enters the hydrocarbon core of the membrane. Thus photoaffinity labeling of mitochondria by uncoupler anions can be expected to result in labeled phospholipids was well as proteins.

The labeling pattern of polypeptides in NPA-labeled mitochondria as revealed by polyacrylamide gel electrophoresis in the presence of NaDodSO$_4$[1]–mercaptoethanol is shown in Figure 9. It is seen that two peaks corresponding to molecular

[1] Abbreviations used: NaDodSO$_4$, sodium dodecyl sulfate; Hepes, $N$-2-hydroxyethylpiperazine-$N'$-2-ethanesulfonic acid; Tricine, $N$-tris-(hydroxymethyl)methylglycine.

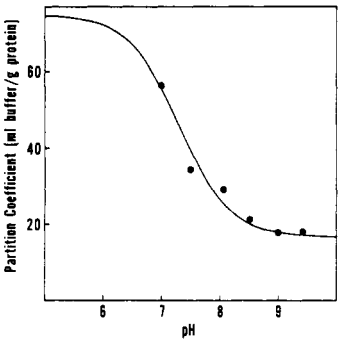


FIGURE 8: Partitioning of NPA between the aqueous and the mitochondrial phases as a function of pH. The partition coefficients were obtained from binding experiments with beef heart mitochondria (Hatefi & Lester, 1958) and NPA at 2 °C in a medium containing sucrose (0.25 M), ATP (1 mM), $MgCl_2$ (1 mM), succinate (1 mM), Hepes (72 mM), and Tricine (72 mM). The initial pH of the buffers was adjusted with NaOH. The final pH values were measured in the supernatants of the binding experiments at 2 °C. For comparison, pH values were also determined at 22 °C. These data were used to calculate values at 2 °C as expected from the temperature dependence of the p$K_a$ values (Good et al., 1966). The observed and the calculated data agreed with each other within 0.02 to 0.03 pH unit. The results shown in this figure can be fitted to curves calculated with p$K_a$ values ranging from 6.9 to 7.6, maximum values of 68–105, and a minimum between 16 and 18. The curve shown is based on the results of a least-squares procedure with p$K_a$ = 7.3, and maximum and minimum partition coefficients of 75 and 17, respectively.

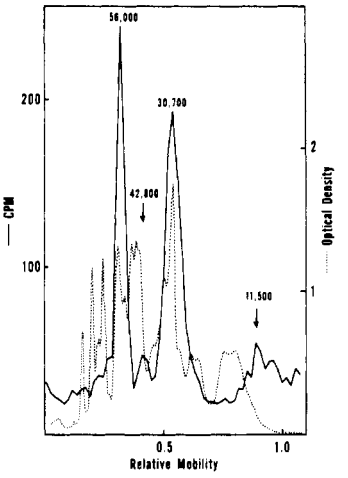


FIGURE 9: Molecular weight distribution in NPA-labeled beef heart mitochondria. Beef heart mitochondria (0.6 mg of protein) containing 0.19 nmol of covalently bound radioactive NPA/mg of protein were treated with NaDodSO$_4$–mercaptoethanol, electrophoresed, and analyzed as described in the experimental part. The specific radioactivity of NPA was 21 mCi/mmol. The optical density trace was obtained with a gel stained with Coomassie Blue.

weights of 56 000 and 31 000 contain about 60% of the radioactivity incorporated with the label. Minor peaks are detected at calculated molecular weights of 43 000 and 12 000. Little radioactivity is found around the dye front where phospholipids tend to migrate (Hanstein, 1978). Nevertheless, the presence of small amounts of NPA-labeled phospholipids was shown as follows.

Chromatography on silica gel (Ansell & Hawthorne, 1964) of the purified acetone extract obtained under conditions of the experiment shown in Figure 7 yielded two main radioactive peaks containing mainly phosphatidylethanolamine and phosphatidylcholine. Under conditions where phospholipids are selectively hydrolyzed at the fatty acyl ester bonds (Dittmer & Wells, 1969; Duncan et al., 1971), radioactive products were obtained which eluted in the fatty acid and the glyceryl phosphoryl ester fractions (Table III). The latter contained

Table III: Selective Hydrolysis of NPA-Labeled Phospholipids

| | cpm | phosphorus (μmol) | cpm/nmol of phosphorus |
|---|---|---|---|
| acetone extract | 19 400 | 11.0 | 1.8 |
| radioactive peak from silica gel column containing mainly phosphatidylethanolamine (peak I) | 10 860 | 0.97 | 11.2 |
| selective hydrolysis of peak I at 37 °C for 15 min | | | |
| glyceryl phosphoryl ester fraction | 4 180 | 0.50 | 8.4 |
| fatty acid fraction | 560 | | |
| phospholipid fraction | 5 100 | 0.33 | 15.5 |
| selective hydrolysis of phospholipid fraction at 60 °C for 15 min | | | |
| glyceryl phosphoryl ester fraction | 2 510 | 0.19 | 13.2 |
| fatty acid fraction | 350 | | |
| phospholipid fraction | 2 080 | 0.09 | 23.1 |

most of the radioactivity of the cleaved products, and only about 14% was found in the fatty acid fraction. After two consecutive selective hydrolyses, the specific radioactivity of the unsaponified material (23 cpm/nmol of phosphorus) corresponded to a molar ratio of label:phospholipid of roughly 1:1000. Although further identification of the cleavage products (Dawson, 1976) was not attempted, the data suggest that mitochrondrial phospholipids are labeled by anionic NPA under the conditions employed, and that labeling occurs most extensively in the glyceryl phosphoryl ester moiety, i.e., in the most polar region of the molecule. Preliminary experiments have shown that liposomes containing phospholipids without proteins can also be labeled by NPA under similar conditions.

## Discussion

In general, photoaffinity labeling with an aromatic azide is likely to be successful if the following three conditions are satisfied. (1) The azide should produce predominantly singlet nitrene upon irradiation; (2) the insertion reaction of the singlet nitrene should be considerably faster than the conversion to the triplet state (intersystem crossing; Lwowski, 1967); and (3) the product should be stable, at least under the conditions used in the labeling reaction. The present data indicate that NPA satisfies all of these conditions and promises to be a versatile reagent for photochemical labeling.

Data pertaining to the relative abundance of singlet and triplet nitrene (Figures 2 and 4) suggest that irradiation of NPA with visible light generates 85–90% singlet and 10–15% triplet nitrene. This conclusion is based on the assumption that 2-amino-4-nitrophenol is the only major reaction product of the triplet nitrene, and that all NPA-labeled BSA is a product of (protein bound) singlet nitrene. This is consistent with the general rule that insertion into C–H bonds and hydrogen abstraction are typical reactions of singlet and triplet nitrenes, respectively (Reiser & Wagner, 1971).

The chemical stability of NPA-labeled proteins is, of course, determined to a large degree by the reactivity of the covalently attached 2-amino-4-nitrophenol moiety. 2-Amino-4-nitrophenol itself is completely stable in the presence of high intensities of visible light over prolonged periods of time. In addition, hydrolysis conditions employed for amino acid analysis are expected to be tolerated by the label, since the tritiation of 2-amino-4-nitrophenol carried out under harsher conditions is accompanied by only minor decomposition. However, treatment of NPA-labeled BSA or NPA-labeled mitochondria with detergents in the presence or absence of salts leads to substantial or complete loss of the label. Autoxidative reactions are apparently involved, since absence of oxygen or the presence of reagents such as 2-mercaptoethanol or 2,6-di-*tert*-butyl-4-methylphenol prevent the dissociation of the label from the macromolecules.

For many aspects of photoaffinity labeling, it is necessary to use a radioactive labeling reagent with relatively high specific activity. The radiochemical synthesis described in this paper employs a simple and perhaps generally applicable method of tritiation which allows the preparation of millimolar amounts of material with a specific radioactivity (21 mCi/mmol) high enough for many purposes. In these respects, the new method compares favorably with other methods published during the preparation of this paper (Cyboron & Dryer, 1977; Kurup & Sanadi, 1977).

Although photoaffinity labeling experiments with BSA were done in order to study the course of the photochemical reactions of NPA with a protein, some of the data are interesting in their own right, and suggest that useful information about structure and function of serum albumin may be obtained in this type of study. Spectral data obtained with NPA (Figure 3) reveal the existence of one strong uncoupler binding site which can be covalently labeled with NPA (Figure 4). In NPA-labeled BSA, a 2-amino-4-nitrophenol moiety appears to be bound to the protein through the amino nitrogen. At neutral pH, the apparent $pK_a$ of this chromophore is close to 8 (Figure 6B), whereas free 2-amino-4-nitrophenol has a $pK_a$ of 6.9. Such a shift to higher $pK_a$ values is expected from the transfer of a neutral acid into a more lipophilic environment with a lower dielectric constant (Robinson & Stokes, 1965). At higher pH, the apparent $pK_a$ increases linearly with the average net charge of the protein, as predicted by eq 1. In addition to direct electrostatic interactions, it is also possible that the data in Figure 6 reflect other environmental changes brought about by the N–B transition which occurs between pH 7 and 9 (Leonard et al., 1963; Zurawski & Foster, 1974). Indeed, there are definite similarities between the change in $pK_a$ as a function of the pH (Figure 6B, ordinate vs. upper abscissa) and the changes in specific rotation (Leonard et al., 1963) and the chemical shift of trifluoroacetonylated serum albumin (Zurawski & Foster, 1974) observed in the pH region of the N–B transition. In further studies of ligand binding and conformational changes in serum albumin, NPA-labeled BSA may be useful because of the apparent specificity of the labeling and the ease with which absorption spectroscopic data may be obtained.

Photoaffinity labeling of mitochondria occurs mainly on two polypeptides with approximate molecular weights of 56 000 and 31 000. The former is identical with subunit 1 of the soluble mitochondrial ATPase ($F_1$) (Hanstein, 1976) and the latter is a hydrophobic polypeptide which appears to be a component of the membrane sector of the oligomycin-sensitive ATPase complex (Hanstein, 1975, 1977). Data regarding solubilization and identification of labeled mitochondrial polypeptides and the effects of pH, effectors, and inhibitors on photoaffinity labeling of the mitochondrial inner membrane will be presented elsewhere.

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