Rapid Photolytic Release of Adenosine 5′-Triphosphate from a Protected Analogue: Utilization by the Na:K Pump of Human Red Blood Cell Ghosts†

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ABSTRACT: 2-Nitrobenzyl phosphate and 1-(2-nitro)phenylethyl phosphate have been synthesized and demonstrated to be suitable as photolabile sources of inorganic phosphate. The same protecting groups were attached to the terminal phosphate of adenosine 5′-triphosphate. These "caged ATP" compounds released adenosine 5′-triphosphate on illumination at 340 nm in aqueous solution and P^3-1-(2-nitro)phenylethyl-ATP gave about a 70% yield in under 30 s. The unphotolyzed caged ATP was neither a substrate nor inhibitor of purified renal Na,K-ATPase (EC 3.6.1.3). Following photolysis in the presence of the enzyme, the liberated ATP was hydrolyzed but at an inhibited rate. The photo-dependent inhibition could be eliminated by prior addition of glutathione or bisulfite to the irradiated solution. Caged ATP was incorporated into resealed human erythrocyte ghosts prepared from red blood cells depleted of internal energy stores. While the Na:K pump was unable to use incorporated caged ATP as a substrate, the ATP liberated by photolysis activated the pump as evidenced by measurements of K-dependent, ouabain-sensitive Na efflux. Thus the caged ATP can be used as a stable source of ATP unmetabolizable by intracellular ATPases until the ATP is released following photolytic irradiation.

In many studies of cellular processes it would be useful to be able to generate significant intracellular levels of substrates or inhibitors in such a way that the process may be turned on rapidly and a predetermined level of intracellular substrate or inhibitor quickly attained. This paper describes a photochemical approach designed to be of use in such experimental situations.

Several protecting groups for carboxylic acid, alcohol, amine, ketone, or phosphate residues have been employed in recent years in synthetic chemical procedures, where deblocking of the functional group has been achieved by photolytic irradiation (Barltrop et al., 1966; Hebert & Gravel, 1974; Kirby & Vagelos, 1967; Patchornik et al., 1970). We have investigated the potential of this approach for the rapid release of biological substrates in situ. Specifically, we prepared several adenosine phosphate analogues in which the terminal phosphate was protected by a light-sensitive residue. In order to be useful in biological systems, such compounds should be nonutilizable substrates and noninhibitory prior to photolysis, but after photolysis, the substrate should be released rapidly and in good yield. The photolytic conversion should be demonstrable in an intracellular environment when, following photolysis, the substrate-dependent process can then be studied.

We have demonstrated these features using photosensitive ATP analogues, called herein "caged ATP", and have studied their effects on the purified Na,K-ATPase (EC 3.6.1.3) prepared from pig renal medulla together with their effects on Na:K transport associated with this enzymatic activity as seen in resealed human red blood cell ghosts.

Materials and Methods

Thin-layer chromatography of organophosphate compounds was carried out on precoated plastic sheets of PEI-cellulose (Macherey-Nagel) using aqueous 1 M LiCl (where ATP, ADP, and AMP ran with R_f values of 0.08, 0.17, and 0.39, respectively). Compounds were visualized under short or long wave light from a hand-held ultraviolet lamp (Mineralight UV SL-25). Total phosphate determinations were performed following the method of Ames & Dubin (1960). Absorption spectra were recorded on an Aminco DW2 spectrophotometer. All evaporations were carried out at a bath temperature of 30 °C using a Buchi flash evaporator, the cooling tubes of which were held at −20 °C with circulating refrigerated aqueous methanol. Triethylammonium bicarbonate for column chromatography was prepared by passing CO_2 through a cooled stirred solution of aqueous triethylamine (redistilled from 4-toluenesulfonyl chloride) until the pH fell to pH 7.5. All synthetic procedures were performed under subdued safe-light conditions.

Proton NMR spectra were recorded on a HX 270 Bruker instrument. The solvents were either dimethyl-d_6 sulfoxide or dimethyl-d_6 sulfoxide plus D_2O with Me_4Si as an internal standard. Shifts are reported in ppm. All phosphate salts were converted to the Na form by ion-exchange chromatography on Dowex resin. The proton resonances originating from the ribose ring were obscured by broad solvent peaks, with the exception of the H_1' proton, and were not assigned. The H_2, H_8, and H_1' resonances of the adenosine moiety were assigned by comparison to the data of Gatlin & Davies (1962).

Synthetic Procedures. An outline of the synthetic route to the caged ATP compounds is shown in Figure 1.

2-Nitrobenzyl Phosphate. 2-Nitrobenzyl phosphate was prepared by a modification of the procedure used by Verheyden et al. (1965) to synthesize the 4-nitro isomer. 2-Nitrobenzyl alcohol (recrystallized from water, 5.4 g, 36 mmol) was added to anhydrous phosphoric acid (1.16 g, 12 mmol, prepared from 85% phosphoric acid by the method of Ferrel et al., 1948) dissolved in acetonitrile (9 mL). Triethylamine (3.33 mL) and trichloroacetonitrile (5.6 mL) were then added and the mixture was stirred at room temperature for 2 h. The solution was then cooled and evaporated under reduced pressure to remove volatile components and the residue dissolved in

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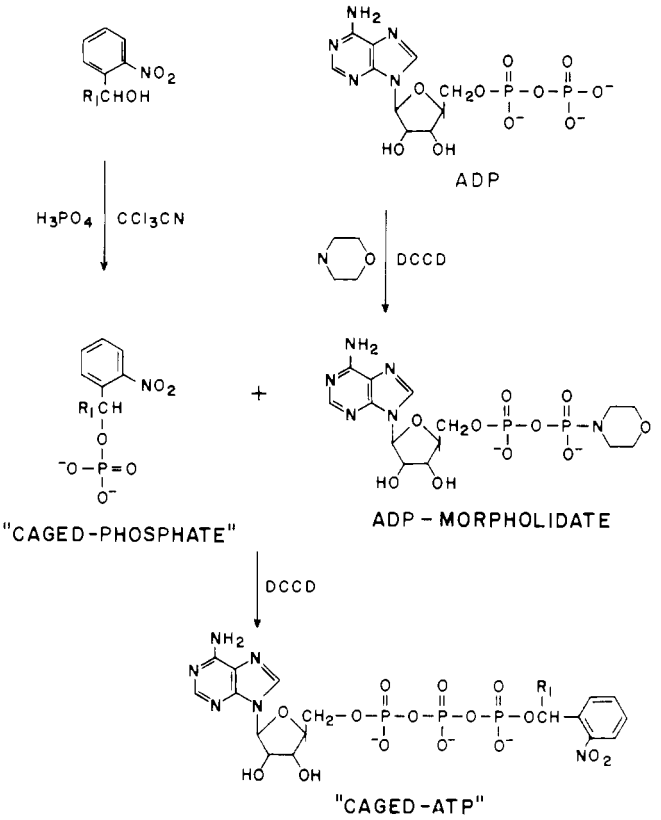

FIGURE 1: Outline of synthetic route to caged ATP compounds, where R_1 is H for the 2-nitrobenzyl series and CH_3 for the 1-(2-nitro)phenylethyl series.

water (25 mL). The aqueous solution was then extracted three times with 25 mL portions of benzene, concentrated by evaporation, and the pH adjusted to pH 8 with triethylamine. The solution was then applied to a DEAE-A25 Sephadex column (HCO_3^- form), washed with about 10 column volumes of water, and eluted with a linear gradient of 0 to 0.2 M triethylammonium bicarbonate (total volume was 2 L). The eluate was collected using an automated fraction collector with the aid of an ultraviolet absorption monitor. The absorbing fractions were collected and bulked. Separation from P_i was confirmed by adding a trace of $^{32}P_i$ to the reaction mixture prior to column separation and counting aliquots of eluate in a liquid scintillation spectrometer (Searle Mark III). The bulked samples were evaporated to dryness; residual traces of triethylamine were removed by resuspending the residue in methanol and drying several times. The final product was obtained as a concentrated solution of the triethylammonium salt in 18% yield (based upon ϵ = 5400 at 265 nm for the parent alcohol). Thin-layer chromatography revealed a single spot R_f 0.41. The proton NMR spectrum was compared with the parent alcohol [2-nitrobenzyl alcohol: aromatic 8.064–7.427 m (4 H); benzylic 4.899 s (2 H); 2-nitrobenzyl phosphate: aromatic 8.148–7.479 m (4 H); benzylic 5.226–5.202 d (2 H), $^3J_{P\text{-}O\text{-}C}$ = 6.62 Hz]. Assuming an unchanged molar extinction of the alcohol moiety in the phosphorylated product a ratio of 2-nitrobenzyl residue:P of 1:1 was obtained using the phosphate assay of Ames & Dubin (1960). Attempts to increase the yield by altering temperatures (25 to 70 °C) and reaction time (2 to 48 h) were not successful; a major competing reaction yields 2-nitrobenzaldehyde. This side product presumably comes about as a result of removal of benzylic protons from a complex which is produced from the alcohol and trichloroacetonitrile immediately on addition of the latter (unpublished observations).

4-Nitrobenzyl Phosphate. 4-Nitrobenzyl phosphate was prepared following the procedure of Verheyden et al. (1965) from 4-nitrobenzyl alcohol (recrystallized from water). Phosphate assay of the product gave a ratio of 1:1 for P:4-nitrobenzyl residue and product ran as a single spot in 1 M LiCl, R_f 0.36. The proton NMR spectrum was compared with the parent alcohol [4-nitrobenzyl alcohol: aromatic 8.22–7.485 m (4 H), benzylic 4.711 s (2 H); 4-nitrobenzyl phosphate: aromatic 8.268–7.634 m (4 H); benzylic 5.000–4.973 d (2 H), $^3J_{P\text{-}O\text{-}C}$ = 6.62 Hz].

1-(2-Nitro)phenylethanol. 2-Nitroacetophenone (5.2 g, 31.5 mmol) was dissolved in dioxane:methanol (75 mL, 2:3 by volume) and sodium borohydride (3.5 g, 92.5 mmol) was added. The mixture was stirred in ice for 30 min and at room temperature (23 °C) for a further 1.5 h. The reaction is strongly exothermic and the cooling period is necessary. The solvents were removed under reduced pressure, water (250 mL) was added, and the suspension was stirred at room temperature for 30 min. The solution was then extracted with methylene chloride (3 × 75 mL), the organic phase dried over anhydrous magnesium sulfate, and the filtered solution evaporated to dryness. The product (4.9 g, 94%) was obtained as a yellow oil [bp 105 °C at 1.05 mmHg (128–130 °C at 2.0 mmHg (Lesniak, 1962); 129–133 °C at 5 mmHg (Nagui et al., 1965))]. Thin-layer chromatography on silica gel in chloroform gave a single spot, R_f 0.23. The proton NMR spectrum revealed the following resonances, aromatic 7.931–7.527 m (4 H), benzylic, 5.370–5.302 q (1 H), methyl 1.538–1.519 d (3 H), which was in accord with the proposed structure. The absorption spectrum of an aqueous solution (containing 0.1% dimethyl sulfoxide) gave ϵ = 4240 at 265 nm.

1-(2-Nitro)phenylethyl Phosphate. 1-(2-Nitro)phenylethyl phosphate was prepared essentially as described above for 2-nitrobenzyl phosphate but starting from 1-(2-nitro)phenylethanol and anhydrous phosphoric acid. The reaction was allowed to proceed for 1 h at room temperature and after evaporation the aqueous solution was extracted with ether (three times) and methylene chloride (twice). Following ion-exchange chromatography as described above, using a linear gradient of 0 to 0.3 M triethylammonium bicarbonate the product was obtained as a concentrated solution of the triethylammonium salt in 20% yield based upon ϵ = 4240. Phosphate analysis revealed a ratio of 1:1 for 1-(2-nitro)-phenylethyl residue:P. Thin-layer chromatography revealed a single spot R_f 0.42. The proton NMR spectrum showed: aromatic 7.993–7.492 m (4 H); benzylic 5.782–5.681 m (1 H); $^3J_{P\text{-}O\text{-}C}$ = 5.88 Hz; methyl 1.555–1.530 d (3 H).

ADP Morpholidate. ADP morpholidate was prepared according to the procedure of Wehrli et al. (1965) and isolated using a linear gradient of triethylammonium bicarbonate (0.1–0.4 M) on DEAE-Sephadex A-25 (HCO_3^- form) as described above. The desired product and the minor product (AMP morpholidate) were converted to the 4-morpholino-*N,N'*-dicyclohexylcarboxamidino salts as previously described (Wehrli et al., 1965) and stored over P_2O_5 in a desiccator.

P^3-(2-Nitro)benzyladenosine 5'-Triphosphate. The concentrated solution of triethylammonium 2-nitrobenzyl phosphate (see above) was dried under reduced pressure using several washes with dry pyridine followed by a similar treatment with benzene; the residue (2.57 mmol) was dissolved in dry dimethyl sulfoxide (12 mL). The 4-morpholine-*N,N'*-dicyclohexylcarboxamidino salt of ADP morpholidate (2.79 g, 2.57 mmol) was added and the solution left at 60 °C for 50 h. After cooling, water (12 mL) was added and the mixture eluted from DEAE-Sephadex A-25 (HCO_3^- form), following a wash with 10 column volumes water, using a linear gradient

of triethylammonium bicarbonate of 0 to 1 M (total volume was 3 L). The eluate was monitored and peaks showing 260 nm absorbance were further examined by thin-layer chromatography. Fractions showing both long wavelength and short wavelength absorption within a peak when visualized on TLC plates were pooled. Amongst these were unreacted 2-nitrobenzyl phosphate and product which had eluted at about 0.5 M salt and gave a single spot R_f 0.32 in 1 M LiCl. The triethylammonium salt was obtained as a concentrated solution as described above; the absorption spectrum indicated a yield of 18%. The absorption spectrum could be constructed from the adenine absorption (ϵ = 15 400 at 263 nm) and a contribution from the nitrobenzyl moiety (2-nitrobenzyl alcohol, ϵ = 5400 at 265 nm). The absorbance at 300 nm agreed on a molar basis with the nitrobenzyl contribution at 260 nm. Adenine nucleotides have zero absorbance at this higher wavelength. Phosphate analysis yielded a P:nitrobenzyl ratio of 3:1. The proton NMR spectrum was in agreement with the proposed structure and was composed of the sum of ATP and 2-nitrobenzyl phosphate.

An initial attempt to prepare this compound, following the procedure of Eckstein et al. (1975) for the P^3-methyl ester of ATP, gave after column chromatography an impure product in about 0.5% yield, which eluted from DEAE-Sephadex at about 0.43 M salt. Another product absorbing in the long wavelength and short wavelength regions was eluted at about 0.26 M salt. This material gave adenosine:P:nitrobenzyl of 1:1:1 from a combination of spectroscopic and total phosphate determinations and appeared to be 2-nitrobenzyl AMP, obtained in about 2% yield.

P^3-1-(2-Nitro)phenylethyladenosine 5'-Triphosphate (Caged ATP). This compound was prepared essentially as described above for the 2-nitrobenzyl analogue, from 1-(2-nitro)phenylethyl phosphate and the 4-morpholine-*N,N'*-dicyclohexylcarboxamidino salt of ADP morpholidate. The product obtained in about 25% yield gave a single spot R_f 0.21 in 1 M LiCl. The absorption spectrum showed the characteristic longer wave absorption due to the nitrobenzyl moiety superimposed upon the adenosine chromophore (Figure 2) as described above for the 2-nitrobenzyl analogue. The proton NMR spectrum of the sodium salt was in agreement with the proposed structure and showed the following absorptions: 8.565–7.432 m (6 H); H_1' 6.029–6.101 d (1 H); benzylic 5.860–5.768 m (1 H); $^3J_{P-O-C}$ = 5.88 Hz; methyl 1.522–1.500 d (3 H). Phosphate analysis gave a P:1-(2-nitro)phenyl ratio of 3:1.

P^2-2-Nitrobenzyladenosine 5'-Diphosphate. This compound was prepared following the same procedure described above for the ATP analogue, using the minor product from the preparation of ADP morpholidate, AMP morpholidate, as starting material and was obtained in 25% yield. The adenine:P:2-nitrobenzyl ratio was 1:2:1 from spectrophotometric and total phosphate determinations.

Photolysis. Photolytic irradiation was carried out using a 1000-W mercury arc lamp (Ushio 1005D Oriel Corp.). Between the lamp housing (Oriel 6119 and condensing lens) and sample holders were placed in series, a jacketed heat filter (8-cm H_2O path length), 4.2% $CuSO_4$ solution (1.3-cm path length), an ultraviolet glass filter (UG-11, 3 mm, Schott, Duryea, Pa.), and finally a 2-in. lens. This filter arrangement gave light of peak wavelength at 342 nm (73% transmission at peak) and a half bandwidth of 60 nm. When experiments were performed using a concentration of <0.2 mM irradiated chromophore, photolysis was carried out with a defocused spot of 2.5-cm diameter (approximately 0.44 μE cm^{-2} s^{-1}, determined by the method of Hatchard & Parker, 1956), using

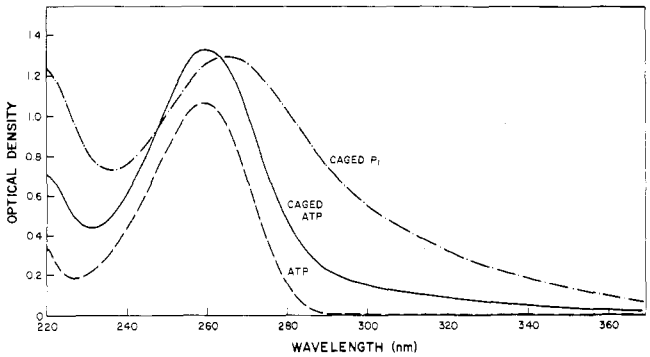

FIGURE 2: Absorption spectra of caged compounds and ATP [(- -) 6.67 × 10^{-5} M ATP; (—) 5.0 × 10^{-5} M caged ATP; (·····) 3.1 × 10^{-4} M caged P_i].

polystyrene tubes (1-cm i.d.). For experiments using higher concentrations of chromophore (up to 2.0 mM), a spot of 4.2-cm diameter was used and the irradiated solutions or ghost suspensions were contained in glass cells (ca. 1-mm path length, capacity 1.2 mL). Photolysis was carried out at room temperature (23 °C) without stirring. The maximum attenuation of the 340-nm light by absorption in the samples was less than 50%.

Isolation and assay of Microsomal Na,K-ATPase. Purified Na,K-ATPase was prepared by the method of Jorgensen (1974) with two minor modifications: (1) outer medulla dissected from bisected fresh pig kidneys was homogenized for 15 s in a Waring Blendor and filtered through cheesecloth before homogenization in the Teflon pestle homogenizer; (2) the density gradient centrifugation was performed in a Sorvall Ti 865 angle rotor (2 h, 65 000 rpm) on a 20–36% sucrose gradient. Fractions between 30% and 33% were pooled and treated as the zonal rotor fractions of Jorgensen. The specific activity of this preparation was 800–1000 μmol of P_i released per mg of protein per h, the ouabain-insensitive component constituting less than 1% of the total activity. Protein was determined by the method of Lowry et al. (1951), without precipitation.

Enzyme activity was assayed using a modification of the procedure of Martin & Doty (1949). The assay medium routinely contained 125 mM NaCl, 25 mM KCl, 4 mM $MgCl_2$, 4 mM Na_2ATP, 1 mg/mL bovine serum albumin, when desired 10^{-3} M ouabain, 60 mM Tris-Cl, 0.75 mM EDTA, pH 7.5, and approximately 1 μg of Na,K-ATPase in a final volume of 0.6 mL. After 10 min incubation at 37 °C, the reaction was stopped by the addition of 1 mL of freshly prepared 1.5% ammonium molybdate, 2% silicotungstate in 1.4 N H_2SO_4, and 2 mL of 2-butanol:hexane (1:1, by volume) and immediately vortexed for 10 s. An aliquot (0.8 mL) from the upper phase was diluted with 1.6 mL 2% H_2SO_4 in 95% ethanol and the phosphomolybdate in this extract reduced with 0.1 mL of freshly diluted 1.2% $SnCl_2$ in acid ethanol. The optical density of the resulting blue solution was determined at 670 nm. The optical density at 670 nm was linear with P_i concentration in the assay medium to beyond 1 mM ($\simeq$1 OD). Na,K-ATPase activities referred to in this paper are the ouabain-sensitive component amounting to greater than 99% of the total enzymatic ATPase activity.

Preparation of Resealed Ghosts and Measurement of ^{24}Na Efflux. Freshly drawn blood was collected in heparinized tubes and centrifuged at 12 000*g* in a Sorvall RC-5 centrifuge. The supernatant was removed by aspiration and the cells resuspended at about 20% hematocrit in 153 mM KCl, 11 mM sodium phosphate solution (pH 7.5 at 0 °C) containing a trace of chloramphenicol (1–2 mg/100 mL). The suspension was

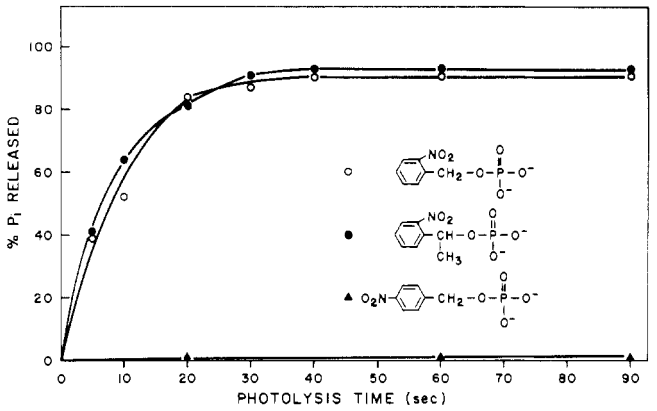

FIGURE 3: Comparison of photorelease of P_i from phosphate esters. Aqueous solutions (2×10^{-4} M) of the phosphate esters were photolyzed as described in Materials and Methods, and the photolyzed solution was then assayed for free P_i.

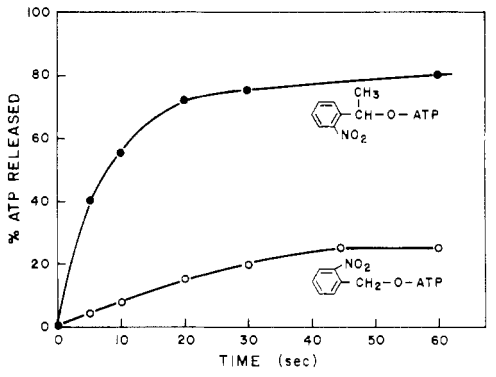

FIGURE 4: Comparison of photorelease of ATP using P^3-2-nitrobenzyl-ATP and P^3-1-(2-nitro)phenylethyl-ATP. Aqueous solutions (2×10^{-4} M) of two analogues were photolyzed in polystyrene tubes (see Materials and Methods), and the irradiated solution was assayed for free ATP.

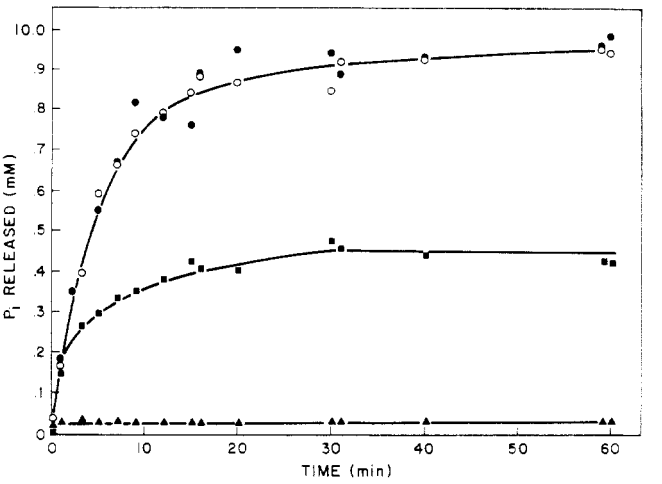

FIGURE 5: Utilization of photoreleased ATP by renal Na,K-ATPase. Incubation was initiated by the addition of 70 μg of the purified enzyme to the experimental medium, at 37 °C, which was composed of 6 mM imidazole, 150 mM NaCl, 30 mM KCl, 0.86 mM $MgCl_2$, 0.25 mM EDTA, 0.25% bovine serum albumin, 65 mM Tris (pH 7.5), total volume 4 mL with either 1 mM ATP or 1 mM caged ATP (circles). Aliquots (0.25 mL) were removed at the indicated times and assayed for P_i content (see Materials and Methods). Irradiation was carried out for sufficient time to release approximately 0.5 mM ATP from caged ATP [(○) 1 mM ATP + 1 mM caged ATP; (●) 1 mM ATP; (■) 1 mM caged ATP following photolysis; (▲) 1 mM caged ATP without photolysis].

then incubated at 37 °C with gentle shaking for 24 h in order to deplete the cells of their endogenous energy stores such as ATP and 2,3-diphosphoglycerate (Blum & Hoffman, 1971). The depleted cells were then centrifuged and washed three times in about 10 volumes of 166 mM NaCl solution. The packed cells were resuspended to 50% hematocrit in 166 mM NaCl and cooled to 0 °C in ice. In order to hemolyze the red cells, one volume of this cold suspension was then added rapidly to 10 volumes of $MgCl_2$ (2 mM) with either ATP, disodium salt (2 mM), or P^3-1-(2-nitro)phenylethyl ATP, sodium salt (caged ATP, 2 mM) also at 0 °C, together with a trace of ^{24}Na. After 5 min, the tonicity was raised by the addition of sufficient cold (0 °C) NaCl (3 M), choline chloride (3 M), and KCl (3 M) so that the final composition of the medium was 10 mM KCl, 10 mM NaCl, 150 mM choline chloride. After a further 5 min at 0 °C, the suspension was transferred to a water bath and gently shaken for 40 min at 37 °C. The resealed ghosts were then centrifuged (49 000g, 5 min) and washed three times with about 20 volumes of cold (0–4 °C) buffer (150 mM NaCl, 10 mM KCl, 10 mM Tris-Cl, pH 7.5). The washed ghosts were then resuspended in 9 volumes of the buffer and portions (1 mL) were either photolyzed (see above) or held on ice. In order to initiate the measurement of Na efflux, samples of the ^{24}Na-loaded ghosts (10% suspension) were added to 10 mL portions of the incubation medium (150 mM NaCl, 10 mM KCl, 10 mM Tris-Cl, pH 7.5) at 37 °C. Ouabain, when present, was at a concentration of 10^{-4} M. The incubating cells were gently shaken at 37 °C, samples were withdrawn at 5, 35, and 65 min and immediately centrifuged at 49 000g. The radioactivity in each supernatant was then compared with the radioactivity in an equal volume of ghost suspensions from that sample using a gamma counter (Nuclear Chicago). Rate constants for the loss of intracellular ^{24}Na were calculated from the fractional loss of radioactivity over the indicated time periods (Hoffman, 1962). All determinations of efflux rates were carried out in duplicate samples.

ATP Assays. ATP was assayed by a modification of the procedure described by Strehler (1968), using an extract from firefly lanterns (Sigma Co., FLE-50). Each vial of extract was shaken with water (5 mL) and then left overnight at 0 °C. The supernatant was decanted and made to 25 mL with a mixture (1:2 by volume) of $MgCl_2$ (40 mM) and sodium arsenate (100 mM). Aliquots (10 μL to 50 μL) of standard ATP solutions were added to 2 mL of arsenate-buffered luciferase solution, mixed by inversion, and rapidly transferred to a Farrand Ratio fluorometer. The light emitted after 30 s was noted and a standard curve relating light emission and quantity of ATP added was constructed. Analysis of the solution where ATP content was to be determined was treated in the same way and the ATP content determined from the standard curve. Concentrations of samples routinely assayed in this way were between 1×10^{-6} and 5×10^{-5} M. When the ATP content of resealed red cell ghosts was determined, a sample of the suspension used for the determination of Na efflux was taken at the beginning of the flux measurements. The suspension was then placed in a boiling water bath for 2 min and stored at 0 °C prior to assay by the procedure described above. Control experiments showed that the 2-min incubation in boiling water did not cause a detectable fall in the ATP concentration of standard solutions, in the absence or presence of resealed ghosts.

Results

Photolytic Release of P_i. Aqueous solutions of 2-nitrobenzyl phosphate, 1-(2-nitro)phenylethyl phosphate, and 4-nitrobenzyl phosphate were photolyzed for increasing lengths of time in plastic tubes (see Materials and Methods). The irra-

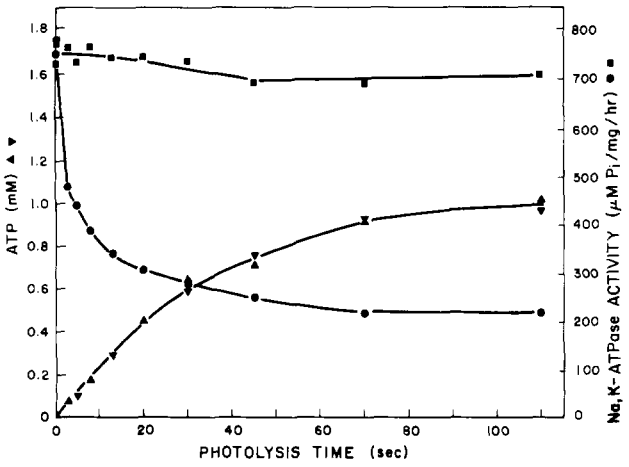

FIGURE 6: Photorelease of ATP in the presence of purified Na,K-ATPase. A buffered solution (1.2 mL) containing 0.1% bovine serum albumin, 20 mM imidazole, 0.8 mM EDTA, and the purified enzyme (3 μg/mL) with either 1.8 mM caged ATP alone or 1.8 mM caged ATP (circles) and 2 mM glutathione was photolyzed for the indicated time periods. Aliquots (0.5 mL) of the irradiated solutions were then assayed for ATPase activity by the addition of 0.1 mL of assay solution containing 750 mM NaCl, 150 mM KCl, 24 mM $MgCl_2$, 24 mM NaATP, 6 mM EDTA, 360 mM Tris (pH 7.5), at 37 °C. A second aliquot of the irradiated solution was used for the determination of the concentration of photoreleased ATP. Enzymatic activity: (■) 1.8 mM caged ATP + 2 mM glutathione; (●) 1.8 mM caged ATP. ATP release: (▲) 1.8 mM caged ATP; (▼) 1.8 mM caged ATP + 2 mM glutathione.

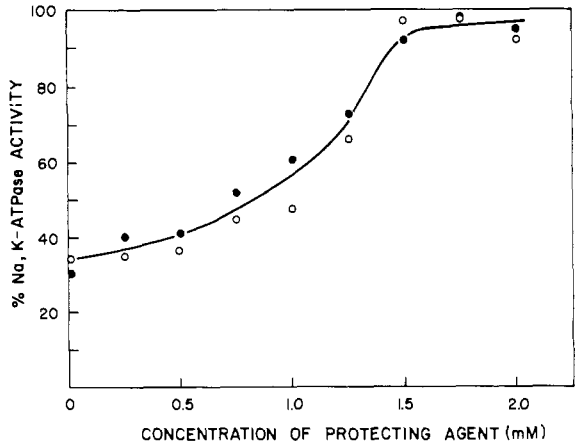

FIGURE 7: Effects of glutathione and bisulfite on the photoinduced inhibition of Na,K-ATPase activity. A buffered solution (1.1 mL) containing 0.15% bovine serum albumin, 14 mM imidazole, 0.55 mM EDTA, 2 mM caged ATP, and the purified enzyme (3 μg/mL) with the indicated concentrations of glutathione or bisulfite (0 to 2.0 mM) was photolyzed for 30 s and assayed for enzymatic activity as described in the legend to Figure 6. Under these conditions 1.43 mM ATP was photoreleased.

diated solutions were then assayed for P_i by the method of Martin & Doty (1949). The results of these experiments are shown in Figure 3. As can be seen the two phosphate esters containing 2-nitro-substituted benzyl groups showed a rapid release of P_i on illumination, while photolysis of the 4-nitro-substituted isomer was without effect on the free P_i content of the irradiated solution. The time for release of half of the available P_i is about 6–9 s for both photosensitive compounds. The quantum yield for the photolytic release of P_i from 1-(2-nitro)phenylethyl phosphate was determined using the ferrioxalate actinometer system described by Hatchard & Parker (1956). Separate estimates of the quantum yield gave values of 0.50 and 0.58 under the usual (see Materials and Methods) illuminating conditions (peak transmittance 342 nm); using the Mineralight UV SL.25 lamp, values of 0.27 and 0.33 (254 nm) were obtained.

Photolytic Release of ATP. Aqueous solutions of P^3-1-(2-nitro)benzyl-ATP were photolyzed as described above and the irradiated solutions were then assayed for free ATP (see Materials and Methods). Photolysis of this compound resulted in the release of ATP (see Figure 4) but the yield was never greater than about 25% of that theoretically available. A similar experiment with P^3-1-(2-nitro)phenylethyl-ATP showed a marked increase in the efficiency of photodeprotection of ATP. In Figure 4 are shown the results of an experiment where equal concentrations of the two protected ATP analogues were photolyzed and the free ATP measured as a function of illumination time. The secondary alcohol derivative released about 80% of the theoretically available ATP in less than 60 s, whereas the primary alcohol analogue produced maximally 25% release. The time for release of 50% of the available ATP from the P^3-1-(2-nitro)phenylethyl ATP, that is, "caged ATP", was about 6–9 s.

Release of Substrate for Na,K-ATPase. Aqueous solutions of caged ATP (P^3-1-(2-nitro)phenylethyl-ATP) were irradiated for increasing lengths of time and the rate of production of P_i by the microsomal Na,K-ATPase preparation from the irradiated solutions was measured. As shown in Figure 5 without illumination no P_i is detected, but after illumination ATP is released and the rate of P_i production from the enzymatic breakdown of the free ATP can be followed. This is in contrast to the photoaffinity labeling approach previously described (Haley & Hoffman, 1974) where an essential property of the ATP analogue is its suitability as a substrate prior to photoactivation. Prior to photolysis, concentrations of caged ATP up to 2 mM did not affect the rate of hydrolysis of ATP by the purified enzyme.

In a series of experiments the enzyme was present during irradiation; the irradiated caged ATP plus enzyme were then transferred to the assay solution which contained additional ATP and the amount of hydrolysis of ATP measured over the next 10 min of incubation. As can be seen in Figure 6, an inhibition of the Na,K-ATPase activity was observed, the extent of which is related to the duration of irradiation. This inhibition was unaffected by the presence of ATP in the irradiated suspension (not shown). Since previous work (see Discussion) suggested that a nitroso ketone would be the likely cleavage product following photolysis of the caged ATP, various compounds (hydroxylamine, hydrazine, sodium bisulfite, and glutathione) were tested to see whether or not a protection against the post-irradiation inhibition could be achieved. Protection against the photolytically induced inhibition was achieved with either sodium bisulfite or glutathione. The results obtained with glutathione are shown in Figure 6. Measurements of the rate of release of ATP from caged ATP showed that gluthathione had no effect on the photolytic process per se (Figure 6). Figure 6 also shows that, in the absence of glutathione, the concentration of the photoreleased inhibitor increased with increasing time of radiation as indicated by the change in inhibition of the Na,K-ATPase activity. Figure 7 shows that, when the concentration of glutathione or bisulfite present in the irradiated suspension was varied, almost complete protection was observed at concentrations of protecting agent that were stoichiometrically related to the amount of ATP released. This is consistent with the observation (see Figure 6) that most of the inhibition occurs at short irradiation times, that is, low concentration of the photoreleased inhibitor.

Utilization of Intracellular ATP. Resealed ghosts prepared to contain either ATP or caged ATP and a trace quantity of

TABLE I: Effect of Photolysis of Suspensions of Resealed Ghosts with Incorporated ATP or Caged ATP.[a]

Nucleoside triphosphate incorporated	Photolysis	$^{o}k_{Na}$	$^{o}k_{Na}^{ouab}$
ATP	−	0.379	0.129
ATP	+	0.390	0.138
Caged ATP	−	0.133	0.132
Caged ATP	+	0.289	0.129

[a] Resealed ghosts were prepared from depleted human red blood cells and the rate of ^{24}Na efflux determined as described in Materials and Methods. The ghosts containing caged ATP were photolyzed to yield approximately 1 mM ATP. $^{o}k_{Na}$ and $^{o}k_{Na}^{ouab}$ are the rate constants (h^{-1}) for efflux calculated from the isotope loss over the first 30 min in the absence and presence of ouabain (10^{-4} M), respectively. The difference between $^{o}k_{Na}$ and $^{o}k_{Na}^{ouab}$ represents the ouabain-sensitive Na efflux and indicates the extent of activation of the Na pump by incorporated ATP and photoreleased ATP from incorporated caged ATP.

TABLE II: Comparison of ATP Contents of Resealed Ghosts Prepared by Incorporating ATP or Caged ATP at Hemolysis.[a]

Nucleoside triphosphate concentration		
Incorporated at hemolysis (μM)	Determined after resealing and photolysis (μM/L ghosts)	
	With ATP	With caged ATP
250	10.2 ± 3.3 (n = 3)	40.7 ± 7.2 (n = 3)
500	19.8 ± 3.7 (n = 5)	130.2 ± 20.9 (n = 5)
1000	85.8 ± 61 (n = 5)	291.4 ± 67 (n = 5)

[a] Ghosts were prepared as described in Materials and Methods and the indicated amounts of ATP or caged ATP were incorporated at hemolysis. The free ATP content of the ghosts was subsequently determined (see Materials and Methods) at the time of initiation of the Na efflux measurements.

^{24}Na were suspended to 10% volume suspension and photolyzed in 1-mm path-length cells. The ghosts were then resuspended in appropriate media as specified in Materials and Methods, and the rate of ^{24}Na efflux was determined. Results of such studies are shown in Table I. Photolysis had no effect on the ghosts containing ATP; the ^{24}Na efflux rates with and without ouabain were unaltered by irradiation. However, following irradiation ghosts containing the caged ATP showed an appreciable component of ^{24}Na efflux that was inhibitable by ouabain and absent when irradiation was not carried out. Experiments carried out in a similar way showed that intracellular glutathione (2 mM) had no detectable effect on the rate of ouabain-sensitive or insensitive ^{24}Na efflux from ghosts containing ATP or caged ATP with or without photolysis (not shown).

The procedure used to prepare resealed ghosts with low cation leaks involves an incubation at 37 °C for 40 min; during this period the intracellular ATP level is lowered prior to the measurement of ^{24}Na efflux rates due to the endogenous ATPase activity (see Glynn & Hoffman, 1971). This effect should be minimized in the experiments where the protected ATP analogue was used. When experiments were performed at lower ATP and caged ATP levels, this difference was observed. In Figure 8 the data from a series of such experiments are shown. When 0.25 or 0.5 mM ATP was present at hemolysis, ouabain-sensitive Na efflux was barely detectable; however, when similar concentrations of caged ATP were sealed into the ghosts, and photolyzed, ouabain-sensitive Na effluxes

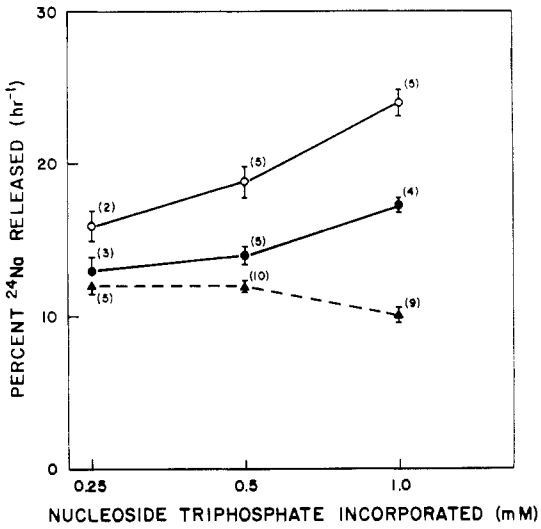

FIGURE 8: Comparison of ^{24}Na efflux rates from resealed red cell ghosts prepared by incorporating either ATP or caged ATP. The preparation of resealed ghosts and determination of ^{24}Na efflux were carried out as described in Materials and Methods. The values of ^{24}Na released per h were obtained by doubling the values obtained at 30 min. Bars indicate the standard errors of the means. The numbers in parentheses indicate the number of experiments performed with the indicated nucleoside triphosphate concentration. The means and errors for the determination in the presence of 10^{-4} M ouabain were the pooled results of determinations using ghosts with ATP and caged ATP since no difference was observed between the two sets of data. The difference in percent ^{24}Na released between the continuous lines and the dotted lines represents the ouabain-sensitive Na efflux via the sodium pump. (○) Caged ATP photolyzed prior to determination of ^{24}Na efflux rate (see Materials and Methods); (●) ATP; (▲) in the presence of 10^{-4} M ouabain.

were observed. At 1 mM caged ATP, ouabain-sensitive Na efflux rates were greater than those observed with 1 mM ATP. The higher rates of ouabain-sensitive Na efflux observed using caged ATP compared with ATP are consistent with the differences in intracellular ATP concentration determined at the initiation of efflux measurements. In Table II are shown the results of such ATP determinations. The ATP content of ghosts following photorelease of ATP from caged ATP is always greater than the ATP content of ghosts which have been resealed to contain free intracellular ATP. The relatively low levels of ATP observed in resealed ghosts following resealing of hemolyzed red cells are in part due to the activation of endogenous ATPase activity on hemolysis of red cells in hypotonic solutions of $MgCl_2$ (unpublished observations).

Discussion

In the present studies we have been able to demonstrate that caged ATP (P^3-1-(2-nitro)phenylethyl ATP) releases ATP in good yield with short irradiation times. Under the illumination conditions described in the present work we can release ATP concentrations in the micromolar range with a reproducibility of about 10% with photolysis times of a few seconds. Since the rate of release of ATP from caged ATP is proportional to the light intensity, by focusing incident light to a smaller area we are also able to attain comparable or higher levels of ATP in less than 1 s.

Prior to photolytic irradiation caged ATP was resistant to the hydrolytic activity of the Na,K-ATPase; after photolysis the enzyme substrate is released and enzymatic hydrolysis is observed. In the experiments where the enzyme was present with caged ATP in the irradiated suspension, the enzymatic hydrolysis of the liberated ATP proceeded at an inhibited rate. Irradiation of the enzyme solution in the absence of caged ATP

did not cause inhibition of the enzymatic activity. The onset of inhibition was rapid and the greater part of the inhibition took place at short irradiation times. The inhibition resulting from the photodeprotection process could be prevented by the presence of glutathione or bisulfite in the irradiated suspension. This protection probably occurs via an interaction with the nitrosoketone fragment of the photocleaved caged ATP. Indeed maximal protection against the inhibition was achieved only in the presence of bisulfite or glutathione at concentrations that were equal to the concentration of liberated ATP (and presumably the nitrosoketone).

The findings with resealed ghosts established the feasibility of generating free ATP within the cell by deprotection of caged ATP with a brief pulse of light. Irradiation is carried out at wavelengths that have no detectable effect on the transport functions of the cell and the brief exposure to light supplies the intracellular compartment with the required energy source for active Na transport.

During the preparation of this paper a report appeared on the synthesis of a "caged" cyclic adenosine 3′,5′-monophosphate and the subsequent photorelease of the cyclic monophosphate (Engels & Schlaeger, 1977). There have been several reports on the uses of photosensitive protecting groups in synthetic organic chemistry. Baltrop et al. (1966) suggested the usefulness of 2-nitro-substituted benzyl groups as protecting residues for carboxylic acids utilizing an internal redox mechanism which produced a nitrosoaldehyde and a free carboxylic acid on photolysis. However, under these conditions nonaqueous solvents and extended photolysis times (several hours) were needed. Subsequently Patchornik et al. (1970) showed that such protecting groups could be used for amino acids and peptides, glycosides (Zehavi et al., 1972), and amino sugars (Amit et al., 1974). Rubinstein et al. (1975) were able to show that irradiation of bis(2-nitrobenzyl) phosphate in ethanolic solution gave a high yield of inorganic phosphate. However, while small scale procedures were apparently successful in alcoholic solvents, preparative scale procedures failed because of the reactivity of the by-product of photolysis, 2-nitrosobenzaldehyde. In the present work we have confirmed that the 2-nitrobenzyl group is a photo-labile protecting group for phosphate and the failure to obtain photolytic release of P_i from 4-nitrobenzyl phosphate (Figure 3) suggests that the internal redox mechanism postulated by Barltrop et al. (1966) from their studies on carboxylic acids probably also applies to phosphate photodeprotection. The subsequent failure to obtain high yields of ATP from P^3-(2-nitro)benzyl-ATP was surprising. Since the photochemical step is the same in both phosphate and ATP deprotections, presumably in the case of P^3-(2-nitro)benzyl-ATP a subsequent reaction occurs between the photoliberated nitrosoaldehyde and the adenosine moiety of ATP. Patchornik et al. (1970), following the findings of Barltrop et al. (1966), had suggested that better yields following photodeprotection may be expected using a secondary alcohol protecting group. To this end we synthesized the 1-(2-nitro)phenylethyl analogues. The time course for the release of P_i from this ester was the same as observed previously with the primary alcohol but the yield of ATP from the P^3-ATP modified analogue was dramatically increased by using the secondary alcohol protecting group.

Rapid photorelease of ATP from a stable precursor should facilitate studies performed on membrane vesicles or reconstituted systems where ATP-dependent transport is measured after lengthy procedures during which endogenous ATPase activities break down large amounts of the substrate. Furthermore photolysis of caged ATP also makes possible studies involving the kinetic effects of predetermined and variable intracellular levels of ATP or radiolabeled ATP in those systems where the intracellular compartment is accessible by hemolysis (red cell ghosts), by injection (axons), or by iontophoresis (neurons). It is hoped that this approach is in principle applicable to the development and use of other caged substrates for the study of cellular transport and metabolic processes.

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