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# Rate of Release of Ca$^{2+}$ following Laser Photolysis of the DM-nitrophen–Ca$^{2+}$ Complex[†]

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ABSTRACT: The determination of the rate of release of Ca$^{2+}$ by pulsed photolysis of the photolabile chelator DM-nitrophen is important for its use in time-resolved physiological studies: the rate of substrate or effector release should be faster than the processes they initiate. Flash photolysis of DM-nitrophen using a 50-ns pulse from a frequency-doubled ruby laser (with emission at 347 nm having energy of ca. 10–20 mJ) yields short-lived photochromic or aci-nitro intermediates. At pH 6.9, double-exponential decay of a photochromic intermediate was observed for DM-nitrophen itself and its Ca$^{2+}$ complex ($\tau_{1/2}$ values of 24 and 570 $\mu$s, and 32 and 220 $\mu$s respectively), while only monoexponential decay of the DM-nitrophen–Mg$^{2+}$ complex was detected ($\tau_{1/2}$ = 31 $\mu$s). Only the photochemistry of DM-nitrophen–Ca$^{2+}$ was found to be pH sensitive (monoexponential decay, $\tau_{1/2} \sim$ 115 $\mu$s at pH 7.9 and 8.9). Use of the Ca$^{2+}$-sensitive metallochromic dye antipyrylazo III in conjunction with pulsed photolysis of DM-nitrophen–Ca$^{2+}$ enabled an *upper* limit of the half-time of release of Ca$^{2+}$ to be established of ca. 180 $\mu$s (the rate of association of Ca$^{2+}$ with the dye was probably rate determining). The rate of Ca$^{2+}$ photorelease may, however, be faster than this. Thus, the DM-nitrophen–Ca$^{2+}$ complex releases Ca$^{2+}$ on photolysis sufficiently rapidly for the study of many Ca$^{2+}$-dependent physiological processes with improved kinetic resolution over conventional mixing methods.

Many physiological processes take place in time periods of short duration (microsecond to millisecond time domain). Muscle contraction, synaptic transmission, hormone secretion, etc., are some of the processes which respond to brief, localized fluctuations in various effector molecules. In many situations, kinetic resolution of the process is limited by diffusional mixing delays or the need to initiate such processes inside closed compartments or ordered structures. During the past 14 years or so, a technique has been developed which enables such processes to be studied. The idea is that the physiological substrate is rendered inactive or "caged" by the use of a photochemical protecting group; the substrate is then released (and the process under study "switched on") by a brief pulse of ultraviolet light. This approach was developed first with ATP (called caged ATP) and P$_i$ (Kaplan et al., 1978) and has subsequently been applied successfully for other nucleotides [CTP (Somlyo et al., 1988), cAMP and cGMP (Nargeot et al., 1983), GTP-$\gamma$-S and GMP-PNP (Dolphin et al., 1988), ATP-$\gamma$-S (Walker et al., 1988), IP$_3$ (Walker et al., 1987)], neurotransmitters (Wilcox et al., 1990), protons (McCray & Trentham, 1985), and fluorophores (Mitcheson, 1989). All of these substrates are released by the photochemical cleavage of the protecting group which is covalently linked to the substrate [for reviews, see Kaplan (1990), McCray and Trentham (1989), and Kaplan and Somlyo (1989)]. Recently, a new class of photolabile chelators has been developed (Ellis-Davis & Kaplan, 1988; Kaplan & Ellis-Davies, 1988) using a strategy which is similar to that developed with caged ATP. In this case, since no covalent bond can be formed with divalent cations to render them inert, it was reasoned that rapid, localized changes in divalent cations could be achieved by the photochemical cleavage of the *chelator itself.* DM-nitrophen[1] is an *o*-nitrophenyl derivative of EDTA, a tetracarboxylic chelator having high affinity for divalent cations at physiological pH (Ca$^{2+}$ $K_d$ = 5 nM, Mg$^{2+}$ $K_d$ = 3 $\mu$M; Kaplan & Ellis-Davies, 1988). The photolabile chelator is bifurcated upon ultraviolet irradiation to yield two dicarboxylates of known low affinity for divalent cations (iminodiacetic acids $K_d$ = ca 3 mM for Ca$^{2+}$ and Mg$^{2+}$). Thus, on cleavage the affinity for Ca$^{2+}$ (for example) decreases by about 5 orders of magnitude and the free [Ca$^{2+}$] rises. The quantum yield for Ca$^{2+}$ release is 0.18 (Kaplan & Ellis-Davies, 1988). The *chemical* yield can be higher due to the re-excitation of the DM-nitrophen–Ca$^{2+}$ complex which was not split upon initial excitation. Such "multi-excitation" can occur because excitation and de-excitation processes of such aromatic species take place very rapidly in comparison to the length of the laser pulse. For an alternative strategy for a caged Ca$^{2+}$, see Adams et al. (1988). The two approaches are compared and discussed in a recent review (Kaplan, 1990).

In order to make use of this experimental strategy to initiate Ca$^{2+}$- and Mg$^{2+}$-dependent physiological processes, it is necessary to know how rapidly the free divalent cation concentration is increased. The present work was performed to obtain estimates of the rate of release of Ca$^{2+}$ and the rates of associated photochemically induced molecular conversions. A preliminary report of some of this work has appeared previously (Fidler et al., 1988).

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[1] Abbreviations: Ap III, antipyrylazo III; EDTA, ethylenediaminetetraacetic acid; TMX, tetramethylmurexide; DM-nitrophen, 1-(2-nitro-4,5-dimethoxyphenyl)-*N*,*N*,*N'*-*N'*-tetrakis[(oxycarbonyl)methyl]-1,2-ethanediamine; Ar III, arseno III; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; nitr-2, 1-[2-amino-5-[1-methoxy-1-(2-nitrophenyl)methyl]phenoxy]-2-(2'-amino-5'-methylphenoxy)ethane-*N*,*N*,*N'*-*N'*-tetraacetic acid.

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## EXPERIMENTAL PROCEDURES

DM-nitrophen was synthesized as reported by Ellis-Davies and Kaplan (1988). Typical sample conditions for measurement of the aci-nitro intermediates were 2 mM DM-nitrophen, 130 mM KCl, 10 mM Hepes buffer, and 2.2 mM $CaCl_2$ at pH 6.9 and 21 °C. For the Ca$^{2+}$ dye uptake experiments, typical conditions were 250 $\mu$M DM-nitrophen, 140 mM KCl, 10 mM Hepes buffer, 350 $\mu$M $CaCl_2$, and 100 $\mu$M Ap III at pH 6.9 and 23 °C.

DM-nitrophen and its Ca$^{2+}$ and Mg$^{2+}$ complexes were photolyzed by a frequency-doubled Q-switched pulse from a Holobeam Series 634 ruby laser. The primary 694-nm output of the laser was frequency-doubled with a potassium dihydrogen phosphate (KDP) crystal to give a 50-ns, 30-mJ, 347-nm pulse. A Hoya U-350 filter was placed between the sample and the KDP crystal to minimize the pump light and the primary beam striking the sample.

The sample was contained in a 2 mm × 10 mm × 10 mm quartz cuvette, angled at 45° with respect to the laser beam. The path length was thus 2.8 mm. The sample area typically irradiated was about 5 mm × 3 mm.

The light source for measuring absorbance changes of the sample was a tungsten–iodide lamp positioned at a right angle to the photolyzing laser beam. A monochromator for selecting the detecting wavelength was positioned between the sample cuvette and the light source. Absorbance changes were detected with an EMI 9601B photomultiplier. Spectral changes due to the rapid formation and decay of the aci-nitro intermediate were measured at 425–435 nm where the aci-nitro intermediate absorbs strongly. Blue glass filters (2.4 cm thick, with a $\lambda_{max}$ transmission at 415 nm and a half-maximal bandwidth of 125 nm) were placed directly in front of the photomultiplier to reduce the scattered primary and secondary laser light reaching the photomultiplier. For the calcium uptake experiments with Ap III, a red-sensitive photomultiplier (EMI 9792B) was used to increase the signal to noise ratio with measuring wavelength at ca. 660 nm. In these experiments the blue glass filters were removed.

The analog output of the photomultiplier was converted into a digital signal by a Biomation 802 A/D converter (500 kHz sampling rate, 1024 words at 8 bits per word) and then stored on flexible disks which were used with an IBM type PC for analysis. The data could be digitally filtered and fit with a single or double exponential of the following form:

$$y(t) = a_1 + a_2e^{-k_1t} + a_3e^{-k_2t}$$

where $a_1$, $a_2$, $k_1$, $a_3$, and $k_2$ are all variable parameters in the minimization routine.

## RESULTS

Two types of time-dependent absorption measurements were made in order to obtain information on the rate of release of Ca$^{2+}$ from DM-nitrophen upon photolysis. The first type of time-dependent measurements analyzed involved life-times of photochromic intermediates intrinsic to DM-nitrophen photochemistry itself. Such transients are well-characterized species in *o*-nitrobenzyl photochemistry and photophysics (Wettermark, 1962; Mosher et al., 1960; Wettermard, 1962; McCray et al., 1980; Atherton & Craig, 1986; Yip et al., 1985) and were also found to exist in the case of the photolysis of DM-nitrophen and its metallo-complexes with Ca$^{2+}$ and Mg$^{2+}$. Typical absorption transients at 425 nm for these compounds (at pH 6.9) are shown in panels a, b, and c of Figure 1, respectively. (The absorption signal does not return to the initial level as the photoproducts absorb at 425 nm.) The solid lines are digitally fitted with either a mono- or double-exponential decay to the data points (dots) with the equation in the Experimental Procedures section. The effect of pH on these rates of decay was also examined and the data averaged from several experiments are summarized in Table I.

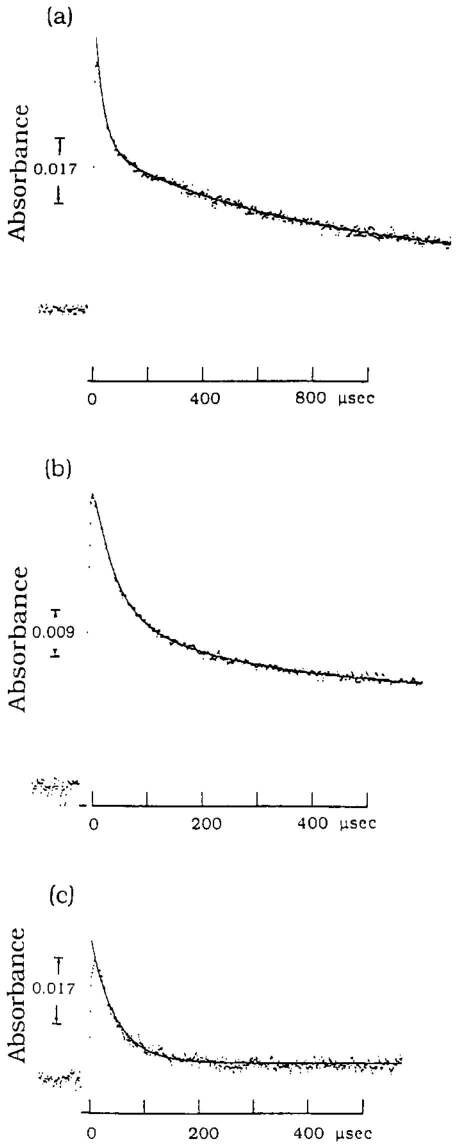


FIGURE 1: Aci-nitro transient absorbances generated by flash photolysis of (a) DM-nitrophen, (b) DM-nitrophen–Ca$^{2+}$, and (c) DM-nitrophen–Mg$^{2+}$. Conditions: pH 6.9 (Hepes, 10 mM); KCl, 130 mM; Ca$^{2+}$ or Mg$^{2+}$, 2.2 mM; 21 °C. The continuous lines represent the digitally fitted curve to the data points (dots) using the equation in the Experimental Procedures section.

The second method used to characterize the rate of Ca$^{2+}$ release was the absorption change generated using the metallochromic indicator Ap III. The increase of absorption seen at 663 nm when Ca$^{2+}$ is added to dye solutions corresponds to the formation of the dye dimer–calcium complex (Hollingsworth et al., 1987). At this wavelength, the signal is virtually free of interference from the 1:1 dye–calcium complex and suffers no interference at all from the photochromic intermediates of DM-nitrophen itself. Figure 2a shows the absorption increase at 663 nm when DM-nitrophen is photolyzed in the presence of saturating Ca$^{2+}$. The half-time for Ca$^{2+}$ release was derived by single-exponential digital fitting of these data, an example of which is shown in Figure 2d, and the average from six experiments was ca. 180 $\mu$s. If a smaller,

Table I: Rates of Decay (Half-Times in Microseconds) of Aci-nitro Intermediates of DM-nitrophen and Its $Ca^{2+}$ and $Mg^{2+}$ Complexes[a]

| | decay half-times ($\mu$s) | | |
|---|---|---|---|
| pH | DM-nitrophen | DM-nitrophen–$Ca^{2+}$ | DM-nitrophen–$Mg^{2+}$ |
| 6.9 | 24 ± 1[a] 570 + 40 (12) | 32 ± 4[b] 220 ± 70 (12) | 31 ± 4 (4) |
| 7.9 | 27 ± 3[c] 490 ± 160 (4) | 110 ± 20 (10) | 36 ± 1 (2) |
| 8.9 | 30 ± 3[d] 370 ± 70 (4) | 120 ± 10 (7) | 36 ± 1 (4) |

[a] The fast component constitutes 63% total amplitude. [b] The fast component constitutes 61% total amplitude. [c] The fast component constitutes 67% total amplitude. [d] The fast component constitutes 80% total amplitude. Numbers in parentheses are the number of experiments performed for each determination. Conditions of photolysis: DM-nitrophen, 2 mM; KCl, 130 mM; buffers, 10 mM (pH 6.9, Hepes; pH 7.9, Taps; pH 8.9, Ches); divalent cation, when present, 2.2 mM; temperature, 21 °C.

nonsaturating amount of $Ca^{2+}$ is used, $Ca^{2+}$ is still released, but some rebinding by unphotolyzed chelator occurs, thus generating a pulse of $Ca^{2+}$. This is shown in Figure 2c.

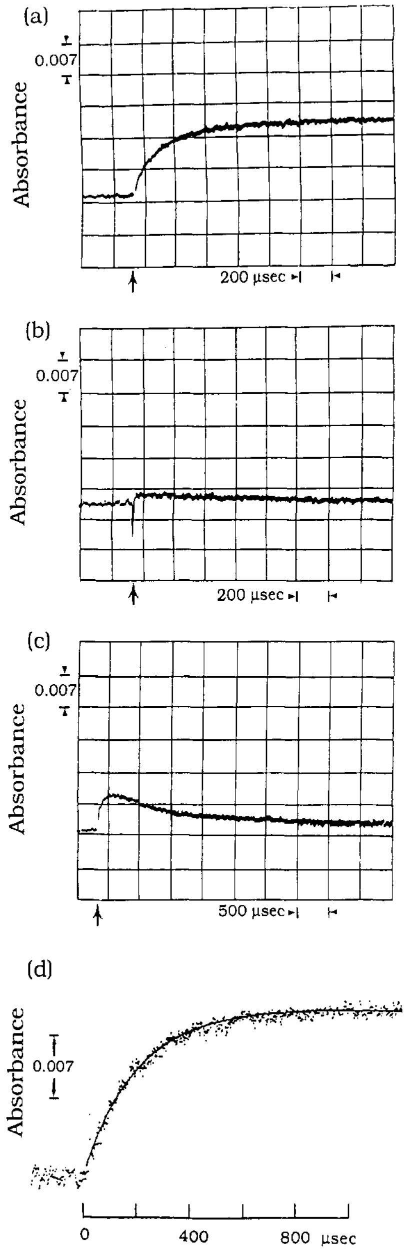


FIGURE 2: Time course of $Ca^{2+}$ release by laser flash photolysis of DM-nitrophen–$Ca^{2+}$ reported by the $Ca^{2+}$-sensitive indicator antipyrylazo III. Conditions: (a) DM-nitrophen, 250 $\mu$M; $CaCl_2$, 350 $\mu$M; Ap III, 100 $\mu$M; (b) same as (a) without $Ca^{2+}$; and (c) DM-nitrophen, 500 $\mu$M; $CaCl_2$, 250 $\mu$M; Ap III, 100 $\mu$M. Each experiment was at pH 6.9 (Hepes, 10 mM) and 130 mM KCl at 21 °C. Arrows indicate laser flash. Digital fitting of the time-dependent increase in (a) is shown in (d) using the equation in the Experimental Procedures section. The continuous line is the fitted curve to the data points (dots).

## DISCUSSION

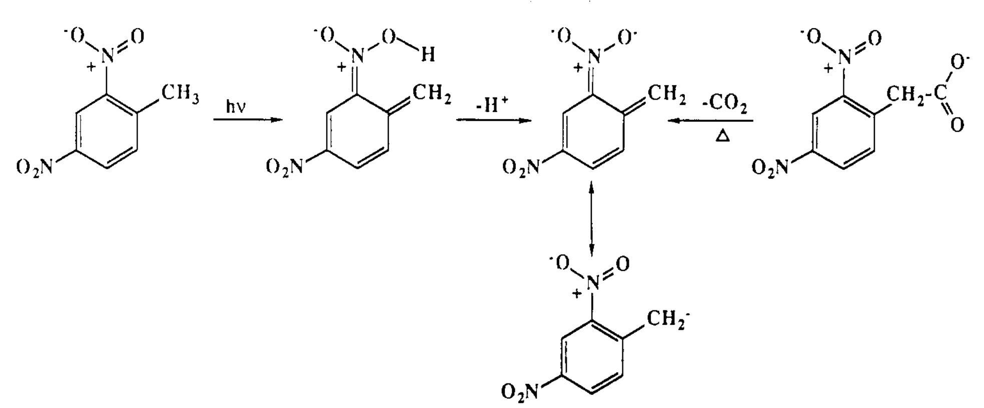
The technique of flash photolysis has been used to obtain an estimate of the rate of release of $Ca^{2+}$ from its complex with a photolabile chelator, DM-nitrophen. In addition to this, rates corresponding to the postulated structural changes which take place during the photochemical cleavage of the chelator have been determined in the pH range 6.9–8.9. These measurements were made to attempt to establish if there was a correlation between the rate of $Ca^{2+}$ release and the decay of the aci-nitro transients intrinsic to DM-nitrophen photochemistry. The determination of the existence of such a correlation would be useful for the development of other substituted nitrophens, since it would provide estimates of their $Ca^{2+}$ photorelease rates from aci-nitro transient spectroscopy alone, without the need for extensive use of independent measures of such rates.

The photochemistry of related *o*-nitrotoluene systems has been studied extensively since flash photolysis techniques first became more widely available. Initially, it has been the photochromism of such systems which has been investigated [for example, see Wettermark (1962), Atherton and Craig (1986), Wettermark and Ricci (1963), Weinstein et al. (1966), and Mosher et al. (1960)]. In a recent important study, McClellan and Steenken (1987) demonstrated that the spectra of the photochromic intermediates in water and organic solvents (acetonitrile) differ greatly and that in the latter there is a great similarity between a photochemically produced transient intermediate and the ground state anion generated by thermal decarboxylation of the corresponding acetic acid reported by Buncel co-workers (Buncel et al., 1984; Buncel & Menon, 1980) (see Scheme I).

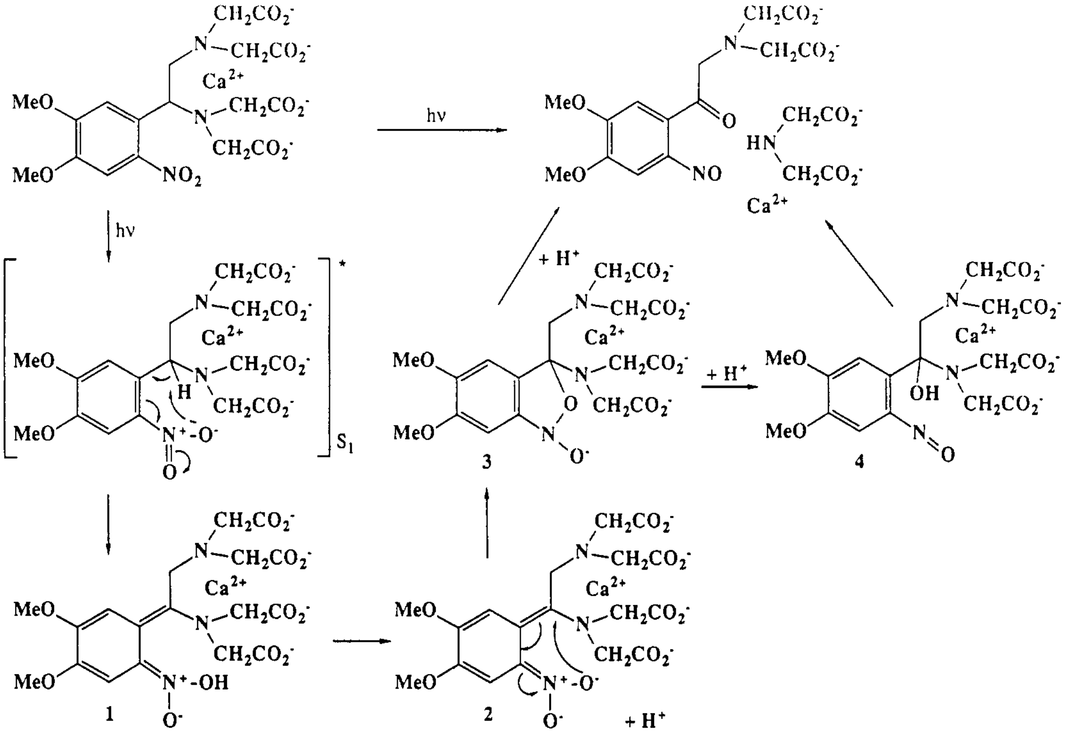
A second type of study has been reported in which rates of covalent bond breaking and effector release have been correlated with rates of transient aci-nitro decay. During the past 10 years, the investigations of Trentham, McCray, and co-workers have shown that the rate of photorelease of ATP from caged ATP is concomitant with the decay of an aci-nitro intermediate. They did this by showing a correlation between an independent measurement for the rate of appearance of ATP, the ATP-induced dissociation of actomyosin subfragment 1, and the decay of the aci-nitro intermediate (McCray et al., 1980). Further evidence was provided by the demonstration that the rates of appearence of long-wavelength absorption at 740 nm ascribed to aromatic nitroso species (Walker et al., 1988) and of a fluorescent analogue of ATP ($1,N^6$-etheno-2-azaATP) from its caged precursor are the same as the rate of aci-nitro decay (Wootton & Trentham, 1989). In the case of caged nucleotides, it now seems reasonable to infer that the rate of decay of the transient aci-nitro intermediates is indicative of the rate of appearance of the released substrate.

It is now widely accepted that when substituted *o*-nitrotoluenes are excited to the state $S_1$, rapid deprotonation of the benzylic position ensues from either the $S_1$ or $T_1$ state; this is effected by electronic rearrangement to a colored aci-nitro intermediate (Yip et al., 1985; Wan & Yates, 1986). Decay of this highly energetic species can occur via return to starting materials by re-protonation (photochromism) or, if there is a heteroatom adjoining the benzylic carbon, an intramolec-

Scheme I: Photochemical and Thermal Generation of Aci-nitro Intermediates



Scheme II: Proposed Photochemical Reaction Mechanisms of DM-nitrophen

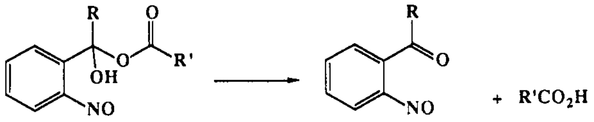


ular redox reaction can take place, which results in covalent bond rupture (and release of the caged substrate; McCray & Trentham, 1989).

*Decay of the DM-nitrophen Aci-nitro Transient Is Biphasic.* Laser flash photolysis of the DM-nitrophen–$Ca^{2+}$ complex results in transient, aci-nitro intermediates similar to those of the other substituted *o*-nitrotoluenes discussed above (intermediates **1** and **2** in Scheme II). Inspection of the decay profile reveals that two transient species are resolved within the time course of our analysis (6 μs–500 ms). There is only one other literature report showing biphasic decay of the aci-nitro intermediates of a caged compound, that of caged carbamoylcholine (Milburn et al., 1989). In that work, the $\tau_{1/2}$ value of the fast component only was reported. Close inspection of the time course of the appearance of fluorescence from caged 1,$N^6$-etheno-2-azaATP (Wootton & Trentham, 1989) reveals that this process may also be biphasic in nature.

There are several possible interpretations of the biphasic decay of the photochemical intermediate observed with the DM-nitrophen–$Ca^{2+}$ complex. For example, it could be that intermediate **3** (see Scheme II) not only decays directly to products but also decays to products via the hemiaminal **4**. This follows the original suggestion of Baltrop and co-workers (1966), who, in their pioneering work on photochemical deprotection of carboxylates, postulated the intermediacy of hydroxyesters along the pathway to free-acid products (shown in Scheme III). This mechanism was adopted by Tsien and Zucker (1986) in their paper on $Ca^{2+}$ photorelease from nitr-2. In the case of phosphate esters, Walker et al. (1988) have shown that such a two-path mechanism does not pertain, by the use of an $H^+$ indicator which enabled them to determine that the initially released protons were not taken up by reaction intermediates during the photochemical release process. In the case of DM-nitrophen, we think that the intermediacy of hemiaminal **4** (Scheme II) can be ruled out a priori as such species eliminate *water* to give *enamines* (March, 1985) and not ketones.

Scheme III: Proposed Intermediate in the Photochemical Deprotection of Carboxylic Acids (Baltrop et al., 1966)



Another interpretation of the biphasicity is that only one of the two transients resolved in the flash photolysis of DM-nitrophen and the DM-nitrophen–$Ca^{2+}$ complex leads to products. This possibility is suggested by the fact that in the case of the DM-nitrophen–$Mg^{2+}$ complex only a fast transient decay could be resolved. It might be that it is this fast decay, which is common to all three systems studied, that should be associated with the fragmentation of DM-nitrophen and the concomitant increase in cation concentration in the case of the two chelator–cation complexes. The slow component of the decay of DM-nitrophen and DM-nitrophen–$Ca^{2+}$ might be due to a nonphotochemical mode of de-excitation of the $S_1$ excited state (see Scheme II), which was not resolved in our photolysis system for the DM-nitrophen–$Mg^{2+}$ complex.

A third alternative is that there are two sequential species on a single pathway from starting material to products. Any two of species **1**, **2**, and **3**, for example, might be colored transients which give rise to the double-exponential decay we observe. However, by analogy with caged ATP, the conjugate acid **1** (Scheme II) can be ruled out, as caged ATP was found to release protons very rapidly upon photolysis ($>10^5$ $s^{-1}$; Walker et al., 1988), in agreement with the earlier work of Buncel and co-workers (1980, 1984), who found using transient conductance measurements that conjugate acids like **1** deprotonate extremely rapidly in water ($k = 10^8$–$10^9$ $s^{-1}$). If the double-exponential decay is indicative of a two-step sequential mechanism, then obviously the lower limit of the $Ca^{2+}$ release rate cannot be any faster than the slower exponential aci-nitro decay.

The fact that simple kinetic analysis of the aci-nitro decay of DM-nitrophen and its divalent cation complexes cannot differentiate between the latter two interpretations of the data illustrates that it is important to use an independent means to calibrate the release of the effector molecule from its caged precursor. Analyzing the decay of the aci-nitro intermediate(s) will not necessarily give an accurate value for the rate of release of the caged substrate, because if the aci-nitro decay is biphasic, it is not clear a priori which intermediate gives rise to products. Such flash photolysis studies are important, however, in gaining an understanding of the overall reaction mechanism of the photolysis of DM-nitrophen and related compounds.

*Effect of pH on DM-nitrophen Photochemistry.* The effects of changing pH in the 6.9–8.9 range on the rate of decay of the aci-nitro intermediates of DM-nitrophen and its metallocomplexes are subtle but relatively minor compared to those reported for other caged compounds [for a discussion of these effects, see McCray and Trentham (1989)]. In the light of almost all such literature reports, it would have been predicted that the rate of disappearance of the aci-nitro intermediates would be acid catalyzed in the pH range studied. Such a dependence is not observed for DM-nitrophen and its $Mg^{2+}$ complex: there is no effect of changing pH on aci-nitro decay (Table I). Thus, for these two species, the decay of the photochromic intermediates apparently does not have a rate-determining acid-catalyzed step; i.e., it is a unimolecular process. The data for DM-nitrophen–$Ca^{2+}$ is, however, somewhat different: at higher pH's (7.9 and 8.9) only a single exponential, of intermediate value, (110–120 μs) was resolvable in our experiments. This would imply that the rate-determining step is partially acid catalyzed (if it was fully acid catalyzed, the rate should be reduced by 100-fold from pH 6.9 to 8.9 rather than 4-fold). The process could be a mixture of unimolecular and bimolecular reactions or else some intermediate mechanism is in operation. Similar results have been observed for caged carbamoylcholine (Wilcox et al., 1990) and caged formycin 3′,5′-cyclic phosphate (Wootton & Trentham, 1989) in the same pH range. There seems to be no simple explanation for why partial acid catalysis is observed for these few caged compounds. Our results do suggest that if the decay rate of an aci-nitro intermediate is concomitant with the release of $Ca^{2+}$, then the DM-nitrophen–$Ca^{2+}$ complex releases $Ca^{2+}$ with a half-time of no more than about 200 μs in the pH 6.9–8.9 range.

*Rate of $Ca^{2+}$ Release: The Use of Metallochromic Dyes.* The principle goal in these studies was to determine the rate at which the DM-nitrophen–$Ca^{2+}$ complex releases $Ca^{2+}$ upon laser flash photolysis. The approach we chose utilizes a $Ca^{2+}$-sensitive metallochromic dye to measure the rate of appearance of the liberated cation. Three categories of indicators were considered: murexides (e.g., TMX), arylazo dyes (e.g., Ar III and Ap III), and BAPTA-types dyes (e.g., fluo-3). BAPTA-type dyes were ruled out because their response times were considered to be too slow (millisecond time domain; Kao & Tsien, 1988; Jackson et al., 1987). TMX and Ap III, on the other hand, have useful response times, being about 2 μs and 180 μs, respectively (Geier, 1968; Scarpa, 1979). Other factors to consider in this selection of a dye are its analysis wavelengths, $K_d$ and $\Delta\epsilon$. The $\Delta\epsilon$ for Ap III and TMX are 6.5 and 12.8 $mM^{-1}$ $cm^{-1}$ at 720 nm and 540 nm, respectively (Ogawa et al., 1980), and the best working concentration ranges are 50 nM–20 μm and 20 μM–1 mM, respectively (Scarpa, 1979). Therefore, both can produce a measurable signal response from $Ca^{2+}$ photoreleased from DM-nitrophen (although TMX ideally requires a good deal more $Ca^{2+}$ than Ap III, an increase in [$Ca^{2+}$] of 100 μM from DM-nitrophen photolysis is quite feasible). The increase in absorbance of TMX in response to an increase in [$Ca^{2+}$] is in the 400–500-nm region (with a maximum at 480 nm). However, this part of the visible spectrum is also the region where the DM-nitrophen aci-nitro transient absorption appears. Thus, we selected Ap III to monitor the rate of $Ca^{2+}$ released from DM-nitrophen–$Ca^{2+}$ complex as this can be monitored at 660 nm, without any interference from DM-nitrophen transients.

The results shown in Figure 2 for this experiment indicate that the DM-nitrophen–$Ca^{2+}$ complex does indeed release $Ca^{2+}$ rapidly upon laser flash photolysis. The half-time for $Ca^{2+}$ release in the case when saturating $Ca^{2+}$ is present was determined by digital fitting of the time-dependent absorption and was found to be about 180 μs (see Figure 2d). This is at the *upper limit* of the response time for the dye Ap III (Scarpa, 1979). Thus, this experiment only enables a lower limit to be placed on the rate of photorelease of $Ca^{2+}$ from the DM-nitrophen–$Ca^{2+}$ complex. If a dye with a faster response time could be used, then it could be determined whether or not the Ap III response time is indeed rate-limiting in our experiment.

Figure 2c shows the time-dependent response of Ap III when the DM-nitrophen concentration is greater than the total $Ca^{2+}$ concentration (in a ratio of 2:1). Under such conditions, the absorbance change of Ap III shows an increase followed by a decrease in free $Ca^{2+}$; a pulse of free $Ca^{2+}$ is produced rather than a step increase. (Pulses of $Ca^{2+}$ from photolysis of DM-nitrophen have also been noted by Delaney and Zucker (1990) but could not be furthr characterized because the response rate of the $Ca^{2+}$ indicator (fura-2) used in their experiments is too slow for these purposes.) This phenomenon occurs because the $Ca^{2+}$ which is released initially from the DM-nitrophen–$Ca^{2+}$ complex is rebound by nonloaded, unphotolyzed DM-nitrophen; under the conditions of photolysis only a fraction (approximately 5–10%) of the chelator is photocleaved. The amplitude and duration of the pulse are determined by (i) the amount of Ca$^{2+}$ released; (ii) the pH of the solution; (iii) the concentration of free DM-nitrophen; and (iv) the rate constants of association and dissociation of the chelator and the other processes which remove Ca$^{2+}$ from the solutions. It should be noted that in some physiological processes it is likely that activation occurs via pulses rather than a sustained increase in the Ca$^{2+}$ concentration. Both protocols are potentially accessible using the photorelease strategy.

## CONCLUSIONS

Laser flash photolysis of DM-nitrophen, DM-nitrophen–Ca$^{2+}$, and DM-nitrophen–Mg$^{2+}$ yields short-lived aci-nitro intermediates. At physiological pH, all three systems share an exponential decay in the 20–30-μs time domain (the first two systems also possess a further exponential decay of hundreds of microseconds). The life-times of the DM-nitrophen–Ca$^{2+}$ intermediates are the only ones which show any sensitivity to pH in the 6.9–8.9 range; only one component is resolvable in our system at pH 7.9 and 8.9. Our attempt to use an independent means to determine the rate of cation release (viz. the Ca$^{2+}$ sensitive dye Ap III) enabled us to establish what may be only an *upper* limit of ca. 180 μs for the half-time of photorelease of Ca$^{2+}$; this half-time is at the *upper* limit of the response time of the dye itself. However, this rate should prove sufficiently rapid for the study of many Ca$^{2+}$-dependent physiological processes with significantly improved kinetic resolution in comparison to rapid mixing techniques. It may indeed prove to be the case that the absolute value of Ca$^{2+}$ release is much faster than this (e.g., concomitant with the rapid aci-nitro exponential decay of 20–30 μs), and further studies are underway to determine if this is the case.

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**Registry No.** Ap III, 14918-39-9; Ca, 7440-70-2; DM-nitrophen–Ca complex, 126386-82-1.