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Photolysis quantum yield measurements in the near-UV; a critical analysis of 1-(2-nitrophenyl)ethyl photochemistry

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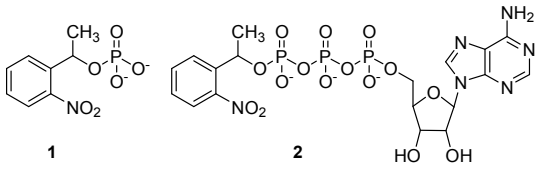
Introduction

Recently a series of three short papers and a Corrigendum appeared that related to photolysis quantum yield, Q_p, determinations of 1-(2-nitrophenyl)ethyl phosphate **1** (caged P_i) and the P^3-1-(2-nitrophenyl)ethyl ester of ATP **2** (caged ATP).[1-3] Q_p is an important parameter in photolysis, relating the chemical yield to the number of photons absorbed and quantifying possible dark fragmentation reactions that may follow light absorption. These topics form the focus of this Forum article. In essence Q_p values for **1** (0.54)[4] and **2** (0.63 based on Q_p value for **1**)[5] were challenged first with values of 0.04 for **1** and 0.02 for **2**[1] and later revised to values ranging from 0.19 to 0.47 for **1**.[3] These are major discrepancies over measurements that at first sight should be relatively straightforward to resolve. However beneath the surface there are traps for the unwary and it seems appropriate to air some of these in a Forum article. We will confine ourselves to one photon photolysis and to compounds discussed in the above papers.



1 **2**

There are two general approaches for Q_p measurement; actinometry and comparison with Q_p values of other caged compounds.[6] Actinometry provides the most fundamental measurement as the number of molecules photolyzed (or formed) is directly compared with the number of photons absorbed. It becomes more technically demanding in situations where a fraction of photons passes through the sample. The comparative approach is more straightforward to perform and permits multiple crosschecking with compounds whose Q_p values have been measured by actinometry. However the different spectral properties of compounds being compared can generate problems. It is a *sine qua non* that the light source and filter system used for photolysis are critical in determining a Q_p value and consideration of these together with the nature of the absorption spectrum of the photolyzed compound are especially important in comparison measurements. In general it is important to restrict the extent of photolysis over a linear range of photoproduct formation with time and this is best attained by only allowing photolysis to proceed to a limited extent. Accurate quantification either of substrate photolyzed or of product formed is also essential. Furthermore in the case of comparative determinations, precise quantification of both test and reference compounds is required. Additionally as shown in an example below, disappearance of caged starting

material does not necessarily result in a stoichiometric amount of product formation because of side reactions.

Discussion

In a recently published paper, the description of earlier literature on the Q_p determination of **1** by Kaplan et al.[4] was misconstrued by Anstaett et al.[3] because they considered an accurate absorption coefficient of **1** was crucial. In fact the key measurement for determination of Q_p was the colorimetric measurement at 670 nm of the photolysis product (P_i) as reduced phosphomolybdate just as determination of Q_F, the quantum yield of ferrioxalate photolysis, is a colorimetric measurement at 510 nm of product Fe^{2+} as a ferrous ion complex with 1,10-phenanthroline.[7] Reference 4 was written prior to the days when Supplementary Material was available and experimental protocols were of necessity concise leading to possible difficulties in understanding. Because of the fundamental and historic importance of **1** and its Q_p value in the caged compound literature, it is timely to expand on the published account[4] of the protocol. The procedures used were based on those established by Hatchard and Parker.[8]

First, successive solutions of **1** (molar absorption coefficient, also called extinction coefficient, ε = 540 $M^{-1}cm^{-1}$ at 342 nm), transmitting over a range of 15 – 50% incident light, were placed between a ferrioxalate solution and the source of the irradiation (342 nm peak with 60 nm half bandwidth).[4] The cell containing **1** was located immediately in front of that containing the ferrioxalate solution to ensure any divergence of the irradiation beam falling on the two cells was minimized. Concentrations of P_i and Fe^{2+} formed were determined as outlined above. The irradiation was repeated with the same intensity at 342 nm for the same time but with blank aqueous samples substituted for **1**. This procedure was repeated for various irradiation times. The protocol was repeated at different light intensities with and without **1** present, following the method of Hatchard and Parker.[8] For each light intensity resulting $[P_i]$ and $[Fe^{2+}]_B$ were measured, giving the $(P_i/nmol)$ and $(Fe^{2+}/nmol)_B$ with **1** present, and resulting $[Fe^{2+}]_A$ was measured without **1** present giving $(Fe^{2+}/nmol)_A$. Graphs were plotted of $(P_i/nmol)$, $(Fe^{2+}/nmol)_A$ and $(Fe^{2+}/nmol)_B$ against time. At any given time *t*, the same number of photons would have been delivered in the two set-ups and so:

$$(P_i/nmol)/Q_P + (Fe^{2+}/nmol)_B/Q_F = (Fe^{2+}/nmol)_A/Q_F.$$

Regression lines were drawn through the graphs giving slopes and

$$(\text{slope for } P_i)/Q_P + (\text{slope for } Fe^{2+})_B/Q_F = (\text{slope for } Fe^{2+})_A/Q_F.$$

On rearrangement

$$Q_P = Q_F \{(\text{slope for } P_i) / [(\text{slope for } Fe^{2+})_A - (\text{slope for } Fe^{2+})_B]\}.$$

Note that the above equation used to determine Q_p does not involve knowledge of the accurate concentration of **1**. In addition there was a second arrangement at 254 nm in which all irradiation was absorbed. In this case **1** and ferrioxalate solutions in identical cuvettes were placed side by side and Q_p was directly calculated from the relative amounts of P_i and Fe^{2+} formed. Linearity of graphs obtained of photolysis product formed both with respect to time and light intensity was a condition for data to be used in the determination of Q_p.

In the 342 nm measurements described above correction has to be made for the sample of **1** being 1 cm closer to the lamp than the ferrioxalate sample. The former was exposed to slightly

higher intensity light because of beam divergence. Originally this correction was not done and leads to a systematic error. Now however the correction has been made and the Q_p value for **1** is revised from 0.54 to 0.53 (see Footnote[32] at the end of the literature references). It follows that the Q_p value for **2** is revised from 0.63 to 0.62.

We now turn to protocols and results obtained by Anstaett et al.[1,3] The principal approach used by Anstaett et al.[1,3] was to determine Q_p values for **1** and **2** by comparison with Q_A, the quantum yield for the photoisomerization of *trans*- to *cis*-azobenzene. Their first Q_p values[1] obtained using a 355 nm Nd-YAG laser were 0.036 for **1** and 0.02 for **2**. Subsequently using illumination at 360 nm (< 10 nm bandwidth) Q_p values for **1** between 0.19 and 0.29 were obtained in water and pH 7.2 buffer.[3] [Q_p values as high as 0.47 based on the yield of photoproduct 1-(2-nitrosophenyl)ethanone (also called 2-nitrosoacetophenone as used below) were considered unreliable.][3] The authors claimed their revised values were due to a calculation error. No correction was made to the Q_p value for **2**.

An alternative independent approach[1] was measurement of Q_p of **1** at 355 nm (Nd-YAG laser) by comparison with Q_F of ferrioxalate. A value of 0.04 was obtained for Q_p. That low value of Q_p is clearly in error as later demonstrated by the authors, when subsequently they obtained much higher values for Q_p by comparison with Q_A.[3] These later results also cast doubt on other results in the Communication such as those in Table 1.[1]

Measurement of the Q_p values for **1** described above have all been performed by direct comparison with Q_p values of compounds (ferrioxalate or *trans*-azobenzene) determined by actinometry. This approach is readily extended to obtain Q_p values of new compounds by comparison with those already determined by the more direct approach.[6] For example Q_p of **2** was determined from Q_p of **1**.[5] One important advantage of this method is that it allows for cross-checking of Q_p values. For example Q_p for 1-(2-nitrophenyl)ethanol was 0.67($\pm$ 0.10) when measured in aqueous media when *trans*-azobenzene was used as an actinometer and equalled (within experimental error) that of **1**.[2,9] Other examples support the Q_p value of 0.53 for **1** from such networks.[2] Critical remarks about this approach have been made[3], but with due care it is an important asset particularly when cross-checking of Q_p values is possible, as in the example quoted. In these cross-checking experiments accurate concentrations of **1** were required. These were determined by equating ε of **1** at its λ_{max} with that of 1-(2-nitrophenyl)ethanol in water, for which ε = 4,700 $M^{-1}cm^{-1}$ at 263 nm measured in a solution prepared from a weighed sample (0.1% accuracy using a μg-sensitive balance).[10]

When determining Q_p of a new compound by comparative photolysis with a previously measured standard as in the above paragraph, consideration must be given to differences in the chromophore of the standard and test compounds. If these are not identical and a broadband irradiation source is used, assessment of the proportion of incident light absorbed by the standard and test compound will be non-trivial. A simpler protocol (but see Footnote[33]) is to use a monochromatic light source and a solution that contains equal concentrations of the test and reference compounds.[11] The absorbance of the test and reference compound at the irradiation wavelength A_p and A_{ref} must be measured separately. The ratio of absorbed photons (N_p and N_{ref} respectively) is then calculated from the Beer-Lambert law:

$$\frac{N_p}{N_{ref}} = \frac{1 - 10^{-A_p}}{1 - 10^{-A_{ref}}}.$$

Measurement of the extent of photolysis for the test and reference compounds (E_p and E_{ref} respectively) can be made, for example by HPLC, and the quantum yield of the test compound (Q_p) relative to the quantum yield (Q_{ref}) of the reference compound can be obtained from:

$$E_p/Q_pN_p = E_{ref}/Q_{ref}N_{ref}.$$

A similar correction to that described in the Footnote[33] applies to the Q_p of caged GABAs containing 7-nitroindoline substituents, so that the Q_p value of 0.088 of the DPNI-caged GABA (compound **6** in reference 12) obtained by actinometry[13] should now be compared with a value of 0.067. Thus the agreement between these two Q_p values is less good than previously claimed[2], if anything suggesting the Q_p for **1** is slightly larger than 0.53 (though different photolysis conditions[4,12] could also be relevant).

The paper[11] describing the photochemistry of substituted 1-acyl-7-nitroindolines also reports an example of the important difference that may occur between Q_p values of the compound photolyzed and of the product formed (see page 39 of reference 14). In that case the glutamate photoproduct was only ≈71% of the caged glutamate (1-[(4*S*)-(4-amino-4-carboxybutanoyl)]-4-methoxy-5,7-dinitroindoline) photolyzed. By contrast it is noteworthy that photolysis of **1** generated > 90% P_i suggesting Q_p values for photolysis of **1** and for P_i formation are equal.[4] That photolysis experiment (Figure 3 in ref. 4) has the additional merit of providing a cross-check on the concentration of starting concentration of **1** against that of the standard solution of P_i used in calibration of P_i formation on photolysis of **1**.

Q_p values may or may not vary with wavelength and illustrative examples can be found for compounds used in chemical actinometry where invariance is desirable.[15] However some specific points are relevant to the above discussion. Anstaett et al.[1] argued that variations in Q_p values for **1** and **2** between 342 to 350 nm range were significant. But that seems improbable, as in each case the principal absorption band is due to an n → π* transition of the 1-(2-nitrophenyl)ethyl group. In accordance with Kasha-Valivov rules (normally applied to light emission) we can expect Q_p not to vary between these two wavelengths.[14,16,17] It is though expected that Q_p values will vary with wavelength when other transitions exist. Thus Q_p for **1** has a mean value of 0.30 at 254 nm[4] where a π → π* transition likely predominates.[18,19] In a related example a caged proton containing a 1-(2-nitrophenyl)ethyl group has a Q_p value of 0.29 when photolyzed using a UG-11 filtered xenon arc lamp with illumination centred at 350 nm (64% of total transmission 320-380 nm) compared to 0.095 at 309 nm.[20] In the latter case a greater π → π* contribution to the absorption spectrum of the caged proton is probable.[20,21]

Why Anstaett et al.[1] obtained erroneous Q_p values for **1** and **2** using azobenzene isomerization as a chemical actinometer is explained in part by a calculation error.[3] The potential use of azobenzene as an actinometer stems at least from the 1950s[22] and its development has been dominated by research in Gauglitz's laboratory.[15,23,24] Care both in experimentation and calculation is needed in the region of 360 nm, the wavelength used by Anstaett et al.[3], because of the steeply declining absorption spectrum and low extinction coefficient of *trans*-azobenzene.[22] Nevertheless protocols for doing this have been firmly established by Gauglitz and others.[24] On the other hand a problem from another source can be identified. In choosing to measure a Q_p value for **1** by following its photolysis using NMR spectroscopy[3], the amount of 2-nitrosoacetophenone formed (up to 60% in one instance, Figure S6 in reference 3 Supporting Information) means a fraction of photons will be absorbed by this photoproduct. At 360 nm ε = 535 $M^{-1}cm^{-1}$ for 2-nitrosoacetophenone[25] while ε = 340 $M^{-1}cm^{-1}$ for **1**. So only 30% of the 360 nm illumination is available to photolyze **1**. Even at the time the first sample is taken for analysis (10% photolysis), the percentage of photons available to

photolyze **1** has dropped from 100% to 85%. It is not surprising that the average Q_p value claimed for **1** of 0.245 as recorded in Table 2[3] is just 46% of Kaplan's et al. revised value of 0.53.[2] How to correct for an absorbing photoproduct is straightforward[16], but its extinction coefficient must be known. (2-Nitrosoacetophenone can dimerize spontaneously and this reversible reaction has been studied by NMR spectroscopy.[26] However, the reaction is photosensitive with efficient photodissociation to monomer[26] meaning that the presence of dimer is unlikely to interfere with the experiment recorded in Figure S6 of reference 3.)

Conclusion

In summary the principal focus of this Forum article has been to address the discrepancies evident in three recent papers addressing Q_p values of **1** and **2**. In the course of this it has been useful to speculate why these discrepancies occurred. These reflect on the wider use of caged compounds and the theoretical and experimental underpinning that is the basis of this use. It should also be emphasised that **1** and **2** were never designed to be set up as standards for Q_p measurements; by the 1970s there was already a rich source of compounds with associated protocols suitable to serve in chemical actinometry.[8,23,24] A much broader perspective is to be gleaned from in depth review articles and books such as references 27 and 14 as well as comprehensive multiauthored compendia.[28,29] Finally it is worth emphasising that, for most experimental applications of caged compounds to biological research, the experimentalist is more concerned with the extent of photolysis, and the released ligand concentration, than the Q_p value.[21] However, the former is instrument dependent and should be calibrated by photolysis and analysis of a standard, such as **1, 2** or a caged fluorophore in separate photochemical measurements.[20,30] The product of Q_p and the molar absorption coefficient measures the efficacy (εQ_p) of photolysis at a particular wavelength[27] and may be used to compare efficiencies between different caged compounds. These, together with kinetic data, can predict the spatially and temporally defined ligand concentrations expected at sites of biological action in photolysis experiments.

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32. A correction of Q_p for **1** to 0.51 from 0.54 had been made previously based on rough estimate of photolysis beam divergence.[2] A referee requested that this be looked into. Fortunately the optical equipment used in the original publication[4] was found, permitting an accurate estimate of divergence of the collimated beam. From this analysis Q_p is revised to 0.53.
33. We are grateful to a referee who pointed that the value of Q_p in reference 11 had been based on the equation $E_p/Q_pA_p = E_{ref}/Q_{ref}A_{ref}$ rather than on $E_p/Q_pN_p = E_{ref}/Q_{ref}N_{ref}$. The Q_p for the MNI-caged glutamate (1-[*S*-(4-amino-4-carboxybutanoyl)]-4-methoxy-7-nitroindoline)[31] is 0.067 (corrected from 0.085).

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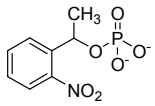

The photolysis quantum yield, Q_p, of 1-(2-nitrophenyl)ethyl phosphate (caged P_i) measured in the near-UV (342 nm peak with 60 nm half-bandwidth) is 0.53. Some general principles relating to measurement of Q_p values are discussed.