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Reply to Commentary by Trentham et al. on "Caged Phosphate and the Slips and Misses in Determination of Quantum Yields for Ultraviolet-A-Induced Photouncaging" by Gasser et al.

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Following a recent communication on the determination of quantum yields for ultraviolet-A-induced photouncaging written by a few of us (P.A., A.L., and G.G.),[1] Dr. Trentham contacted *ChemPhysChem* because he doubted the experimental results presented in that article. In a commentary co-authored with several colleagues,[2] Dr. Trentham has argued in favor of the initial quantum yields determined by Kaplan et al. 37 years ago,[3] that our measurements disproved. The arguments of the commentary are based on several photouncaging studies conducted since the pioneering work of Kaplan et al. However, the commentary by Trentham and his colleagues could not pinpoint the errors that could have led to the markedly different results obtained in our group, apart from suggesting an instrumentation failure. This explanation can be ruled out with a relative certitude, as different instruments were used in our (P.A., A.L., and G.G.) initial study to exclude this as possible source of error.[1] Nevertheless, this commentary has sparked our motivation to continue our investigations and to find an explanation for the issues around the determination of uncaging quantum yields. We therefore contacted a colleague with experience in the field of caged compounds, Prof. Christian Bochet (C.B.), and asked him and his coworker Dr. Elia Janett (E.J.) to independently determine the uncaging quantum yield of caged phosphate in his laboratories, using his experimental protocols and equipment, and this without the presence of P.A, A.L., or G.G. We would like to point out at this stage that both project investigators (C.B. and G.G.) are not related and have not been involved in any common projects to date. In this article, we (P.A., A.L, E.J., C.B., and G.G.) not only present these new results, but also speculate on potential explanations for the divergences in the determined uncaging quantum yields.

The new measurements were performed on an Atlas Photonics LUMOS 43 A photoreactor equipped with LEDs with a center-wavelength of 360 nm and using a standard 1 cm absorbance quartz cell. The emission spectrum of these LEDs is quite narrow (bandwidth <10 nm) and thus close to monochromatic. The total photon flux was determined by phenylglyoxylic acid actinometry.[4] Generally, quantum yields are defined as described by Equation (1):

$$\Phi = \frac{\text{number of events}}{n_{\text{photons abs.}}} \quad (1)$$

with $n_{\text{photons abs.}}$ the number of photons absorbed by the sample. In the case of photouncaging quantum yields, the events are of course the uncaging reactions. If the total photon flux of a light source has been determined, for example, by actinometry, and the sample concentration is adjusted to absorb all the photons entering the irradiated solution (n_{photons}), the uncaging quantum yield can be calculated following Equation (2):

$$\Phi = \frac{n_{\text{sample},0} \times m_{\text{sample}}}{n_{\text{photons abs.}}} = \frac{n_{\text{sample},0} \times m_{\text{sample}}}{n_{\text{photons}}} \quad (2)$$

with $n_{\text{sample},0}$ the sample amount (in mol) at time $t=0$, and m_{sample} the slope of percent of reacted sample, or the inverse of the slope of percent of remaining sample, over time.[5] Thus, concentrated solutions, that is, OD($\lambda=360$ nm) >2, of a known concentration and volume of caged phosphate were irradiated for certain time intervals. Notably, although the use of concentrated solutions offers the advantage of not having to deal with the fraction of absorbed photons, it has the significant disadvantage that much material is needed, limiting its application to readily available caged compounds. The measurements were carried out in pure water and in PBS (pH 7.2), as it has been performed in our (P.A., A.L., and G.G.) communication.[1] Two analytical techniques were used to determine the extent of uncaging. In either case, the appearance of the photouncaging product 1-(2-nitrosophenyl)ethan-1-one was monitored by HPLC. In Method 1, 1-(2-nitrosophenyl)ethan-1-one was separately prepared and a calibration curve was estab-

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lished, allowing the determination of the amount of 1-(2-nitrosophenyl)ethan-1-one in the uncaging mixtures. In Method 2, the 1-(2-nitrosophenyl)ethan-1-one peak with the largest observed area in an uncaging series was considered as point of total conversion. The peak areas at time points with less than 20% conversion were set in relation to this peak and considered for analysis. In both cases, m_{sample} was determined and the quantum yield was calculated with Equation (2) (see Table 1). The values in water and PBS differ only moderately, but there is a large difference between the two methods. This indicates that the photoproduct 1-(2-nitrosophenyl)ethan-1-one is unstable, and thus not suitable for accurate monitoring of the progress of the uncaging reaction. Thus, Method 1 is likely closer to the actual value than Method 2. However, the obtained values can be seen as lower and upper limits for a range in which the actual uncaging quantum yield can lie.

Table 1. Lower and upper limits for the uncaging quantum yields of caged phosphate, determined by HPLC measurements detecting the uncaging product 1-(2-nitrosophenyl)ethan-1-one in aqueous solutions upon irradiation at 360 nm.

	Lower limit (Method 1)	Upper Limit (Method 2)
Water	0.24	0.47
PBS (pH 7.2)	0.19	0.37

In a subsequent series of measurements, the uncaging process was independently monitored by ^{1}H and ^{31}P NMR measurements. To this end, the integral of the signal stemming from the protons of the benzylic methyl group of caged phosphate was monitored relative to an internal standard, namely the signal of CH_3 of ethanol. The integral of the ^{31}P signal of free phosphate was determined relative to the sum of caged phosphate and free phosphate. No other ^{31}P signals were observed. The measurements were again carried out in pure water and in HEPES buffer (pH 7.2). HEPES was chosen as a buffer system at the same pH as PBS (7.2), but excluding the presence of extra phosphorus to allow for the ^{31}P NMR measurements. Utilizing Equation (2), quantum yields in the range of 0.19–0.26 were determined for caged phosphate (see Table 2).

These values significantly differ from our previously found value of 0.04,[1] but also from the values from Kaplan et al. of 0.54[3] or 0.51,[2] respectively. We (P.A., A.L., and G.G.) therefore repeated the experimental procedures laid out in our communication,[1] using the LUMOS photoreactor in C.B.'s laboratories.

Table 2. Uncaging quantum yields of caged phosphate determined with NMR techniques in aqueous solutions upon irradiation at 360 nm. Each value is the average of the results of two independent sets of experiments.

	^{1}H	^{31}P
Water	0.29	0.19
HEPES buffer (pH 7.2)	0.26	0.24

To evaluate whether the azobenzene actinometry protocol had been established correctly, dilute solutions, that is, OD($\lambda =$ 360 nm) < 0.2, of known concentration and volume of *trans*-azobenzene were irradiated for certain time intervals. The amount of photons absorbed by a dilute solution $n_{photons\ abs.}$ is not equal to the amount of photons reaching the irradiated solution, but given by Equation (3):

$$n_{photons\ abs.} = n_{photons} \times F_{sample} = n_{photons} \times (1 - 10^{-A}) \quad (3)$$

with F_{sample} the fraction of photons that are being absorbed by the sample, and A its absorbance. Thus, Equation (2) can be rewritten for an incompletely absorbing solution in the following manner [Eq. (4)]:

$$\Phi = \frac{n_{sample,0} \times m_{sample}}{n_{photons\ abs.}} = \frac{n_{sample,0} \times m_{sample}}{n_{photons} \times F_{sample}} \quad (4)$$

If the quantum yield of the sample is known, as it is for *trans*-azobenzene, solving the equation for $n_{photons}$ gives the total photon flux of the light source [Eq. (5)]:

$$n_{photons} = \frac{n_{sample,0} \times m_{sample}}{\Phi \times F_{sample}} \quad (5)$$

The photon flux determined by the azobenzene-measurements (5.48×10^{-7} E min^{-1}) matched the one determined by phenylglyoxylic acid actinometry (5.44×10^{-7} E min^{-1}, thus 1% deviation). This shows that the azobenzene protocol was not a source of error.

As final experiment, dilute solutions of known concentration and volume of caged phosphate were irradiated for certain time intervals. Using Equation (4), a quantum yield of 0.25 was obtained, which matches the values obtained with the above described methods. Thus, the experimental procedure that uses *trans*-azobenzene as reference for caged phosphate is in principle working.

In our previous quantum yield calculations, however, we used a different formula, which obsoletes the knowledge of the total photon flux [Eq. (6)]:

$$\Phi_{sample} = \Phi_{reference} \times \frac{m_{sample}}{m_{reference}} \times \frac{F_{reference}}{F_{sample}} \quad (6)$$

Of note, Equation (6) has been used in several works reported by several prominent groups in the field.[6] If employing this formula, the absorbances of sample and reference are typically adjusted to the same optical density, bringing the quotient of the fractions of absorbed photons close to unity. Thus, this factor can be neglected and the formula be further simplified. The slopes m are usually obtained from a plot showing the percent of remaining caged compound vs time or vs light dose. The formula itself can be derived from Equation (2) by dividing it for the sample by the same equation for the reference [Eq. (7)]:

$$\frac{\Phi_{\text{sample}}}{\Phi_{\text{reference}}} = \frac{n_{\text{sample},0} \times m_{\text{sample}}}{n_{\text{photons abs.,sample}}} \times \frac{n_{\text{photons abs.,reference}}}{n_{\text{reference},0} \times m_{\text{reference}}} = \frac{n_{\text{sample},0} \times m_{\text{sample}}}{n_{\text{photons}} \times F_{\text{sample}}} \times \frac{n_{\text{photons}} \times F_{\text{reference}}}{n_{\text{reference},0} \times m_{\text{reference}}} \quad (7)$$

The lighting conditions for sample and reference are identical, thus n_{photons} drops out. Solving for the quantum yield of the sample then gives Equation (8):

$$\Phi_{\text{sample}} = \Phi_{\text{reference}} \times \frac{n_{\text{sample},0} \times m_{\text{sample}}}{n_{\text{reference},0} \times m_{\text{reference}}} \times \frac{F_{\text{reference}}}{F_{\text{sample}}} \quad (8)$$

Equations (8) and (6) are similar, but they only become identical if $n_{\text{sample},0}$ is equal to $n_{\text{reference},0}$. This, however, is only the case for two solutions with the same optical density if their molar extinction coefficients are identical. Thus, results obtained by using the simplified Equation (6) can be incorrect. Indeed, the error is proportional to the quotient of the molar extinction coefficients of sample and reference. Table 3 shows the extinction coefficients of *trans*-azobenzene and caged phosphate at 355 nm and 360 nm, which highlights the magnitude of the factor for this example, as well as its wavelength dependence. Notably, if broadly emitting light sources were employed, the emission spectrum and absorption spectra had to be considered over the whole range, making such a correction difficult. The use of monochromatic light sources is therefore once again strongly advised when uncaging quantum yields are measured. Markedly, also differences in the refractive indices of different solvents used for the sample and reference could lead to errors, if sufficiently divergent.

Table 3. Extinction coefficients of *trans*-azobenzene and caged phosphate at relevant wavelengths. All values are given in [$M^{-1}cm^{-1}$].

Compound	ε(355 nm)	ε(360 nm)
trans-Azobenzene	2990	1100
Caged phosphate	486	400

Table 4 shows a comparison of uncaging quantum yields obtained from Equation (6), as done in our initial communication (P.A., A.L., and G.G.),[1] and correctly calculated values via Equation (8). As can be seen, the resultant quantum yields of caged phosphate are significantly greater than previously determined following Equation (6). However, these new calculated values fit perfectly with the results independently obtained by E.J. and C.B. At this stage, we (P.A., A.L., and G.G.) would like to apologize for any inconveniences this mistake may have caused to the readers of *ChemPhysChem*. However, taken into account this new finding, we (P.A., A.L., and G.G.) would like to strongly emphasize that this finding confirms that no experimental errors were involved in our initial communication. Nevertheless, it appears that all obtained values are still lower than the value for caged phosphate reported by Kaplan et al.[3] and corrected by Trentham and his colleagues[2]—roughly by a factor of 2. Thus, we strongly believe that our initial communication[1] has opened up an important scientific discussion that has finally led to the discovery that a quantum yield which has been used by the scientific community for 37 years was most probably overestimated and that an equation was often inappropriately used by the community.

Table 4. Uncaging quantum yields of caged phosphate upon irradiation of dilute solutions. Note, that the values obtained from Equation (6) are incorrect.

	Φ(355 nm)	Φ(360 nm)
Following Equation (6)	0.038	0.10
Following Equation (8)	0.23	0.27

The commentary by Trentham and colleagues contains several arguments that have been meant to back up the initial value against a tenfold smaller value, not a twofold one. Nevertheless, those arguments need to be considered in this discussion as well. The first cited evidence concerns nitrophenyl-caged compounds, which release protons instead of phosphate.[7] These compounds can be expected to have quantum yields different from caged phosphate, as the nature of the leaving group has an influence on the efficiency of photolysis.[8] Thus, this study does not provide evidence regarding the quantum yield of caged phosphate. Other quoted literature examples indeed used caged phosphate, but as sole reference compound, and thus the studies also provide no data for the evaluation of the quantum yield of caged phosphate itself.[9,10] Notably, most of the experiments carried out in these publications were done in equimolar, not in "equi-absorbant", solutions. Assuming that the extinction coefficients of the tested compounds and caged phosphate are similar in the complete broad irradiated range of 300–400 nm, the ratio of quantum yields should be roughly correct. Thus, the quantum yields reported therein for the new compounds are likely affected by our new uncaging quantum yield to a similar extent as caged phosphate itself. The data presented by Morrison et al.[11] is not suitable either for the determination of the uncaging quantum yield of caged phosphate on the scale of a factor of two, due to differences in the compounds and irradiation conditions. Combining the results of Morrison et al.[11] and Papageorgiou et al.,[10] a doubly indirect comparison of caged phosphate with an actinometer is possible, but factors such as differences in the light sources, the assessed compounds, and the double indirectness itself prevent a sufficiently precise result. The same is true for a report by Anderson and co-workers, which Dr. Trentham alerted us of after publishing his commentary.[12] All in all, the arguments presented in the commentary by Trentham and his colleagues[2] do not deliver any valid additional evidence that the uncaging quantum yield of caged phosphate is above 0.5. Furthermore, the initial publication by Kaplan et al.[3] has certain shortcomings that might explain an error by a factor of 2. First, the extinction coefficient of a precursor, 1-(2-nitro)phenylethanol, has been used as approximate extinction coefficient of caged phosphate. As it is shown above, an accurate extinction coefficient is crucial for the determination of quantum yields. Seven years after the paper of Kaplan et al., a 30% higher value has been determined for

caged phosphate by Bamberg and coworkers (5500 $M^{-1}cm^{-1}$ instead of 4240 $M^{-1}cm^{-1}$ at 265 nm),[13] and our own measurements are in agreement with this value. Moreover, the use of a broad band light source hinders a precise determination. In fact, Trentham and colleagues themselves called the use of mercury arc lamps, which was used to obtain the uncaging quantum yield of caged phosphate,[3] "sub-optimal".[2] Moreover, judging from the data shown in the article of Kaplan et al.,[3] only few measurements have been performed in the first few percent of decomposition, which is the time frame used for the quantum yield determination. Unfortunately, neither the original paper by Kaplan et al.,[3] nor the commentary by Trentham and his colleagues[2] supply full details on experimental methodology for the quantum yield measurements and calculations, nor provide the associated data. For instance, the commentary has introduced, but has not elaborated on the "additional geometric factor" used to slightly correct the original 1978 quantum yield values.

In summary, while so far only one study has reported the caged phosphate quantum yield to reach above 0.5,[3] our (P.A., A.L., E.J., C.B., and G.G.) multiple measurements under a number of different conditions consistently resulted in lower values. Due to the arguments presented above, we strongly believe that the quantum yield of caged phosphate has been over-estimated and is indeed around 0.25 in buffered solution at pH 7.2 and upon irradiation in the 355–360 nm range. Very importantly, this value has been now confirmed by two independent laboratories using state-of-the-art apparatus and analytical methods. Admittedly, our data does not rule out the wavelength dependence of the caged phosphate quantum yield. Indeed, the original 0.51 value has been obtained upon broad-band irradiation around 342 nm, while our results were recorded at 355–360 nm. Nonetheless, as mentioned by Trentham and colleagues in their commentary, it is extremely unlikely that there is such an important wavelength dependence.

Finally, we emphasize once again that the evaluation of quantum yields of caged compounds remains analytically challenging, as can be seen by the typically relatively large error margins. Therefore, using a caged compound as a reference for the determination of quantum yields of other caged compounds should be strongly discouraged—this only further increases the uncertainty! Other references, such as azobenzene, or other actinometric measurements, should be preferentially used, as previously mentioned in our (P.A., A.L., and G.G.) initial communication.[1] Also, monochromatic light sources should be used to avoid problems due to different wavelength dependencies of reference and sample. Readers especially interested in this and further possible pitfalls are referred to the literature.[14] Very importantly, we would like to express our consent with the statement of Trentham and colleagues, that the consequences of the previously incorrectly reported quantum yields in the literature have only very small influences towards biological studies.

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