Reply to "Comment on 'An Apparent General Solution for the Kinetic Models of the Bacteriorhodopsin Photocycle' "

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Lozier's and Nagle's main concern[1] regarding my recently published paper[2] has three parts. 1. Actinic light, even in the absence of polarizers, imposes significant positional photoselection on ground state chromophores. 2. Reorientations of the chromophores due to nonphotocycle events, such as Brownian motion, tumbling, rotational, and translational diffusions contribute kinetic information that obscures and distorts true photocycle kinetics. 3. Only polarizers set at the magic angle (54.7°) can provide clean, photocycle-related kinetics. This issue is addressed in more detail in the Supporting Information. In addition, Lozier and Nagle maintain that my finding of more than the six exponentials found by van Stockkum and Lozier[3] using the data of Xie et al.[4] is due to an unjustified over-fitting of the data. There is no basis for such a claim. Statistical parameters designed to detect over-parametrization[5] (i.e., dependency values and standard errors) were presented and these showed no indication of superfluous exponentials. It is important to note that under-parametrization is to be avoided as much as over-parametrization.

There is no evidence that nonphotocycle events lead to contamination of photocycle kinetic information. Lozier and Nagle cite eight publications to document their contention that physical motions, independent of specific photocycle transitions, contribute kinetic data that adds to and obscures those of the photocycle process per se. On close reading of all eight of these citations, no experiments were performed or described that demonstrate erroneous kinetic information emanating from nonphotocycle-related reorientations of the retinal chromophore which led to contamination of the kinetics resulting from normal transitions between intermediates. In most cases the authors stated that magic angle techniques were used to avoid the possibility that such undesirable effects may be present.

There is evidence that no nonphotocycle events occur which lead to contamination of bacteriorhodopsin photocycle kinetics. The most direct evidence is found in the unprocessed raw data of Xie et al.[4] All reorientations of the chromophore associated with transitions of the photocycle are reversible, and the anisotropy at the end of the cycle returns to that at the start. Anisotropy-changes associated with nonphotocycle-related, physical changes in the location or orientation of the chromophore are nonreversible. This is because these changes in orientation reflect the randomization of the uniquely photoselected population. In the former (i.e., reversible) case, the absorbance obtained with polarizers in parallel orientation ($A_{||}$) and that with perpendicular orientation ($A_{\perp}$) will return to their starting values. If physical motions occur, some of the photoselected molecules will be lost from the parallel plane and will appear in the perpendicular plane. This will result in a decrease in the $A_{||}$ at the end compared to its value at the start. Similarly, the value of $A_{\perp}$will increase to a higher value at the end of the cycle compared to the start. In the Supporting Information, it is shown that there are no significant differences in the starting or final values of either $A_{||}$ or $A_{\perp}$. This finding is consistent with the absence of significant nonphotocycle reorientations of the chromophore during the photocycle. Song et al.[6] studied PM both in suspension and embedded in polyacrylamide gels and determined the angle θ for the position of the absorption dipole in each of the intermediates as approximately 0°, 8°, 15°, 17°, 20°, and 3°, respectively for BR, K, L, M_1, M_2, and O. The changes in anisotropy were reversible and followed the same time dependency as the photocycle intermediates. This means that all changes in anisotropy are accounted for by reorientations of the chromophore associated with the photocycle itself. Similarly, no evidence of the suspected changes due to nonphotocycle reorientations was found by Groma et al.[7] who studied time-dependent anisotropy for PM embedded in polyacryalamide gel using partially saturating polarized light to establish photoselection. They examined six different photocycle models, one of which was a parallel model consisting of two separate cycles. They concluded, as did Song et al., that the kinetics of anisotropy correlated with the photocycle and not with rotational diffusion. Their main conclusion was that an analysis of the fitted amplitudes of exponentials favors a model with parallel processes rather than those invoking nonphotocycle chromophore reorientations. In our own work, we find that the kinetics for bR are essentially the same whether suspensions or polyacrylamide gels are used (Table 1). That is, the variations encountered are within the range obtained using many different samples of suspension alone. If repeated determinations find that there is a small but significant increase in the slowest time constant for membranes encased in gel, a likely explanation is that the recovery of BR from M entails the greatest conformational change of the bR or membrane, as indicated by the anisotropy values of Song et al.[6] and the high viscosity of the gel impedes this transition. The idea that there is significant rotational diffusion of PM in suspensions that can lead to distortions of kinetic data emanating from reorientations of the retinal chromophore in photoselected populations seemed to be supported by Czege et al.[8] who reported that embedding PM in agar gels removed the changes in anisotropy observed in suspensions. They concluded "the direction of the retinal chromophore (and consequently of the retinal itself) does not change even during the transitions of the photocycle." These statements and conclusions are opposite from the findings of both Song et al.[6] and Groma et al.[7] Additional and more detailed evidence of the absence of contamination of photocycle kinetics by nonphotocycle reorientations of chromophores are presented in the Supporting Information.

TABLE 1: Fitted Taus for Suspensions and Gels

suspensions		7.2% polyacrylamide gels	
published[a]	unpublished	random	oriented[c]
6[b]	8	<1	<1
34	53	35	32
123	157	115	115
460	368	368	366
1830	1833	1783	1749
4371	4412	5130	5109

[a] Hendler, R. W.; Shrager, R. I.; Bose.[9] [b] Taus in microseconds. [c] As described in Keszthelyi and Ormos, *FEBS Lett.*, **1980**, 189−193.

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In the early history of kinetic studies of the BR photocycle it was important to be concerned about possible artifacts arising from photoselection and nonphotocycle reorientations of chromophores. However, in the absence of any convincing evidence over the past thirty years that significant nonphotocycle processes occur during the photocycle and with more recent evidence that all of the kinetics during the photocycle can be attributed to transitions of one intermediate to another, there is no apparent reason for using polarizers and magic angles. In fact there is reason to suspect that the use of polarizers may introduce small but significant distortions in the data (Supporting Information). The supporting informtion provided by Lozier and Nagle focuses on two issues: 1. That kinetics taken with parallel, perpendicular and magic angle polarizations differ. 2. An explanation is offered as to why measured anisotropies exceed allowable theoretical limits. As to issue one, there is no disagreement. The meaning of this difference is the whole point of the exchange of views discussed here. As to issue two, I do not intend to discuss or criticize it further, mainly because of the limitations of space and the fact that the out-of-bounds anisotropies and a possible explanation for them play a very small part in the fundamental discussion as to whether magic angle data is more or less reliable than nonmagic angle data. In view of all of the considerations discussed here and in my Supporting Information, it seems safer to avoid the use of MA procedures for the study of BR photocycle kinetics.

Supporting Information Available: A fuller discussion of the issue of photoselection presents additional evidence of the absence of kinetic misinformation due to nonphotocycle motions of the chromophores, and demonstrates that magic angle data may be undependable. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Lozier, R. H.; Nagle, J. F. *J. Phys. Chem. B* **2006**, *110*, 15041−15042.
(2) Hendler, R. W. *J. Phys. Chem. B* **2005**, *109*, 16515−16528.
(3) van Stokkum, I. H. M.; Lozier, R. H.. *J. Phys. Chem. B* **2002**, 106, 3477−3485.
(4) Xie, A. H.; Nagle, J. F.; Lozier, R. *Biophys. J.* **1987**, *51*, 627−635.
(5) Shrager, R. I.; Hendler, R. W.; *J. Biochem. Biophys. Methods* **1998**, *36*, 157−173.
(6) Song, Q.; Harms, G. S.; Wan, C.; Johnson, C. K. *Biochemistry* **1994**, *33*, 14026−14033.
(7) Groma, G. I.; Bogomolni, R. A.; Stoeckenius, W. *Biochim. Biophys. Acta* **1997**, *1319*, 69−85.
(8) Czege, J.; Der, A.; Keszthelyi, L. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 7273−7277.
(9) Hendler, R. W.; Shrager, R. I.; Bose, S. *J. Phys. Chem. B* **2001**, *105*, 3319−3328.