Supporting material to the Comment by R. H. Lozier and J. F. Nagle on R. W. Hendler's paper "An Apparent General Solution for the Kinetic Models of the Bacteriorhodopsin Photocycles"

1. Demonstration that kinetics of data taken with parallel, perpendicular, and magic angle polarization are all different.

Figure 1 shows a representative sample of the data of Xie et al[3], a small subset of our data analyzed by R. W. Hendler[1]. The dashed lines in the top panel shows the kinetics of the optical absorption changes of bacteriorhodopsin at 600 nm, 20°C, and pH 7 for three polarization conditions (parallel, perpendicular and magic angle) following a 490 nm 7 ns laser flash of moderate intensity. The most obvious difference in the data under the three polarization conditions is in the absolute amplitudes, as expected from theory[13]. Kinetic differences in the three traces can be best seen in the normalized differences (normalized parallel minus magic, P-m; normalized perpendicular minus magic, p-m). Also shown is parallel + 2 perpendicular – 3 magic)/3 which should be zero[13] ignoring a small effect described by Lewis and Kliger[14]. The bottom panel of Fig. 1 shows the difference traces with higher sensitivity on the absorbance axis. It is clear that: (i) the kinetics at all three polarizations differ; (ii) the kinetic differences in the normalized parallel – magic and in the normalized perpendicular – magic traces are of similar shape but opposite sign, consistent with motions out of and in to the polarization of the measuring beam, respectively; (iii) the (parallel + 2 perpendicular – 3 magic)/3 trace is essentially zero within the noise level, as expected from theory[13]. The kinetics in the parallel and perpendicular traces are clearly influenced by chromophore motions. The magic or (parallel + 2 perpendicular)/3 data must be used for analyzing the kinetics of the photochemical cycle unperturbed by physical motions of the chromophores.

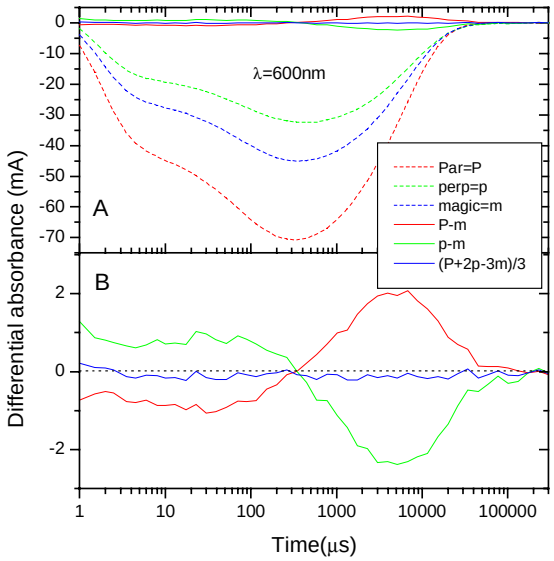

Fig. 1. Kinetics of absorption changes of bacteriorhodopsin and difference traces between the data taken with parallel, perpendicular, and magic angle polarization (see details in text).

2. Behavior of the polarization anisotropy when difference absorption changes are measured.

As is well known, it is the differences in absorption under different polarization conditions that reveal the motions of the chromophore[13]. When a polarized actinic flash is applied, it is convenient to define the absorbance of those chromophores that are activated as $A_{ac,parallel}$ when the polarization of the measuring beam is parallel to the actinic beam and as $A_{ac,perpendicular}$ when the polarization of the measuring beam is perpendicular to the polarization of the actinic beam. The photocycling anisotropy of only those activated chromophores is given by

$$r_{ac} = (A_{ac,parallel} - A_{ac,perpendicular})/(A_{ac,parallel} + 2A_{ac,perpendicular}). \quad (1)$$

When the distribution of chromophores is orientationally isotropic before the actinic flash and when the linear absorption dipole moment of each chromophore reorients from its initial orientation by an angle whose magnitude is θ, then

$$r_{ac} = (3\cos^2\theta - 1)/5 \quad (2)$$

in the limit when the actinic beam is weak so that the fraction of chromophores that photocycle is small. (See Nagle et al., *Photochem. Photobiol.* **1983**, *38,* 331-339 for the saturation effect that occurs with strong actinic beams.)

Obviously, r_{ac} is constrained to the interval [–0.2,0.4] by Eq. 2. If one takes the values of θ published by Song et. al., (*Biochemistry* **1994**, *33,* 14026-14033 for the different intermediates in the bR photocycle, then r_{ac} decreases, achieving a minimum value ~0.33-0.36 at the time when the M state is prevalent. It is important, however, to realize that one does not measure the absorbances $A_{ac,parallel}$ and $A_{ac,perpendicular}$ of only the activated chromophores. One measures the total absorbances $A_{parallel}$ and $A_{perpendicular}$ and one usually reports and analyzes the absorbance differences $\Delta A_{parallel} = A_{parallel} - A_{bR}$ and $\Delta A_{perpendicular} = A_{perpendicular} - A_{bR}$, where A_{bR} is the absorbance of the non-activated sample. It is these quantities that were supplied by us to Dr. Hendler. The absorbance differences include the absorbances of the photocycling chromophores, but subtracting A_{bR} also means that 'deficit' absorbances are included because A_{bR} includes absorbances from molecules that are no longer in the bR state. This is evident because the ΔA quantities are negative for many wavelengths and times. If one now defines a difference anisotropy as

$$r_{diff} = (\Delta A_{parallel} - \Delta A_{perpendicular})/(\Delta A_{parallel} + 2\Delta A_{perpendicular}),$$

there are no constraints on its value. For example, the r_{diff} data in Wan et al.[11], from which the θ and r_{ac} values mentioned above were obtained, have values less than –0.4 and many of the r_{diff} data have values near zero, even though none of the θ values reported in Song et al. give values of r_{ac} less than 0.33. It is even possible for r_{diff} to become positively or negatively infinite (Nagle et al, 1983, see above) because the total absorbance given by the denominator can become zero at a time during the photocycle when $\Delta A_{parallel}$ does not equal $\Delta A_{perpendicular}$. Indeed, Fig. 2 shows experimental values of r_{diff} well outside the range [–0.2,0.4] that occur when the difference absorbance crosses zero.

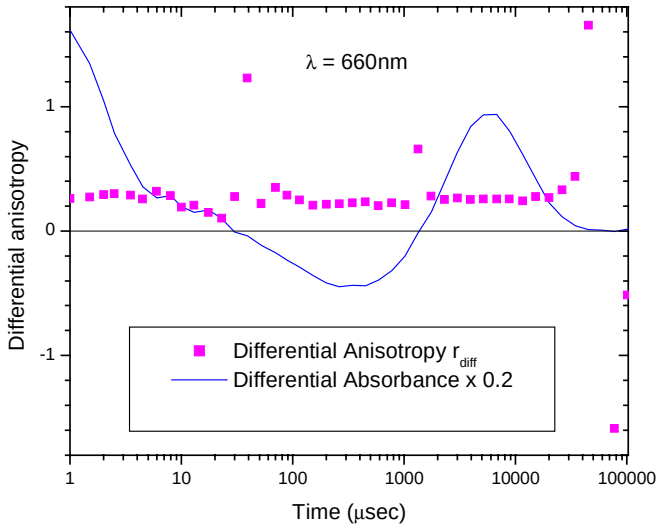

Fig. 2. Difference anisotropy (squares) and the magic angle absorbance (times 0.2 and in mA) versus time after the actinic flash for wavelength 660 nm, and 20°C, and pH 7. Large magnitudes for r_{diff} occur when the absorbance crosses zero, as predicted by theory (Nagle et al., 1983).

Widely varying values of r_{diff} have apparently been a source of concern to Dr. Hendler, but they are the expected behavior, even for the case of photocycles in which the motion of the absorption dipole moment is rigidly prescribed by the chemical intermediates, which was the case treated theoretically by Nagle et al. (1983). Fortunately, all complications due to motions are removed when the total absorbance $\Delta A_{parallel} + 2\Delta A_{perpendicular}$ is analyzed to find the time course of the chemical intermediates. Because magic angle polarization obtains the total absorbance in the limit of low actinic intensity (Lewis and Kliger[14]), we have tended to prefer it because it is experimentally expeditious to take one data set rather than adding two data sets. In any case, it is clearly important to have well defined polarization conditions for high quality photocycle studies in biology. As noted by a reviewer of our Comment and the Reply, this has been clearly recognized by workers such as Song et al. and Groma et al. (*Biochem. Biophys. Acta* **1997**, *1319*, 69-85), as well as by authors of other papers we refer to in our Comment and undoubtedly by others in other subareas of photobiology.