BLEACHING OF RHODOPSIN IN THE DARK*

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Urea acts on cattle rhodopsin to produce a urea-bleached product (Kito and Takezaki, 1966). We have demonstrated that this process occurs not only in the light but also in the dark, and shows a marked dependence on urea concentration. Moreover, at low urea concentrations, dark-urea bleaching is reversible, the regenerated rhodopsin being a manifestly different product from the native rhodopsin. The total bleaching can be made to occur in two stages by exposure to urea in the dark, followed by exposure to light. The second step takes place at a rate much increased over light bleaching in the absence of urea.

MATERIALS AND METHODS Rhodopsin was prepared from bovine retinas and kept in the dark at 0°C before use.† A new and direct technique for extracting the rhodopsin involved homogenizing 100 retinas with 0.066 M phosphate buffer, pH 6.8 at 0°C in the dark and centrifuging for 20 minutes at 7,700 x g. This washing step was repeated 4 or 5 times, reducing hemoglobin and soluble proteins with no loss in rhodopsin. The pellet was then extracted with 5% cetyl trimethyl ammonium bromide (CTAB) in buffer at 0°C for 18 hours with stirring, giving a good sample of rhodopsin (stage 1). Further purification was achieved at 35% saturation in ammonium sulfate (stage 2) followed by precipitation at 90% saturation in ammonium sulfate (stage 3) when OD 280/)D 500 = 4-5. Rhodopsin preparations at stage 1 were used in this work.

Urea was reagent grade and used as obtained. All spectrophometric work was carried out on a Beckman DBG recording instrument and samples were handled either in the dark or under a deep red light.

RESULTS Figure 1 shows a typical visible spectrum of rhodopsin at stage 1 and that of its totally light bleached product. Absorption

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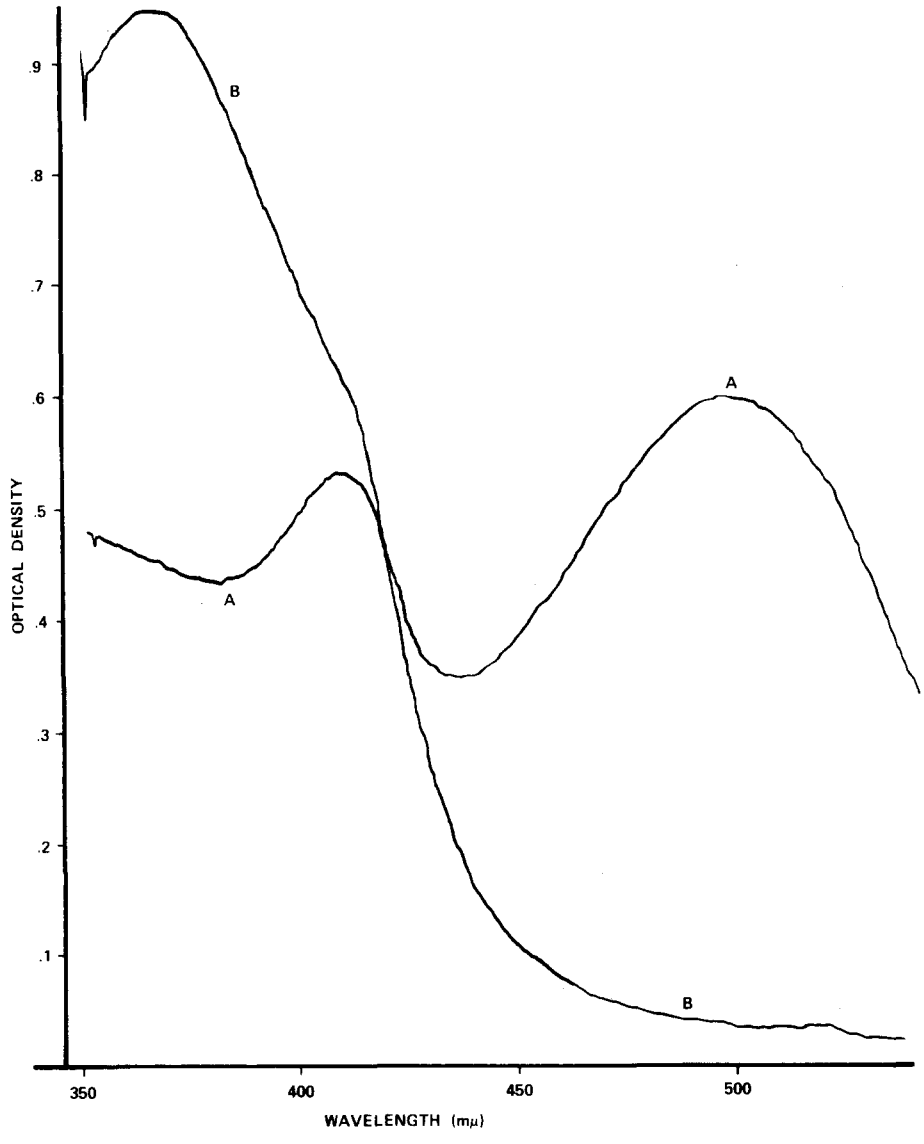

Figure 1 - Absorption spectrum of rhodopsin solution (A) before photobleaching and (B) after complete photobleaching.

at 500 mμ was reduced to less than 6% while absorption increased maximally at 375 mμ corresponding to the appearance of retinal. Figure 2 shows how such an unbleached rhodopsin sample may be bleached by urea to a limiting OD 500 one-half the value of the unbleached sample. The conditions for this, 5 M urea for 2 hours in the dark, were considerably longer than required for complete dark-urea bleaching. Also shown is the difference spectrum of this dark-urea bleached sample, after it had been exhaustively dialyzed, versus the light-bleached sample. For comparison, a difference

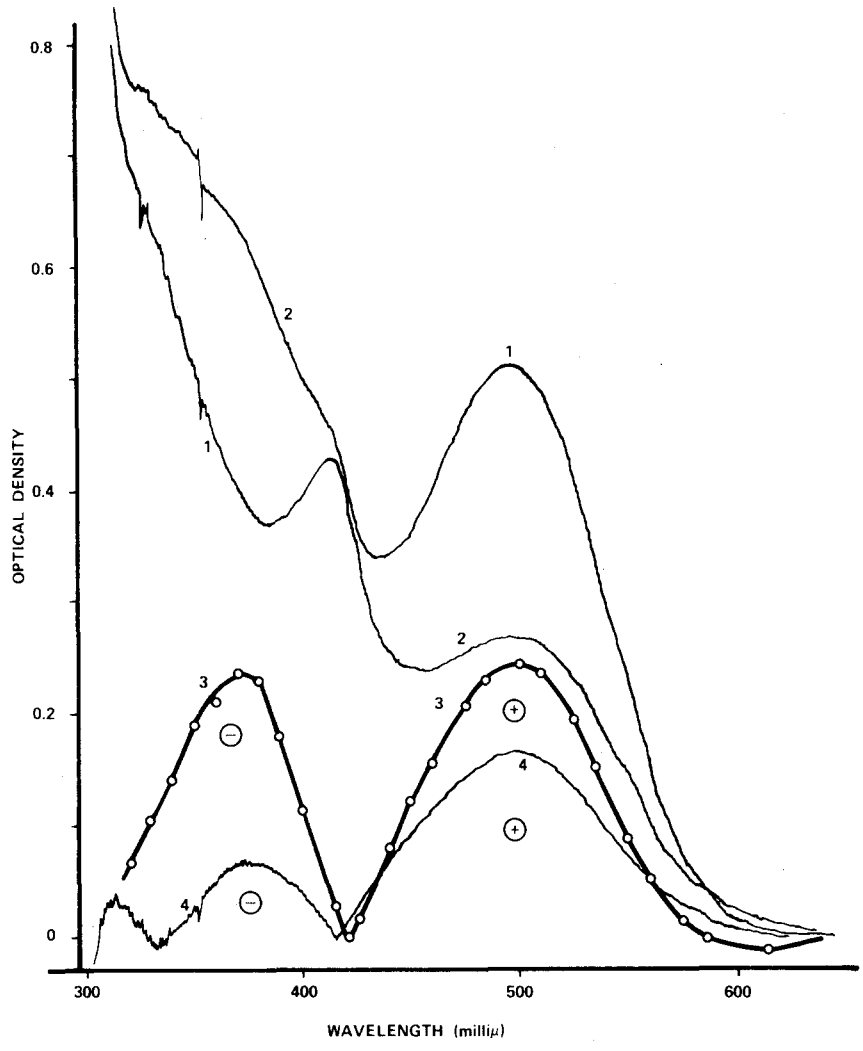

Figure 2 - Absorption spectrum of rhodopsin solution (1) before bleaching; (2) after complete urea-dark bleaching in 5M urea, pH 6.8, for 2 hours. Difference spectrum of (3) unbleached versus completely dark-urea bleached rhodopsin; (4) dark-urea bleached rhodopsin, after exhaustive dialysis, versus completely photobleached rhodopsin.

spectrum of the unbleached sample versus the dark-urea bleached sample is drawn. It is clear that the absorption properties of the chromophore bleached in the dark by urea are different from those that remain and which can be bleached by light.

The existence of two stages in the bleaching process is unequivocally demonstrated in Figure 3, where the dark-urea bleaching of a rhodopsin

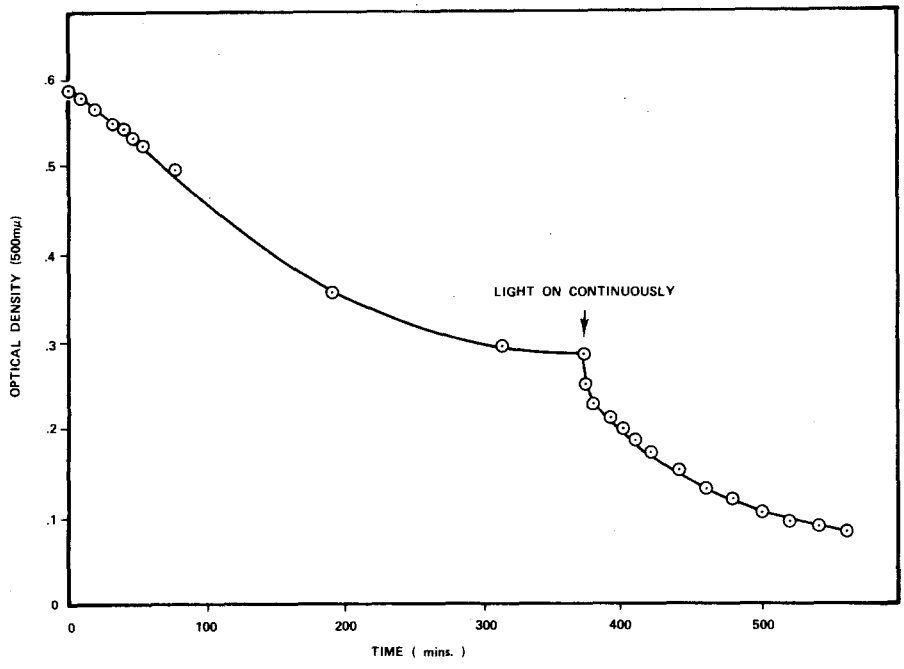

Figure 3 - The 2-stage bleaching of rhodopsin. Initial dark-urea bleaching in 2M urea reaching asymptote. After light is turned on continuously, bleaching progresses at increased rate.

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sample, made 2 M in urea, was followed by observing the OD 500 at intervals of time by momentary exposure to the spectrophotometer tungsten source. By the end of 3 hours, the dark-bleaching had reached an asymptote, one-half the initial value, yet when the sample was then exposed continuously to the tungsten source, the bleaching resumed at an accelerated rate. This rate is many times greater than that of rhodopsin bleaching in 2 M urea in light ab initio (see Figure 5).

Figure 4 shows the relationship between the pseudo first order rate constant $k = k_o U^{\alpha}$ and the urea concentration, U, for the dark-urea bleaching, plotted as a log-log relationship, so as to evaluate α which was determined to be 2.4. The rate constant is defined by the relationship

$$(OD - OD_o/2) = OD_o/2.e^{-kt}$$

where OD_o is the initial OD. This relationship, applied to the data, gave good straight lines.

Similar data for the light-urea bleaching did not give straight lines when plotted as a simple first order reaction. Values for k were obtained

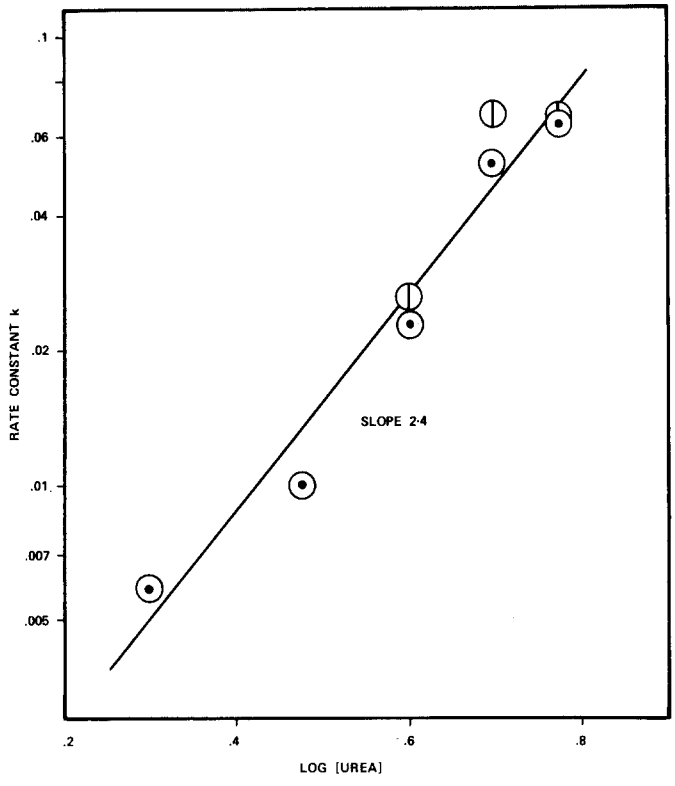

Figure 4 - Dependence of the pseudo-first order rate constant for dark-urea bleaching of rhodopsin on urea concentration.

from the initial slopes of these plots and are shown in Figure 5. It is clear that the kinetics of light-urea bleaching are more complex than those of dark-urea bleaching. However, at high urea concentrations, 4-7 M, we can observe a good linear relationship, resulting in a value of $\alpha = 7.8$.

DISCUSSION It would appear that there are two chromophores in native rhodopsin (Akhtar and Hirtenstein, 1969) which show different susceptibilities to light and urea bleaching. It would seem logical to identify these two chromophores respectively as the 11-cis retinal/lysine Schiff base belonging to the opsin (Akhtar et alia, 1968) and the 11-cis retinal/phosphatidyl ethanolamine (PE) Schiff base (Poincelot et alia, 1969) said to be the source of stored 11-cis retinal (Anderson, 1970).

To account for dark-urea bleaching, we propose the hypothesis that retinal is bound to opsin by way of a substituted aldimine bond, involving the ϵ-amino group of lysine (Bownds, 1967) and the sulfhydryl group of cysteine. It has been demonstrated (Mizuno, Ozawa and Kuno, 1966) that

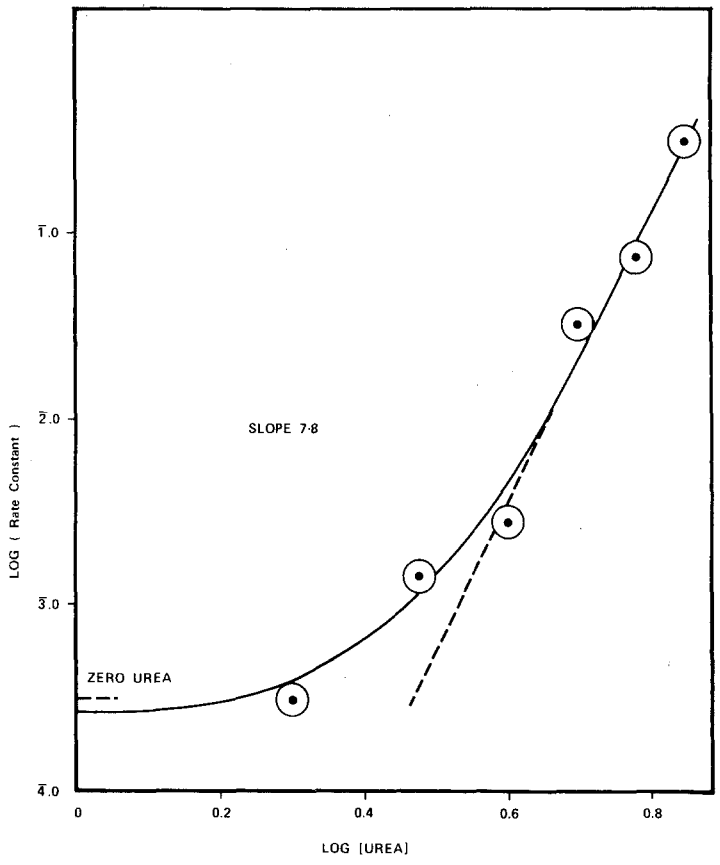

Figure 5 - Dependence of the pseudo-first order rate constant for light-urea bleaching of rhodopsin on urea concentration.

both cysteine and thiazolidine-4-carboxylic acid form Schiff bases with retinol, but only the latter, which was shown to involve a sulfur-substituted aldimine bond, was photosensitive. No colored product was formed between retinal and simple thiols. Moreover, they showed (Mizuno, Kuno and Ozawa, 1966) that the thiazolidine compound with retinal was (a) unstable in the presence of water and (b) underwent photolysis at an increased rate in the presence of water. In the presence of urea, the PE chromophore may be exposed to the aqueous environment and be hydrolytically cleaved to 11-cis retinal and PE, leaving the still photosensitive substituted aldimine chromophore intact, thus giving half bleaching. Exposure to light completes the bleaching process at an accelerated rate due to the ready availability of water at the chromophoric site. The different dependence on urea concentration of dark-urea and light-urea bleaching at high urea concentrations, suggests a different mechanism for the two processes, the latter probably leading to denaturation.

Studies are being pursued on both the mechanisms of urea-bleaching and on model light sensitive compounds.

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