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# Direct Measure of the Rate of Decay of Intermediate B in the Bleaching of Rhodopsin in the Excised Eye of the Albino Rat

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## Abstract

Kinetic studies on the fast photovoltage (FPV) of the intermediate B (B) in the bleaching process of the excised eye of the albino rat were performed. By using some new combinations of the blue and green test flashes the half-lives and several kinetic constants of B were obtained. Two different methods of experiments gave a similar value of the half-life of B, which is smaller by a factor of three or four than the value reported by Ebrey. The photo-regeneration of B from its daughter C was demonstrated, which explains the discrepancy.

## Introduction

When rhodopsin (Rh), either in retina or in solution, is excited by a photon, it is bleached through a series of several intermediates in the following scheme,

$$\text{Rh} \rightsquigarrow \text{PrelumiRh} \longrightarrow \text{LumiRh} \longrightarrow \text{A} \longrightarrow \text{B} \longrightarrow \text{C} \longrightarrow \text{Bleached substance.}^{**}$$

A and B stand for the intermediates A and B or metarhodopsins I and II, respectively. C stands for the intermediate C (Hagins, 1957). The wavy line denotes a photo-chemical reaction and straight lines denote thermal reactions. In the living system regeneration back to Rh takes place in the dark. Brown and Murakami (1964) discovered the fast photovoltage (FPV,*** originally named as early receptor potential, ERP) in the very early stage of the electroretinogram (ERG) from the excised monkey retina and called attention of many researchers studying the visual excitation mechanism. Similar electrical responses were

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** See the reviews by Wald, Brown and Gibbons (1963), by Hubbard, Bownds and Yoshizawa (1965) and by Abrahamson and Ostroy (1967). See also Cone (1967) and Ebrey (1968).

*** The nomenclature of FPV is by Hagins and McGaughy (1967).

found by Arden and Ikeda (1965) from the excised albino rat eye in some intermediate stage of bleaching. Later Cone (1967) and Pak and Boes (1967) showed that these responses are assigned as due to the intermediates A, B and C. B and C give a blue-sensitive cornea-positive signal and a green-sensitive cornea-negative signal, respectively. In this paper they will be called the B- and C-signals. Quantitative studies on these electrical signals have been made by several groups of researchers (Cone, 1965, 1967; Arden, Ikeda and Siegel, 1966; Pak and Ebrey, 1966; Pak and Boes, 1967). Ebrey (1968) has been able to measure several kinetic quantities of the three intermediates, A, B and C in excised eyes of the albino rat at different temperatures. The results are compatible with other spectroscopically determined data.* He obtained the decay rate of B indirectly from the analysis of the C signal by the mother-daughter relation of B and C. Originally this work was suggested and initiated by Ebrey to see if the same decay rate of B can be obtained by a direct measurement of the B-signal.**

## Method

In order to describe the individual features of and the relations among the series of experiments clearly and in a condensed form, it is convenient to adopt simple notations for several different types of operations. Thus,

*Bleach:* the operation *Bleach* means that a strong continuous 100 W tungsten light was focused into an excised eye in order to bleach all of the Rh. The wavelengths of the light above about 900 nm and below about 460 nm were cut off with Corning CS 1-69 and CS 3-71 filters. A diffuser was used to give a uniform illumination and was made out of a sheet of glass (2″×2″) coated with a half-transparent "Scotch Magic" tape. All of the Rh was bleached after a fifteen second illumination.

*Blue:* the operation *Blue* means that a 0.5 msec blue test flash was directed into the eye with a Honeywell Strobonar 600 electronic flash and a Corning CS 7-51 narrow band-path filter (365±30 nm) and the diffuser. An FPV was recorded across the eye.

*Green:* the operation *Green* is the same as *Blue* except that a green filter (Kodak 58B, 530±30 nm) was used instead of a blue filter.

*$\Delta t$:* the operation *$\Delta t$* means to wait for a certain length of time $t$ between two operations.

Bracket: a pair of solid brackets, [ ], means to repeat several times the processes in them.

* See the references cited in Ebrey (1968).

** Most of the equipments used in this experiment are the same as by Ebrey (1968).

Underline: the underline of an operation means that a series of experiments were done by changing the underlined operation in various ways.

The eyes were excised from dark-adapted albino rats (Sprague-Dawley, male, about 150 g) and were mounted between two wick electrodes soaked with saline solution and touching the cornea and the back of the eyeball. The FPV signals were displayed on a Tektronix 564 storage-type oscilloscope amplified by a Princeton Applied Research CR-4 or a Tektronix 122 low-level preamplifier.

## Results and Discussion

*Experiment i)* *Bleach* *Δt* *Blue.*

The object of this experiment was to measure the decay of the B-signal versus time and to compare this to Ebrey's measurement. The half-lives of PrelumiRh, LumiRh, and A are so short (Wald *et al.*, 1963; Hubbard *et al.*, 1965; Abrahamson and Ostroy, 1967) even at room temperature that several seconds after the operation *Bleach* all of the Rh has decayed into blue-sensitive B which slowly changes into blue-insensitive C.* If the maximum amplitude of the FPV (*R2*, the cornea-negative component) was measured with the *Blue* before *Bleach*, the ratio $B_{max}/R2$ obtained from different eyes can be plotted as a function of the time interval $t$. $B_{max}$ is the maximum amplitude of the B-signal. This set of data gives the tnermal decay rate of B at a specific temperature. This experiment was conducted at five different temperatures

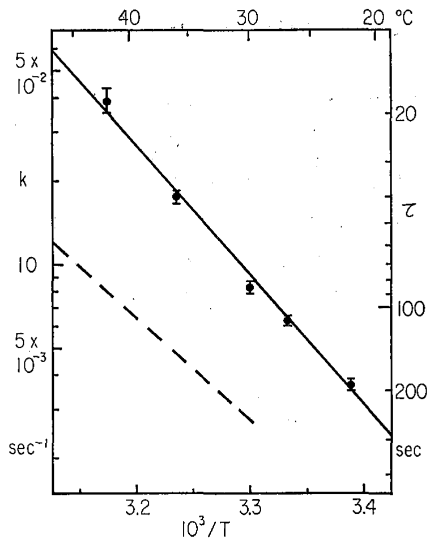



Fig. 1. Arrhenius plot of decay rate of the intermediate B in the bleaching process of excised eyes from the dark-adapted albino rat (*Experiment Bleach Δt Blue*). After a 15-sec illumination of a strong yellow light (>480 nm) on the excised eye, the B-signal responding to a blue test flash (365±30 nm) was measured with different time intervals $t$. The ratio of the $B$ and $R2$ component of the FPV, which has been measured with the same blue test flash before the bleaching is getting smaller with the increase of $t$, according to a first-order decay equation. Each decay constant $k_B$ of B at a certain temperature was obtained from about five different fresh eyes. The broken line was taken from Ebrey (1968), who measured the same quantity $k_B$ indirectly from the analysis of the grow-and-decay curve of the C-signal responding to a series of successive green test flashes (530±30 nm) on C.

* The term blue-sensitive means here that the 365 nm of light used in this experiment does not appreciably induce the C-signal.

from 20 to 42°C. Each plot (four~eight points) of $\log(B_{max}/R2)$ versus $t$ gave a fairly good fit to a straight line, from which the decay rate $k$ was obtained. Fig. 1 shows an Arrhenius plot ($k$ versus $T^{-1}$), from which the following thermodynamic quantities were obtained: $\Delta H^* = 20.7$ kcal/mole, $\Delta S^* = 0$ e. u., and at 30°C $\Delta F^* = 20.6$ kcal/mole and $Q_{10} = 3.2$.

The decay rate of B obtained by this direct method is systematically larger by a factor of three or four than the decay rate of B inferred by Ebrey (1968) from the growth rate of C, although his experimental procedure, when carefully followed, gave almost the same results as he got. Because of this discrepancy between these two experiments it seemed necessary to seek a third experiment to measure the decay rate of B.

*Experiment ii)* *Bleach* [*Blue* $\underline{\Delta t}$].

With all the equipments in *Experiment i* being used, successive blue flashes (time interval, $t$) were given to a single eye after *Bleach*. The B-signal was found to decay much faster than in *Experiment i*. This observation can be explained if a fraction, $\alpha$, of B is photodecomposed by each operation of *Blue*. If the observed apparent half-life and the thermal half-life of B are denoted by $\tau_B^{obs}$ and $\tau_B$ respectively ($\tau = 0.693/k$), these quantities are related to each other by the following equation,

$$t/\tau_B^{obs} = t/\tau_B - 3.322 \log(1-\alpha).$$

A value of $\tau_B^{obs}$ is observable from each experiment on a single eye with each experiment at a different $t$. The experiment was performed at 23°C with four different values of $t$ ranging from one to three minutes. As long as the size of the B-signal was larger than the experimental error, the B-signal shape remained constant (peaking at $1.2 \pm .1$ msec and decaying with a half-life of 1.5 msec) and the peak intensity decreased exponentially for at least one log-unit (see Fig. 2). This means that B is not appreciably contaminated by C at least in the early stage of the decay of B. When the values of $t/\tau_B^{obs}$ are plotted against $t$, a good fit to a straight line is found as shown in Fig. 3. Each point represents an average of two or three different measurements. From the slope and intercept of the line the values of $\tau_B = 260$ sec and $\alpha = 0.2$ were obtained. The former value agrees fairly well with the value of 180 sec at 23°C obtained in *Experiment i*. This small discrepancy might be explained by a photo-regeneration of Rh from B. However, with my equipment no measurable amount of regenerated FPV could be detected.* Ebrey (1968) showed that B can probably be photo-decom-

* Cone (1967) has shown the partial regeneration (nearly twenty per cent) of FPV by a blue flash on B. However, in my experiment the peak wavelength of transmittance of the filter is shorter and the test flash is much weaker than his experiment.

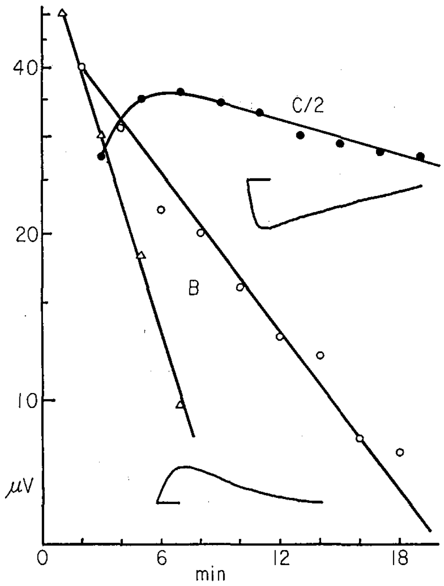



Fig. 2. Comparison of the decay rates of the intermediate B observed by a series of successive blue flashes and by alternating blue and green flashes in the bleaching process of eyes excised from the albino rat. In both the experiments all the rhodopsin was bleached by a continuous illumination of a strong yellow light (>480 nm) shortly before a series of test flashes, while the temperature was kept at 23°C. The peak intensity of the B-signal obtained with a blue flash (365±30 nm) was decaying exponentially as marked by triangles ($\tau_B^{obs}$=160 sec for a time interval of two minutes). If between every two blue flashes a green test flash (530±30 nm) was given, the half-life of the B-signal (open circle) was increased by a factor of more than two ($\tau_B^{obs}$=380 sec) showing the photo-regeneration of B from C by a green light. The rising and decaying stages of the C-signal (filled circle) responding to the green flash were also observed. Traces of typical B- and C-signals are also given. The calibration lines are one msec.

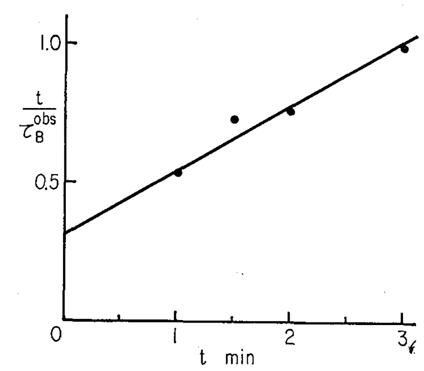



Fig. 3. Experimental plot for obtaining the half-life ($\tau_B$) of the intermediate B and the fraction ($\alpha$) of photo-decomposing B by each blue test flash on a bleached eye excised from the albino rat (*Experiment Bleach* [*Blue* $\Delta t$]). By a successive series of blue flashes (365±30 nm) with a constant time intervals ($t$), the B-signal is decaying with a half-life of $\tau_B^{obs}$ which is shorter than a thermal $\tau_B$ because of the photochemical decay of B to C. Shortly before shining these blue flashes the eye has been exposed to a continuous strong yellow light (>480 nm) for 15-sec to bleach all the rhodopsin. If a set of $\tau_B^{obs}$'s are measured with different $t$, the values of $\tau_B$ and $\alpha$ can be obtained graphically on a plot of $t/\tau_B^{obs}$ versus $t$ by use of the following relation,

$$t/\tau_B^{obs}=t/\tau_B-3.322\ \log(1-\alpha).$$

Each point is an average of data from two or three different eyes at 23°C. From the slope of the straight line $\tau_B$ (260 sec) is obtained. The intercept at the ordinate gives $\alpha$=0.2.

posed into C by an intense blue light.* (Therefore the following scheme is being followed by this experiment, B $\rightleftarrows$ C) On the other hand, the value of about 480 sec extrapolated from his experiment is again too large to be interpreted as the half-life of the same species. Before discussing this discrepancy, let us consider his experiment.

* Personal communication.

*Experiment iii*) (Ebrey's experiment) *Bleach* [*Green* $\underline{\Delta t}$].

By successive operations of *Green* on the bleached eye, the C-signal comes out instead of the B-signal in *Experiment ii.* Ebrey (1968) found that in the initial stage of the flashing the C-signal was increasing to a certain value and then decreasing slowly. He also found that the apparent decay rate is decreasing with the increase of the time interval between every two green flashes. These results were analyzed by him based on the mechanism

$$\mathrm{B} \longrightarrow \mathrm{C} \rightleftarrows \text{Bleached substance.}$$

From the rising and decaying parts of the observed curve, log (peak intensity of the C-signal) versus time, the decay rates of B and C were obtained, respectively. As mentioned earlier his results were confirmed by the present author and the values of $k_B$ obtained by this indirect method is much more smaller than that obtained in *Experiments i* and *ii.* However, it should be pointed out here that the C-signal shape was found to be changing in the rising stage. This might suggest some complicated mechanism and/or existence of some unknown factor making the result of this experiment erroneous.

One possible explanation for the discrepancy is that there must be some mechanism which retards this decay scheme during successive operations of *Green.* In this regard Cone (1967) has suggested the photo-regeneration of B from C, although no experimental evidence seems to have been given. The following experiments were conducted to show this regeneration, which can explain the discrepancy.

*Experiment iv*) *Bleach* [*Blue* $\Delta t$ *Green* $\Delta t$] or *Bleach* [*Green* $\Delta t$ *Blue* $\Delta t$].

Since the essential features of their results are almost the same, only the former experiment will be described. By the alternating operations of *Blue* and *Green,* we can observe both of the decay rates of the B- and C-signals from the same eye. As expected, the B-signal was monotonously decaying, while the C-signal rose and then decayed as in *Experiment iii.* At first sight they seemed to show a good superposition of the results of *Experiments ii* and *iii.* The half-life of C is in the order of an hour at room temperature approximately the same as in *Experiment iii.** However, the decay rate of the B-signal was retarded by a factor of more than two when a comparison was made between the results of this experiment and *Experiment ii* both with the same time interval between two operations of *Blue* (see Fig. 2). This slower decay of the B-signal can be explained as the photo-regen-

* In principle the two $\tau_C$ values from *Experiments ii* and *iv* are different from each other. However, analysis was not tried, since at room temperature they are too large and erroneous.

eration of B from C by the operation of *Green* as in the following scheme,

$$B \rightleftarrows C \rightleftarrows \text{Bleached substance.}$$

There is further evidence for the photo-regeneration of B from C. That is, in *Experiment ii* after the B-signal decayed away, it could be regenerated just by the operation of Green on that eye. The regeneration was not complete, but the more intense the flash the more the B-signal recovered, which in turn obeyed the original decay curve. Although the ratio of the photo-conversion of C to B and to the bleached substance could not be measured, most C seemed to be regenerated back to B by *Green*.

*Kinetic consideration*

The above discussion is based on the standpoint that the result of *Experiment iii* is erroneous unless it is interpreted by a mechanism more complicated than what was adopted by Ebrey (1968). On the other hand the following discussion is based on the standpoint that both of his and the present experimental results are correct but they correspond to different reactions.

Many possible mechanisms involving successive and/or parallel decaying schemes of B and C were assumed and the rate equations were solved. It was deduced that in any mechanism where B is the precurser of C the concentration of C is increasing in the initial stage with the decay rate of B, $k_B$. The following is the only mechanism in which C is increasing with the rate different from the decay rate of B.

$$B \xrightarrow{k_B} X \xrightarrow{k_X} C \xrightarrow{k_C} \qquad k_B \gg k_X \gg k_C\,,$$

where X is an intermediate, or a precurser of C. Under the condition above the concentrations of B, X and C at time $t$ are expressed as

$$[B]=[B]_0 \exp(-k_B t)$$

$$[X]\fallingdotseq[B]_0\{\exp(-k_X t)-\exp(-k_B t)\}$$

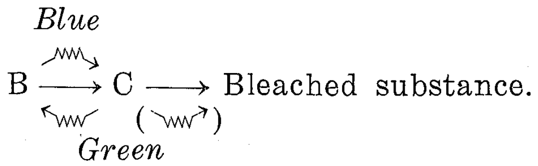
$$[C]\fallingdotseq[B]_0\{-\exp(-k_X t)+\exp(-k_C t)\}\,.$$

At time zero they are assumed to be $[B]_0$, 0, and 0.

Thus it is inferred that *Experiment iii* by Ebrey gives the rate constants $k_X$ and $k_C$, but not $k_B$. However, there has been no spectral evidence for the existence of any intermediate between B and C.

*Conclusion*

All of the reaction happening in *Experiments i–iv* can be represented by the following scheme, in which the wavy arrow in the parentheses indicates a weak effect,*




The most probable explanation for the observed discrepancy between the results of *Experiments i* and *ii* and *Experiment iii* by Ebrey is the photo-regeneration of B from C.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* In this scheme *Blue* and *Green* mean the most effective wavelength regions of light.