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THE EFFECT OF TEMPERATURE ON THE BIPHASIC PHOTORESPONSES OF AN ALL-*TRANS*-RETINAL BIMOLECULAR LIPID MEMBRANE

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SUMMARY

1. The bimolecular lipid membranes formed from lipid solutions containing all-*trans*-retinal have been studied. These membranes can generate, upon illumination, temperature sensitive biphasic photopotentials which are seen to be quite similar to the early receptor potentials of visual receptors.

2. The photoresponse consists of the fast component (R1), and the slow component (R2) which has the sign opposite to R1.

3. Under certain experimental conditions a complete phase shift from R1 to R2 occurs as the temperature is increased from 9 to 45°.

4. R1 and R2 are not influenced by changes in the ionic strength of K^+, Na^+ and Cl^-, nor by generation of the concentration gradient of these ions across bimolecular lipid membrane.

5. The mode of biphasic photoresponses, however, can be influenced by pH gradient across the membrane, the buffer capacity of the aqueous solutions, and light intensity.

6. A possibility of applying the above observations to the elucidation of the mechanism of generation of the early receptor potentials is discussed.

INTRODUCTION

In recent years experimental bimolecular (bilayer) lipid membranes have been studied extensively as a model for a variety of biological membranes. The results of these studies and the relevance of bimolecular lipid membranes in the understanding of biological membrane structure and function have been summarized in two comprehensive reviews[1,2]. Recently, it has been shown that the pigmented membranes can exhibit interesting photovoltaic effects, and the photoelectric action spectra of the membranes parallel closely with the adsorption spectrum of the involved pigments[3]. Experiments carried out by Hesketh[4], Kay and Chan[5], Ullrich and Kuhn[6], Mauzerall and Finkelstein[7], Läuger and co-workers[8,9], Pant

Abbreviations: R1 and R2 are, respectively, the fast and slow components of the photoresponse.

and Rosenberg[10], and by Cherry *et al.*[11] have demonstrated a number of new and related photoelectric effects in pigmented membranes. We have reported the use of membranes containing carotenoid pigments as a model for the visual receptor membrane[12]. The present paper is concerned with studies on the temperature dependence of the biphasic photo-emf's of an all-*trans*-retinal bimolecular lipid membrane. Specifically the following questions were asked: (1) would it be possible to demonstrate temperature-dependent biphasic photoresponses with all-*trans*-retinal bimolecular lipid membranes? and (2) if so, what would be the likely mechanism by which temperature could influence the mode of the photoresponses?

EXPERIMENTAL

Experimental details are essentially the same as those given elsewhere[12,13]. Briefly, the membrane forming solution used in the present work was prepared by dissolving lecithin and oxidized cholesterol in *n*-octane. The major lipid constituents of the present membrane are the following surface active materials: lecithin, oxidized cholesterol, and all-*trans*-retinal (Eastman Kodak Co., Rochester, N.Y.).

The cell arrangement may be represented as follows: salt bridge/outer solution/bimolecular lipid membranes/inner solution/salt bridge. The potential difference across the membrane was measured with an electrometer of high input impedance (Keithley, Model 610B). The output of the electrometer was fed into a strip chart recorder (Servowriter II, Texas Instruments, Houston, Texas).

The photoexcitation of the membrane was achieved by illumination with a Sylvania tungsten halogen lamp (DWY, 650 W, Sylvania Electric Products, Salem, Mass.) filtered through 2''-$CuSO_4$ solution. An Alphax shutter (Wollensak, Rochester, New York) was used for controlling the duration of the illumination.

The temperature of the aqueous phases was measured with thermometers with 0.1° divisions and a range from −1 to 50°. The thermometers were placed in each of the chambers and checked repeatedly throughout the experiments. The temperature of the chambers was controlled by running water thermostatically regulated by the use of a Haake circulator (Type Re, Berlin, Germany), through the bottom chamber of the cell assembly. The temperature was maintained within ±0.05°. All experiments were carried out at equilibrium in temperatures.

In the absence of electrical and chemical asymmetry across the membrane, it was found in the early stages of this work that the photoresponses were small and not very reproducible. This could be due to the variation of microenvironment around the membrane or around the Plateau–Gibbs border[2] which supports the membrane. Asymmetric charge distribution could also originate from the uneven distribution of lipid at the border or slight hydrostatic pressure gradient across the membrane[3]. In order to remove this difficulty a number of substances for the generation of membrane asymmetry were tested. $FeCl_3$ was selected since it gave a significant enhancement of the photoresponse as well as reproducible dark potentials.

RESULTS

The effect of temperature on the photoresponses

All photoresponses were obtained at equilibrium temperatures. The aqueous phases were symmetric with respect to 0.1 M KCl and only the inner solution con-

tained 0.001 M $FeCl_3$. The photoresponses for 1-sec illumination at various temperatures ranging from 9 to 44° are presented in Fig. 1. At room temperature (25°) the photoresponse showed a distinct biphasic reponse. When the temperature was raised to 44° only R2 component could be seen. On the other hand the lowering of temperatures down to 9° abolished the R2 component completely and only the R1 component remained.

The magnitude of R1 and R2 varied from membrane to membrane, even at a given temperature, so much that a direct comparison of the magnitudes of the photoresponses at various temperatures could not be carried out meaningfully. The ratio of the magnitude of R2 to that of R1, however, was relatively free from membrane variations and was the major concern. This ratio was strongly dependent on temperature. It can be seen that the complete disappearance of the R1 component occurred above 36° and that of R2 below 16°. R2 exhibited the same amplitude to R1 at a slightly lower temperature than room temperature.

The general observations about the effect of temperature on the mode of photoresponses were strengthened by the studies carried out with single membranes subjected to various temperatures. In this work it became possible to relate the photopotential to the dark membrane properties as a function of temperature.

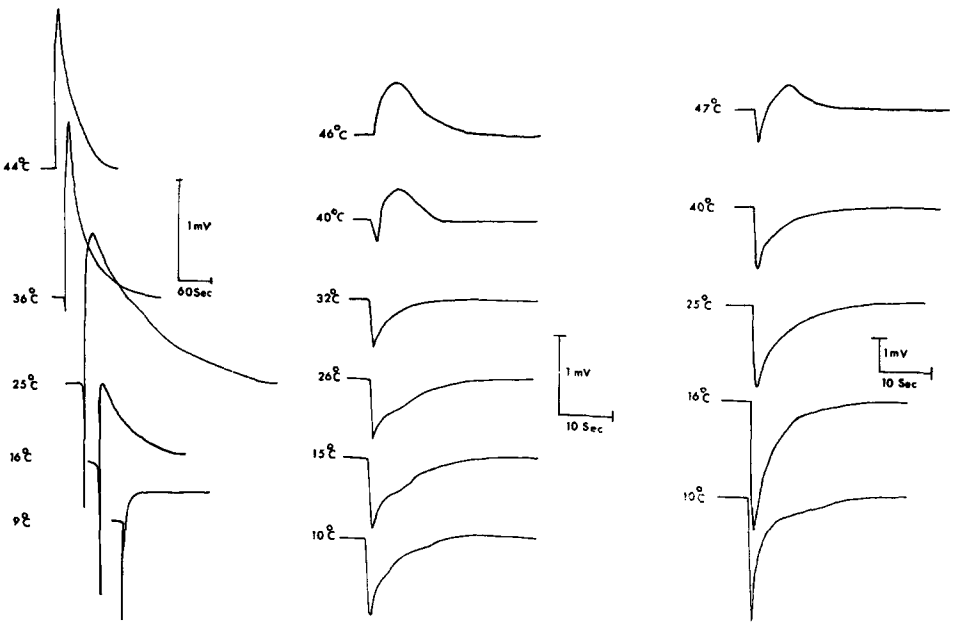

Fig. 1. The time course of the photoresponse of all-*trans*-retinal bimolecular lipid membrane. 0.001 M $FeCl_3$ was placed in the inner chamber. The duration of the illumination was 1 sec. The outside pH was 5.4 and the inside pH was 3.3.

Fig. 2. The time course of the photoresponses of all-*trans*-retinal bimolecular lipid membrane as a function of temperature: HCl system. The aqueous phases contained only HCl with the inside pH at 2.8 and outside pH at 3.5. The duration of the illumination was 1 sec.

Fig. 3. The time course of the photoresponses of all-*trans*-retinal bimolecular lipid membrane as a function of temperature: $FeCl_3$ system. The aqueous phases contained only HCl with the outside pH at 3.5. The inside chamber contained, in addition to HCl, 0.001 M $FeCl_3$ to lower pH to 2.8. The duration of illumination was 1 sec.

The time course of the photoresponses obtained with the system which contained only HCl with pH difference of 0.7 are shown in Fig. 2. The same features observed in Fig. 1 can be seen but the temperatures for the appearance of R2 and the disappearance of R1 were higher than those of Fig. 1. This difference may be due to a smaller pH difference across the membrane system in the latter case.

The data shown in Fig. 3 were obtained in the presence of Fe^{3+} in the inner solution while maintaining the same pH difference as the system used for obtaining the data presented in Fig. 2. The magnitude of the photoresponses was increased by approx. 4 times. The temperature for the appearance of the R2 component was elevated to about 40° and the R1 component could be seen even at 47°.

The dark membrane potentials of both HCl and $FeCl_3$ systems increased with increasing temperature (see Fig. 1, ref. 12). The thermal coefficients of these two systems were similar (Table I). Due to the same magnitude of the dark membrane potential development and the identical thermal coefficient between the HCl system and the $FeCl_3$ system, it is very likely that the differences of photoresponses between the two systems may be attributed to the presence of Fe^{3+}, which are excellent electron acceptors.

TABLE I

THE THERMAL COEFFICIENTS OF DARK MEMBRANE POTENTIAL, MEMBRANE RESISTANCE, AND R1 AND R2 COMPONENTS

Parameter observed	*Thermal coefficient (kcal)*	
	*HCl system**	*$FeCl_3$ system***
Dark membrane potential	5.1	4.9
Membrane resistance	6.9	6.9
R1 component	9.1	6.9
R2 component	18	15

* Experimental details are given in Fig. 4a.
** Experimental details are given in Fig. 4b.

The semilog plots of the R1 component and the dark membrane resistance against the reciprocal temperature (Fig. 4) revealed the close relationship between the two properties. Under this particular experimental condition the thermal coefficients of the dark membrane potential, dark membrane resistance, and the R1 component were almost identical (Table I). The R2 components had relatively higher thermal coefficients.

Light intensity dependence of the photoresponses

Due to the fact that the photoresponses could be influenced by the dark membrane potential and/or pH differences across the membrane, the intensity dependence was studied at various pH differences as a function of temperature.

The data shown in Figs. 5 and 6 were taken by making a membrane at a given temperature, varying the pH difference and measuring the intensity dependence of the photoresponses at each pH difference. The inner solution contained 1 mM $FeCl_3$ in 1 mM KCl solution and the pH of the outer chamber was varied by the addition of HCl to the original 1 mM KCl solution. R1 and the ratio of R2

to R1 were plotted against the light intensity as a function of temperature for a given pH difference.

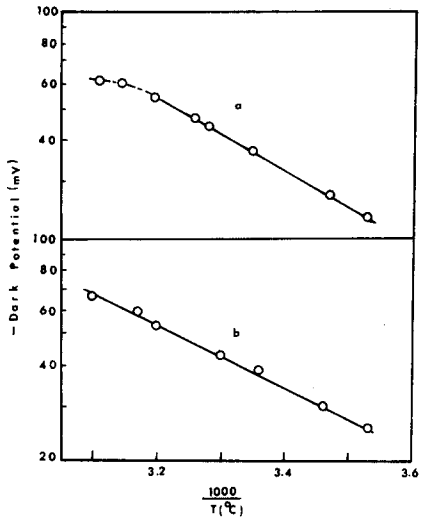

Fig. 4. The temperature dependence of the dark membrane potential of all-*trans*-retinal bimolecular lipid membrane. (a) The aqueous phase contained only HCl with the inside pH at 2.8 and the outside pH at 3.5. The thermal coefficient obtained from the slope of the straight line is 5.1 kcal. (b) The aqueous phases contained only HCl with the outside pH at 3.5 and the inside chamber contained, in addition to HCl, 0.001 M $FeCl_3$ to lower pH to 2.8. The thermal coefficient obtained from the slope of the straight line is 4.9 kcal.

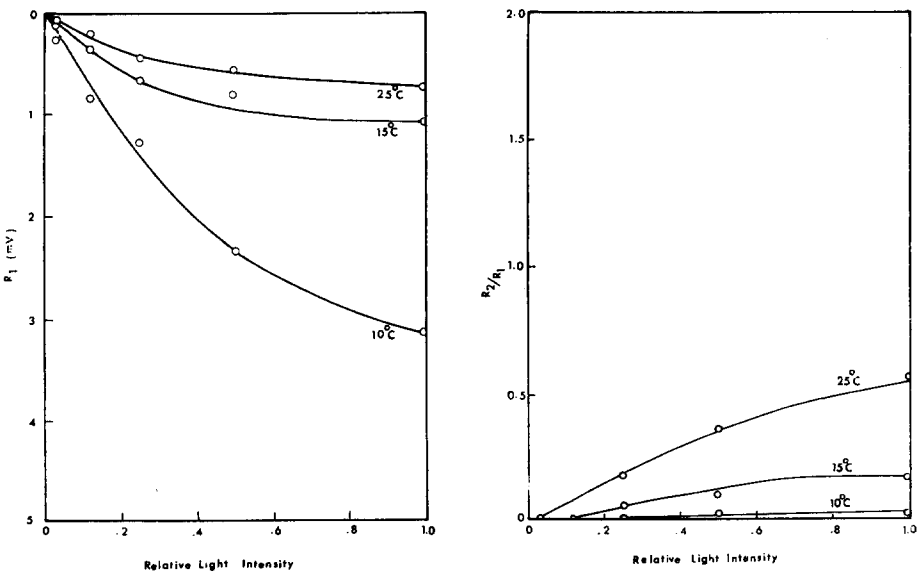

Fig. 5. The effect of relative intensity of light on R1 component at ΔpH 0.3 as a function of temperature. The inner pH was 3.3 and the outer chamber contained HCl and the same concentration of KCl.

Fig. 6. The effect of relative intensity of light on R2/R1 at ΔpH 0.3 as a function of temperature. The inner pH was 3.3 and the outer pH was higher. The inner chamber contained about 1 mM $FeCl_3$ in 1 mM KCl while the outer chamber contained HCl and the same concentration of KCl.

The intensity dependence of the R1 component can be seen as a monotonously increasing (or decreasing) curve in all cases. At a proper pH difference the ratio of R2 to R1 had a constant value within a limited range of the intensity variation.

The effect of pH difference across the membrane was remarkable. The R1 component was almost insensitive to a change in pH gradient while the R2 component increased with an increase in pH gradient. This can be seen clearly in the plot of R2/R1 against the light intensity where the values increased generally with increasing pH gradient (Fig. 6).

Temperature dependence of R1 was evident, since at lower temperatures higher photoresponses were observed. The effect of temperature on the ratio of R2 to R1 could be seen clearly: at higher temperatures the positive peak increased with respect to the magnitude of the negative peaks.

At lower pH difference it can be seen that the generation of the R2 component requires higher intensity than that of the R1 component. In order to observe the relationship more closely, the intensity dependence of the photoresponses of the symmetric 0.1 M KCl system was investigated. Shown in Fig. 7, the magnitude of R1 and R2 could be fitted to a straight line in semilog plot of the photopotential against the light intensity. The threshold intensity was higher in the case of R2. On the other hand, after its appearance, the R2 component had greater intensity dependence than the R1 component.

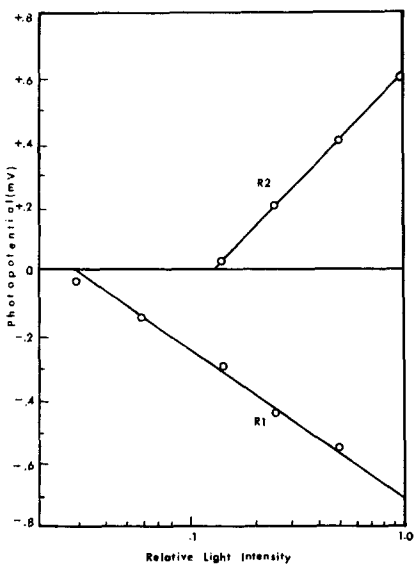

Fig. 7. The effect of light intensity on the photopotential of all-*trans*-retinal bimolecular lipid membrane. The membrane was formed in 0.1 M KCl solution without making any chemical asymmetry. The photopotentials were recorded from a single membrane.

DISCUSSION

Background of the early receptor potential

Using light flashes Brown and Murakami[14] observed in 1964 a new photoresponse in the monkey eye, which had a latency about 1 μsec and a duration in

the msec range. This fast photoeffect is now known as the "early receptor potential"[15]. At room temperature the early receptor potential had two components in the frog eye, as was shortly reported by Cone[16]. The first corneo-positive phase was observed to reach a peak value in about 100 μsec, and was followed by the second corneo-negative phase with a peak time about 900 μsec. Since the sign of two components depends on the position of the recording electrodes, Cone proposed the use of R1 and R2 for the first and second components of the early receptor potential, respectively. Pak[17] used R1 and R2 for the two components of "rhodopsin responses". It is due to this discovery of the biphasic nature of the early receptor potential that the understanding of the mode of its generation has been strengthened, since various experimental conditions are now known to influence R1 or R2 independently.

Temperature is the most effective and first discovered parameter in controlling the mode of generation of the early receptor potential. Pak and Cone[18] and later Pak and Ebrey[19] demonstrated the different temperature dependence of R1 and R2 in the albino rat eye, and discovered that lowering the temperature sufficiently abolished R2 and isolated R1, which could be detected even at a subzero temperature of $-35°$. Similar results were observed in the frog eye by Brindley and Gardner-Medwin[20].

The occurrence of temperature-sensitive biphasic photoresponse is not restricted to the retina. It is now established that the early receptor potential can be found in non-ocular pigment epithelium and the leaves of green plants[15]. In spite of the minor differences in response time and sensitivity toward temperature variations, all photoresponses observed in these pigmented tissues show essentially the same features of the early receptor potential and, hence, indicate the common mechanisms of the origin of the initial photoresponse in all pigmented biological tissues.

The necessity of the membranous structure for the generation of the early receptor potential

At the present time the early receptor potential has not been obtained with aqueous suspension of rhodopsin or any other pigments or even with the aqueous suspension of rods and cones[22]. Instead, the optimum early receptor potential can only be observed in membranous structures with high electric resistance and proper orientation of the pigment molecules. Hagins and McGaughy[21] have suggested that the organized pigment arrangement in membrane structure is required for the generation of the early receptor potential. Under these conditions it was found that, although the absorption spectrum of rhodopsin was not affected, the microspectrophotometric measurement indicated a complete disorientation of rhodopsin molecules from the intact membrane, as if rhodopsin molecules were in a solution.

The high efficiency of cone early receptor potential was explained by Goldstein and Berson[23] as an indication of the structural difference between cones and rods. In rods the presence of outer membrane separated from pigmented discs was rather inefficient for generation of the early receptor potential. From this observation Goldstein concluded that the membranous structure was necessary and that the early receptor potential was considered to be generated across the pigmented membranes. The involvement of the cell membrane in generation of the early receptor potential was also indicated by the observation with the pig-

ment epithelium[15]. After penetrating a pigment epithelium cell, the membrane potential declined because of possible membrane damage by the microelectrode, and R2 of the responses decreased more rapidly than R1. This could suggest that the second phase was specifically dependent upon the integrity of the cell membranes.

There seem to be differences in the functions of the membranous structures in generation of the early receptor potential in various types of photoreceptors as well as the structural differences. In the pigment epithelium and the leaves, the cellular membrane is not primarily pigmented and the initial photoevent has to be transferred to the cell membrane from the discontinuous pigment granules. On the other hand, in the rods and cones the disc membrane contains the pigment molecules without any auxiliary structures. The consequence of this difference between two types of photoreceptors on the function of the membranous structure in the generation of the early receptor potential may be seen in the following two points.

The first point is the necessity of the mediator of the early receptor potential generation, which not only can develop the cross-membrane potential but also can interact or be transferred to the membranous structure located away from the original site of the formation of the mediator. The nature of the mediator is totally unknown at the present time. Nevertheless, the identification of such a mediator may help us to understand the mechanism of the generation of the early receptor potential in all photoreceptors. The second point is that, although the understanding of the mechanism of generation of the early receptor potential of the pigment epithelium and the plant cells would certainly contribute to the understanding of that of the rods and cones, the above characteristic difference has to be taken into consideration. Furthermore, this difference could be so important that the substitution of the former photoreceptors for the study of the latter photoreceptors may not be desirable. This point has to be seen in future works with various photoreceptors.

Significance of present experimental findings

For the purpose of discussing the effect of temperature on the biphasic photopotentials using the theory developed previously[12], it will be useful to recall the relationship between temperature and protonic membrane conduction, which controls both dark membrane potentials and mode of biphasic photopotentials. In this section the relevant findings are considered in some detail by referring to biological observations, in relation to the mechanism of the generation of the early receptor potentials and its relationship to the generation of electroretinograms.

Since the early receptor potentials are most likely to be membrane phenomena[24], it seems probable that the transition from "non-ionic" phenomenon to "ionic" phenomenon (electroretinogram) can take place across the disc membrane, *i.e.* the electroretinograms may be explained as the induced potentials of Na^+, K^+, and Cl^- by the initial rapid protonic potentials (early receptor potentials) as diagrammed in Fig. 8.

The basic idea to be examined is that the mechanism for the explanation of the biphasic photoresponses of all-*trans*-retinal membranes may have a biological role as the primary process leading to generation of the receptor potentials. Refer-

ring to Fig. 8, the proposed mechanism may be summarized as follows: the photo-induced redox reactions generate, by the diffusion of holes, the fast phase of the biphasic early receptor potentials, and the transport of the photogenerated protons is responsible for the slow phase of the potentials. The proton transport may further induce ionic transport as a sequentially coupled transport phenomena. This mechanism may be called a redox–protonic–ionic excitation mechanism.

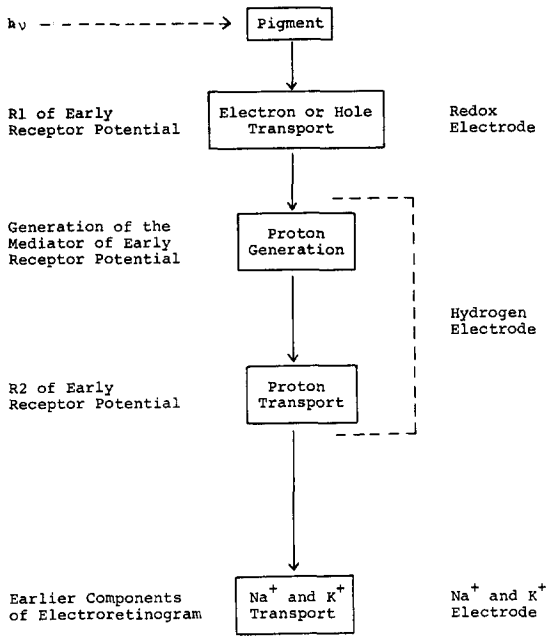

Fig. 8. The schematic diagram of photoelectric transduction showing how H^+ generation and transport can function as the intermediate stages (early receptor potentials) leading to the ultimate Na^+ and K^+ transport (the earlier component of electroretinogram) across the photoreceptor membrane.

The possibility of coupling electron movement across the biological membrane to ionic movement has been suggested by many investigators. In particular, the coupling of electron transport to ionic transport in mitochondrial and photosynthetic phosphorylation proposed by Mitchell[25] is of interest. Jahn[26] has included non-enzymic electron pathways (such as β-carotene and vitamin A derivatives) which could be coupled to ion transport. Further, Jahn[27] has extended the possibility of the similar mechanism to visual excitation. Jahn's theory of visual excitation may be summarized as follows: light absorption leads to the conformation change of retinal from 11-*cis* to all-*trans*, thereby increasing the probability of the transport of the electrons made available through redox systems which could exist across the membrane. However, since the discovery of the early receptor potential of the photoreceptors, the application of Jahn's theory to the interpretation of generation of the potentials has not been attempted[2]. Judging from the observations made in the present work, it appears likely that Jahn's theory can be applied to the explanation of the generation of the early receptor potentials.

Furthermore, the three-step excitation mechanism outlined above may, indeed, function in visual photoreceptors if electronic movement can initiate protonic movement, which, in turn, generates potassium and sodium movement as proposed in the present work.

The successful demonstration of the biphasic photoresponse in an all-*trans*-retinal membrane clearly indicates that this model membrane can be used for the study of the early receptor potential. Further, the temperature dependence of the mode of the generation of this biphasic photoresponse closely resembles that of the early receptor potential. The similarities between the early receptor potential and the photoresponse of all-*trans*-retinal membranes as a function of temperature can be seen in the following two points: (i) at the lower temperature the fast component (R1) dominates the photoresponse while at the higher temperature the slower component (R2) dominates, and (ii) the shift from R1 to R2 can occur within the temperature range from 0 to 50° under proper experimental conditions.

It is likely, however, that the type of pH changes which generate the proton concentration across the photoreceptor membrane can influence the mode of generation of the early receptor potential, as demonstrated in the present work in the case of the photoresponse of all-*trans*-retinal membrane separating two aqueous phases which are different in their ionic composition.

Finally, the present findings demonstrate that it is possible in principle, in view of available evidence concerning the early receptor potentials, to explain the effect of temperature on the mode of generation of the early receptor potentials without considering the photochemistry of rhodopsin and its photoproducts. Those workers who subscribe to the rhodopsin derivative hypothesis have reached the conclusion that the early receptor potential is not the main pathway of the visual excitation[17]. The present work, however, provides a rational basis for constructing an alternative mechanism of the generation of the early receptor potential by taking account of the active function of the membranous structure to its origin, and the proton as the mediator of the generation of the early receptor potential. At the present time, the connection between the early receptor potential and the generation of the electroretinogram as coupled consecutive membrane phenomena is obscure. Perhaps future investigations using the bimolecular lipid membranes system may provide us with the necessary insight.

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