THE EFFECT OF TEMPERATURE ON THE BIPHASIC PHOTOPOTENTIALS OF ALL - TRANS - RETINAL BIMOLECULAR LIPID MEMBRANES

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ABSTRACT

THE EFFECT OF TEMPERATURE ON THE BIPHASIC PHOTOPOTENTIALS OF ALL-TRANS-RETINAL BIMOLECULAR LIPID MEMBRANES

By

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The bimolecular lipid membranes formed from lipid solutions containing all-trans-retinal could generate, upon illumination, temperature-sensitive biphasic photopotentials which were quite similar to the early receptor potentials of visual receptors and pigmented animal and plant tissues: (1) The photoresponses consist of the fast component (R1), and the slow component (R2) which showed the sign opposite to R1. (2) At proper experimental conditions a complete phase shift from R1 to R2 took place as the temperature increased from 9°C to 45°C. (3) R1 and R2 were not influenced by changes in the ionic strength of K^+, Na^+, and Cl^-, nor by generation of concentration gradient of these ions across the membrane.

R1 was concluded to be the potential which was mainly determined by hole diffusion from the following observations: (1) The sign and magnitude of R1 could be influenced only by strong electron acceptors such as ferric ions. (2) R1 was independent of pH gradient and buffer capacity. (3) The side containing ferric ions showed negative photopotentials.

R2 was identified as the potential which was strongly dependent on protonic diffusion by the following observations: (1) Changing proton concentration gradient across the membrane could control R2 in such a manner that the magnitude of R2 was linearly proportional to the logarithm of the proton concentration gradient. (2) R2 was abolished in the presence of strong buffer capacity.

The effect of increasing temperature could be seen as an enhancement not only in R2 but also in dark membrane potentials and membrane conductances. This observation was interpreted to mean that the effect of temperature was to increase proton mobility in the hydrocarbon layer of the membrane. When this finding was applied to the theory developed in this work to account for the time course of the photopotentials, the temperature dependence of the biphasic photopotentials could be explained satisfactorily as the consequence of an increase in the proton conductance relative to the hole conductance.

The mode of biphasic photopotential could also be influenced by pH gradient across the membrane, the buffer capacity of the aqueous solutions, and light intensity. The theory developed in this work could account for these observations as well as the temperature effect.

A possibility of applying the observations carried out in this work to the elucidation of the mechanism of generation of the early receptor potentials was examined.

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CHAPTER I

INTRODUCTION

Since the first report on the techniques of the formation and properties of black lipid membranes in aqueous solutions by Mueller, Rudin, Tien, and Wescott (1962a, b, 1963, 1964), quite a new area of membranology has been developed. The revival of colloid chemistry was inevitable, since the structure, stability, and formation processes of this ultra-thin membrane had to be understood in terms of interfacial thermodynamics and molecular interaction between polar or non-polar portions of surface active lipid molecules. Indeed, at the time of the first publication, the above workers knew not only most of the basic properties of this new type of membrane but also the biological implication of their model membrane as the manifestation of the Gorter and Grendel (1925) theoretical model of the plasma membrane of red cells, which was then known as the unit membrane model generalized by Danielli and Davson (1935). In less than one decade this model membrane provided an enormous amount of information in the field of colloid science and biological membranology.

The fundamental importance of the Mueller-Rudin-Tien-Wescott model membrane is that it can have stability for hours in aqueous solutions. This fact is a strong support for the Gorter-Grendel-Danielli-Davson membrane model, in which the hydrophobic-interior and hydrophylic-exterior type molecular arrangement was proposed to have enough strength to function as a cellular barrier, since the electron microscopic, optical, and electrical determinations of the membrane thickness and the molecular arrangement of phospholipids in this membrane now leave no doubt on the bimolecular leaflet nature of this model membrane.

This in vitro formation of cellular membrane, i.e. Mueller-Rudin-Tien-Wescott model membrane, can be achieved by painting a hole or ring (about 3 mm diameter hole in Teflon or polyethylene support) with a minute amount of biological or abiological surfactants dissolved in normal alkanes of carbon length from six to fourteen. If the surfactant concentration is proper, the thinning process takes place immediately and a completely black membrane surrounded by the Plateau-Gibbs border (the lipid reservoir) can be formed within minutes. When this thinning process is viewed under a proper arrangement of light source and a low power (about 20x) microscope, vivid and bright colorful fringes can be seen. An initially colored film thins down by drainage of the lipid to the border. After the colored film turns to a bright silver, the nucleation of the black spot appears, usually near the border of the silver film. It pushes away the colored portion toward the opposite border region, and finally covers the total film area. This state of the lipid film has been called a "black lipid membrane," "bilayer lipid membrane," or "bimolecular lipid membrane," abbreviated quite often as BLM (Tien and Diana, 1968).

Excellent review articles in the general field of BLM are now available. The works up to 1967 covering the techniques of membrane formation and the basic physicochemical properties were extensively reviewed by Tien and Diana (1968). Similar review was presented by Bangham (1968) in conjunction with various other membrane models. Rothfield and Finkelstein (1968) covered the works on the membrane model from the view point of membrane biochemistry. Reeves (1969) and Castleden (1969) reviewed the value of the BLM as a model of the biological membrane in comparison with other membrane models. The general aspect of this field from the point of view of the biological application of BLM were reviewed by Henn and Thompson (1969). A recent review by Goldup, Ohki, and Danielli (1970) is comprehensive and fascinating in that one of the proponents of the bilayer membrane structure examines the progress made in the field.

A recent unique application of the BLM system is successful formation of photosensitive membranes by incorporating commercial chlorophyll, xanthophyll, and chloroplast extracts from spinach and algae (Tien, 1968a, b). This pigmented model membrane can show photovoltaic effect and photoconductivity. Studies on the mechanisms of these processes may contribute significantly toward the understanding of the initial processes of photosynthesis. For the explanations of the observed photoresponses, Tien (1968a, b) proposed a possibility of the existence not only of ionic but also of electronic charge carriers. It was further proposed that if electrons and holes could be generated by light, the chemical work also could be done by these charge carriers. Because of a lack of knowledge of the nature of the charge carriers in the dark state as well as in the light state, these alternative explanations still remain undetermined.

In the following year, Hesketh (1969) confirmed Tien's observations and proposed that similar electronic charge separation would result in the differential charging-up of the electric double layers under the effect of an applied electric field. Tien and Verma (1970) strengthened the possibility of the existence of the electronic charge carriers, observing the enhancement of the photovoltaic effect by placing proper electron acceptors and/or donors. Indeed, Tien and Verma (1970) pointed out the similarity between the mode of the photoresponses of chlorophyll BLM and those of solar cells.

In an attempt to form photosensitive BLM, inorganic ions were used by several workers. These include ferric ions (Tien, 1968d; Rosenberg and Pant, 1969) and iodide ions (Kay and Chan, 1969; Mauzerall and Finkelstein, 1969). In all of these cases electronic charge transfer processes were assumed to take place, presumably at lipid/water interfaces of BLM, similar to the suggestion made by Tien (1968a, b) in the case of chlorophyll BLM.

If, indeed, BLM are capable of having photogenerated electronic charge carriers, then the coupling of such an electronic potential to ionic potential (if any exists) may exhibit biphasic photopotentials similar to the early receptor potentials of photoreceptors.

In spite of the recognition of the fact that a membranous structure plays an essential role in the generation of early receptor potentials, the temperature dependence of the biphasic mode of the early receptor potentials in visual receptors has been interpreted only as the direct manifestation of the different rate of the formation of rhodopsin derivatives by the action of light at various temperatures. Studying the linkage between the photoregeneration of rhodopsin from its photoderivatives to that of the early receptor potential with elaborated techniques, Cone (1967) and Cone and Cobbs (1969) proposed that the fast component of the biphasic responses was spectrophotometrically related to the transition from rhodopsin to metarhodopsin I, and the slow component to that from metarhodopsin I to metarhodopsin II. A similar proposition was presented independently by Pak and Boez (1967).

Although Arden, Bridges, Ikeda, and Siegel (1968), from thermodynamic arguments, preferred the formation and decay of prelumirhodopsin rather than metarhodopsin I, an essentially same mechanism was also proposed by Haggins and McGaughy (1967) for the squid retina. However, this mechanism is still unsatisfactory since it failed to explain how such an intramolecular charge rearrangement can develop not only the membrane potential but also the potential which can be recorded by the electroretinogram techniques, as pointed out by Wald (1968). This one-sidedness of the studies on the early receptor potential was inevitable, due to the experimental limitation in using whole eyes and retinas.

The ubiquitous nature of the early receptor potential of the photoreceptors in the pigmented animal and plant tissues strongly suggests that such a photoresponse could be demonstrated by a model membrane system with a proper biological pigment. One of the best biological membrane models at the present time is the BLM, especially for the purpose of measuring electrical responses. The incorporation of all-*trans*-retinal into BLM would provide an excellent membrane model for studying the mechanism of the generation of the early receptor potential of visual receptors (Tien, 1968a). A close similarity to the early receptor potential and not to the early components of the electroretinogram (the early phases of P-III) of the photoresponses of all-*trans*-retinal BLM can be easily recognized, since the membrane model lacks the proteinous component. Thus, it may not be capable of inducing selective transport of K^+ or Na^+ across the membrane. Such an ionic selectivity can be achieved after a specific modification of the BLM, as extensively reviewed by Mueller and Rudin (1969). Furthermore, the absence of phosphatidylethanolamine in the present model membrane also excludes the ionic selectivity as shown by Bonting and Bangham (1967). If this similarity is demonstrable, the photoresponse of all-*trans*-retinal BLM will show the biphasic response whose mode of generation would depend strongly on temperature, in a similar manner to the early receptor potential.

The purpose of this work is to explore the possibility of the concept that the early receptor potential is a membrane phenomenon and is to be understood not only in terms of the photochemistry of the pigment present in the membrane but also in terms of the factors which affect the membrane properties. Specifically the following questions are asked:

1. Is it possible to demonstrate temperature-dependent biphasic photoresponses with all-*trans*-retinal BLM?
2. What is the mechanism by which temperature can influence the mode of the photoresponses?

It was successfully demonstrated that all-*trans*-retinal BLM showed temperature-sensitive biphasic photoresponses with striking resemblance to the early receptor potential of visual receptors and pigmented animal and plant tissues. It was also shown that the mobility of hydrogen ions was the major factor which influenced the mode of biphasic photoresponses as temperature was varied.

CHAPTER II

LITERATURE REVIEW

Early Receptor Potential

Since Brown and Murakami (1964a) discovered the early receptor potential in the monkey retina, this potential has gained the enormous interest of visual physiologists, for this is the earliest electrical response which can be recorded from the eye. An elucidation of its generation mechanism is believed to contribute to the understanding of the initial processes of visual excitation.

Like the intra-retinal recording of the early receptor potential of Brown and Murakami (1964a) the early electroretinogram was recorded by Cone (1964) with the albino rat eye and the frog eye. It was concluded that the two types of recording showed the same electrical response (Cone, 1964; Brown and Murakami, 1964b).

The early receptor potential has the following features distinct from the electroretinogram. The early receptor potential is:

1. unaffected by anoxia (Brown and Murakami, 1964a; Cone, 1964),

2. less sensitive to the ionic environment (Pak, 1965; Brindley and Gardner-Medwin, 1965), and
3. found in the degenerating rat eye, from which no electroretinogram can be recorded (Arden, Ikeda, and Siegel, 1966).

The exact relationship of the early receptor potential to the generation of the electroretinogram is still under investigation, as well as the mechanism of the generation of the early receptor potential itself. At the present time, however, experimental evidence indicates not only that there is a close relationship between two types of photoresponses but also that the early receptor potential could result in the generation of the first wave of the electroretinogram (P-III). For example, Cone (1964) observed that the early receptor potentials had:

1. the same spectral sensitivity as the b-wave of the electroretinogram,
2. its amplitude linearly proportional to the rhodopsin bleached by flash, and
3. been found in the eye which could not generate the electroretinogram as also shown by Brown and Murakami (1964a) and later by Arden, Ikeda, and Siegel (1966).

Particularly, Gage, Crawford, and Brown (1968) observed that while the early receptor potential was independent of ionic changes in the aqueous medium, the early phase of the electroretinogram was clearly ion dependent. From this observation they suggested that the transition from the first two non-ionic potentials to the ionic potential of the third phase would be the fundamental process underlying the excitation of photoreceptor cells.

The early receptor potential was also found in the human eye by Yonemura and Kawasaki (1967) and Galloway (1967). The clinical application of the early receptor potential had the following advantages over many other tests of visual functions: the independence from the neural events would allow a differential diagnosis for pathological conditions affecting the peripheral structures of the visual system and would give direct information on the visual pigment in the rods and cones. And the dark adaptation would not be required.

The Effect of Temperature on the Early Receptor Potential

Shortly after its discovery by Brown and Murakami (1964a) one of the remarkable properties of the early receptor potential was reported. At room temperature the early receptor potential had two components in the frog eye (Cone, 1964) and in the monkey retina (Brown and Murakami, 1964b). The first corneo-positive phase was observed without detectable latency, had a peak time of about 100 μs, and was followed by the second corneo-negative phase with a peak time of about 900 μs. Since the sign of two components depends on the position of the recording electrodes, Cone (1965) later proposed the use of R1 and R2 (R: Receptor Potential) for the first and second components of the early receptor potential, respectively. Pak (1968) 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 (1964) 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°C (Pak and Cone, 1964; Pak, 1965; Pak and Ebrey, 1965). Similar results were observed in the excised goldfish eye (Pak and Cone, 1965), and in the frog eye (Brindley and Gardner-Medwin, 1966).

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 (Brown, 1965; Brown, Watanabe, and Murakami, 1965; Brown and Gage, 1966a, b; Arden, Bridge, Ikeda, and Shiegel, 1966b; Arden, Ikeda, and Shiegel, 1966a, b; Becker and Cone, 1966; Ebrey, 1967, 1968). 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. Instead, the optimum early receptor potential can only be observed in membranous structures with high electric resistance and proper orientation of the pigment molecules.

The requirement of the organized pigment arrangement in membrane structure for the generation of the early receptor potential was indicated by its abolishment after freezing the squid retina to -80°C and rewarming it (Hagins and McGaughy, 1967), or after heating a vertebrate eye to 58°C and cooling it (Cone and Brown, 1967). 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 (Cone and Brown, 1967).

The high efficiency of cone early receptor potential was explained by Goldstein (1967, 1968, 1969) and Goldstein and Berson (1969) 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 made by Brown and Crawford (1966, 1967a, b) with the pigment epithelium. 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.

The Necessity of the Mediator for the Generation of the Early Receptor Potential

The great influence of the front membrane on the mode of generation of the negative phase of the early receptor potential of frog pigment epithelium led Crawford, Gage, and Brown (1967) to the conclusion that the generation of the early receptor potential occurred across the front membranes. They felt that the involvement of the membranous structure was a secondary process following the primary response that had to take place in the melanin granules, which were not the membrane component and were not continuous to the cellular membrane. From this they asked the question of how the primary event could be transferred to the membrane and initiate the R2 phase. Furthermore, they suggested the generality of this type of energy transfer from the pigment granules to the membranous structure in all varieties of photoreceptors.

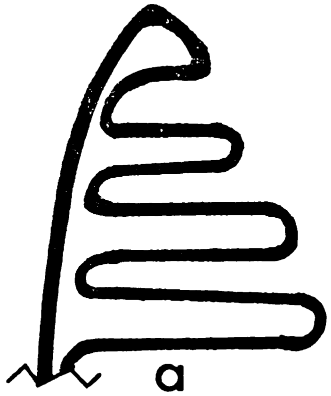
It is interesting to note that in the retina which contains both rods and cones (e.g., frog and monkeys) the early receptor potential has dominant cone origin (Goldstein, 1967, 1968, 1969; Goldstein and Berson, 1969). Because of the fact that cone pigments consist of only 10% of the total pigment content in these eyes, Goldstein (1968) concluded that the dominance of the cone origin in the early receptor potential could be due to the structural difference between cones and rods. The outer membrane of rods could mask the early receptor potential, which otherwise could be detected like that of cones. On the other hand, the eyes with pure rods generate an equally strong early receptor potential as those with pure cones. Indeed, Pak and Ebrey (1966) carefully studied the dependence of the early receptor potential of animals with pure rod retina and pure cone retina as a function of temperature, stimulus intensity, and spectrum, and concluded that there exists an essential similarity between the pure rod retina and the pure cone retina. According to Cohen (1968), the disc membranes are enclosed and separated from the outer membrane of the frog rods. This observation is in support of Goldstein's (1967) proposition; however, it cannot explain that of Pak and Ebrey (1966). The outer membrane of the rods of the pure rod retina may have a somewhat different structure from that of the vertebrate rod structure (Dowling, 1967); however, this view is unlikely. Pedler and Tilley (1967) observed incomplete membrane continuity between the disc and outer membranes of the rods of the guinea pig (*Cavia*), the baboon (*Papio Cynocephalus*), the toad (*Xenopus laevis*), and the albino rat (*Rattus*, v. albino).

Apart from the above electronmicroscopic observations, there is some evidence on the nature of the outer membrane of the rod outer segments. According to Adams and Hagins (1960), the inter-disc space is ionically continuous to the outer space of the frog rod outer segments and seems to be electrically transparent. Dewey, Davis, Blasie, and Barr (1969) observed the existence of a diffusion pathway into the frog rod outer segment of sufficient size to allow molecular species as large as antibody molecules, antirhodopsin serum and fluorescein-labeled sheep anti-rabbit γ-globulin to pass through. If these observations can be taken as indications of the inertness of the outer membrane of the rods, the disc membranes of the rods could function in exactly the same manner as the disc membranes of the cones, which are continuous to the outer membranes (Dowling, 1967).

As described above the outer membrane of the rod outer segment does not seem to play an important role in the generation of the early receptor potential. Furthermore, the intracellular recording closely resembles the extracellular early receptor potential. These observations indicate that it is not likely that asymmetric orientation of the rhodopsin molecules relative to the surfaces of the retina alone leads to the generation of the early receptor potential as suggested by Brindley and Gardner-Medwin (1966) and by Lettvin (1965). Rather, these observations indicate that the orientation of the photopigment relative to the photoreceptor membranes is an important factor in determining the direction and magnitude of the early receptor potential, as suggested by Smith and Brown (1966) and Hagins and McGaughy (1967). Thus, it is very likely that in rods and cones the disc membrane itself is capable of generating both positive and negative components of the early receptor potential, unlike the pigment epithelium. This is in accord with the common knolwedge that the disc membranes contain most of the visual pigments in the rods and cones (Dewey, Davis, Blasie, and Barr, 1969).

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 shown in Figure 1. In the pigment epithelium and the leaves, the cellular membrane (which is referred to as the membranous structure by the investigators) 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 (which is also referred to as the membranous structure by the investigators) 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 can be seen in the following two points.

Figure 1.--The schematic diagrams of the photoreceptor cells from which the early receptor potential has been recorded. Cell membrane, across which the early receptor potential develops, does not always contain pigments. In the outer segment of cones and rods (a) the pigment is incorporated in the cellular membrane; in the pigment epithelium cells of eyes and skins (b) the pigmented granules (g) are isolated from the cell membrane, and in the plant cell (c) the pigment is incorporated in the membranous structure (t) in the chloroplast (e), which is isolated from the cell membrane.



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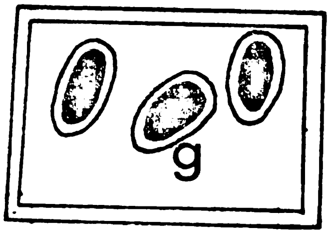

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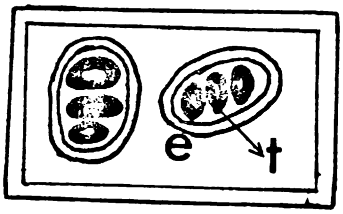

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The first point is that, although the understanding of the mechanism of generation of the early receptor potential of the pigment epithelium and the plant cells would certainly contribute to the understanding of that of the rods and cones, the above 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.

The second 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 Selection of the Membrane Model System

The Importance of Membrane Models in the Studies on the Early Receptor Potential

In order to understand generation of the early receptor potential it is necessary to investigate changes in the membrane properties upon illumination. However, it is almost impossible to carry out intracellular recording of the rods and cones. Brown and Wiesel (1961) feel that the rod outer segment is so small (5-6 μ diameter and 50 μ length) that rods are relatively free to move when contacted by an electrode, due to the hardened structure of multilamellar formation. Furthermore, there is the difficult problem of placing an electrode tip in an intrasaccular width of less than 100 A. Thus, there is no way of being sure of making an "intracellular" recording in the outer segments of rods and cones from any direction with respect to the orientation of the outer segments.

Smith and Brown (1966) reported the intracellular recording of the early receptor potential from Limulus eyes. Even with such a simple eye the complexity of the organization of membranous structure exists. Whether the microvilli are connected to retinular cells is still controversial (Walther, 1966). Assuming the continuity of the cellular membrane from the retinular cell to that of the microvilli as reported by Eguchi and Waterman (1966), the extensive localization of visual pigment on microvilli (Langer and Thorell, 1966) suggests that the intracellular recording from the retinular cells is equivalent to the intracellular recording with an electrode in the inner segment of the rods and cones of vertebrate eyes. More recently, Lasansky and Fuortes (1969) recorded the intraretinular cell and intravacuole electric responses with the leech eye, and proposed that the site of the origin of the electrical response in the visual cells would be the region of the microvilli. Again, since the diameter of the microvilli is less than 1000 A (Eguchi and Waterman, 1966), intramicrovillus recording is extremely difficult.

As shown above, the real photoreceptor systems present technical problems for the investigation of the function of pigmented membranes in generation of the early receptor potential. The other pigmented cells such as epithelium and plant cells contain pigment granules or chloroplasts, but the cell membrane itself does not contain the pigments. The mode of the early receptor potential generation in these cells, therefore, has to be different from that of rods and cones in some aspects. Thus, the model membrane system with visual pigments is invaluable for the investigation of the mode of generation of photoresponses, being sure of a contribution from only the pigments incorporated in the membranous structure.

In the following three sections the structure of the disc membranes, which should be reproduced by ideal membrane models, and the works on photosensitive lipid monolayers and bilayers will be presented in order to help select an ideal membrane model for the study on the early receptor potential.

The Structure of the Membranes of Rods and Cones

From low-angle x-ray diffraction studies Blasie and Worthington (1969) concluded that the pigment molecules of retinal receptor disc membranes would have a core of uniform electron density 38-40 A in diameter with a total diameter of 44-46 A, and would occur in a plane or two dimensional liquid-like arrangement within the disc membranes. Blaurock and Wilkins (1969) specified the position of the pigment on the outer surface of the bilayer lipid membranes in a symmetric manner. Further, they proposed that the thickness of the pigment molecules could be less than 10 A, still occupying the area per particle found by Blasie and Worthington (1969). According to Blaurock and Wilkins (1969), the unhydrated substance in a membrane was about 55 A thick, assuming a uniformity in the membrane, and lipid occupied 26 A of which 18 A was estimated to be hydrocarbon chains.

The low angle x-ray diffraction data of biological membranes provided the thickness of hydrocarbon layers in a range of 20-30 A (Finean, 1969). The minimum thickness of the hydrocarbon layers would exist due to the ionic interaction of the polar head groups of phospholipids, and was estimated as 15 A for phosphatidylethanolamine molecules and phosphatidylcholine molecules (Finean and Millington, 1955). In this liquid crystalline state, phosphatidylethanolamine had a layer thickness of about

35 A and the pure hydrocarbon region would be only 20 A thick. Thus, it may be concluded that the retinal disc membrane has a similar basic membrane structure to the bilayer lipid membrane coated with pigment protein molecules.

It is still uncertain how retinal is arranged in the disc membrane. Since rhodopsin has a hydrophobic core and several binding sites between its opsin part and the retinal chromophore part (Abrahamson and Ostroy, 1967) and since retinal itself is lipid soluble, it is likely that the retinal moiety of rhodopsin molecules is located in the lipid-rich layer of the disc membrane as suggested by Maeda and Isemura (1967). Wolken (1961, 1966), on the other hand, located the retinal molecules at the hydrophilic portion, or the outer layer of the membrane.

Photosensitive Monolayers

Hyono, Kuriyama, Tsuji, and Hosoya (1962) spread rhodopsin extracts with dilute ethanol or digitonin solutions at air/water interfaces. The aqueous phase was a phosphate buffer solution of pH 6.8. Although the rhodopsin solution extracted with a dilute ethanol solution gave a suitable monolayer film, both rhodopsin monolayer films gave an increase in surface pressure upon illumination with a light flash. After the bleaching of rhodopsin, this increase in surface pressure upon illumination completely disappeared. Azuma and Takagi (1966) pointed out that under appropriate experimental conditions illumination had no effect on the surface pressure of the rhodopsin monolayer at air/water interfaces.

The surface active property and the molecular orientation of vitamin A derivatives at air/water interfaces have been well established (Bangham, Dingle, and Lucy, 1964; Anderson, Roels, Dreher, and Schulman, 1967; Roels and Shah, 1969). The conjugated chains are immersed in air or a residual monolayer of hydrophobic solvent, and the aldehyde, hydroxyl, and carboxyl groups are directed toward the aqueous phase. The similar molecular orientation in bilayer lipid membrane was also demonstrated to exist by the studies on lecithin myelinics (Dreher, Schulman, Anderson, and Roels, 1967; Anderson, Roels, Dreher, and Schulman, 1967; Roels and Shah, 1969).

The action of light on the monlayer of retinal was investigated by Maeda and Isemura (1967). All-*trans*-retinal and 11-*cis*-retinal showed changes in the surface pressures required to keep the film area constant after illumination. The maximum change was observed after illumination for 30 seconds. The all-*trans*-retinal film showed an increase in the surface pressure while the 11-*cis*-retinal showed a decrease. They interpreted an increase in the case of all-*trans*-retinal as a film expansion by the isomerization to the most hindered conformation, since the 11-*cis* isomer showed a larger limiting area and lower collapse pressure than the all-*trans* isomer.

Beta-carotene films at air/water interfaces were prepared by spreading a hexane solution of the pigment over 10^{-3} M phosphate buffer solution at pH 8 (Colmano and McGlone, 1961). Colmano (1962) was successful in obtaining the absorption spectrum of the monolayers of the beta-carotene mixed with chlorophyll a and chlorophyll b.

Monolayer studies have been carried out at air/water interfaces and, hence, have been limited to the elucidation of the mechanical properties of the monolayers with rhodopsin and retinals. Investigation of the electric properties of photosensitive films had to wait for the successful formation of BLM.

Photosensitive Bimolecular Lipid Membrane

The formation of photosensitive BLM with visual pigments was tried by several workers. Mueller, Rudin, Tien, and Wescott (1963) observed that the BLM formed from chloroform-methanol solutions of brain lipids or proteolipids became electrically excitable after the adsorption of water-soluble molecules extracted from bacterially fermented retina. Takagi, Azuma, and Kishimoto (1965) formed bimolecular lipid membranes with the digitonin-free rhodopsin lipo-protein extracted from cattle retina. The resistance of the membrane was 10^3 - 10^5 ohm cm^2 with 1 M KCl solutions in both of the chambers, and increased to 10^6 ohm cm^2 when one aqueous phase was replaced with isotonic $CaCl_2$. Excitability, however, was not observed with this preparation. Leslie and Chapman (1967) incorporated beta-carotene, retinal, or retinol into phospholipid BLM and observed that thinning time was affected due to this additional pigment component. The existence of beta-carotene in the membrane was indicated by a specially designed absorption spectroscopy. But, again no photoexcitability was observed with their BLM containing beta-carotene, retinal, or retinol.

The initial account of the present investigation was reported elsewhere (Tien and Kobamoto, 1969). Photovoltaic effect and photoconductivity were observed by incorporating all-*trans*-retinal, 9-*cis*-retinal, all-*trans*-retinol, 9-*cis*-retinol, and beta-carotene in 10^{-1} M KCl solution. Pant and Rosenberg (1970) reported photoconductivity effects in beta-carotene, retinal, and retinols contained in BLM in the presence of iodine-iodide.

CHAPTER III

THE CONSTRUCTION OF THE MODEL MEMBRANE SYSTEM USED IN THE PRESENT WORK

All-*Trans*-Retinal Bimolecular Lipid Membrane

An ideal model BLM would be formed with the lipids and pigment which are known to be the constituents of the visual receptors. Lipids in the outer segment of the rods constitute 20% - 40% of the dry weight, most of which is phospholipid (Wolken, 1962). Cholesterol exists in the disc membrane of the outer segment but only in trace amounts (Feldman, 1967). The membrane forming solution used in the present work was prepared by dissolving lecithin and oxidized cholesterol in the solvent *n*-octane and *n*-dodecane. The use of pure hydrocarbon was inevitable and the trace amount of solvent residue was assumed to remain in the final BLM as in a phospholipid BLM (Henn and Thompson, 1968). Nevertheless, the major lipid constituents of the present membrane are the surface active molecules; lecithin and oxidized cholesterol. Phosphatidylethanolamine was not used in the present membrane forming solution since undesirable ionic selectivity would be induced by the formation of all-*trans*-retinal-phosphatidylethanolamine complexes while lecithin was known to be free from such a complication (Bonting and Bangham, 1967).

All-*trans*-retinal, instead of rhodopsin or 11-*cis*-retinal, was selected as a visual pigment in the present work because the pure commercial product was available, it was the most stable conformer of the retinals, and it lacked the regenerative and degenerative factors found in rhodopsin, which would complicate the mode of generation of the photoresponses. The use of such a simple pigment does not lessen the potential significance of the present model membrane, since the mode of generation of the photoresponse of the type with which the present work is concerned is not dependent on the kinds of pigment investigated, taking into account variations in spectral sensitivity.

In animal and plant tissues only pigmented areas yield responses; for instance, melanin containing skin, or pigmented (non-albino) iris or pigmented epithelium. In these tissues melanin is the light absorbing compound. Likewise, parts of variegated leaves which do not contain chloroplasts do not yield responses (Arden, Bridges, Ikeda, and Siegel, 1966b). Thus, it is clear that the mode of generation of the biphasic responses does not depend on the types of pigments. From this fact it can be concluded that the *cis-trans* isomerization of retinal itself is not responsible for the generation of the early receptor potential unless the isomerization induces the secondary effects which happen to be the common physicochemical effects to all the pigments. It is also clear that the formation of the stepwise rhodopsin derivatives is not likely to be the main mechanism of the generation of the early receptor potential, since such a distinct sequence of photoproducts is not known in melanin and chlorophyll. Thus, a clue to the elucidation of the mechanism of the generation of the early receptor potential has to be present in the early processes of light action common to all photosensitive biological compounds found in membranous structures.

The chemical structure of melanin is still unknown (Hempel, 1966; Mason, 1967), but it is well established that in vivo melanin is strongly associated with proteins (Seiji, Kishimoto, Birbeck, and Fitzpatrick, 1963; Fitzpatrick, Quevedo, Levene, McGovern, Mishima, and Oettle, 1966). Since chlorophyll and retinal also exist in associated form with proteins, one may ask whether protein component can play any significant role in the generation of the early receptor potential. At the present there is no evidence that the absorption of photons by these chromoproteins leads to the dissociation of the chromophores from any protein components except rhodopsin. Cone (1967) observed that a completely bleached retina with dissociated retinal or retinol and opsin could not generate the early receptor potential. On the other hand, Crawford, Gage, and Brown (1967) demonstrated that, after soaking in a solution of synthetic melanin (protein free), filter papers could show the photoresponses similar to the early receptor potential of the frog pigment epithelium. Glutaraldehyde fixation of the retina removes the R2 component selectively (Arden, Bridges, Ikeda, and Siegel, 1968). This evidence could also support the importance of the protein component in the generation of the early receptor potential. However, it is also possible that the membrane structure as a whole could be affected with such a strong protein denaturing agent. The decay of the magnitude of the early receptor potential by extreme illumination also cannot give conclusive evidence since it occurs in all three photosensitive systems regardless of whether the dissociation of the components occurs or not. Thus, at the present time it is not likely that the photodissociation of retinal from opsin is the primary cause of the loss of the early receptor potential generation of the retina. Rather it is more likely that the temporal detachment of retinal from the disc membrane or the random orientation of the retinal molecules in the disk membrane after the bleaching may be the primary cause of the loss of the early receptor potential in the completely bleached retina. This interpretation is in accord with the necessity for organized pigment arrangement in the membranous structure. Furthermore, the nature of the association between the chromophore and the protein is not known in the three pigment systems. Thus, it is more fruitful at the present stage of investigation to examine whether the pigment itself can generate the photoresponse similar to the early receptor potential if the pigment can be incorporated into a membranous structure. It is a matter of interest to note that all three pigments (retinal, chlorophyll, and melanin) have at least one C=O group, which can act as a hydrophilic group when present at a lipid/water interface.

Some of the basic properties of the all-*trans*-retinal BLM in symmetric 0.1 M KCl solutions together with other carotenoid BLM were reported previously (Tien and Kobamoto, 1969). The important feature to be noted is that the electrical properties and the mode of photoresponses are quite similar regardless of the kinds of pigment incorporated, with the exception of beta-carotene. It has been emphasized that not only the presence of photosensitive molecules in the lipid phase, but also the interaction of the molecules with the aqueous phases seemed to play an important role in the generation of the photoresponses (Tien and Kobamoto, 1969).

Aqueous Phases

In the absence of electrical and chemical asymmetry across the BLM it was found at an earlier stage of this work that the photoresponses were small and not very reproducible. This is probably due to the variation of microenvironment around the membrane or around the Plateau-Gibbs border which supports the membrane. Asymmetric charge distribution may originate from the uneven distribution of lipid at the border or slight hydrostatic pressure gradient across the membrane, which can be easily generated by the removal of a droplet of water by the lipid solution injector. In order to remove this difficulty a proper substance for the generation of membrane asymmetry was looked for. $FeCl_3$ was selected since it gave the significant enhancement of the photoresponse as well as reproducible resting potentials. Furthermore, in spite of some controversy over the absence of any trace metal in rhodopsin (Fukami, Vallee, and Wald, 1959) or its presence (Fujishita, 1962), it is almost certain that the outer segment of rods contains some metal: McConnell (1965) observed non-enzymic oxidation of ascorbate and attributed it to the presence of some metal associated with the disc of bovine retinal outer segments. Since Fujishita (1962) identified ferric ions in digitonin-rhodopsin complex, the use of $FeCl_3$ as an agent for making chemical electrical asymmetry not only may be justified but also may elucidate the role of the trace metal in visual excitation. The action spectrum of the $FeCl_3$ system is exactly the same as that of the non-$FeCl_3$ system (Tien and Verma, 1970), implying that $FeCl_3$ functions as a sensitizer for the development of the photoresponse of all-*trans*-retinal BLM.

CHAPTER IV

EXPERIMENTAL

Lipid Solutions

The oxidized cholesterol solution used in the present work was prepared from freshly recrystallized cholesterol obtained from Eastman, Rochester, New York and n-octane (pure grade, 99% minimum) from Phillips Petroleum Co., Bartlesville, Oklahoma by the method of Tien, Carbone and Dawidowicz (1966). In this method 12 gm of the recrystallized cholesterol was added to 300 ml of n-octane in a 1-L flask and heated to the boiling point (125°C) with molecular oxygen bubbled at a rate of 100-125 cc per minute for 5.5 to 6.0 hours. Then, the mixture was transferred to a flask and kept overnight at 5°C. The final solution kept at room temperature was colorless with white precipitation.

A lecithin solution was prepared by dissolving 1 gm of L-alpha-lecithin (chromatographically pure) from egg (General Biochemicals, Chagrin Falls, Ohio) into 100 ml of n-dodecane (pure grade, 99% minimum) from Phillips Petroleum Co., 110 ml of oxidized cholesterol solution, and 90 ml of 1% cholesterol in *n*-octane solution. The stock solution was kept in the freezing compartment of a refrigerator.

Membrane Forming Solution

The all-*trans*-retinal solution prepared with oxidized cholesterol solution formed a BLM with a lifetime of not more than 10 minutes. With lecithin solution the all-*trans*-retinal BLM gave no apparent stable state although it had a lifetime longer than 30 minutes. The most satisfactory membrane forming solution used in the present work was prepared by dissolving 0.025 gm of all-*trans*-retinal to 0.25 ml of a one-to-one mixture of the oxidized cholesterol solution and the lecithin solution. The membrane forming solution was freshly prepared each day since the concentration of all-*trans*-retinal was close to the saturation concentration, and the evaporation of hydrocarbon solvent often resulted in the precipitation of the retinal. The membrane stability was recuded if the solution had been kept over night.

Chemicals

All chemicals used for making aqueous solutions were of reagent grade as obtained from the manufacturers. Double distilled water from an all-glass still was used throughout the present work.

Aqueous Solutions

In all cases the hole for the membrane formation was filled with a drop of lipid solution after placing an aqueous solution into the inside and outside chambers. In order to create the concentration gradient of identical ions or different ionic species at the same or different concentrations across the membrane, 0.05 ml or 0.1 ml of the original solution was removed with a micropipette and the same amount of a proper solution was added each time to insure the hydrostatic equality across the membrane.

Membrane Formation

The membrane forming cell used in this work is similar to that of Mueller, Rudin, Tien, and Wescott (1962a, b). Shown in Figure 2 the outer chamber is a glass cup and the inner chamber is a Teflon cup (Hanai, Haydon, and Taylor, 1964; Tien and Diana, 1967), which separates the inner and the outer chambers except for a small hole for the membrane formation (Figure 2). The volume of aqueous solution put in the chambers in most experiments was 10 ml for the inner chamber and 40 ml for the outer chamber. The diameter of the membrane-forming aperture of the Teflon cup was 0.17 cm. Due to this small diameter of the hole the resulting membrane was minute. The membrane formation was, therefore, observed with a low power microscope under dim red light.

Figure 2.--The apparatus for the formation of BLM and the measurement of membrane potentials. The inner chamber was made from a Teflon cup (10 ml, T). A portion of the wall of the cup was machined down to about 0.0025 cm and a hole (0.17 cm, h) was punched through it. The outer chamber was made of glass (g), the front portion of which was flattened to facilitate the observation of the membrane. The bottom chamber (C) was filled with water for temperature control. For the measurement of potentials a pair of calomel electrodes (e) were connected to an electrometer (E) and a recorder (R).

To form BLM, both chambers were first filled with aqueous solution, and 0.01 ml of all-*trans*-retinal solution was then injected into the hole with a micrometer syringe. The use of the micrometer syringe for membrane formation was first experimented with by Tien and Diana (1967). The best performance of the syringe was achieved when the tip of the fine capillary attached to the syringe was slightly smaller than the diameter of the membrane forming hole. The ease of the formation and membrane stability were also achieved by wetting the hole and the surrounding area with the lipid solution prior to the membrane formation.

Potential Measurements

The apparatus and procedures used in this study for the measurements of membrane potential and resistance were described by Mueller, Rudin, Tien, and Wescott (1962a, b) and Tien and Diana (1967). The membrane potential was studied in a set up as illustrated in Figure 2. The cell arrangement may be represented as follows:

Saturated calomel electrode	Aqueous solution (inside)	Membrane	Aqueous solution (outside)	Saturated calomel electrode

A potential across the membrane was measured with an electrometer of high input impedance (Keithley, Model 610B) through a pair of calomel electrodes via saturated KCl salt bridges. The output of the electrometer was fed into a strip chart recorder (Servowriter II, Texas Instruments, Inc., Houston, Texas).

Resistance Measurements

A d.c. membrane resistance was obtained by applying a potential between the electrodes and introducing a known resistance in series with the membrane determined (Figure 3). Polarizing potentials were applied to the membrane using the instrument purchased from Electronics for Life Sciences, Rockville, Maryland. With the use of this instrument, the input resistance could be varied from 10^5 to 10^9 ohms and polarizing voltage from 0 to 5.4 V.

The membrane resistance is calculated according to Ohm's law for the circuit shown in Figure 3, and is given by:

$$R_m = (E_m \times R_i)/(E_i - E_m) \qquad (1)$$

where R_i is the input resistance in series with the membrane, E_i is the applied voltage (usually 20 mV), and E_m is the voltage in the presence of the membrane. For the best results R_i should be adjusted so that E_m/E_i lies between 0.1 and 0.8.

Figure 3.--Circuit diagram for the measurements of resistance.

E_m - electrometer
R_m - membrane resistance
C_m - membrane capacitance
R_i - input resistors (10^5 - 10^9 ohm)
E - voltage divider and switch for applying polarizing potentials
R_c - recorder

pH Measurements and Control

pH of the solutions was measured with a Beckman pH meter and with Beckman standard pH buffers. The adjustment of pH was done with HCl and KOH and an acetate buffer system when the control of buffer capacity was required.

Temperature Measurements and Control

The temperature of the aqueous phases was measured with a thermometer (Will Scientific, Inc., Rochester, New York) with 0.1°C divisions and a range from -1°C to 50°C. The thermometers were placed in both 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, West Germany), through the bottom chamber of the cell assembly shown in Figure 2. The temperature was maintained within ±0.05°C. All experiments were carried out at equilibrium in temperatures.

Light Sources

The photoexcitation of the membrane system was achieved by illumination with a Sylvania tungsten halogen lamp (DWY, 650 W, Sylvania Electric Products, Inc., Salem, Massachusetts) in a Sylvania "sun-gun" (Model SG55T). An Alphax shutter (Wollensak, Rochester, New York) was used for controlling the duration of the illumination.

Filters

The light beam emerging from the aperture of the lamp housing passed through a heat absorbing filter (water bath of 10 cm light path), a cupric sulfate solution filter (2 cm), two colored glass filters, and a lens before reaching the chamber of membrane formation. The concentration of cupric sulfate was adjusted to achieve the effective elimination of infra-red radiation as demonstrated by Kasha (1948). For the control of light intensity, gray filters (Carl Zeiss, Inc., New York) were used. Except for the intensity dependence experiments, photoresponses were induced without using any gray filters.

Artifacts

The illumination of lecithin-oxidized cholesterol solution did not show any significant photoresponses. The illumination of one calomel electrode while keeping the other out of a light beam also did not show any photoresponses in either the presence or absence of non-pigmented membrane. Indeed, the illumination of one electrode even in the presence of all-*trans*-retinal BLM did not show the photoresponses either. Thus, it was concluded that the photoresponses recorded with the set-up are true photoresponses of all-*trans*-retinal BLM, free from the artifacts of electrodes and the lipid component itself. Similarly, no photoresponse was observed with the pigment-free BLM in the presence of $FeCl_3$ in one aqueous phase.

The possibility that heating of water may cause the charge density gradient across all-trans-retinal BLM was excluded by the observation that there was not significant change in the temperature of the aqueous phases during illumination. After a 60 second illumination at room temperature the temperature changes were less than 0.05°C.

Transference Numbers

The membrane potential, E_m, due to a differential migration of ions through the membrane as a result of salt concentration gradient across the membrane was measured with the same circuit as shown in Figure 2.

The transference number of an ionic species is defined as the fraction of the total current carried by the species. Thus, if t^+ and t^- are the cation and anion transference numbers respectively, and i^+ and i^- the corresponding ionic currents, then, $t^+ = i^+/(i^+ + i^-)$, $t^- = i^-/(i^+ + i^-)$, and $t^+ + t^- = 1$.

Assuming the constancy of the transference number across the membrane, the membrane potential can be expressed by (MacInnes, 1961):

$$E_m = \frac{RT}{F} [t^+ \ln\frac{a^i_M}{a^o_M} - (1 - t^+)\ln\frac{a^i_{Cl}}{a^o_{Cl}}] = \frac{RT}{F}(2t^+ - 1)\ln\frac{a^i_{MCl}}{a^o_{MCl}} \qquad (2)$$

where a_M is the cationic activity, a_{MCl} is the mean ionic activity of the salt MCl, a_{Cl} is the chloride activity, F is the Faraday constant, R is the gas constant, and T is the absolute temperature. The superscripts o and i refer to the aqueous solutions on the outer and inner sides of the membrane, respectively. The ion activities are assumed to be equal to the mean ionic activity of the salt: $a_{Cl} = a_M = a_{MCl}$.

In each of the transference number experiments, only solutions of the chlorides of monovalent cations were used.

CHAPTER V

RESULTS

Dark Membrane Potentials

In order to understand the mechanism of the development of the photoinduced potentials of all-*trans*-retinal BLM, it is necessary to elucidate the mechanism of generation of the dark or resting membrane potentials. For this purpose the nature of the ionic charge carriers was examined by evaluating the transference numbers of the ions used in the present work.

Shown in Figure 4 are changes in the dark membrane potentials due to an increase in the salt concentration in the inner chamber, which was originally set equal to that of the outer chamber (10^{-3} M solutions). HCl, KCl, and NaCl developed negative potentials. The existence of the linearity clearly indicates the applicability of the Nernst equation. With the slope of 58 mV per unit pH, the protonic diffusion potential was far greater than that of other cations and anions used in the present work. The transference numbers calculated from Figure 4 are shown in Table 1.

In the present work ferric ions were used frequently as electron acceptors. Due to the strong acidic nature of

Figure 4.--Cation induced potentials as a function of concentration. The outside concentration was 10^{-3} M for all salts and the inside concentration was increased with the addition of 1 M solutions.

a - HCl
b - KCl
c - NaCl
C_i - concentration in the inner chamber
C_o - concentration in the outer chamber

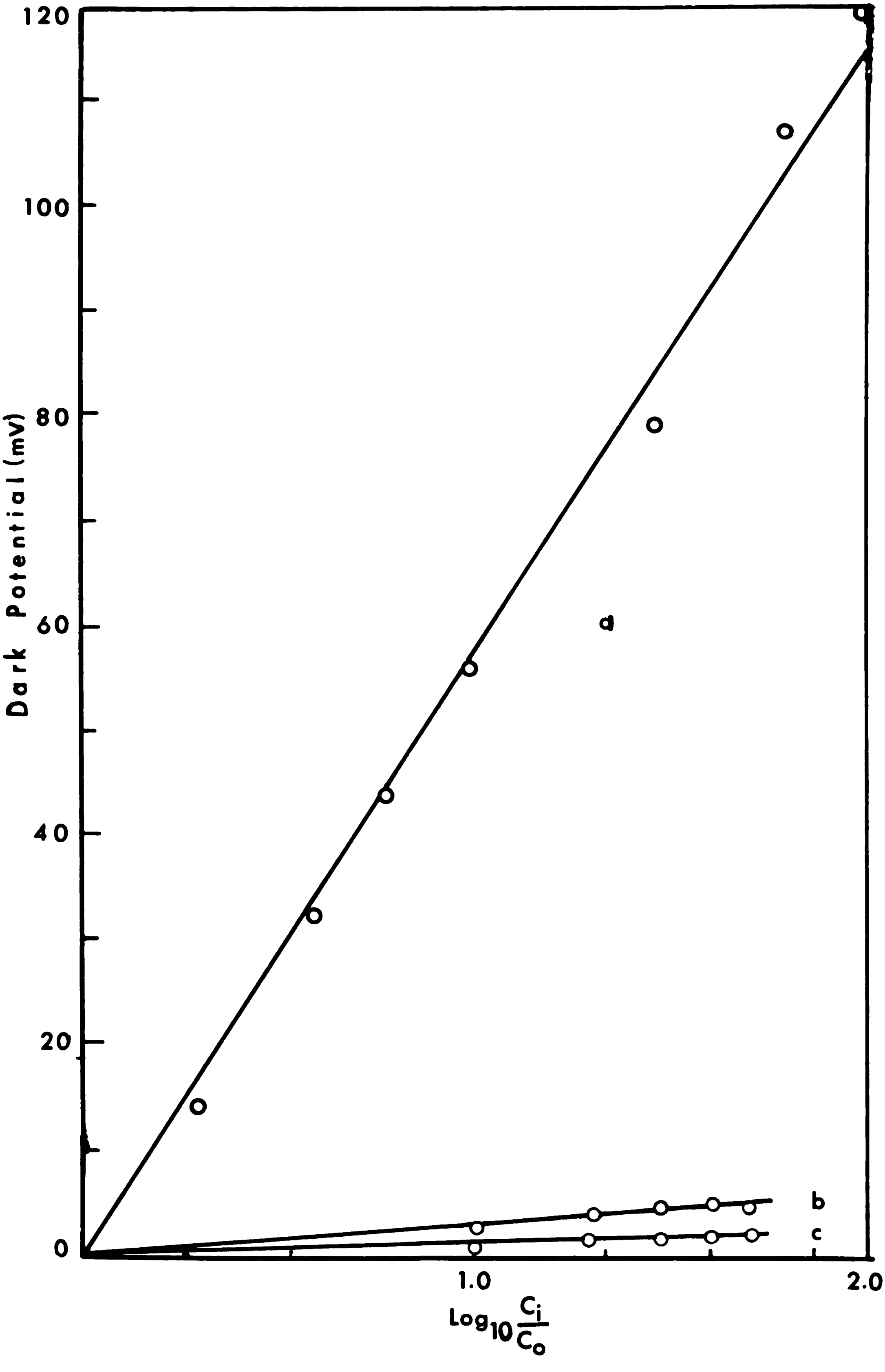

Dark Potential (mV)
120
100
80
60
40
20
0
1.0
2.0
$\mathrm{Log}_{10}\frac{C_i}{C_o}$
a
b
c

TABLE 1.--Cationic transference numbers of all-*trans*-retinal BLM.

Ion	Change in Equilibrium Potential per 10-fold Concentration Gradient (mV)	Transference Number
H^+	-58.5	1.0
K^+	- 3.3	0.53
Na^+	- 1.3	0.51

The values for the potentials were obtained from the slope of the plots of E_m against $\log_{10} C_i/C_o$ shown in Figure 4. The experiment was carried out at room temperature (22°C - 24°C).

the agent, the addition of $FeCl_3$ resulted in the generation of the dark membrane potential. The magnitude of this dark membrane potential was identical to those developed by the addition of HCl. This point can also be seen in the effect of buffer capacity on the development of the dark membrane potential due to the addition of $FeCl_3$ in symmetric acetate buffer solutions. As shown in Table 2, a decrease in the buffer capacity of the aqueous phases increased the magnitude of the dark membrane potential, which could be developed by the addition of a constant amount of $FeCl_3$ in one of the chambers. The adsorption of ferric ions onto the membrane may also cause the development of the membrane potential. This possibility, however, may be eliminated on the basis of the observation that there was no extra effect following the addition of $FeCl_3$ into the aqueous phase other than the previously mentioned pH effect observed due to the addition of HCl. The measurements of the membrane resistance also provided supporting evidence for the above thought: there was no significant effect caused by the presence of ferric ions. These observations now clearly establish the fact that asymmetric addition of $FeCl_3$ induces the dark membrane potentials by generating proton concentration gradient across the membrane.

TABLE 2.--The development of the dark membrane potential as a function of the buffer concentration in the aqueous phases.

Buffer Concentration (M)	Dark Membrane Potential (mV)	pH Gradient
0.1	- 1.8 ± 0.1	<0.1
0.01	- 31 ± 3	0.6
0.001	-110 ±10	1.9

Acetate buffer (pH 5.4)

$FeCl_3$ (0.1 ml of 0.1 M) in the inside chamber

The active electrode is in the inside chamber.

The General Description of the Photoresponses

The sign of the photoresponses depended upon the location of $FeCl_3$ as shown in Figure 5a. The direction of the incident light did not influence the sign of the responses. This can be seen in Figure 5b, which shows the photoresponse recorded with the same electrode arrangement as in Figure 5a, but with the position of $FeCl_3$ shifted to the outer chamber in order to simulate the shift of the direction of the incident light with respect to the preceding time course curve. It is worthy to note that the shift of the direction of the incident light did not change the sign, magnitude, or wave form of the photoresponse.

Unlike the cases of $FeCl_3$ and HCl, the concentration gradient of KCl and NaCl did not show any effect on the mode of the photoresponses. As shown in Figure 6, the ten-fold concentration gradient of KCl had no effect on the photoresponses. This fact is not unexpected since the all-*trans*-retinal BLM is not sensitive to K^+, Na^+, and Cl^- ions. The agreement of the mode of the effect of the salts on the photopotentials and on the dark potentials strongly suggests that the main charge carriers, or protons, in the dark state can interact with the photoexcitation of the membrane selectively, or that protons can function as charge carriers in observed photopotentials.

Figure 5.--The effect of the position of $FeCl_3$ with respect to the incident light on the mode of photoresponse. With $FeCl_3$ (10^{-3} M) in the inner chamber the photoresponse (for 1 second illumination) shown in a was observed while with $FeCl_3$ (10^{-3} M) in the outer chamber the photoresponse shown in b was recorded. Since the outer electrode was grounded in both of the cases, the data indicate that the photoresponses were independent of the direction of the illuminations and solely dependent on the position of $FeCl_3$.

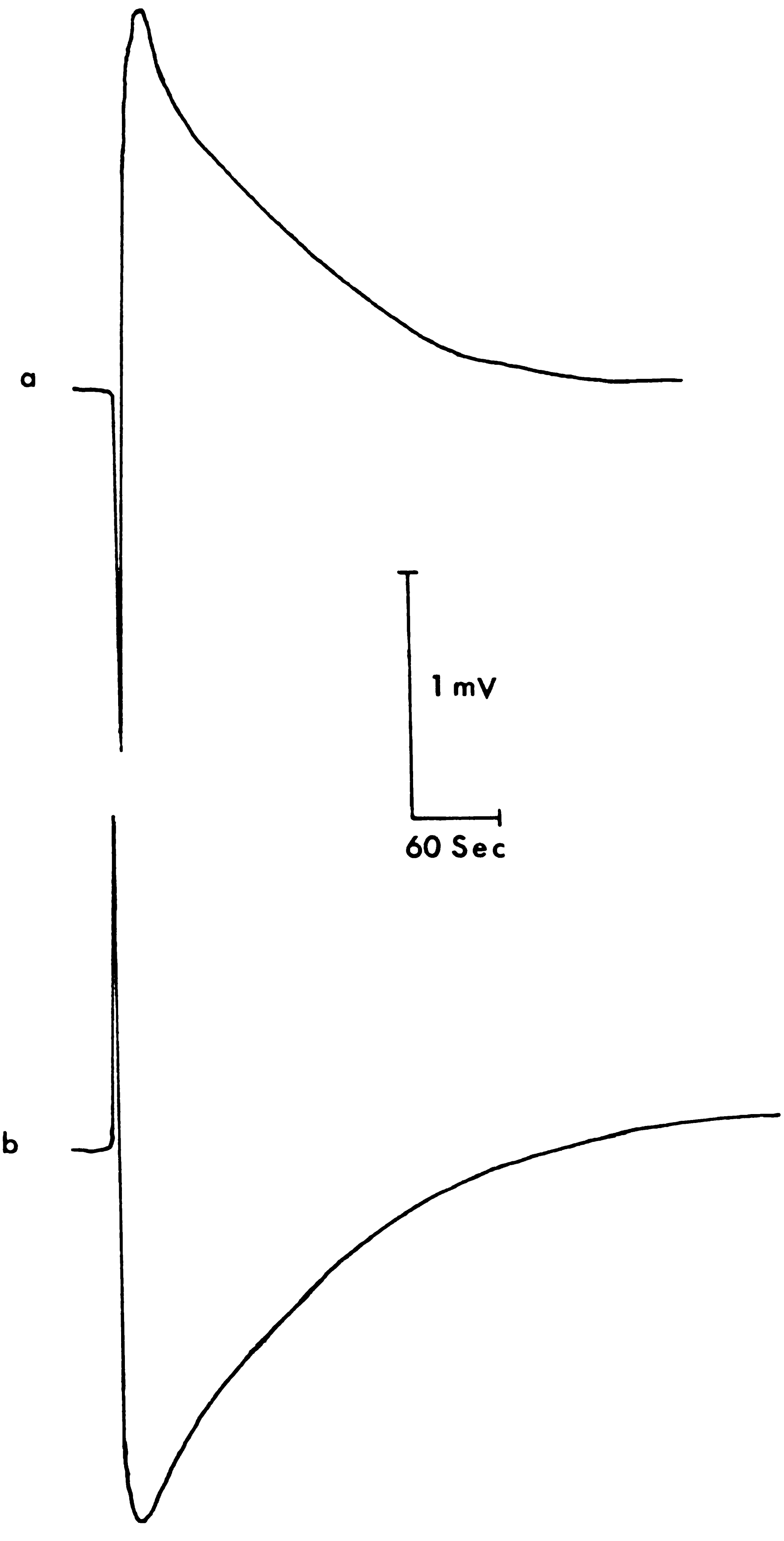
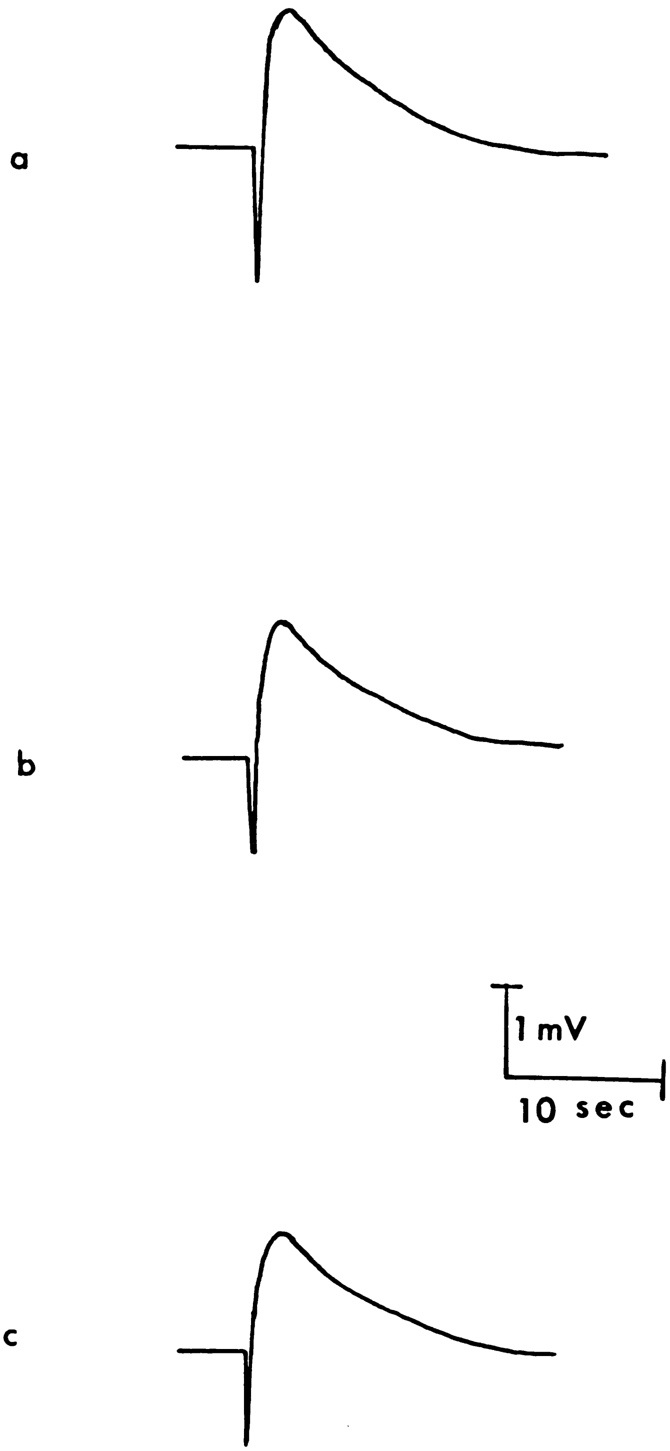

a
1 mV
60 Sec
b

Figure 6.--The effect of KCl concentration gradient across bimolecular lipid membrane on the photoresponses. The responses were recorded with a single membrane in 10^{-3} M KCl (a) with 0.1 ml of 0.1 M $FeCl_3$ in the inner chamber, in 10^{-2} M KCl in the inner chamber (b), and in 10^{-2} M KCl in both of the chambers (c). The concentration of KCl in b and c were made by injecting 0.1 ml of 1 M KCl, first to the inner chamber, and then to the outer chamber. The illumination period was one second for each recording.


a
b
1 mV
10 sec
c

Due to the close similarity between the biphasic mode of the photoresponses of all-_trans_-retinal BLM and that of the early receptor potentials of photoreceptors, the convention of assigning R1 and R2 for the fast component and the slow component respectively was adapted in the present work.

The effect of the duration of illumination was investigated with 0.1 M KCl solution with 10^{-3} M $FeCl_3$ in the inner chamber. Shown in Figure 7, a short time illumination had only the R1 component and prolonging the illumination generated the R2 phase while R1 reached the apparent maximum value. The duration of the illumination for the appearance and disappearance was dependent on the temperature as shown in Table 3.

The illumination time of _one second_ for the present experiments was arbitrarily selected: at one second illumination the saturation of the R1 component did occur along with the reasonably high R2 component. Indeed, the prolonged illumination caused irreversible changes on the membrane potential to such an extent that duration of illumination longer than one second was not desirable: the recovery of the initial membrane potentials was not observed as shown in Figures 7 and 8.

The selection of all-_trans_-retinal as the visual pigment of the present membrane model was made partly because rhodopsin had been proved to have photoregeneration, which would complicate the mode of the photoresponses. In

Figure 7.--The effect of the duration of illumination on the mode of photoresponses. The illumination times were indicated in seconds. The photoresponses were recorded from a single membrane formed in 9.1 M KCl solution with 10^{-3} M $FeCl_3$ in the inner chamber at 25°C.

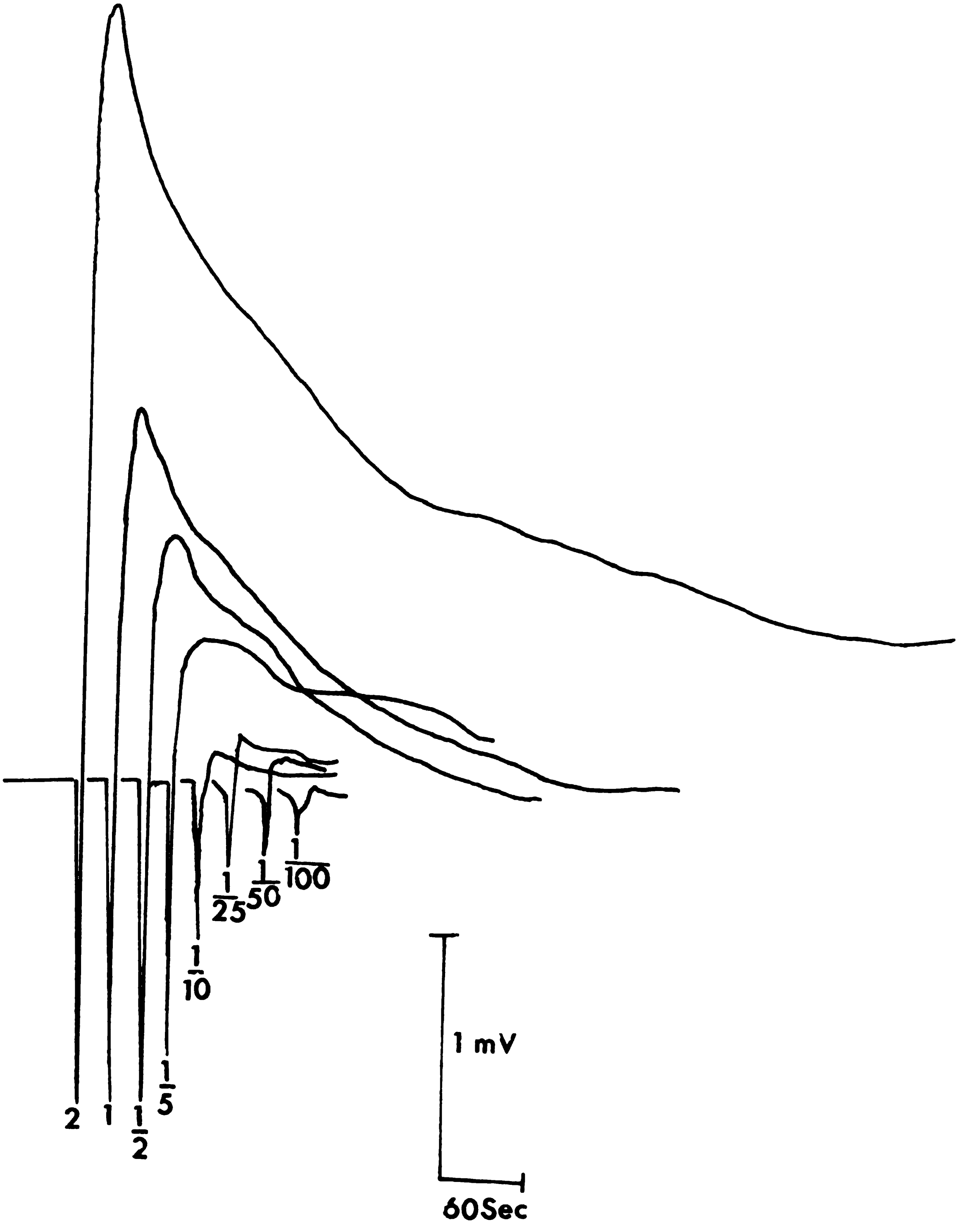

2
1
1/2
1/5
1/10
1/25
1/50
1/100
1 mV
60Sec

TABLE 3.--The effect of temperature on the duration of illumination for the appearance of R2 and the disappearance of R1.

Temperature (°C)	Duration of Illumination for	
	R2 Appearance (sec)	R1 Disappearance (sec)
9.5	>1.0	-
16.0	0.5	>1.0
25.0	0.2	>1.0
36.0	0.04	>1.0
44.0	-	<0.01

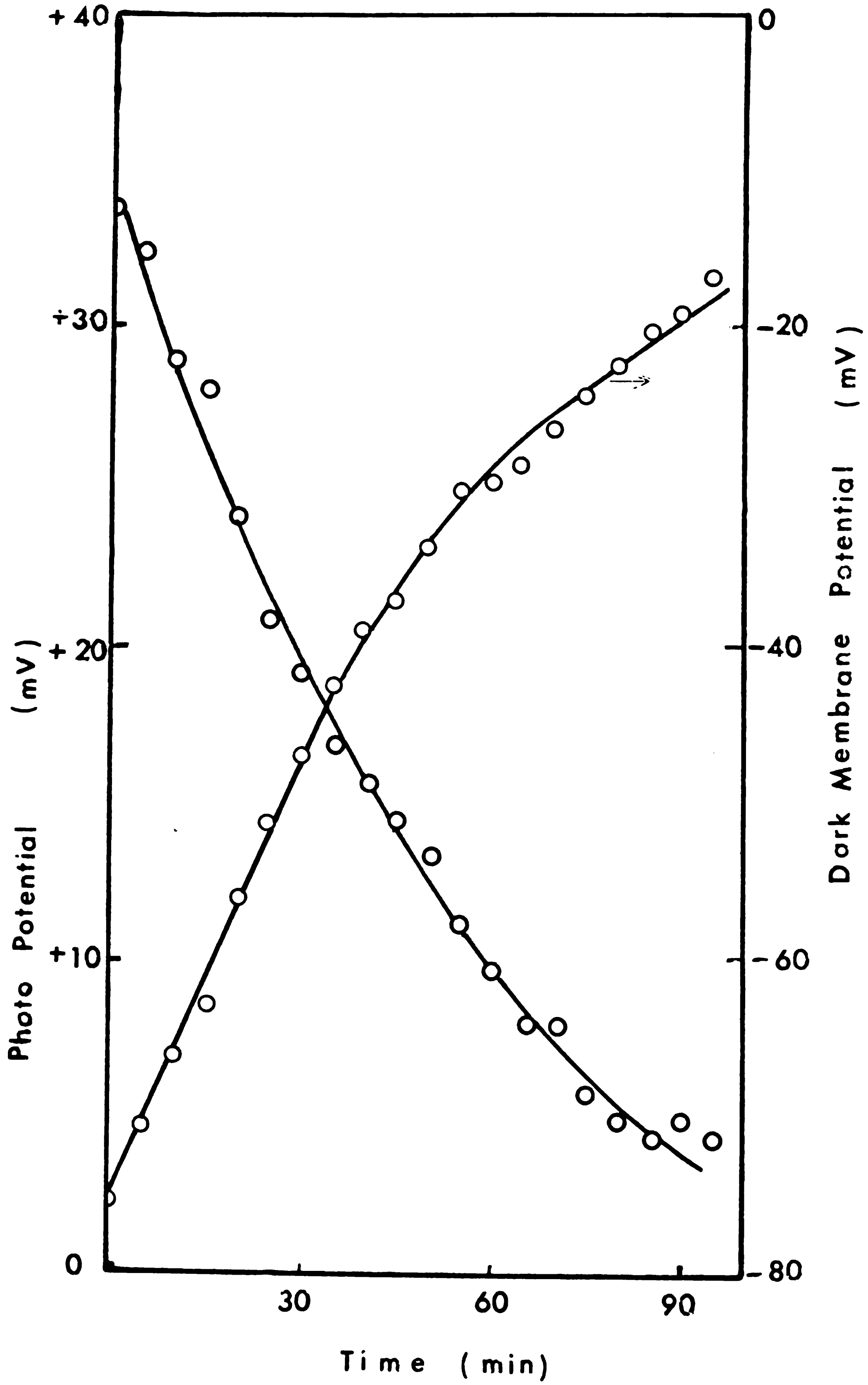
The membrane was formed in 0.1 M KCl solution and $FeCl_3$ was added to the inner chamber to make up 10^{-3} M solution.

Figure 8.--The effect of repetitive illuminations on the photoresponses. The letters shown on the curves indicate times after the first illumination in minutes, as shown below. The duration of each illumination was 30 seconds.

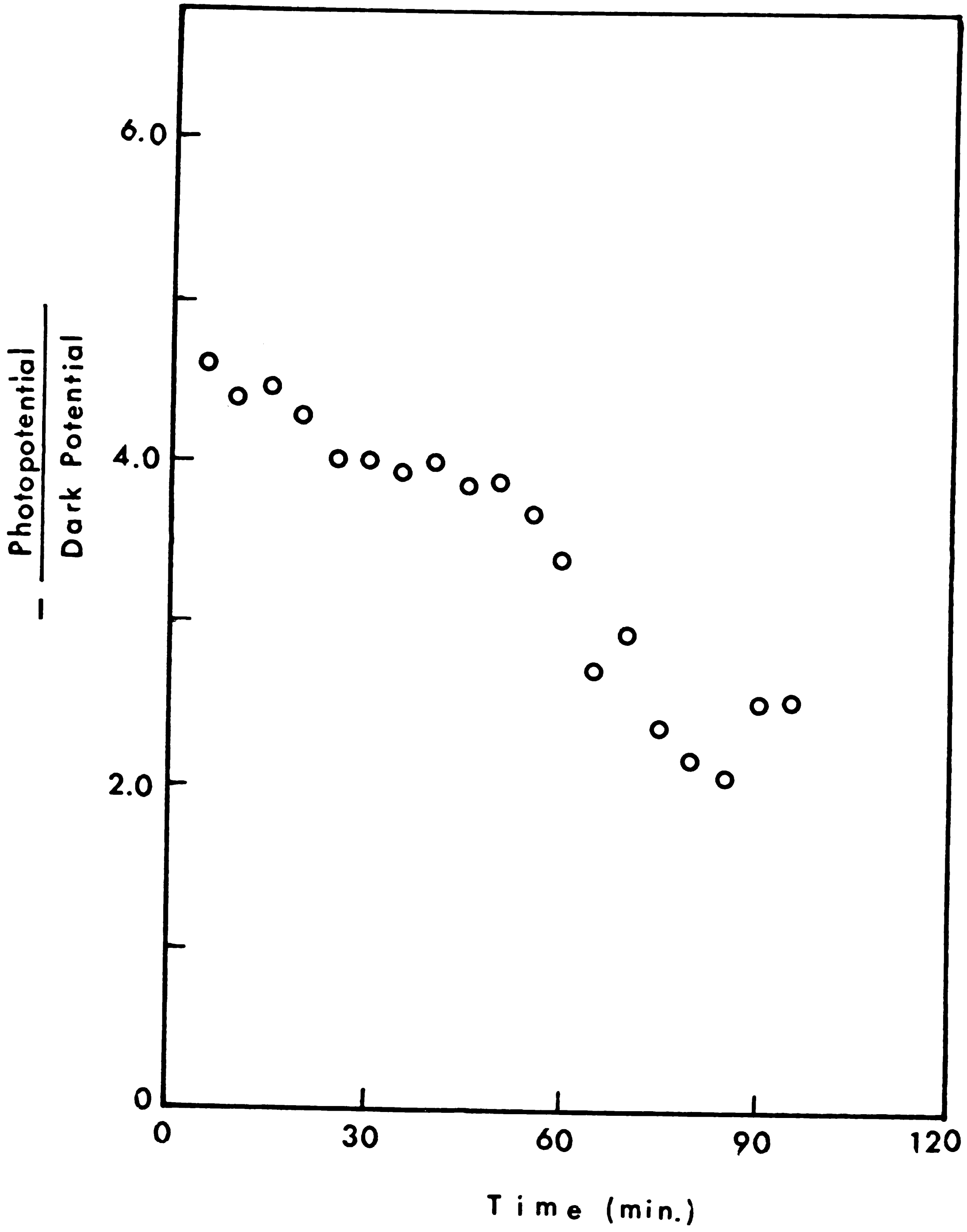
a - 0 minute
b - 10
c - 20
d - 30
e - 40
f - 50
g - 60
h - 70
i - 80
k - 155

order to examine the regeneration problem a membrane was repeatedly exposed to a long-time (30 second) illumination and the dark membrane potential as well as the photoresponse was investigated. In Figure 8 the effect of repetitive illuminations on the photoresponses is shown. The photoresponse decreased in its magnitude with an increasing number of illuminations, without changing the wave form appreciably. Shown in Figure 9 the dark potential also decreased with an increasing number of illuminations. The decrease in the dark membrane potential may indicate the generation of the photoproducts which modify the microenvironment of the membrane. If so, the decrease in the photoresponse should have a strong relationship with a decrease in the dark membrane potential, since the photoproducts cannot be formed without the consumption of the photosensitive pigment molecules, and the presence of the products may decrease the rate of photoreaction via porduct inhibition. A close relationship between the dark membrane potential and the photoresponse is shown to exist in Figure 10. It is interesting that the ratio of the photopotential to the dark potential decreases as the number of illuminations increases. This decrease in the efficiency of the dark potential for generation of the photopotential in an increasing number of illuminations, and the apparent lack of changes in the mode of the time course of the photoresponse with an

Figure 9.--Photopotential (R2) and dark membrane potential as a function of time from the first illumination.


+40
+30
+20
+10
0
Photo Potential (mV)
0
-20
-40
-60
-80
Dark Membrane Potential (mV)
30
60
90
Time (min)

Figurs 10.--The ratio of photopotential (R2) to the dark membrane potential as a function of the time from the first illumination.


6.0
4.0
2.0
0
- Photopotential / Dark Potential
0
30
60
90
120
Time (min.)

increasing number of illuminations, and the apparent lack of changes in the mode of the time course of the photoresponse with an increasing number of illuminations clearly demonstrate that the regeneration problem does not exist in the present model system. The irreversible changes in the membrane potential and photoresponse due to the repetition of long-term illuminations may suggest the possible existence of the photoreaction which leads to the production of membrane active chemical species, possibly protons.

Light Intensity Dependence of the Photoresponses

Due to the fact that the photoresponses could be influenced by temperature and the dark membrane potential could be controlled by pH gradient across the membrane, the mode of the intensity dependence of the photoresponse could also be dependent on the temperature and the pH gradient. In order to examine this point, the intensity dependence was studied at various pH gradients as a function of temperature.

The data shown in Figures 11-18 were taken by making a membrane at a given temperature, varying the pH gradient, and measuring the intensity dependence of the photoresponses at each pH gradient. The inner chamber contained 10^{-3} M $FeCl_3$ in 10^{-3} M KCl solution and the pH of the outer chamber was varied by the addition of HCl to the original

Figure 11.--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 pH was higher. The inner chamber contained about 10^{-3} M $FeCl_3$ in 10^{-3} M KCl while the outer chanber contained HCl and the same concentration of KCl.

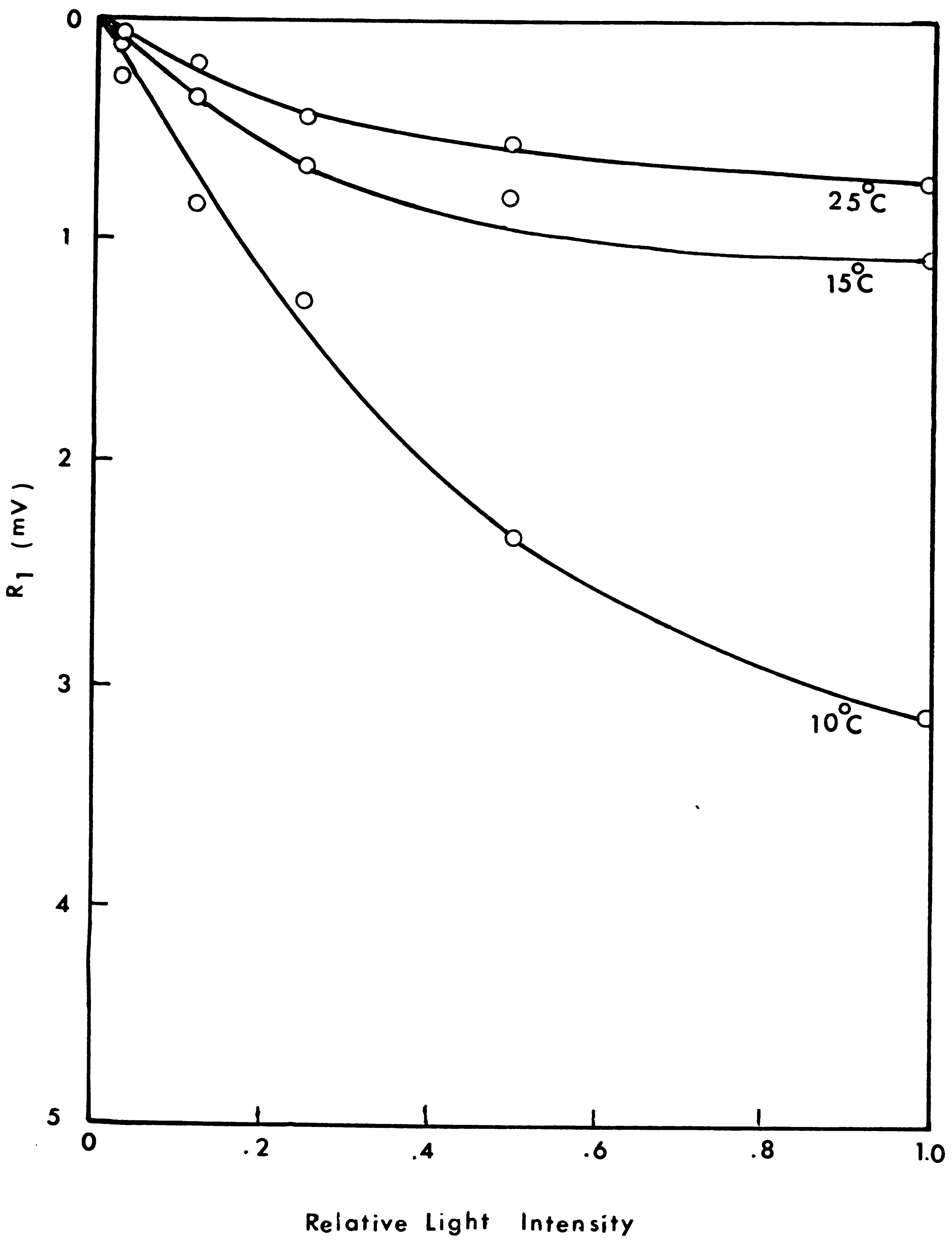

0
1
2
3
4
5
R$_1$ (mV)
25°C
15°C
10°C
0
.2
.4
.6
.8
1.0
Relative Light Intensity

Figure 12.--The effect of relative intensity of light on R1 component at ΔpH 0.6 as a function of temperature. The inner pH was 3.3 and the outer pH was higher. The inner chamber contained about 10^{-3} M $FeCl_3$ in 10^{-3} M KCl while the outer chamber contained HCl and the same concentration of KCl.

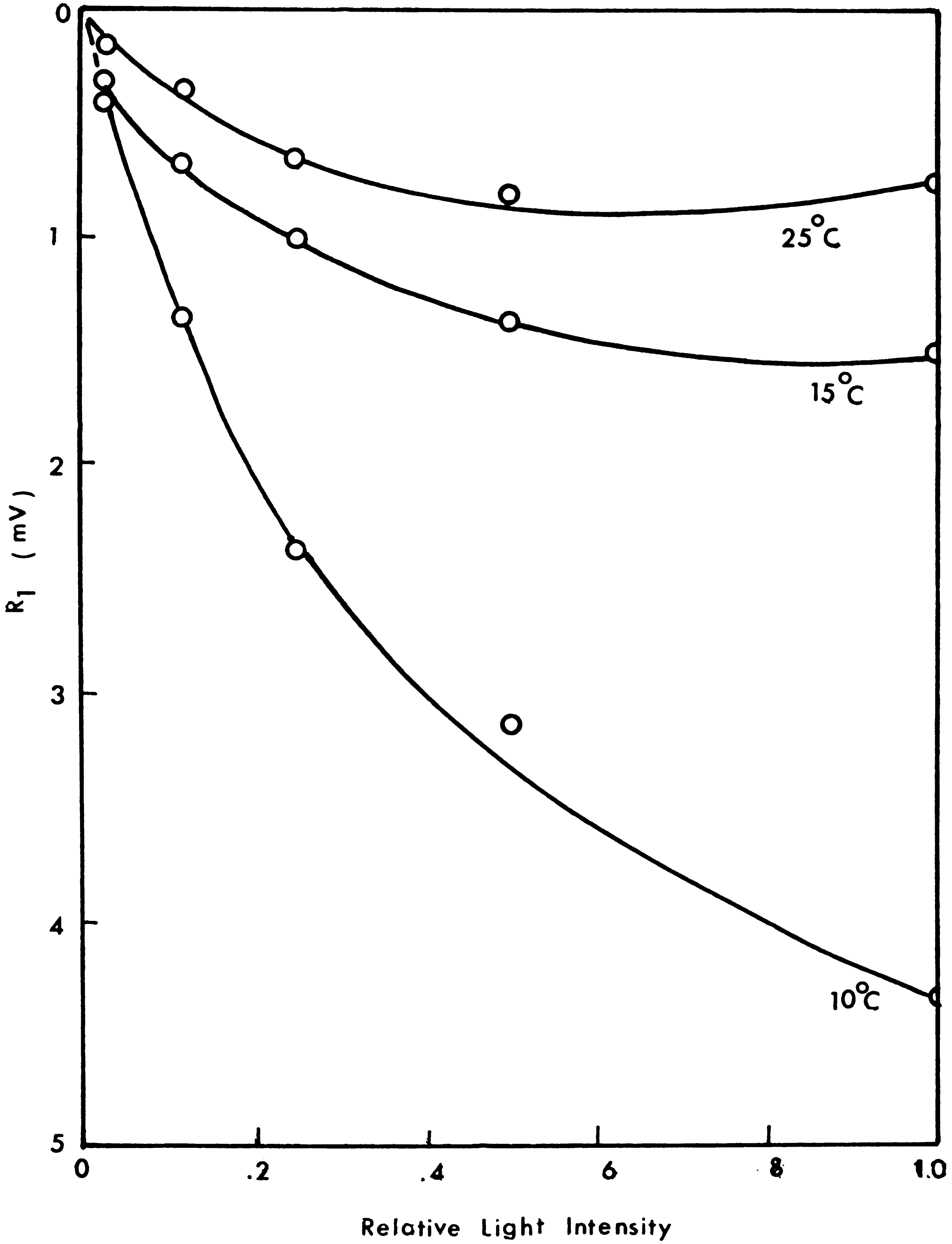

0
1
2
3
4
5
R_1 (mV)
0
.2
.4
6
8
1.0
Relative Light Intensity
25°C
15°C
10°C

Figure 13.--The effect of relative intensity of light on R1 component at ΔpH 1.0 as a function of temperature. The inner pH was 3.3 and the outer pH was higher. The inner chamber contained about 10^{-3} M $FeCl_3$ in 10^{-3} M KCl while the outer chamber contained HCl and the same concentration of KCl.

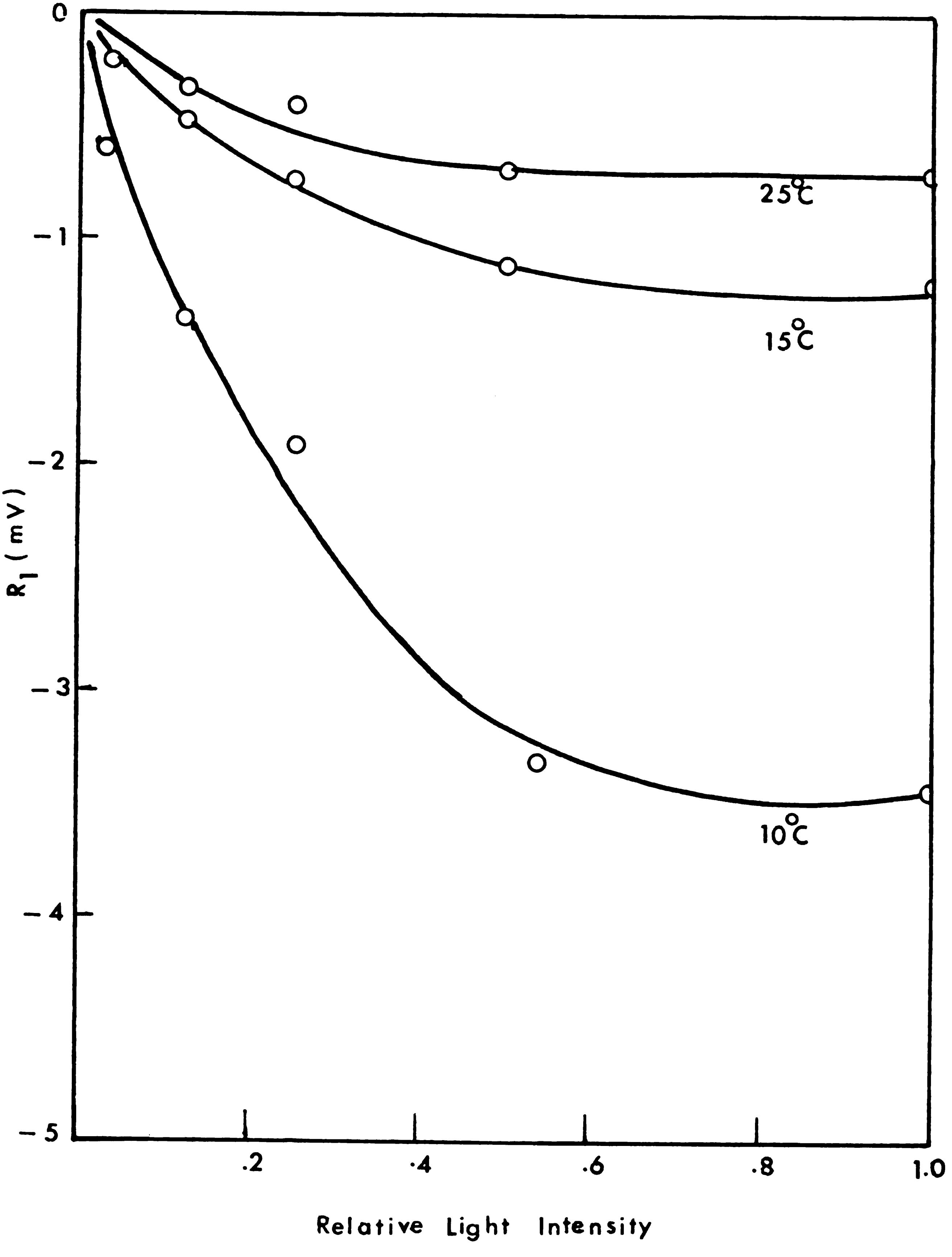

0
-1
-2
-3
-4
-5
R1 (mV)
.2
.4
.6
.8
1.0
Relative Light Intensity
25°C
15°C
10°C

Figure 14.--The effect of relative intensity of light on R1 component at ΔpH 2.0 as a function of temperature. The inner pH was 3.3 and the outer pH was higher. The inner chamber contained about 10^{-3} M $FeCl_3$ in 10^{-3} M KCl while the outer chamber contained HCl and the same concentration of KCl.

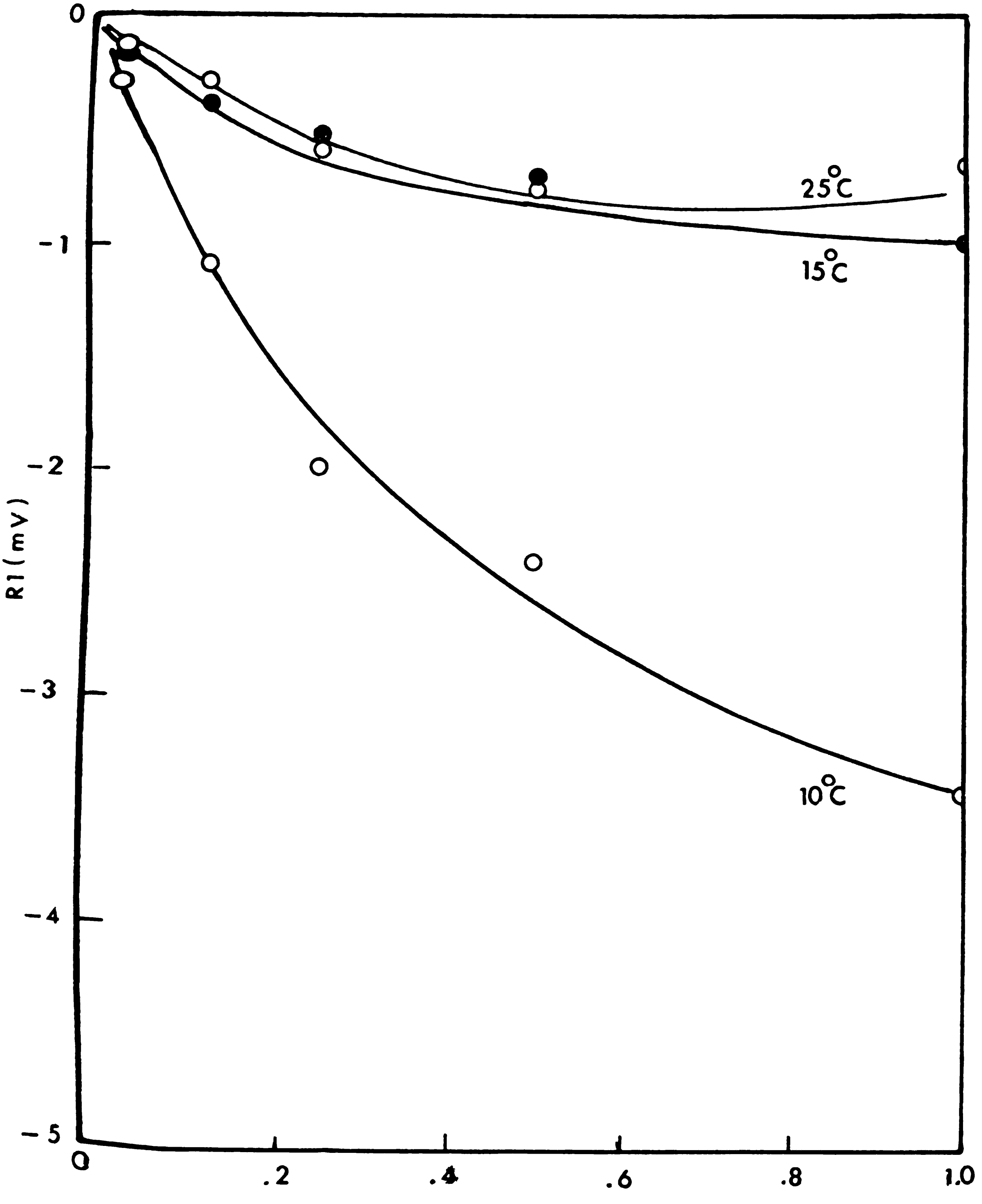

0
-1
-2
-3
-4
-5
R1 (mV)
25°C
15°C
10°C
0
.2
.4
.6
.8
1.0
Relative Light Intensity

Figure 15.--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 10^{-3} M $FeCl_3$ in 10^{-3} M KCl while the outer chamber contained HCl and the same concentration of KCl.

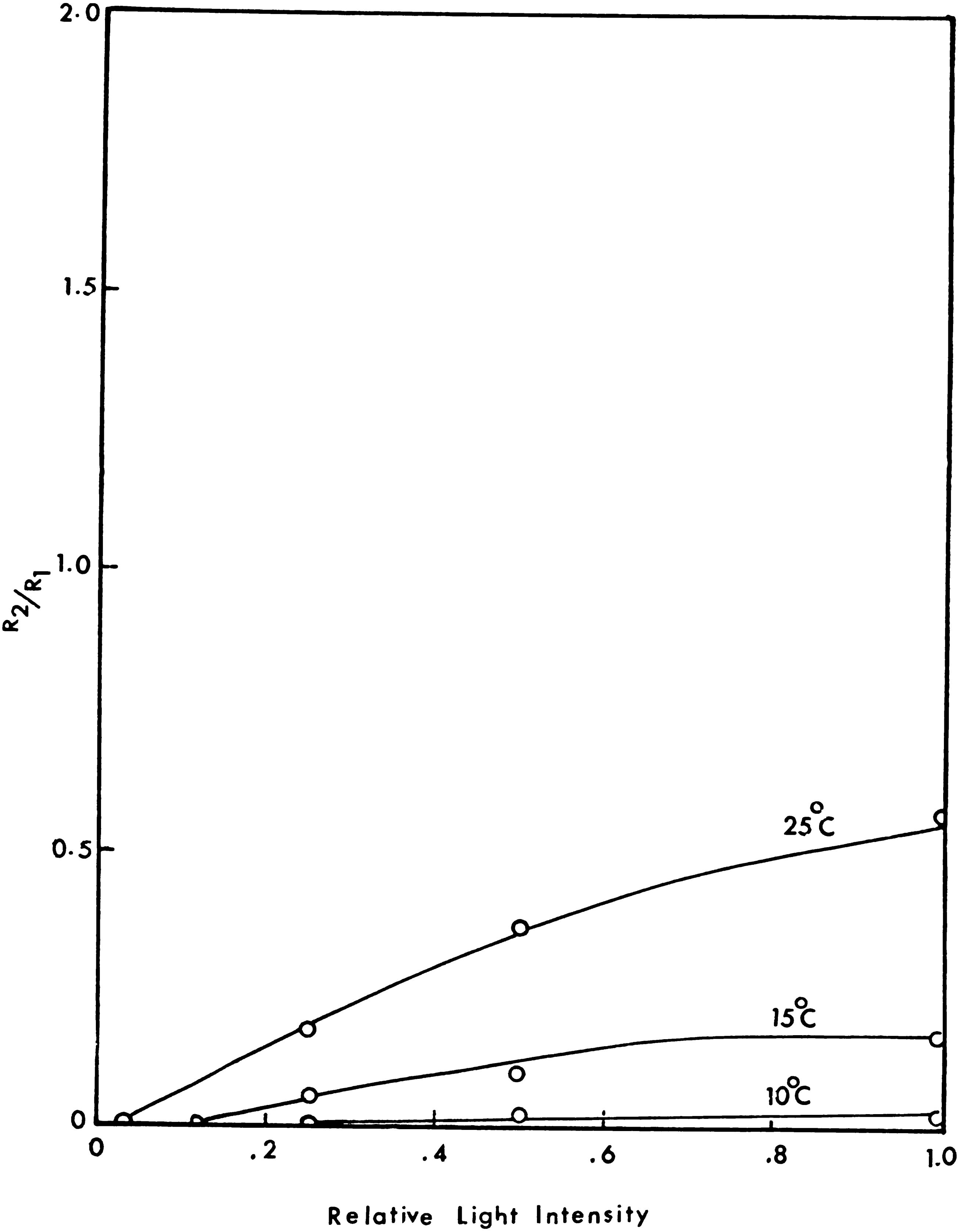

2.0
1.5
1.0
0.5
0
R2/R1
25°C
15°C
10°C
0
.2
.4
.6
.8
1.0
Relative Light Intensity

Figure 16.--The effect of relative intensity of light on R2/R1 at ΔpH 0.6 as a function of temperature. The inner pH was 3.3 and the outer pH was higher. The inner chamber contained about 10^{-3} M $FeCl_3$ in 10^{-3} M KCl while the outer chamber contained HCl and the same concentration of KCl.

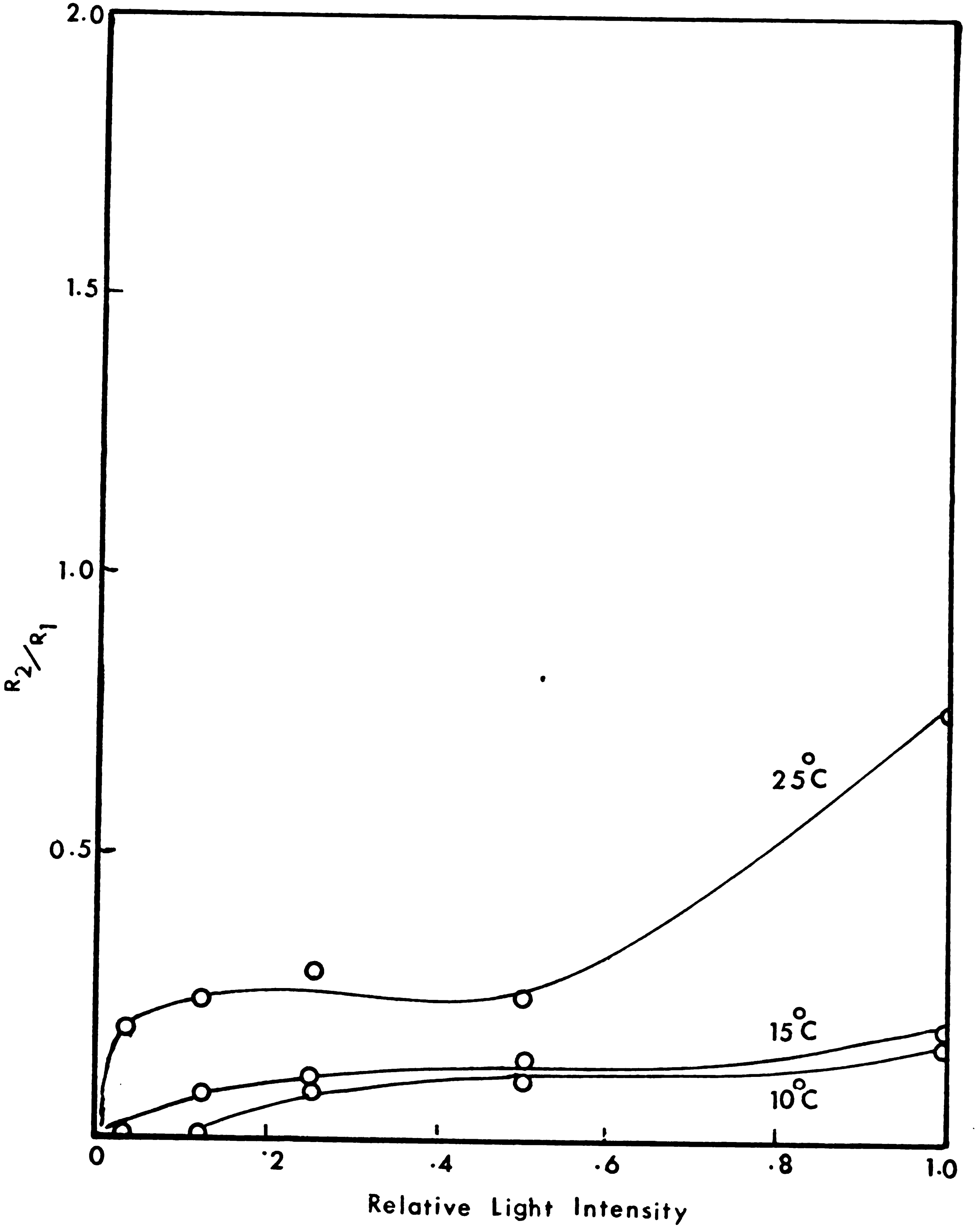

2.0
1.5
1.0
0.5
0
R2/R1
25°C
15°C
10°C
0
·2
·4
·6
·8
1.0
Relative Light Intensity

Figure 17.--The effect of relative intensity of light on R2/R1 at ΔpH 1.0 as a function of temperature. The inner pH was 3.3 and the outer pH was higher. The inner chamber contained about 10^{-3} M $FeCl_3$ in 10^{-3} M KCl while the outer chamber contained HCl and the same concentration of KCl.

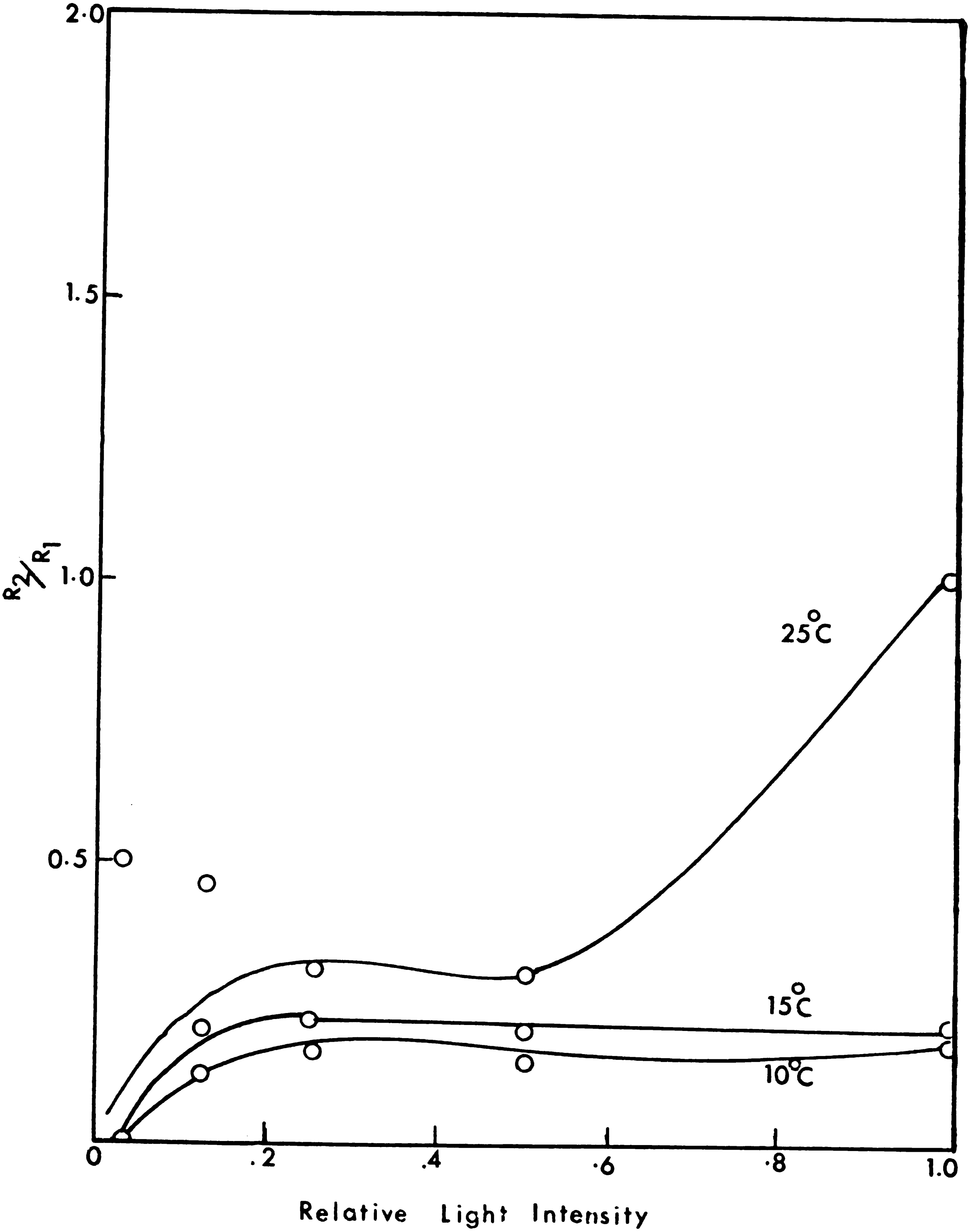
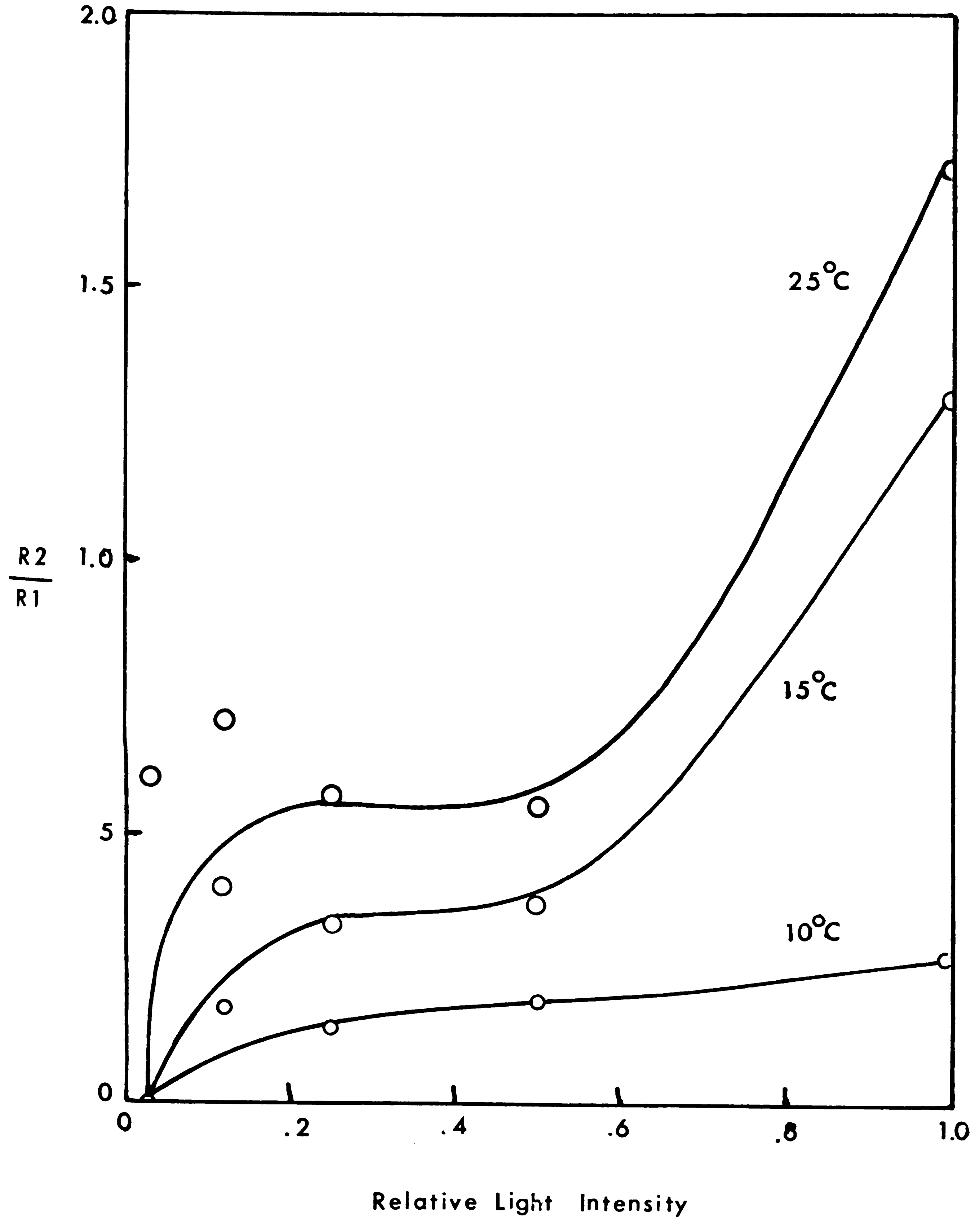

2·0
1.5
1·0
0·5
R_2/R_1
0
.2
.4
·6
·8
1.0
Relative Light Intensity
25°C
15°C
10°C

Figure 18.--The effect of relative intensity of light on R2/R1 at ΔpH 2.0 as a function of temperature. The inner pH was 3.3 and the outer pH was higher. The inner chamber contained about 10^{-3} M $FeCl_3$ in 10^{-3} M KCl while the outer chamber contained HCl and the same concentration of KCl.


2.0
1.5
1.0
5
0
R2
R1
25°C
15°C
10°C
0
.2
.4
.6
.8
1.0
Relative Light Intensity

10^{-3} M 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 gradient.

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

The effect of pH gradient 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 (Figures 15-18).

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 gradient 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 Figure 19, 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.

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 chamber contained 0.1 ml of 0.1 M $FeCl_3$. The photoresponses for one second illumination at various temperatures ranging from 9°C to 44°C are presented in Figure 20. At room temperature (25°C) the photoresponse showed a distinct biphasic response. When the temperature was raised to 44°C only R2 component could be seen. On the other hand the lowering of temperatures down to 9°C abolished the R2 component completely and isolated the R1 component. It is remarkable that this complete simulation of the temperature dependence of the early receptor potential could be achieved within this small range of temperature variation.

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

Figure 19.--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 assymetry. The photopotentials were recorded from a single membrane.

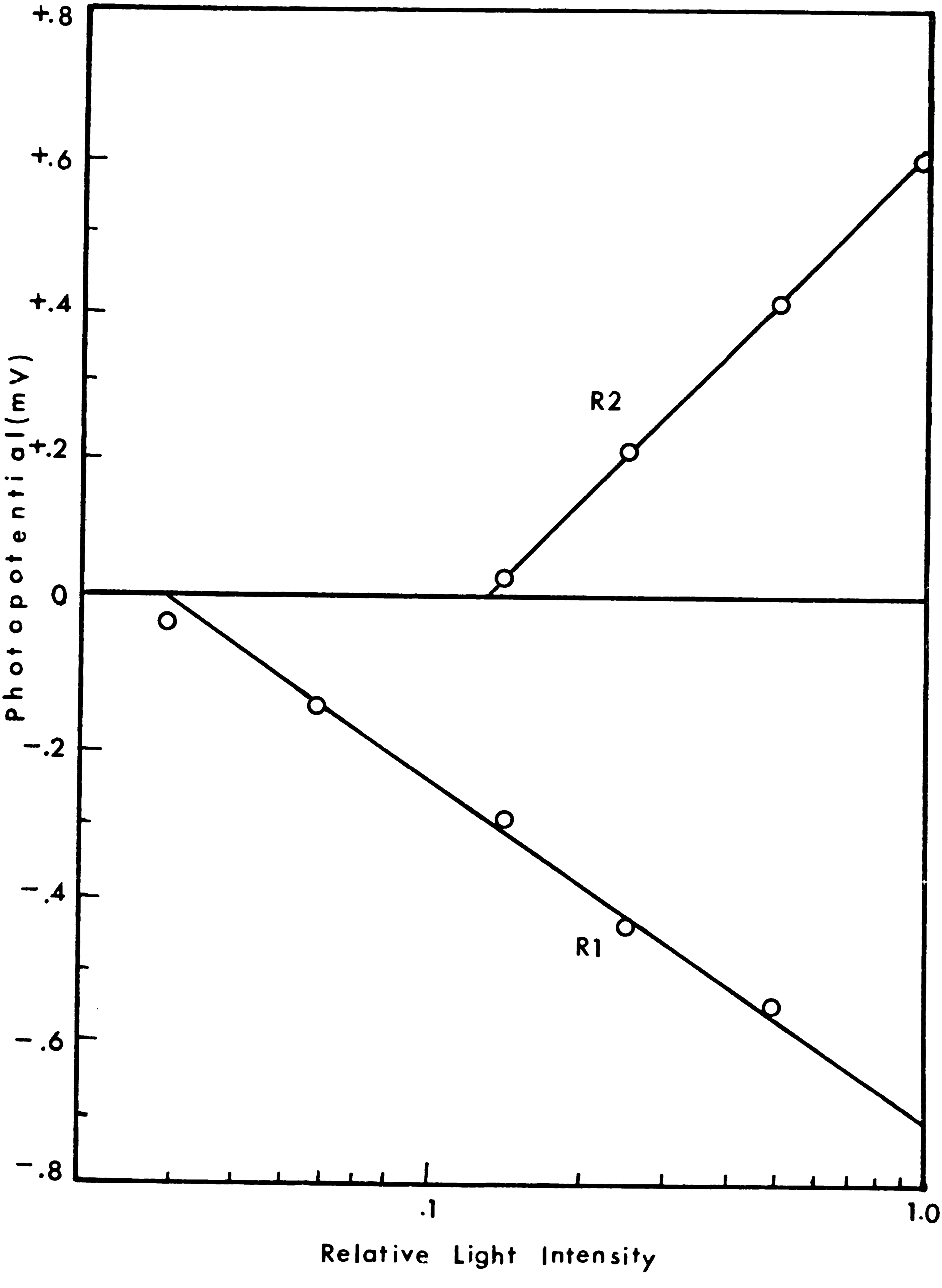
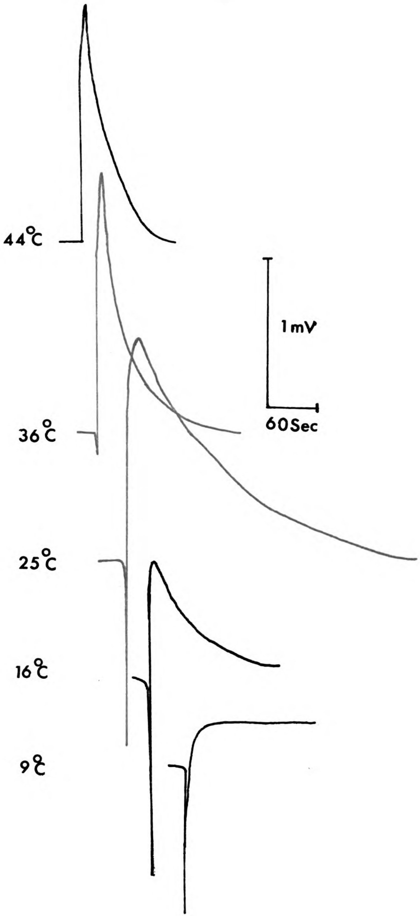

+.8
+.6
+.4
+.2
0
-.2
-.4
-.6
-.8
Photopotential(mV)
R2
R1
.1
1.0
Relative Light Intensity

Figure 20.--The time course of the photoresponse of all-*trans*-retinal BLM. $FeCl_3$ was placed in the inner chamber. The duration of the illumination was 1 second. The outside pH was 5.4 and the inside pH was 3.3.


44°C
36°C
25°C
16°C
9°C
1mV
60Sec

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 as shown in Figure 21. It can be seen that the complete disappearance of R1 component occurred at 44°C and that of R2 at 9°C. 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 for various temperatures. In this work it became possible to relate the photopotential to the dark membrane properties as a function of temperature.

The time courses of the photoresponses obtained with the system which contained only HCl with pH gradient of 0.7 were shown in Figure 22. The same features observed in Figure 20 can be seen but the temperatures for the appearance of R2 and the disappearance of R1 were higher than those of Figure 20. This difference may be due to a smaller pH gradient across the membrane system shown in Figure 22. Indeed as shown in the next section increasing pH gradient could enhance the R2 component significantly.

The data shown in Figure 23 were obtained in the presence of ferric ions in the inner chamber while

Figure 21.--The ratio of R2 to R1 as a function of temperature. The data shown in Figure 20 were used for the calculation of the ratios.

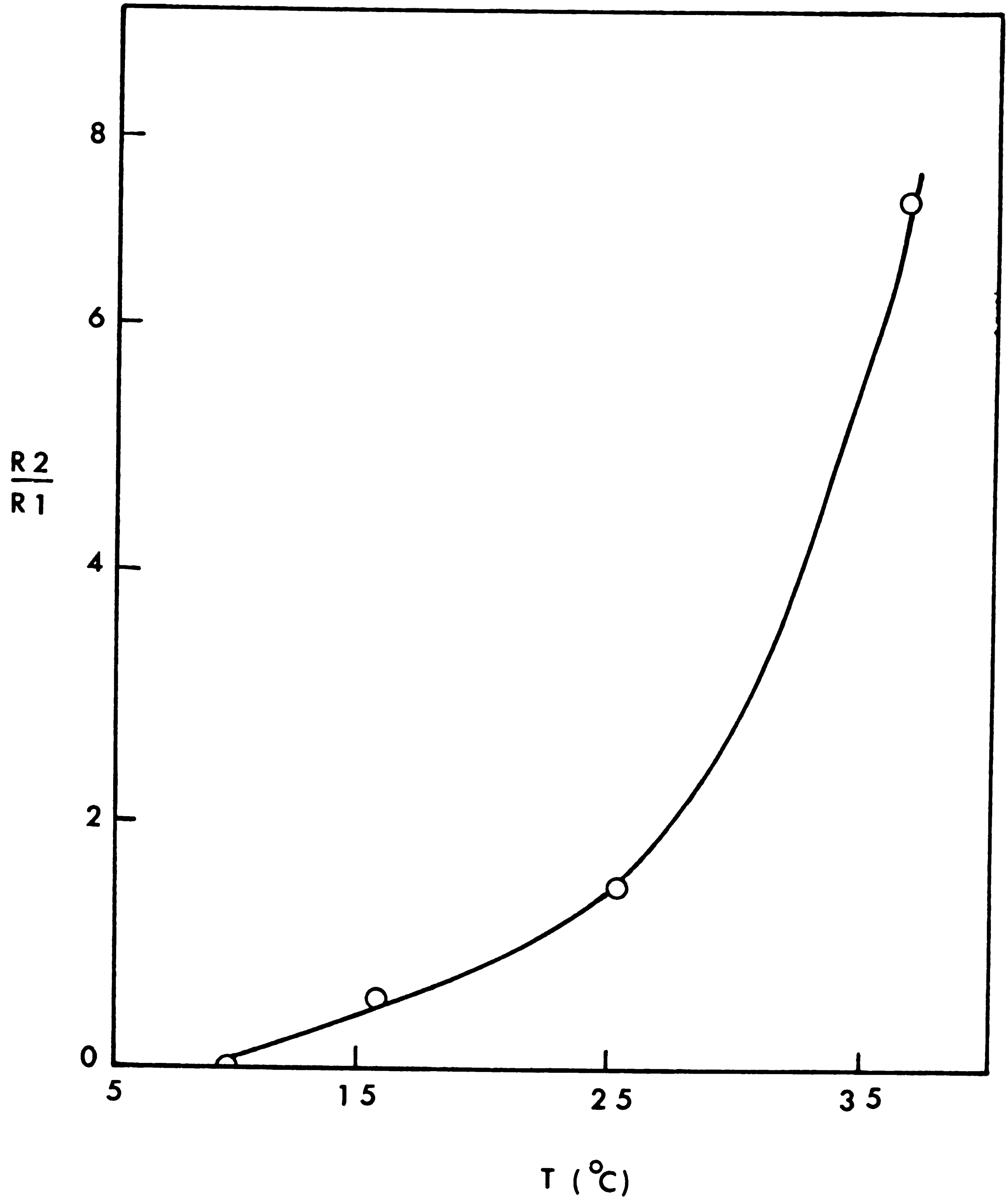

R2/R1
0
2
4
6
8
5
15
25
35
T (°C)

Figure 22.--The time course of the photoresponses of all-*trans*-retinal BLM 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 second.

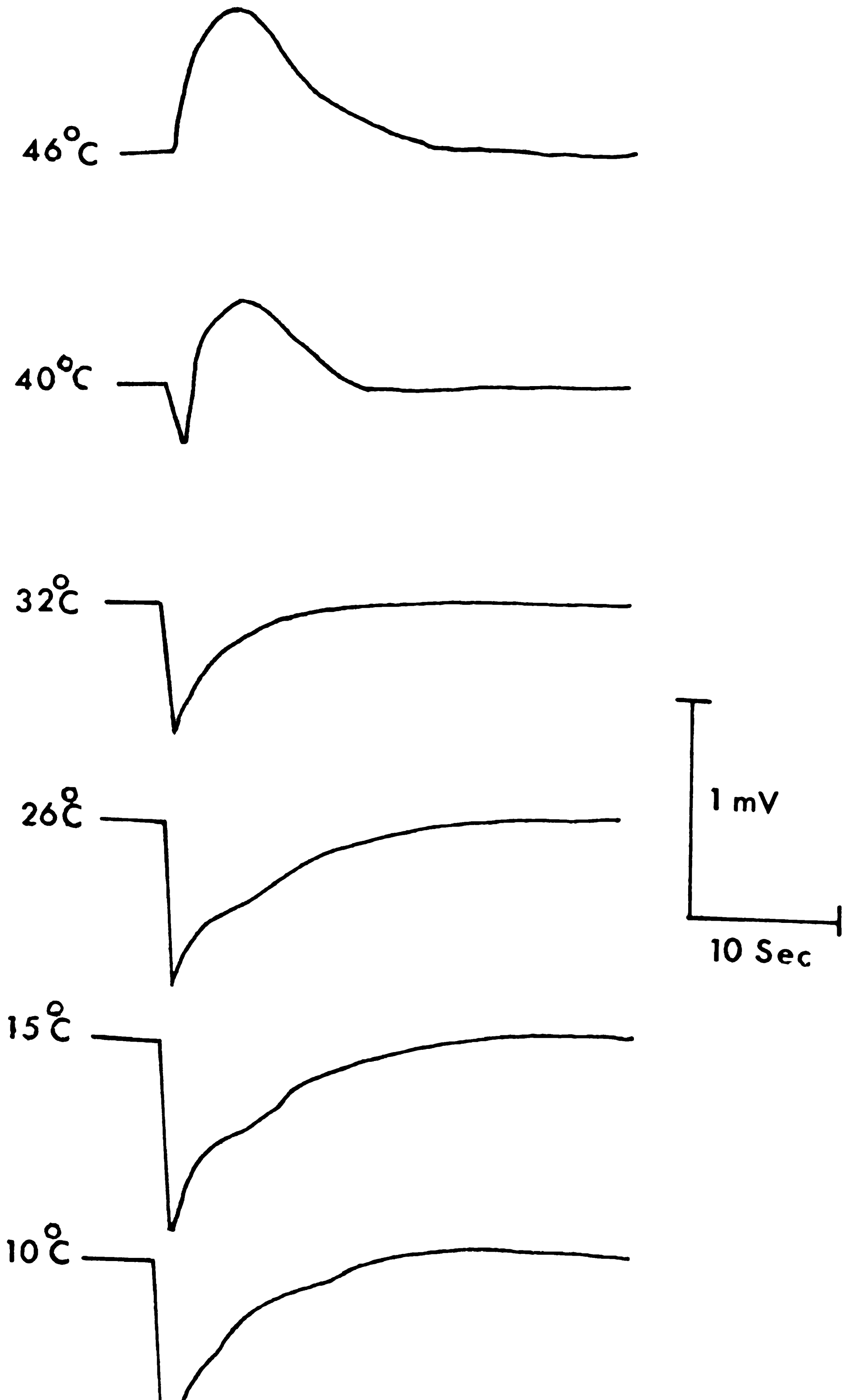
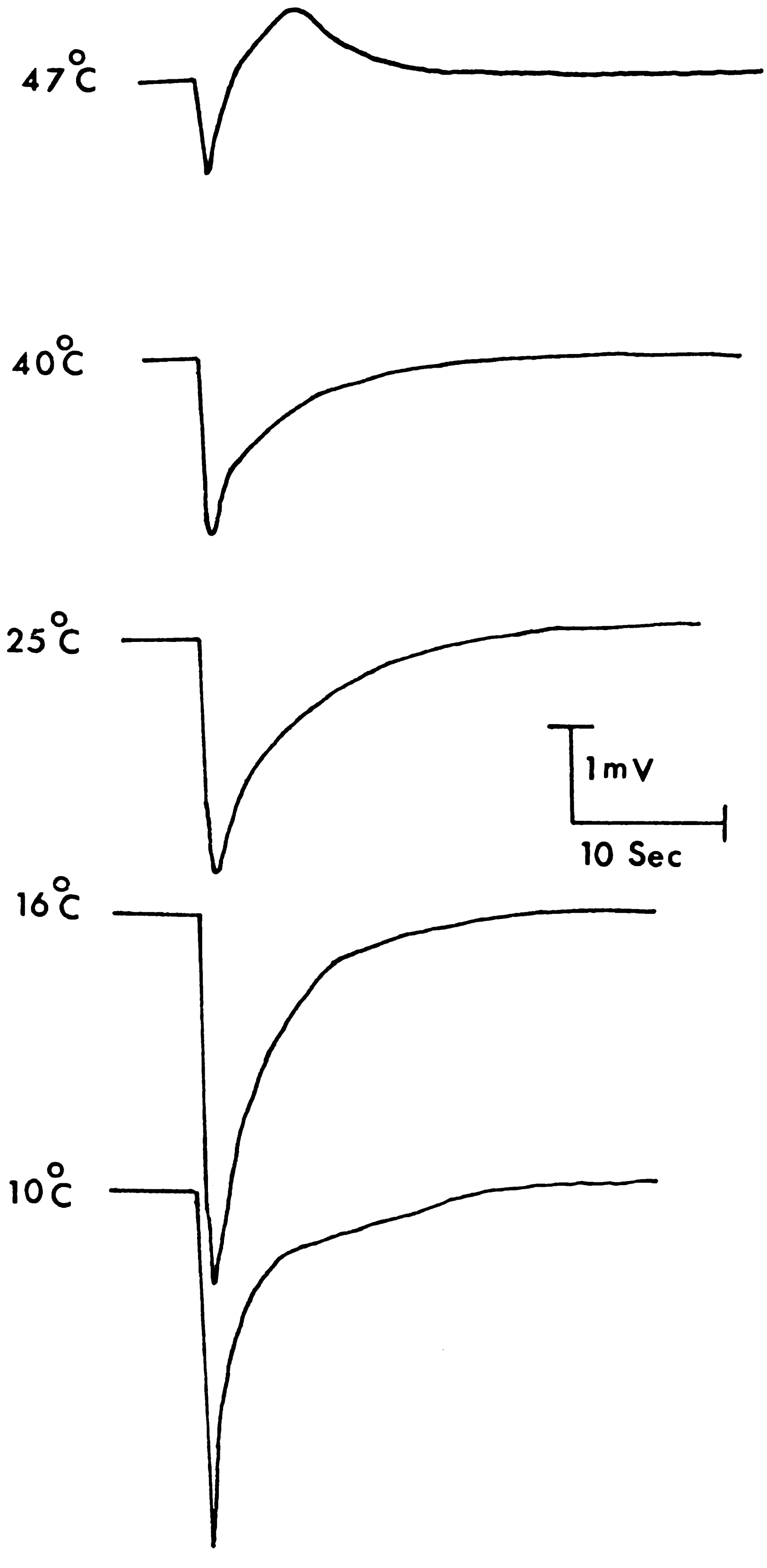

46°C
40°C
32°C
26°C
15°C
10°C
1 mV
10 Sec

Figure 23.--The time course of the photoresponses of all-*trans*-retinal BLM 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, $FeCl_3$ to lower pH to 2.8. The duration of the illumination was 1 second.


47°C
40°C
25°C
1mV
10 Sec
16°C
10°C

maintaining the same pH gradient as the system used for obtaining the data presented in Figure 22. The magnitude of the photoresponses was increased by approximately four times. The temperature for the appearance of the R2 component was elevated to about 40°C and the R1 component could be seen even at 47°C.

The dark membrane potentials of both HCl and $FeCl_3$ systems increased with increasing temperature as shown in Figure 24. The thermal coefficients of these two systems were identical (Table 4). 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 ferric ions, which are good electron acceptors.

The semilog plot of the R1 component and the dark membrane resistance against the reciprocal temperature (Figures 25 and 26) revealed the close relationship between the two properties. Indeed, shown in Figure 27 the R1 component decreased with a decrease in the membrane resistance while the R2 component increased. In this particular experimental condition the thermal coefficients of the dark membrane potential, dark membrane resistance, and the R1 component were almost identical (Table 4). The R2 components had relatively higher thermal coefficients.

Figure 24.--The temperature dependence of the dark membrane potential of all-*trans*-retinal BLM in asymmetric HCl solution or asymmetric $FeCl_3$ solution.

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 was 5.1 kcal/mole.

b - The aqueous phases contained only HCl with the outside pH at 3.5 and the inside chamber contained, in addition to HCl, $FeCl_3$ to lower pH to 2.8. The thermal coefficient obtained from the slope of the straight line was 4.9 kcal/mole.

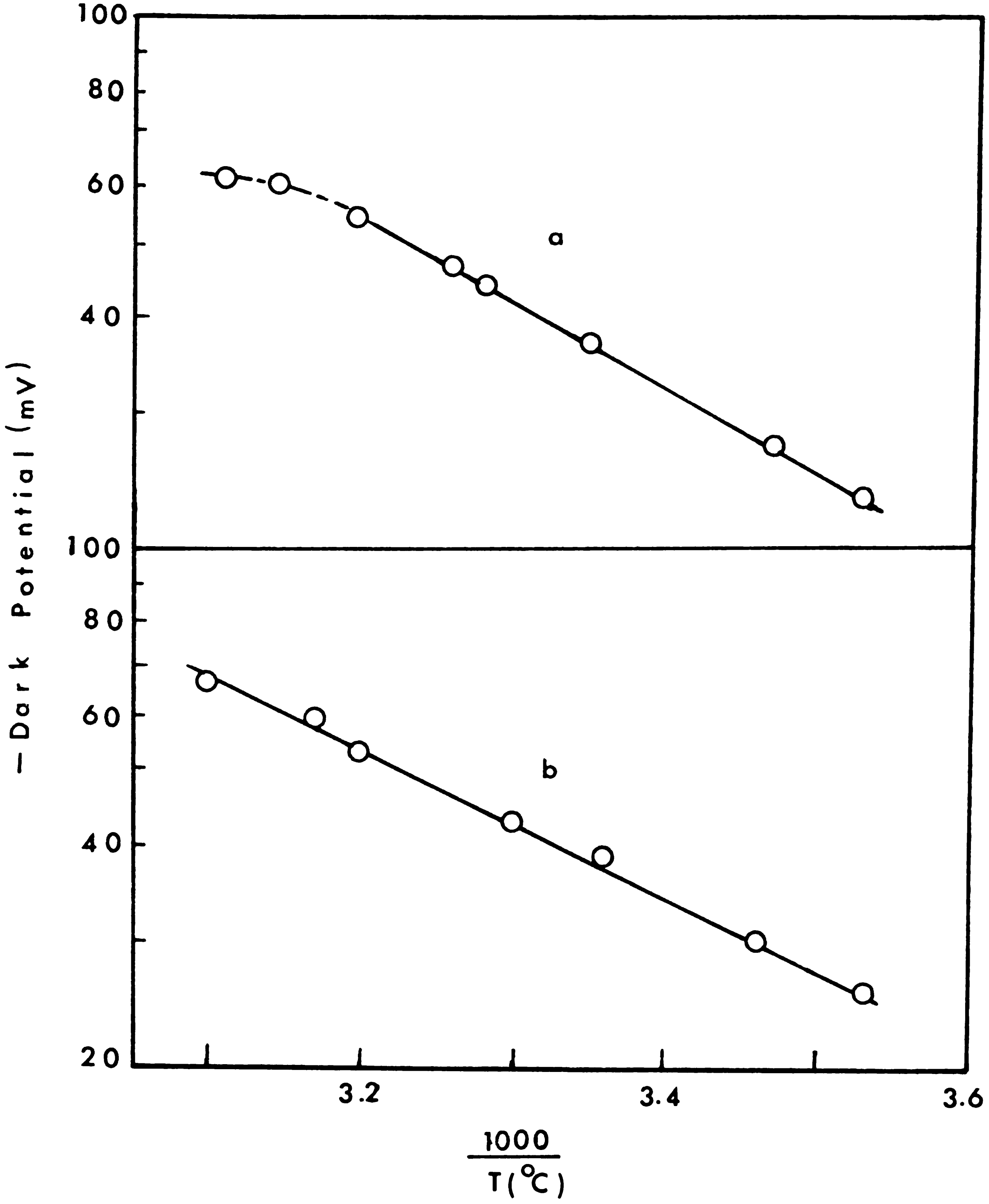

100
80
60
40
a
100
80
60
40
20
b
— Dark Potential (mV)
3.2
3.4
3.6
1000 / T(°C)

TABLE 4.--The thermal coefficients of hydrogen ion transfer number, membrane resistance, and R1 and R2 components.

Parameter Observed	Thermal Coefficient (kcal/mole) in	
	HCl	$FeCl_3$
Dark Membrane Potential	5.1	4.9
Membrane Resistance	6.9	6.9
R1 Component	9.1	6.9
R2 Component	18	15

Figure 25.--Dark membrane resistance and Rl photopotential as a function of temperature in HCl system. See the legend of Figure 24 for the experimental details.

R_m - Membrane Resistance

R_i - Photopotential Rl

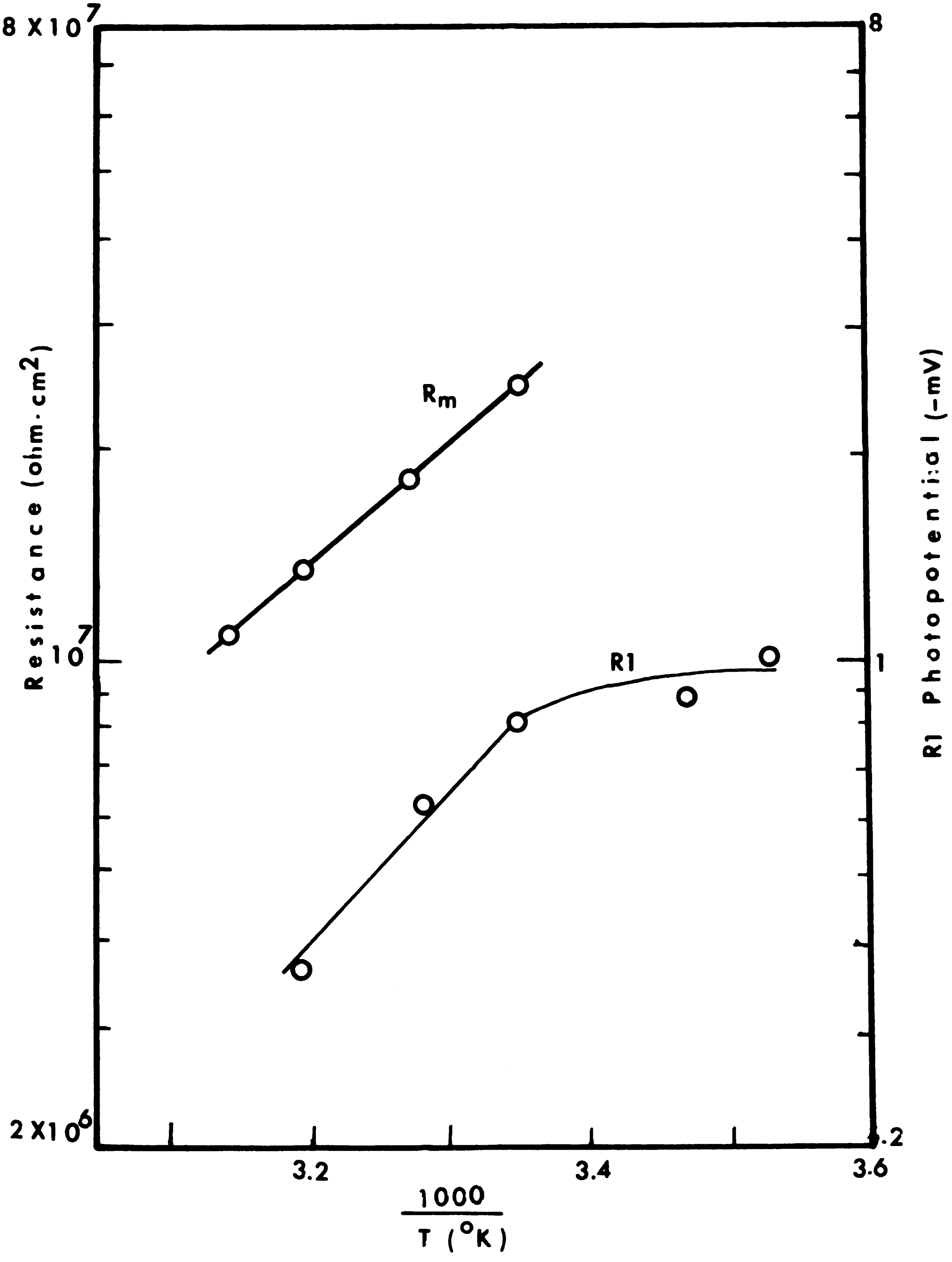
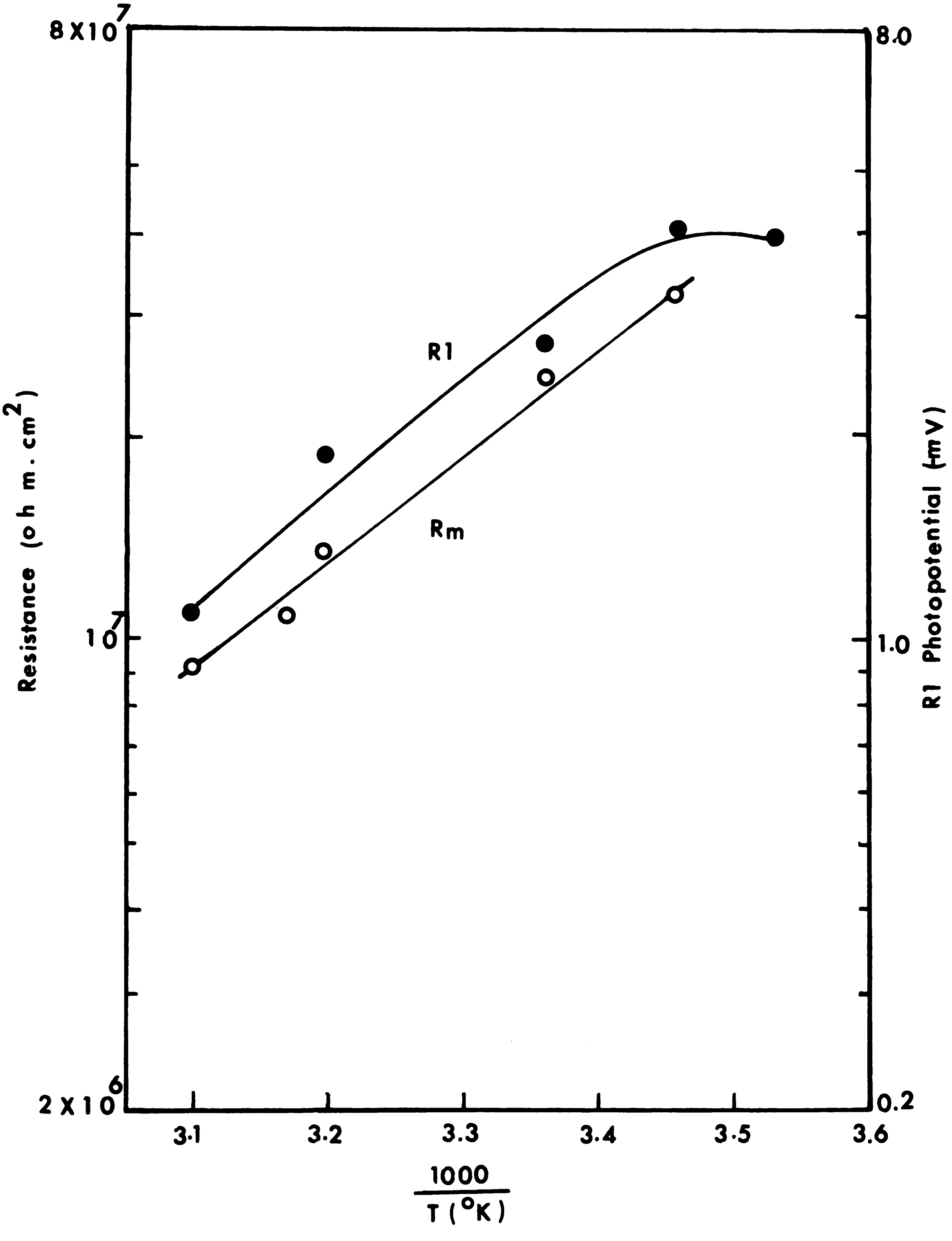

8 X10^7
10^7
2 X10^6
Resistance (ohm·cm^2)
8
1
.2
R1 Photopotential (−mV)
Rm
R1
3.2
3.4
3.6
1000 / T (°K)

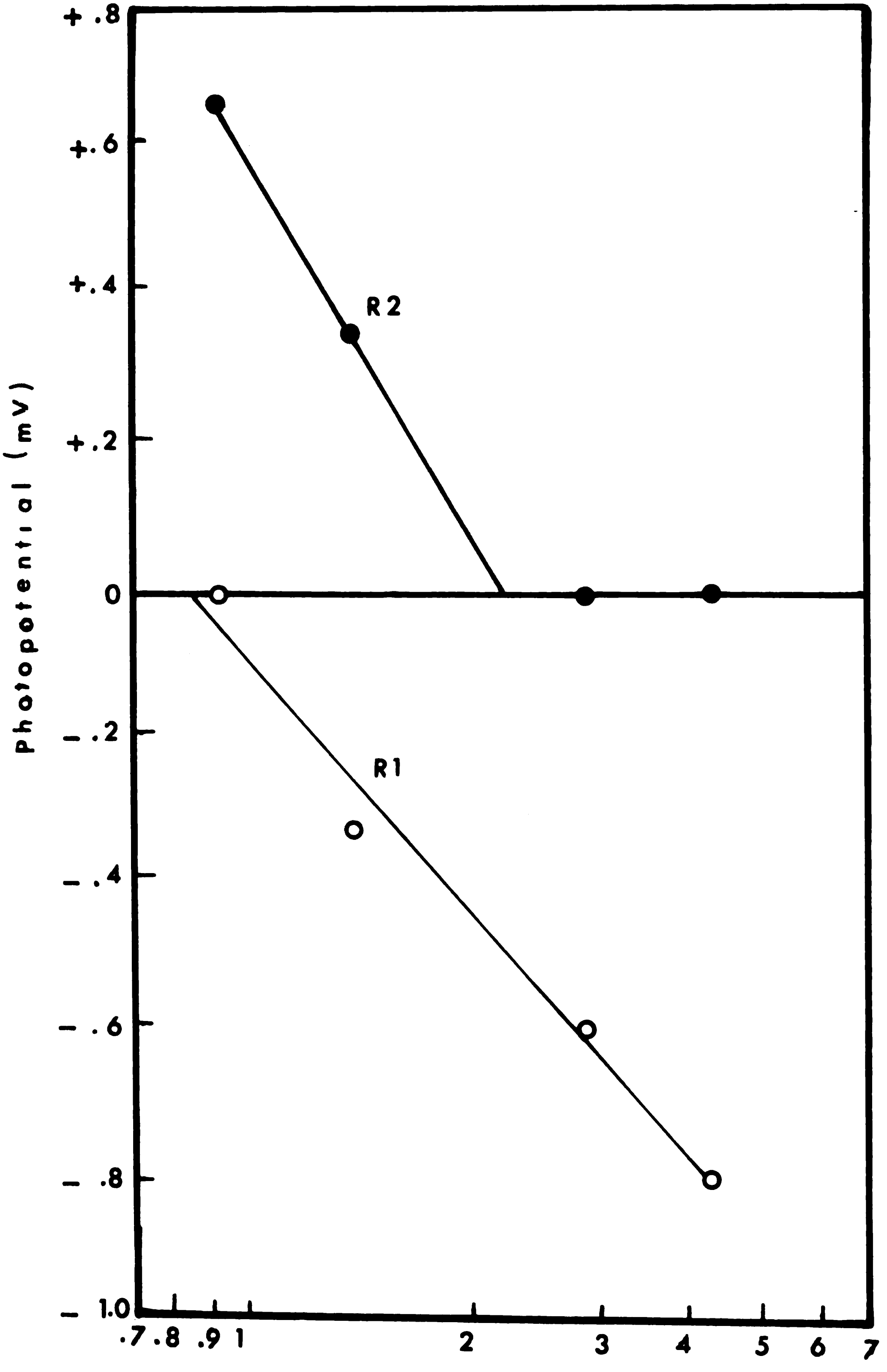
Figure 26.--Dark membrane resistance and R1 photopotential as a function of temperature in $FeCl_3$ system. See the legend of Figure 24 for the experimental details.

R_m - Membrane Resistance
R1 - Photopotential R1



8 X 10^7
10^7
2 X 10^6
Resistance (o h m . cm^2)
8.0
1.0
0.2
R1 Photopotential (-mV)
R1
Rm
3.1
3.2
3.3
3.4
3.5
3.6
1000 / T (°K)

Figure 27.--The relationship between two components of photopotentials and dark membrane resistance. The experimental conditions were the same as those shown in the legend of Figure 24a.


+ .8
+ .6
+ .4
+ .2
0
– .2
– .4
– .6
– .8
– 1.0
Photopotential (mV)
R2
R1
.7
.8
.9
1
2
3
4
5
6
7
Resistance (10^7 ohm.cm^2)

The Effect of pH on the Photoresponses

The fact that resting membrane potential was solely dependent on the gradient of proton concentrations in the bathing solutions separated by the membrane immediately suggests that the mode of photoresponse should show significant variations upon changes in pH gradient. Furthermore, the possible generation of protons as one of the photoproducts may complicate the effect of pH on the photoresponses.

When pH of the outer chamber was increased by the addition of KOH, the magnitude of the slow component (R2) increased, and masking of the negative fast component (R1) could be seen unambiguously (Figure 28). The magnitude of R2 increased with an increase in pH in a linear relationship to pH as shown in Figure 29.

The Effect of Buffer Capacity on the Photoresponses

Due to the essential role of the hydrogen ion concentration gradient in determining the mode of the photoresponses, the effect of buffer capacity was also investigated.

In order to study the effect of buffer capacity the proton concentration gradient has to be kept constant for various buffer capacities. This was best achieved with the use of symmetric KCl systems and symmetric acetate buffer systems. As shown in Figure 30, changes

Figure 28.--The effect of pH on the photoresponses. The values of pH were those of the outer chamber adjusted with KOH and HCl in the 0.1 M KCl solution which was also contained in the inner chamber (pH 5.4).

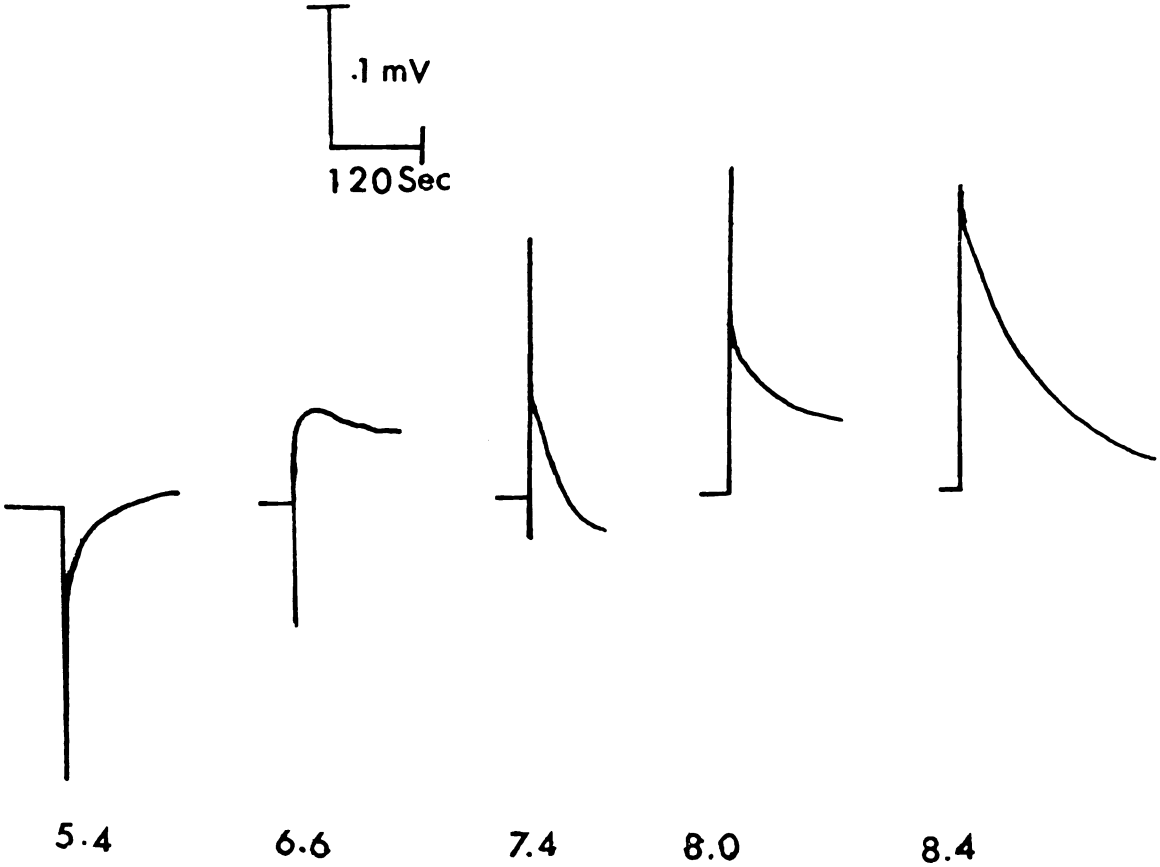

.1 mV
120 Sec
5.4
6.6
7.4
8.0
8.4

Figure 29.--R1 and R2 as a function of pH gradient across the membrane. Without the presence of a strong electron acceptor both R1 and R2 are linearly dependent on the pH gradient. The data used were those of Figure 28.

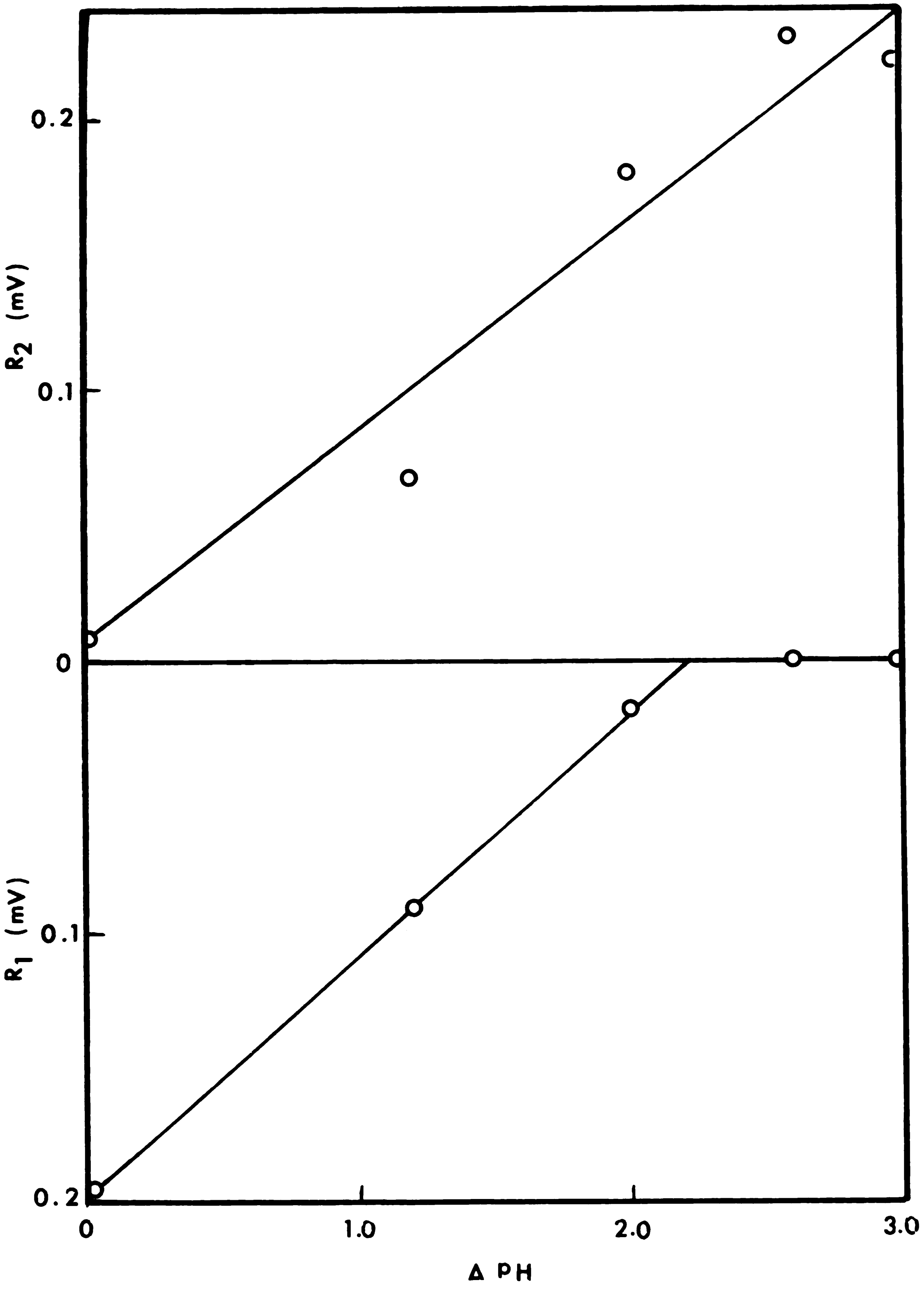

R2 (mV)
0.2
0.1
0
R1 (mV)
0.1
0.2
0
1.0
2.0
3.0
Δ PH

Figure 26.--Dark membrane resistance and Rl photopotential as a function of temperature in $FeCl_3$ system. See the legend of Figure 24 for the experimental details.

R_m - Membrane Resistance

Rl - Photopotential Rl

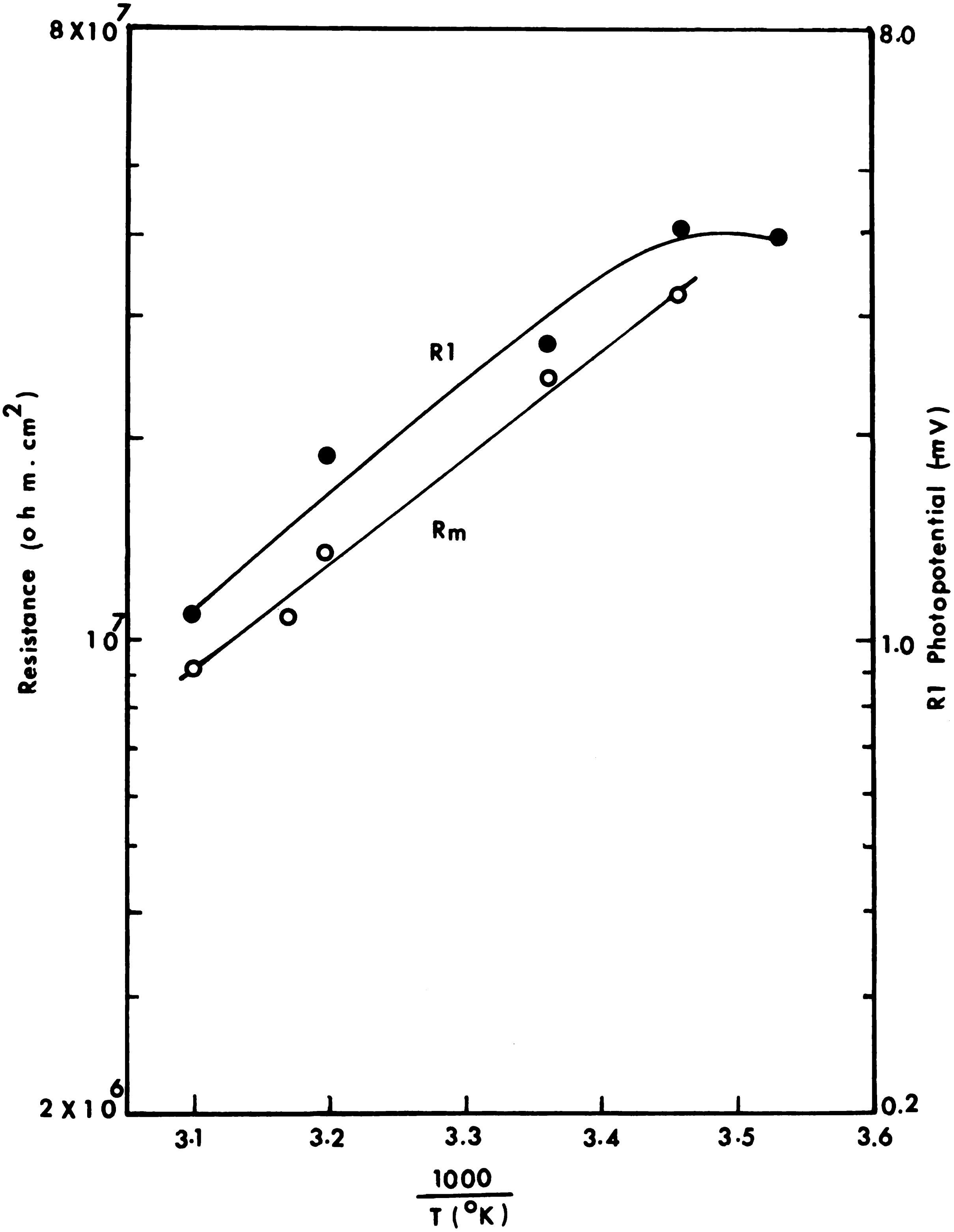
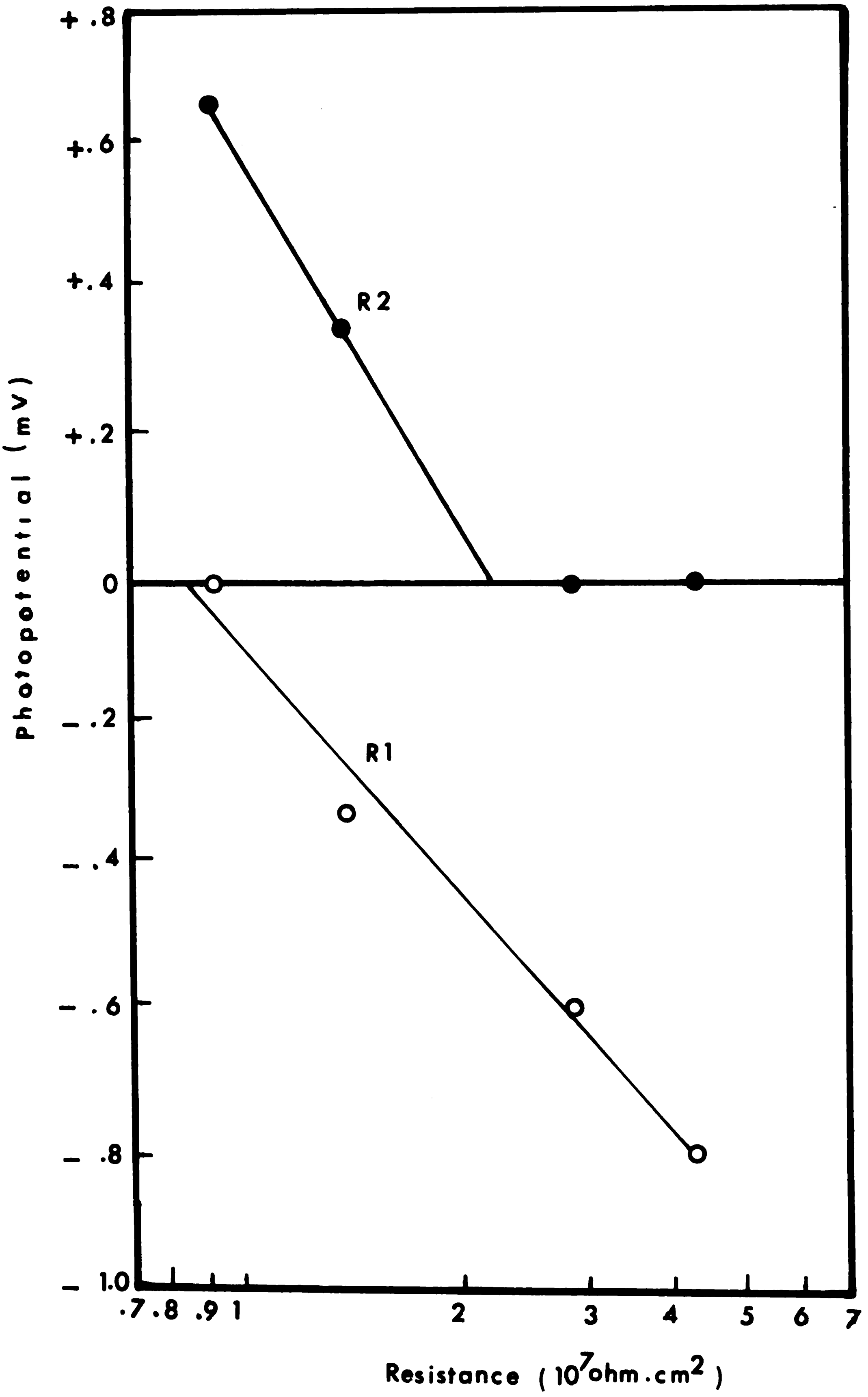

8 X 10^7
10^7
2 X 10^6
8.0
1.0
0.2
Resistance (o h m . cm^2)
R1 Photopotential (-mV)
R1
Rm
3.1
3.2
3.3
3.4
3.5
3.6
1000 / T (°K)

Figure 27.--The relationship between two components of photopotentials and dark membrane resistance. The experimental conditions were the same as those shown in the legend of Figure 24a.


+.8
+.6
+.4
+.2
0
−.2
−.4
−.6
−.8
−1.0
Photopotential (mV)
R2
R1
.7
.8
.9
1
2
3
4
5
6
7
Resistance (10^7 ohm.cm^2)

The Effect of pH on the Photoresponses

The fact that resting membrane potential was solely dependent on the gradient of proton concentrations in the bathing solutions separated by the membrane immediately suggests that the mode of photoresponse should show significant variations upon changes in pH gradient. Furthermore, the possible generation of protons as one of the photoproducts may complicate the effect of pH on the photoresponses.

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In order to study the effect of buffer capacity the proton concentration gradient has to be kept constant for various buffer capacities. This was best achieved with the use of symmetric KCl systems and symmetric acetate buffer systems. As shown in Figure 30, changes

Figure 28.--The effect of pH on the photoresponses. The values of pH were those of the outer chamber adjusted with KOH and HCl in the 0.1 M KCl solution which was also contained in the inner chamber (pH 5.4).

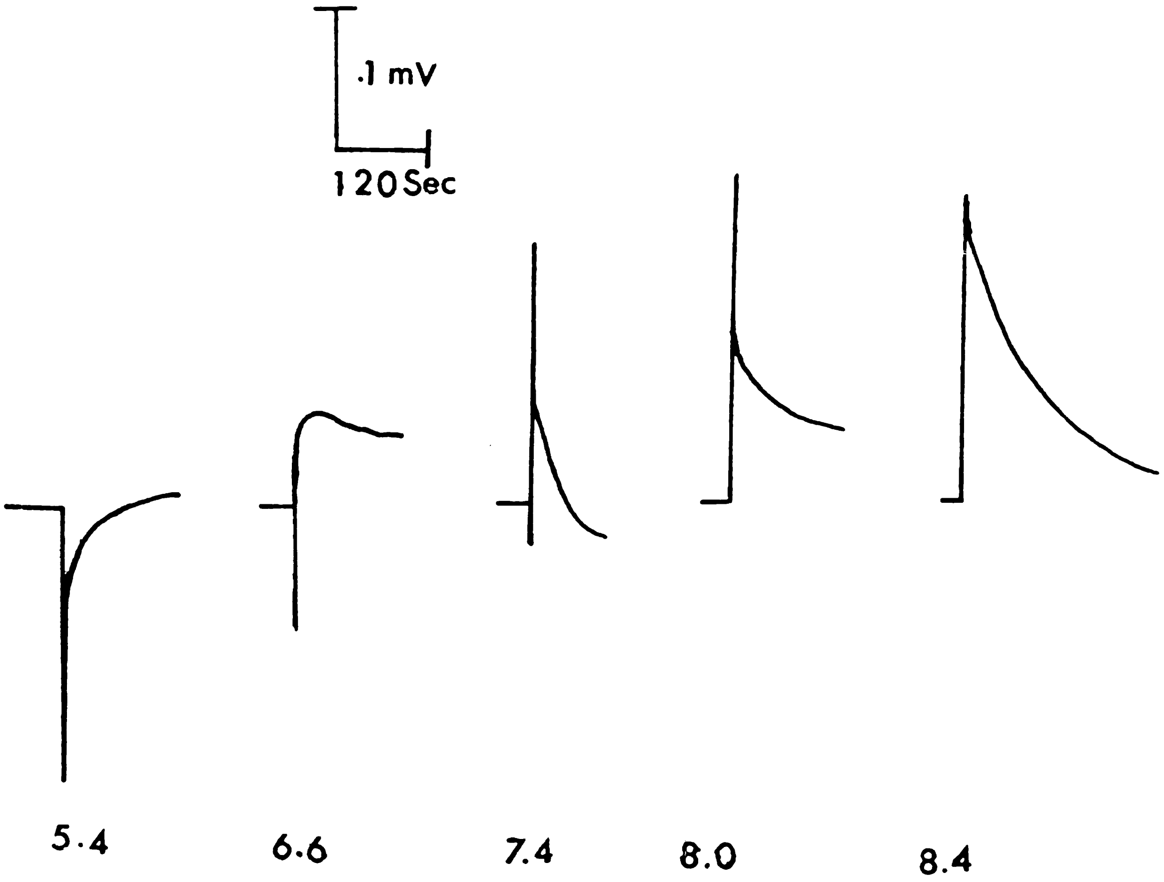

.1 mV
120 Sec
5.4
6.6
7.4
8.0
8.4

Figure 29.--R1 and R2 as a function of pH gradient across the membrane. Without the presence of a strong electron acceptor both R1 and R2 are linearly dependent on the pH gradient. The data used were those of Figure 28.

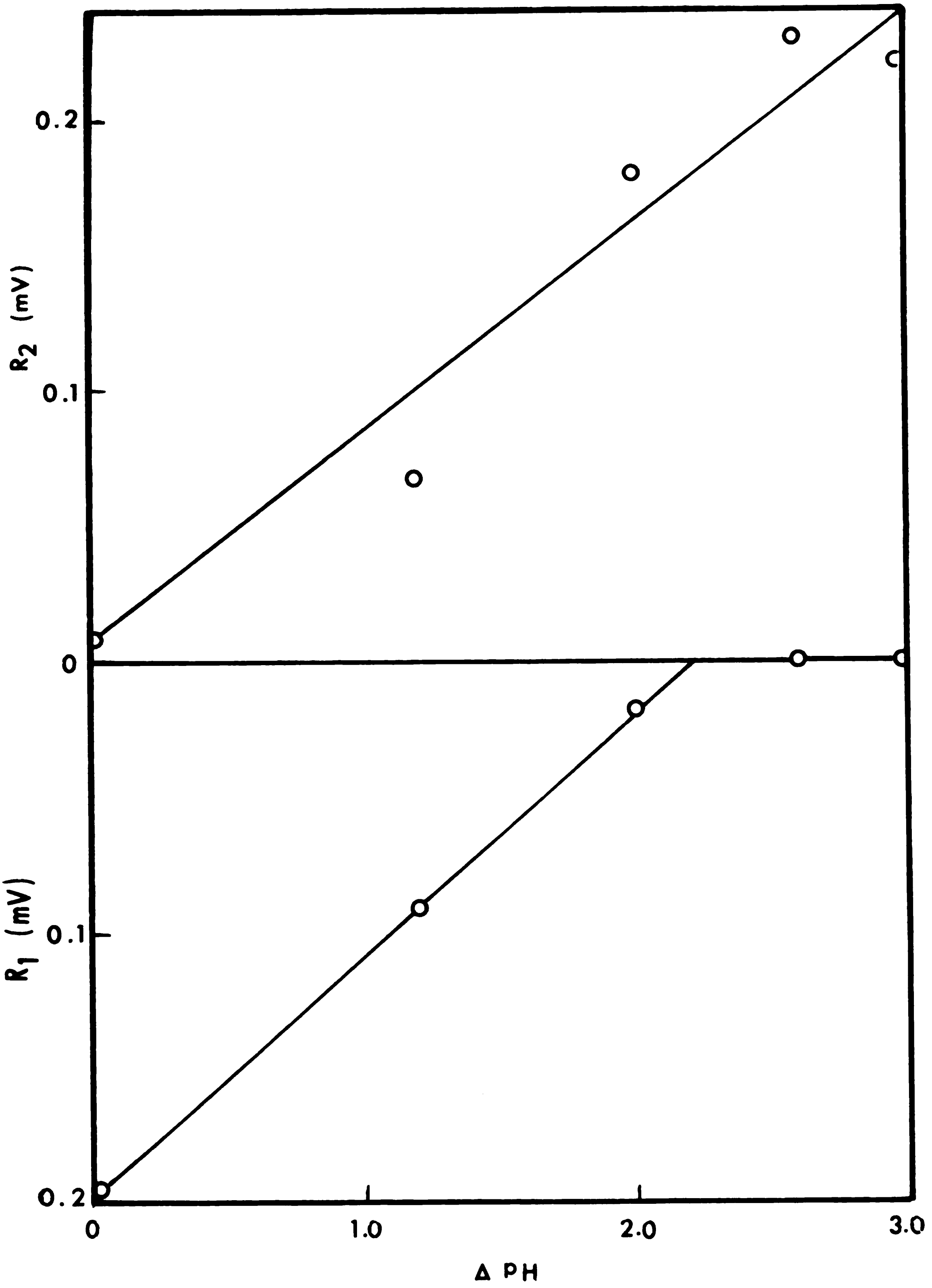
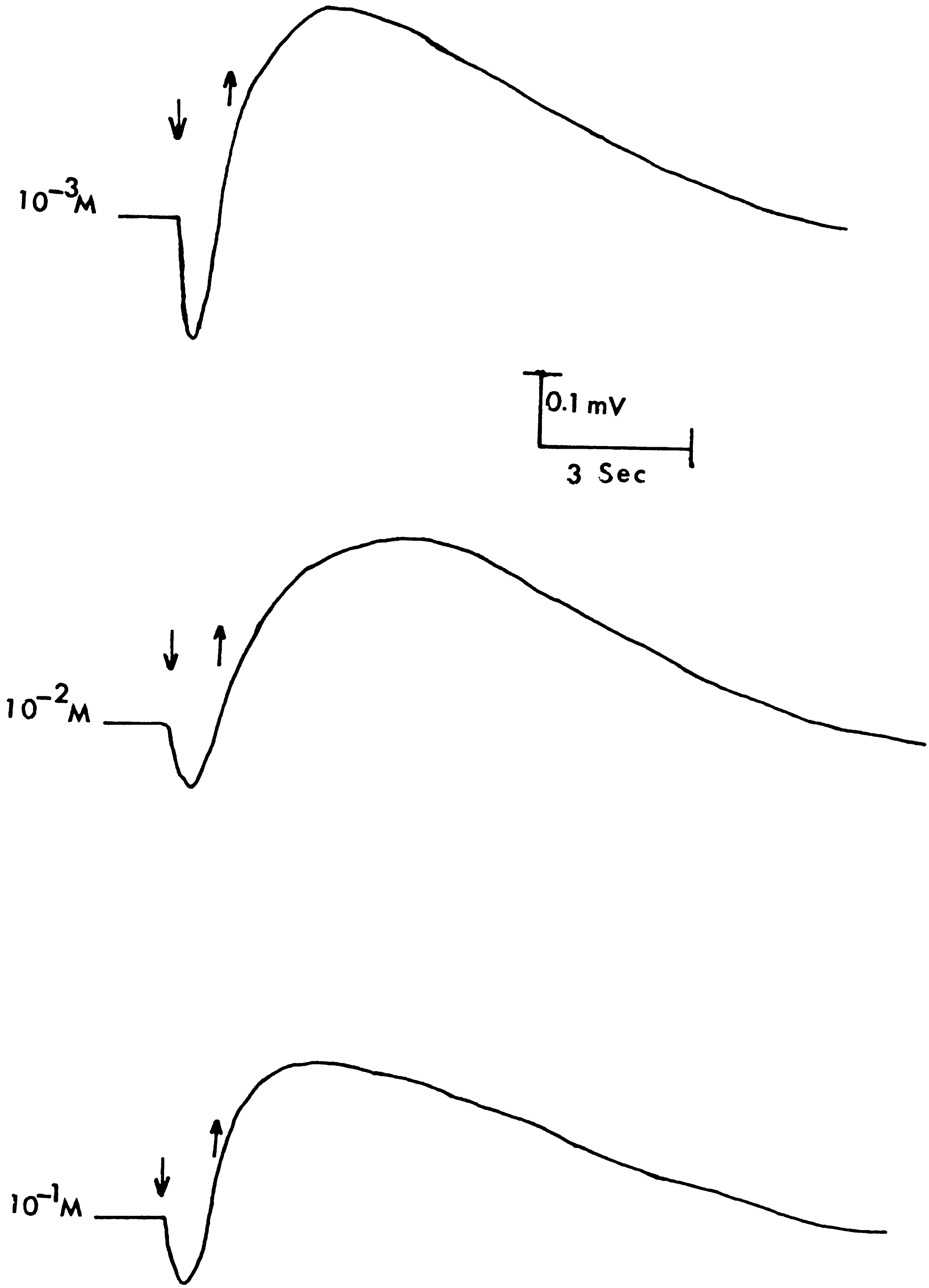

0.2
0.1
0
R_2 (mV)
0.1
0.2
R_1 (mV)
0
1.0
2.0
3.0
Δ PH

Figure 30.--The time course of the photoresponses of all-*trans*-retinal BLM in symmetric KCl solutions as a function of salt concentration. The upward arrow indicates the light-on and the downward arrow the light-off for 1 second illumination.


10^{-3}M
0.1 mV
3 Sec
10^{-2}M
10^{-1}M

in the concentration of KCl had no essential effect on the mode of photoresponses. In acetate buffer systems, however, the concentration effect was remarkable (Figure 31). In a 10^{-3} M system R1 and R2 could be seen in exactly similar mode to those of KCl systems. Indeed, at this buffer concentration there was almost no buffering action as demonstrated in Table 2. In a 10^{-2} M acetate buffer system the R2 component was completely eliminated and the R1 component was slightly enhanced. As the concentration was increased to 10^{-1} M the R1 component was essentially unaffected. These features are shown in Figure 32. In order to separate the effect of buffering capacity from that of the ionic strength the concentration dependence of the dark membrane resistance was compared with the concentration dependence of the photoresponse in KCl systems and acetate buffer systems as shown in Figure 32. In this comparison it can be clearly seen that the buffer capacity influenced mainly the R2 component so that in the presence of 10^{-2} M acetate buffer the complete elimination of the component was observed. The fact that this is the effect of buffer capacity can be recognized in the observation that the concentration dependence of the membrane resistance in acetate buffer systems was identical to the KCl systems, in which R2 was essentially unaffected. The effect of buffer capacity on the R2 component is strong supporting evidence for the

Figure 31.--The time course of the photoresponses of all-*trans*-retinal BLM in symmetric acetate buffer solutions as a function of buffer concentration. The upward arrow indicates the light-on and the downward arrow the light-off for 1 second illumination.

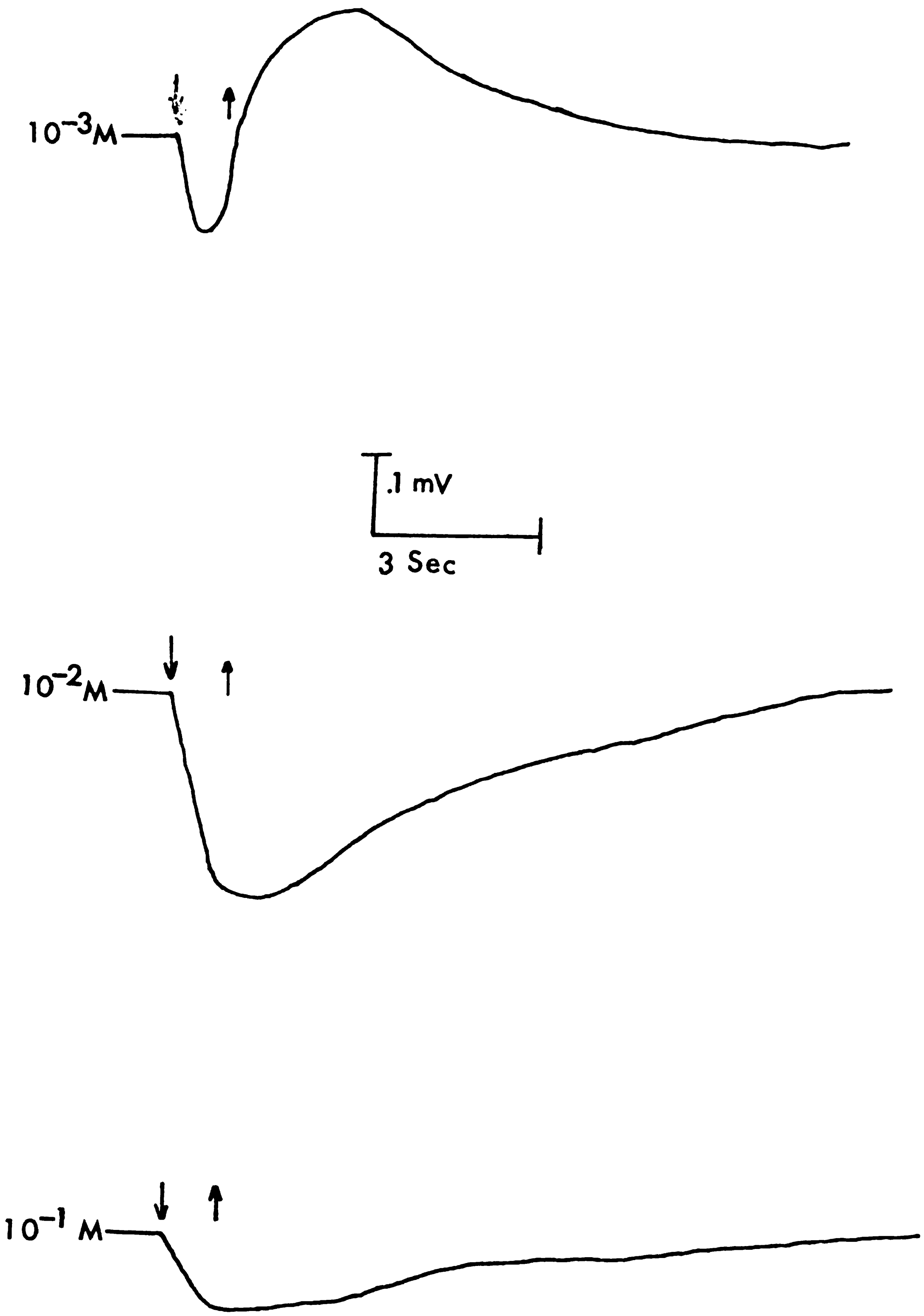
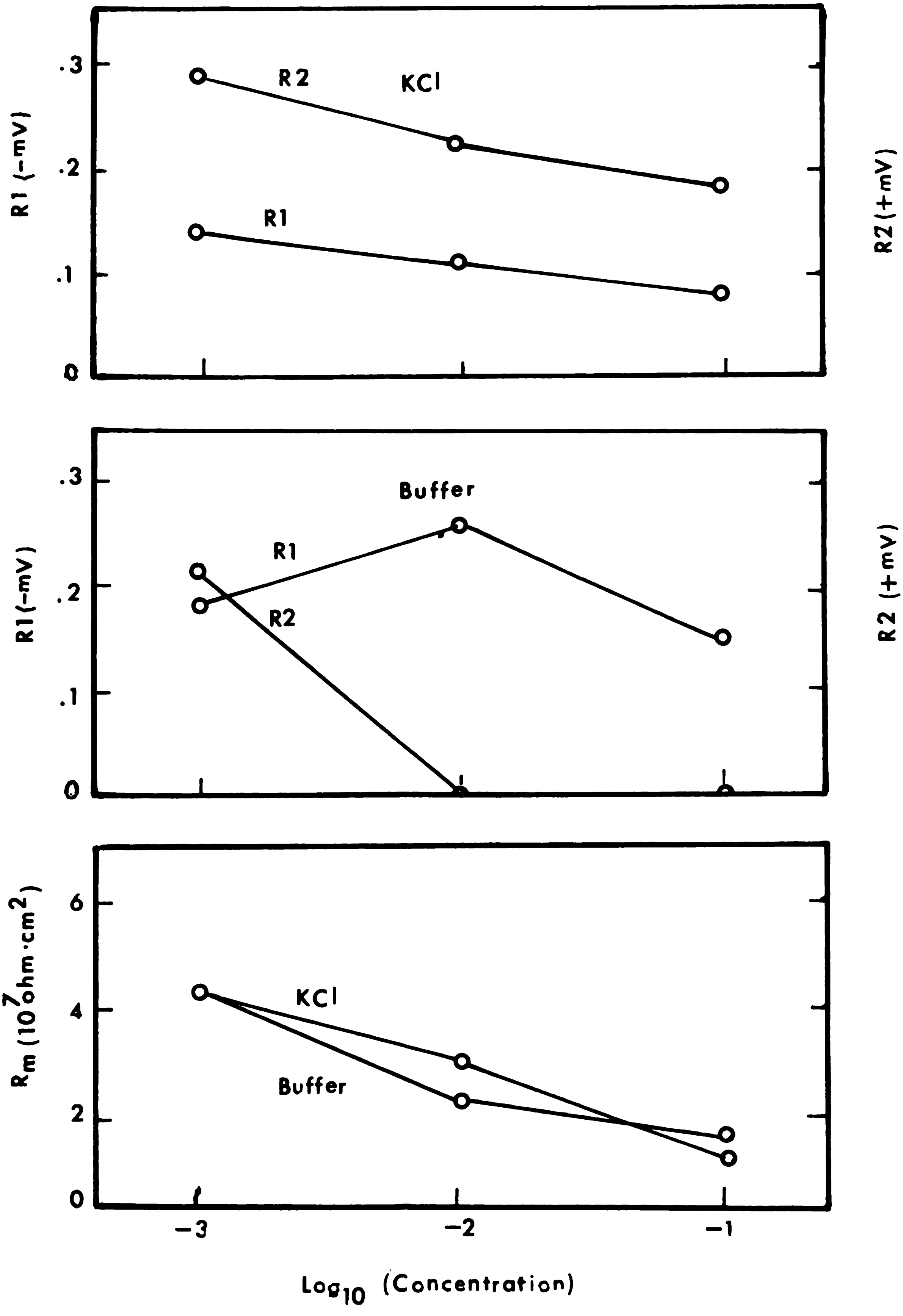

10⁻³M
.1 mV
3 Sec
10⁻²M
10⁻¹ M

Figure 32.--The magnitude of photoresponses and dark membrane resistance as a function of the concentration of KCl or sodium acetate buffer systems.

R1 - R1 Photoresponse

R2 - R2 Photoresponse

R_m - Dark membrane resistance


.3
.2
.1
0
R1 (−mV)
R2 (+mV)
R2
KCl
R1
.3
.2
.1
0
R1(−mV)
R2 (+mV)
Buffer
R1
R2
6
4
2
0
R_m (10^7 ohm·cm^2)
KCl
Buffer
−3
−2
−1
Log_{10} (Concentration)

involvement of the hydrogen ion in generation of the R2 component.

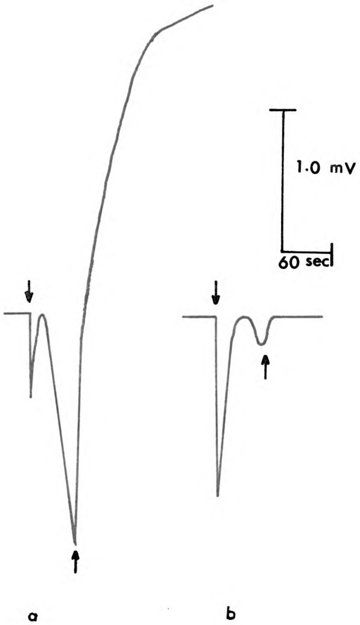
An extremely interesting observation was made on a long-time illumination. In 0.1 M KCl solution with 10^{-3} M $FeCl_3$ in the inner chamber and at zero pH gradient, a 60 second illumination gave triphasic responses which were apparently the modified wave forms of the biphasic responses as shown in Figure 33a. The R1 response was taken over by the R2 component which did not pass over the resting membrane potential developed before the illumination. Instead the R2 component bounced back and developed in the same direction as the R1 component. With a slower rate than R1, this component developed continuously up to the light-off, after which the potential decreased instantaneously to the base line, and then slowly developed the usual R2 component passing over the base line. The final resting potential was approximately the same magnitude to the inverse R2 component but in the opposite direction. This base line change was not reversible.

In the case of high buffer capacity, shown in Figure 33b, the photoresponse was dramatically different from that of Figure 33a. The fast developing R1 was reversed to the base line like the response shown in Figure 33a. After this recovery of the original potential, the reversed R2 component developed very slowly and in a more suppressed manner than that of Figure 33a. Upon the

Figure 33.--The time course of photoresponses with 60 second illuminations at pH 3.3 and ΔpH 0. The downward arrow indicates the light-on while the upward arrow indicates the light-off.

a - 0.1 M KCl solution: the inner chamber contained 10^{-3} M $FeCl_3$ and pH of the outer chamber was lowered with HCl.

b. 0.1 M sodium acetate buffer solution: the inner chamber contained 10^{-3} M $FeCl_3$.


1.0 mV
60 sec
a
b

light-off the response slowly recovered the original base line and never passed over it, unlike the response shown in Figure 33a. These two types of photoresponses following a long time illumination are very important in elucidating the mechanism of generation of the photopotentials.

CHAPTER VI

DISCUSSION

General Discussion

In this section pertinent observations will be discussed to establish an overall view of the physical processes leading to the generation of the photoresponses. Especially, the reasoning for the assignment of hole diffusion potential for the R1 component and proton diffusion potential for the R2 component will be presented after establishing the mechanism of the generation of the dark membrane potentials. A detailed quantitative theory will be given in the next section, followed by the experimental verification of the theory by comparing the theoretical predictions on the mode of the photoresponses to the experimental observations carried out in the present work.

The ionic transference numbers of KCl, NaCl, and HCl show that all-*trans*-retinal BLM is cation selective and the development of the dark membrane potentials can be explained with proton transport alone. When HCl was added in the inner chamber, the negative potential at the inner electrode developed. This may be interpreted as the proton diffusion toward the outer surface leaving chloride ions in the inner side. As to the question of whether the observed protonic potential is actually due to diffusion or adsorption the answer can be given safely as diffusion, since the Nernst equation can hold. If the potential were due to adsorption, the observed negative potential should be due to chloride ions but in the presence of the concentration gradient of KCl or NaCl in two aqueous phases such a large negative potential was not observed.

The elucidation of the mechanism by which this protonic potential develops is certainly interesting. In this work, however, the question was not pursued further since (1) other evidence as presented below indicated that protonic conduction increased with water content in the hydrocarbon layer in BLM, suggesting the possibility of the contribution of water pores to the transport of protons, and (2) the temperature dependence of the membrane potential and resistance was not influenced by the presence of electron acceptors such as ferric ions, suggesting that the potential development observed by creating pH gradient across BLM may be due to the simple diffusion of protons without performing other functions such as catalyzing the electronic charge injection, as reported in the case of anthracene/water interfaces (Beyerle and Gerischer, 1967). The presence of the proton transport channel separated from the electronic conduction channel which is most likely to be a conjugated double bond of all-trans-retinal is assumed to exist in the model membranes used in the present work.

From the sign of the R1 component it is reasonable to consider that this component is likely to be either the diffusion of positive charge carriers (protons and/or holes) to the outer surface and/or the diffusion of electrons to the inner surface. The possibility that R1 may be mediated by protons can be eliminated by the following observations:

1. The sign of R1 was totally dependent on the position of the electron acceptors.
2. The magnitude could be greatly enhanced, as much as 10^3 times, by the addition of electron acceptors.
3. In the presence of the high concentration of electron acceptors the magnitude of R1 was independent of the proton concentration gradient across the membrane.
4. At higher buffer capacity, where the R2 component was completely abolished, the R1 component could still be observed.

Thus, our attention can be focused to electrons and holes as the charge carriers of the R1 component.

As proposed by Tien (1968a, b) in explaining the photopotential of chlorophyll BLM, generation of R1 may be explained as follows: due to the extremely thin structure generation of electrons and holes is symmetric if there is no chemical asymmetry. On the other hand, in the presence of electron acceptors at the inner surface the excited electrons may tend to be withdrawn toward the inner surface, generating a higher concentration of holes in the bulk of hydrocarbon or at the outer surface.

An immediate establishment of the strong electric field across the membrane may have an effect on the space charge distribution across the membrane. The charge carriers which can be influenced by this photoinduced field are protons, since electrons are trapped by ferric ions, the membrane is impermeable to chloride ions, and holes are the sources of the induced field. Thus, the R2 component may be considered as the potential developed by the diffusion of protons toward the inner surface, or anode.

In accord to the above expectation, the R2 component increases with increasing temperature and pH gradient. An increase in temperature influences the R2 component by increasing protonic conductance or increasing protonic mobility whereas the pH gradient increases the magnitude of R2 by increasing the protonic concentration at the outer surface.

In addition to the possible interaction of protonic space charges in the generation of the R2 component, there seems to be another possibility. The accumulation of holes at the outer surface may generate protons via the oxidation of water molecules as proposed by Tien (1968a, b) in the case of chlorophyll BLM. The newly generated protons contributing to pH gradient will cause a decrease in the protonic membrane potentials established at the dark conditions. Indeed, the effect of a strong buffer capacity in abolishing the R2 component cannot be explained without taking into account this photogeneration of protons. The mechanism of the photogeneration of protons is still unknown not only in the present model system but also in well studied chloroplast systems. Nevertheless, since the peak wavelength of the absorption spectrum of all-*trans*-retinal has 80 kcal/einstein and the bond energy of aldehyde-water hydrogen bonds is 2 - 3 kcal/mole (Karyakin, Muradova, Sayenko, 1969), the photogeneration of protons is indeed possible energetically.

Since the membrane is impermeable to chloride and ferric ions, the recovery of the original dark potentials can be achieved by restoration of the chemical equilibrium. Specifically, the dissociation of HCl salts to protons and chloride ions and the reestablishment of equilibrium between ferric ions and ferrous ions may generate protons in the inner chamber and, later, the diffusion of protons will reestablish the original membrane potentials. For a long-time illumination the recovery of the membrane potentials did not take place. This may be due to the disturbance of the electric double layers to such an extent that the distribution of chloride and ferric ions is partially modified from that of the dark state due to the prolonged illuminations.

Theory

In order to set up kinetic equations describing the development of the biphasic photoresponses, it is necessary to establish the physical model of the seemingly complex phenomena. The present model is based on the two basic principles which were confirmed in this work. The first principle is that the membrane is permeable to protons, but not to the other ions studied (e.g., K^+ and Na^+). The second principle is that any external force which disturbs the existing electrochemical equilibrium has to be acted upon by a counter force to maintain microscopic electrochemical neutrality. Based on these principles, it is proposed that the initial electronic phenomenon following light absorption can generate the ionic charge transport, whose major charge carriers are protons.

In this work the resting membrane potential was expressed as the potential of the active electrode which was placed in the inner chamber. The photopotential was expressed as R1 and R2, which were defined as:

$$R1 = V_{min} - V_o \qquad (3)$$

$$R2 = V_{max} - V_o \qquad (4)$$

where V_{min}, V_{max}, and V_o were the value of the membrane potentials at the first peak, the second peak and the resting state, with signs taken in the sense of the inside potential minus the outside potential.

All time courses of the photoresponses could be expressed with the following two equations:

$$V = -A(1 - e^{-at}) + B(1 - e^{-bt}) \tag{5}$$

for $$0 \leq t \leq t_o \tag{6}$$

and

$$V = -A(1 - e^{-at_o})e^{-a(t - t_o)}$$

$$+ B(1 - e^{-bt_o})e^{-b(t - t_o)} \tag{7}$$

for $$t_o \leq t \tag{8}$$

where A, B, a, and b are constants and t_o is the time (t) for the light-off taking $t = 0$ for the light-on. All constants are functions of various experimental parameters and the assignment of numerical values to them requires to specify the exact conditions under which the constants have been evaluated. Because of this it is desirable to evaluate the constants in terms of experimentally measurable parameters.

The sign of A is negative to show that the first term expresses the negative or the major component of R1 while the second term expresses the positive or the major component of R2. In spite of the fact that the observed photoresponses are the results of redox reactions and the diffusion of the products of the redox reactions or ionic charge carriers, the time courses of the photoresponses are determined with two terms of exponential functions. This implies either that the time constant of each component is the sum of the redox reactions and diffusion terms or that it is a pure time constant of either the redox reactions or diffusion processes:

$$a = a_{\mathrm{redox}} + a_{\mathrm{diffusion}} \tag{9}$$

$$b = b_{\mathrm{redox}} + b_{\mathrm{diffusion}} \tag{10}$$

or

$$a = a_{\mathrm{redox}} \tag{11}$$

$$b = b_{\mathrm{redox}} \tag{12}$$

or

$$a = a_{\mathrm{diffusion}} \tag{13}$$

$$b = b_{\mathrm{diffusion}} \tag{14}$$

Due to the fact that these time constants (a and b) are far greater than any of the anticipated rate constants of redox reactions and diffusion processes of holes and protons, it is more fruitful to analyze the kinetic equation in terms of electric circuit theory.

The forementioned two terms of exponential functions are best expressed as the representations of the time courses of two RC circuits. Shown in Figure 34, the hole component may be represented with circuit 1 and the proton component with circuit 2. Then, the following equations can be written down:

$$V_c^1 - V_o = (V_1 - V_o)(1 - e^{-G_1 t/C_m}) \qquad (15)$$

$$V_c^2 - V_o = (V_2 - V_o)(1 - e^{-G_2 t/C_m}) \qquad (16)$$

where

V_c^1 = capacitative potential of hole component

V_c^2 = capacitative potential of proton component
V_o = dark potential
V_1 = saturation potential of hole component
V_2 = saturation potential of proton component
G_1 = conductance of hole component
G_2 = conductance of proton component
C_m = membrane capacitance

The combined system shown in Figure 34c can be expressed as:

Figure 34.--The equivalent circuits for the generation of photoresponses of all-*trans*-retinal BLM.

a - hole system designated with subscript 1

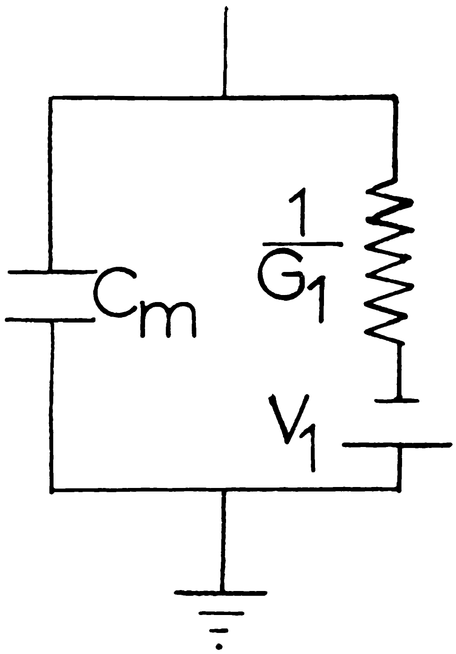
b - proton system designated with subscript 2

c - combined system

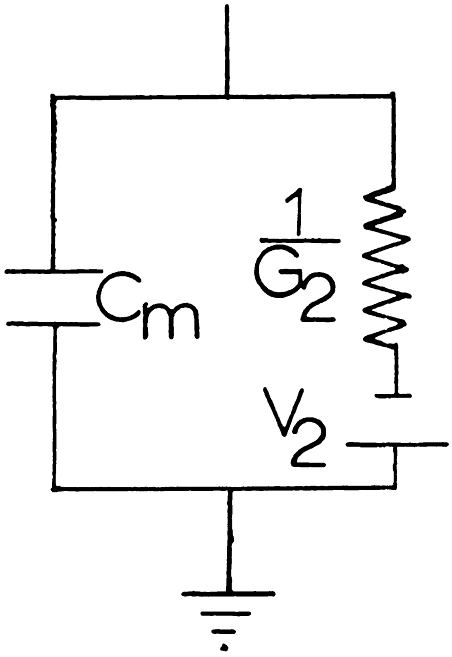
G - membrane conductance

V - membrane potential

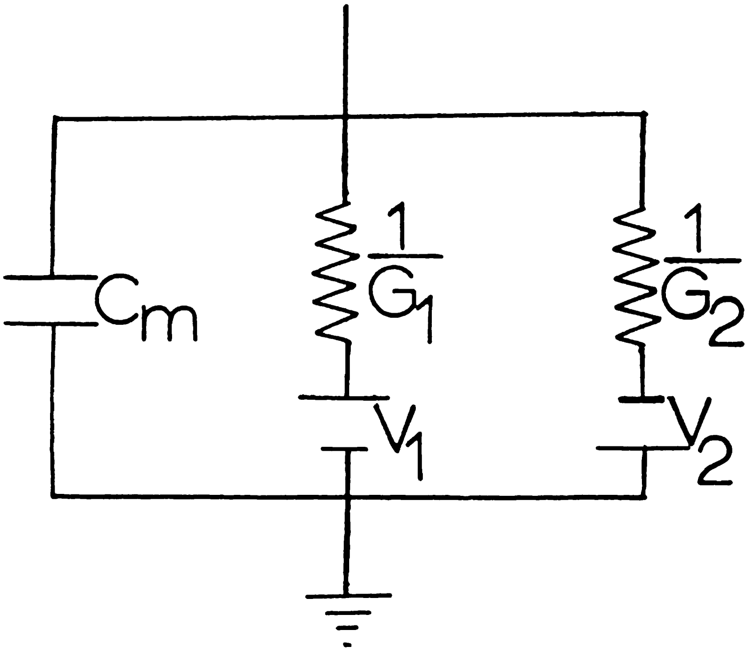
C_m- membrane capacitance



a



b



c

$$V_c^{total} - V_o = \frac{G_1}{G_1 + G_2}(V_1 - V_o)(1 - e^{-G_1 t/C_m})$$

$$+ \frac{G_2}{G_1 + G_2}(V_2 - V_o)(1 - e^{-G_2 t/C_m}) \quad (17)$$

where V_c^{total} is the total capacitative potential. The Equation (17) expresses the time course of the membrane potential for $0 \leq t \leq t_o$. After the light-off, $t_o \leq t$, the time course of the photopotential can be expressed by:

$$V_c^{total} - V_o = \frac{G_1}{G_1 + G_2}(V_1 - V_o)(1 - e^{-G_1 t_o/C_m})e^{-G_1(t-t_o)/C_m}$$

$$+ \frac{G_2}{G_1 + G_2}(V_2 - V_o)(1 - e^{-G_2 t_o/C_m})e^{-G_2(t-t_o)/C_m} \quad (18)$$

Equations (17) and (18) have the same properties of Equations (5) and (7), provided:

$$A = \frac{G_1}{G_1 + G_2}(V_1 - V_o) \quad (19)$$

$$B = \frac{G_2}{G_1 + G_2}(V_2 - V_o) \quad (20)$$

$$a = G_1/C_m \quad (21)$$

$$b = G_2/C_m \quad (22)$$

Our task is now to express V_1 and V_2 in terms of other physical parameters. Since these potentials are saturation potentials, by putting $t = \infty$ in Equations (15) and (16) we get:

$$V_c^1 = V_1 \tag{23}$$

and

$$V_c^2 = V_2 \tag{24}$$

Referring to Figure 35 it is possible to evaluate saturation membrane potentials in terms of the saturation concentrations of charge carriers:

$$\begin{aligned} \Delta V_1^{photo} &= V_1^{photo} - V_1^{dark} \\ &= V_1 - V_o \\ &= \frac{RT}{F} \{[\ln(C_p^{i,h} + C_t^{i,h}) - \ln C_t^{o,h}] - \ln \frac{C_t^{i,h}}{C_t^{o,h}}\} \\ &= \frac{RT}{F} \ln \frac{C_p^{i,h}}{C_t^{i,h}} \end{aligned} \tag{25}$$

where $C_p^{i,h}$ and $C_p^{o,h}$ are the saturation concentrations of photogenerated holes in the inside surface and outside surface, respectively, and $C_t^{i,h}$ and $C_t^{o,h}$ are the saturation concentrations of thermally generated holes in the inner surface and outer surface, respectively.

Figure 35.--The analysis of two components of photopotentials.

V_o - resting membrane potential

V_1 - saturation hole potential

V_2 - saturation proton potential

Similarly we obtain:

$$\Delta V_2^{photo} = V_2^{photo} - V_2^{dark}$$

$$= V_2 - V_o$$

$$= \frac{RT}{F}\{[\ln(C_p^{o,H} + C_d^{o,H}) - \ln C_d^{i,H}] - \ln\frac{C_d^{o,H}}{C_d^{i,H}}\}$$

$$= \frac{RT}{F}\ln\frac{C_p^{o,H}}{C_d^{o,H}} \qquad (26)$$

where C's now stand for proton concentrations at the saturation state.

The terms expressing the concentrations must be represented by more basic parameters. In order to do this the establishment of the whole photochemical reaction starting from the absorption of light to the restoration of the original resting state of electrochemical equilibrium must be carried out. Such an attempt must introduce a great degree of simplification due to the many parameters involved in the reaction sequence, whose mathematical treatment could be unduly complicated for the proper evaluation of the experimental observations. However, there is a risk in attempting such a simplification as it may result in an unrealistic description.

The type of primary excitation species was not determined in the present work; thus, either excitons or excited electrons can serve well for the present discussion as stated below. Whether the excited species is singlet or triplet could not be decided experimentally, but from the time range observed in the present work the species with a longer lifetime is more likely to be involved. The triplet state has a lifetime on the order of one second in the case of carotenoid glasses (Rosenberg, 1958) and less than 10^{-5} second in the case of various organic and aqueous solutions of retinals (Abrahamson, Adams, Wulff, 1959). The longer lifetime of the triplet state in solid is likely to be due to the presence of traps, and the similar possibility may exist in the case of BLM since the pigment molecules are arranged in a regular structure (Tien, 1967). Thus, it is assumed that the primary excitation step is the generation of triplet excitons.

The rate of exciton generation can be expressed by:

$$\frac{dC^e}{dt} = C^r \varepsilon I_o e^{-\varepsilon C^r l} - C^e\left(\frac{1}{\tau_e} + k_1\right) \qquad (27)$$

where C^e is exciton concentration, C^r is retinal concentration, ε is molar extinction coefficient, I_o is the intensity of the incident light, l is the thickness of the membrane, τ_e is the lifetime of excitons, and k_1 is the rate constant of hole generation.

At equilibrium:

$$\frac{dC^e}{dt} = 0 \qquad (28)$$

Then, from Equation (27) we get:

$$C^e = \frac{C^r \tau_e \varepsilon I_o e^{-\varepsilon C^r l}}{1 + k_1 \tau_e} \qquad (29)$$

In deriving Equations (27) and (29) it was assumed that the reaction kinetics of the generation of charge carriers from excitons as the first order. This simplification may be justified as shown in the development of the possible mechanism of charge carrier generation as described in the following paragraphs.

The processes leading to the generation of electronic charge carriers can be considered in the following two ways. The first possibility is the charge separation via the photogenerated excitons and second possibility is the charge transfer complex formation between excited retinal and electron acceptors or electron donors. The first pathway may be written as:

$$e + A \underset{k_b}{\overset{k_a}{\rightleftharpoons}} Ae \xrightarrow{k_c} A^- + \oplus \qquad (30)$$

$$e + D \underset{k'_b}{\overset{k'_a}{\rightleftharpoons}} De \xrightarrow{k'_c} D^+ + e^- \tag{31}$$

where e is exciton, A is electron acceptor, D is electron donor, Ae and De are complexes for acceptor-exciton and donor-exciton, respectively (these complexes may be considered as surface traps), $\oplus$ is hole, e^- is electron, and k's are rate constants for respective processes. For the charge transfer complex pathway reactions may be written as:

$$R^* + A \underset{k_b}{\overset{k_a}{\rightleftharpoons}} AR^* \xrightarrow{k_c} A^- + R^+ \tag{32}$$

$$R^* + D \underset{k'_b}{\overset{k'_a}{\rightleftharpoons}} DR^* \xrightarrow{k'_c} D^+ + R^- \tag{33}$$

where R^* is the excited retinal molecule.

Between these two types of pathways there are similarities. Excited species form a complex with acceptor or donor reversibly and the product formation is irreversible. Donors and acceptors are fixed at the interfaces and can be considered as fixed sites for adsorption. Excitons and excited retinals can be considered as the species colliding onto the fixed sites. It is assumed that excitons and excited retinals can interact with the adsorption sites in the fashion of a random walk. If the above assumptions are correct, then, the kinetics can be studied with the Langmuir adsorption isotherm. Before the development of the kinetic equations using the Langmuir adsorption isotherm, it is necessary to clarify the details of the present model.

Because of the uncertainty on the possible pathway between the proposed two pathways, the rest of the discussion will be focused on the exciton pathway. This selection does not create any inconvenience since the other pathway is quite similar to the former one.

It is expected that water itself can perform the role of electron acceptor as well as electron donor in the absence of better acceptors or donors. The reactions taking place at the inside surface are the summation of the reactions expressed by Equations (30) and (31). The observed net reaction is, then, the difference between the sum of the similar reactions occurring at the outside surface and the sum of the similar reactions at the inside surface. In the case of a symmetric system the rate of the generation of charge carriers is the same between two surfaces, thus, we should expect that potential development is negligible. Indeed, this is the case as commonly observed in the present work. Because of the complexity in treating the total reactions as a general case, and yet the considerable generality of the single reaction, treated in the following paragraphs will be an asymmetric case with ferric ions in the inside chamber. This selection could practically fix the sign of photoresponses and enhance the magnitude, indicating such a simplified treatment is not only desirable but also required. Due to this factor of asymmetry a great simplification can be made in discussing the kinetics of the development of the electronic component.

In the simplest situation ferric ions function as the sites and excitons adsorb onto the surface sites. The reversible adsorption can be expressed by Langmuir's isotherm (1918). Let s be the fraction of surface that is covered and 1 - s the fraction that is bare. The rate of adsorption is then $k_aC(1 - s)$, where C is the concentration of the adsorbate (exciton) and k_a a constant shown in Equation (30) and the rate of desorption is k_bs. At equilibrium the rates are equal, or:

$$\frac{s}{1 - s} = \frac{k_a}{k_b} C = KC \tag{34}$$

where K, equal to k_a/k_b, is a constant. This equation can be written as:

$$s = \frac{KC}{1 + KC} \tag{35}$$

The process of charge separation may be regarded as the process involving the following four consecutive steps. These are:

1. diffusion of the exciton to the surface
2. adsorption of the exciton onto the surface
3. reaction on the surface
4. desorption of the product (hole).

It is assumed that processes 2 through 4 are considered as the rate determining processes as a unit, as treated by Langmuir (1921) in deriving his kinetic equation. This assumption was made since the activation energy may be required for adsorption and desorption. These two processes cannot be separated experimentally, so it is convenient to consider that these steps function as a unit. The rate of reaction (v) is, then, proportional to s and may therefore be written as:

$$v = K' s$$

$$= \frac{K' KC}{1 + KC} \tag{36}$$

where K' is the proportionality constant. This derivation was based on the assumption that the adsorption equilibrium was not disturbed by the occurrence of the reaction.

At sufficiently high reactant concentration the rate is independent of the concentration, which means that the kinetics are zero order. Under these conditions $KC \gg 1$, so that Equation (36) reduces to $v = K'$. At low reactant concentrations, which are the range of concern in the present work, when $KC \ll 1$, Equation (36) reduces to:

$$v = K^{'}KC \tag{37}$$

and the kinetics are first order.

Similarly a first order rate equation can be derived for proton generation at the opposite surface of the BLM by assuming the Langmuir type adsorption of holes at the surface sites as an intermediate step. The diffusion process of holes and protons can be treated as the first order rate reactions. It is also possible to specify that all reactions are biased forwardly and backward rate constants are negligible. From the above considerations it becomes possible to construct the reaction flow diagram composed with first order irreversible reactions as shown in Figure 36. The rate of the reactions of this system shown in Figure 36 can be written as follows:

$$\frac{dC^r}{dt} = - k_o C^r \tag{38}$$

$$\frac{dC^e}{dt} = k_o C^r - (k_1 + k_6)C^e \tag{39}$$

$$\frac{dC_p^{i,h}}{dt} = k_1 C^e - (k_4 + k_5 + D_1)C_p^{i,h} + D_3 C_p^{o,h} \tag{40}$$

$$\frac{dC_p^{o,h}}{dt} = k_6 C^e - (k_2 + k_7 + D_3)C_p^{o,h} + D_1 C_p^{i,h} \tag{41}$$

Figure 36.--The photochemical reactions and diffusion processes involving excitons (e), holes (h), and hydrogen ions (H). k's are reaction rate constants and D's are diffusion coefficients. r is all-*trans*-retinal. L is hydrocarbon layer, o is outside aqueous phase, and i is inside aqueous phase.

$$\frac{dC_p^{o,H}}{dt} = k_2C_p^{o,h} - (k_8 + D_2)C_p^{o,H} + D_4C_p^{i,H} \tag{42}$$

$$\frac{dC_p^{i,H}}{dt} = K_5C_p^{i,h} - (k_3 + k_9)C_p^{i,H} + D_2C_p^{o,H} \tag{43}$$

$$\frac{dC^{HCl}}{dt} = k_3C_p^{i,H} \tag{44}$$

For the sake of simplicity without losing the validity of the discussion, let $k_4 = k_5 = k_6 = k_7 = k_8 = k_9 = 0$ as shown in Figure 37. Then, the above equations become:

$$\frac{dC^r}{dt} = - k_oC^r \tag{45}$$

$$\frac{dC^e}{dt} = k_oC^r - k_1C^e \tag{46}$$

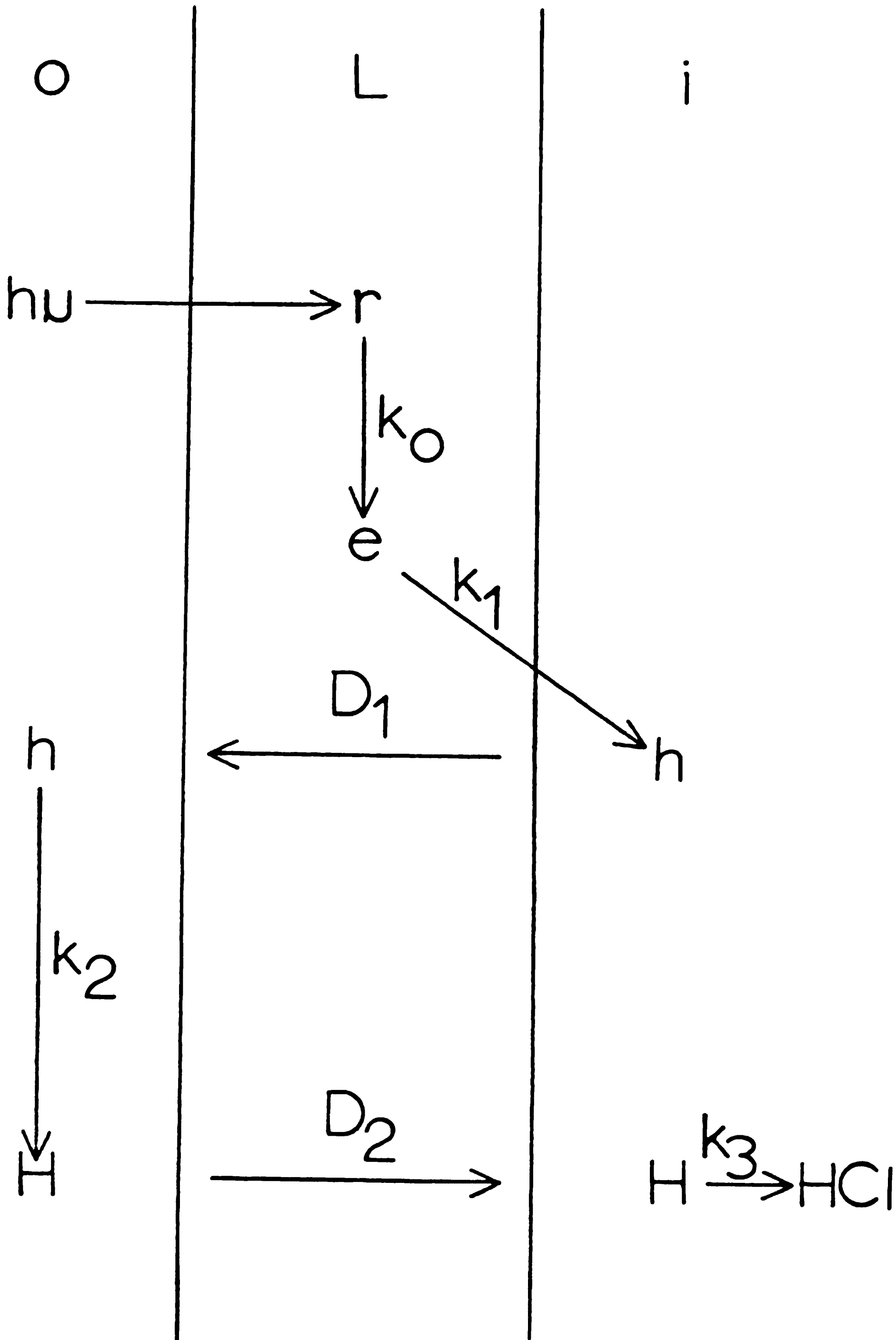
$$\frac{dC_p^{i,h}}{dt} = k_1C^e - D_1C_p^{i,h} \tag{47}$$

$$\frac{dC_p^{o,h}}{dt} = D_1C_p^{i,h} - k_2C_p^{o,h} \tag{48}$$

$$\frac{dC_p^{o,H}}{dt} = K_2C_p^{o,h} - D_2C_p^{o,H} \tag{49}$$

$$\frac{dC_p^{i,H}}{dt} = D_2C_p^{o,H} - k_3C_p^{i,H} \tag{50}$$

Figure 37.--Simplified photochemical reactions and diffusion processes.



o
L
i
hυ
r
k0
e
k1
D1
h
h
k2
D2
H
H
k3
HCl

$$\frac{dC^{HCl}}{dt} = k_3 C_p^{i,H} \qquad (51)$$

The reaction scheme shown above may be expressed as the reactions of most possible reactants (ferric ions as acceptors and water molecules as donors) as shown in Figure 38. Shown in the diagram are four major processes: (1) photon absorption leading to the generation of excitons, then holes, (2) the diffusion of holes across the hydrocarbon layer of BLM in the same sense of the dark current, (3) interaction of holes with water molecules leading to the proton generation, and (4) the back flow of protons due to the newly formed proton or disturbance of the protonic concentration gradient across BLM.

Upon establishing the simplified reaction scheme and the rate equations the equilibrium concentrations of the terms shown in Equations (25) and (26) can now be evaluated:

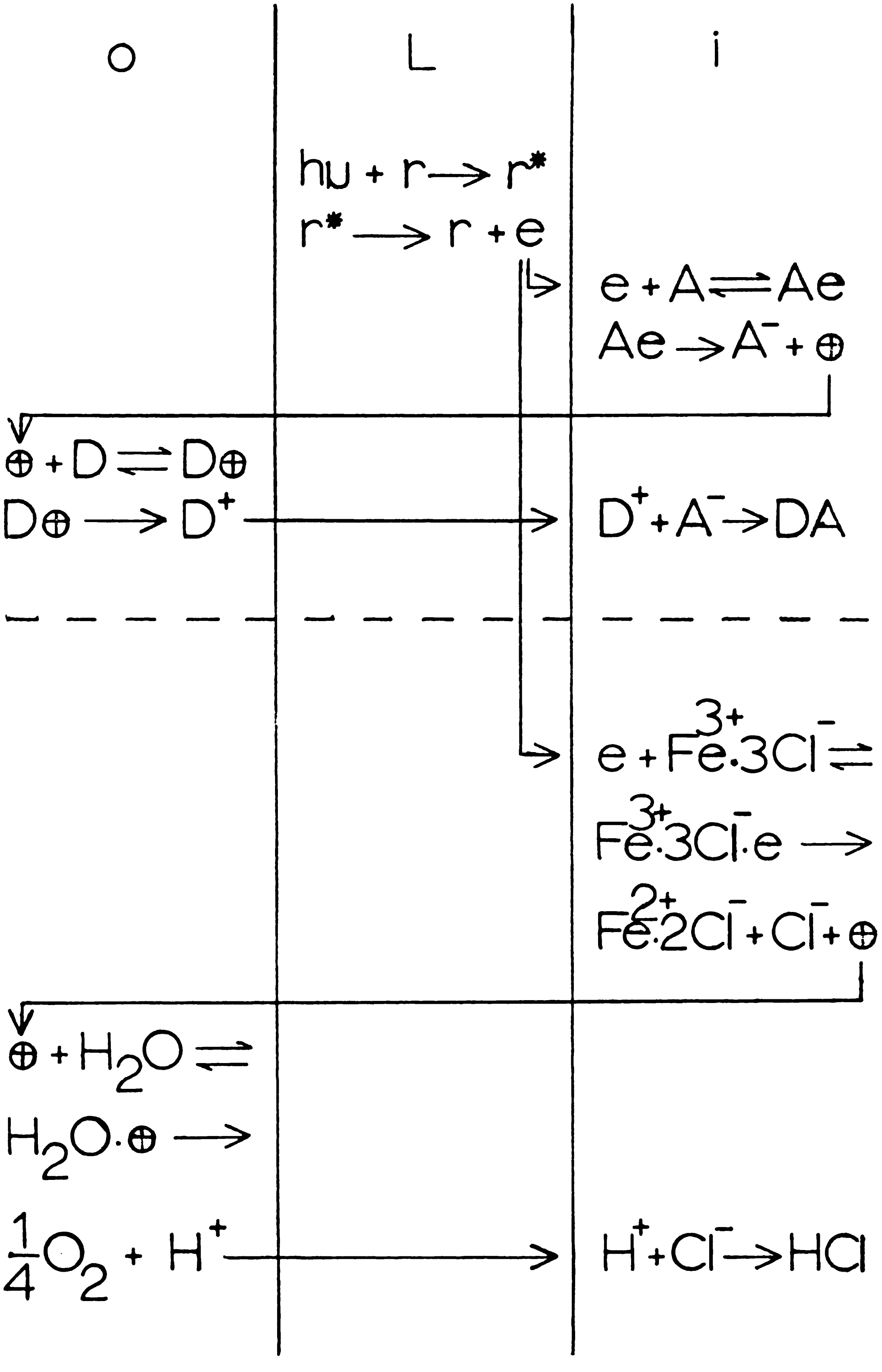
$$\frac{dC_p^{i,h}}{dt} = k_1 C^e - \frac{C_p^{i,h}}{\tau_h} - D_1 C_p^{i,h} \qquad (52)$$

where τ_h is the hole lifetime.

At equilibrium:

$$\frac{dC_p^{i,h}}{dt} = 0 \qquad (53)$$

Figure 38.--Possible redox reactions at the membrane surfaces.


o
L
i
hυ + r → r*
r* → r + e
e + A ⇌ Ae
Ae → A⁻ + ⊕
⊕ + D ⇌ D⊕
D⊕ → D⁺
D⁺ + A⁻ → DA
e + Fe³⁺·3Cl⁻ ⇌
Fe³⁺·3Cl⁻·e →
Fe²⁺·2Cl⁻ + Cl⁻ + ⊕
⊕ + H₂O ⇌
H₂O·⊕ →
1/4 O₂ + H⁺
H⁺ + Cl⁻ → HCl

and

$$C_p^{i,h} = \frac{C^r k_1 \tau_e \tau_h \varepsilon I_o e^{-\varepsilon C^r l}}{(1 + k_1\tau_e)(1 + \tau_h D_1)} \tag{54}$$

Similarly the rate of photogeneration of protons can be expressed as (Equation 49):

$$\frac{dC_p^{o,H}}{dt} = k_2 C_p^{o,h} - D_2 C_p^{o,H} \tag{55}$$

where

$$C_p^{o,h} = \frac{C^r k_1 k_2 \tau_e \tau_h \varepsilon I_o e^{-\varepsilon C^r l}}{(1 + k_1\tau_e)(1 + \tau_h D_1)(1 + k_2\tau_h)} \tag{56}$$

At equilibrium $dC_p^{o,H}/dt = 0$ and the substitution of Equation (56) into Equation (55) gives:

$$C_p^{o,H} = \frac{C^r k_1 k_2 \tau_e \tau_h \varepsilon I_o D_1 \tau_h e^{-\varepsilon C^r l}}{(1 + k_1\tau_e)(1 + \tau_h D_1)(1 + k_2\tau_h) D_2} \tag{57}$$

In a similar fashion, if we define the rate constant of the thermal generation of excitons as k_o^t, we get:

$$C_t^{i,h} = \frac{C^r k_o^t \tau_e k_1 \tau_h}{(1 + k_1\tau_e)(1 + \tau_h D_1)} \tag{58}$$

assuming thermally generated holes also follow the same reaction scheme shown in Figure 37 as in the case of photogenerated holes.

It is known that interfacial pH is determined by the equation:

$$\text{pH (surface)} = \text{pH (bulk)} + \frac{e\zeta}{kT} \qquad (59)$$

where e is the electronic charge, ζ is zeta potential, k is Boltzman constant and T is absolute temperature (Hartley and Roe, 1940). Since zeta potential of BLM (phospholipid) is found to be small, less than 5 mV (Hanai, Haydon, and Taylor, 1965; Bonting and Bangham, 1967), the correction is unnecessary. Thus, the concentration of hydrogen ions at the surface of BLM is assumed to be proportional to the bulk concentration:

$$C_{d}^{o,H} = AC_{\text{dark, bulk}}^{o,H} \qquad (60)$$

where A is the partition coefficient of protons at hydrocarbon/water interfaces.

Finally, the substitution of Equations (54) and (58) into Equation (25), and Equations (57) and (60) into Equation (26), and then the resulting equations into Equations (17) and (18) gives:

$$V_c^{total} - V_o = - \frac{G_1}{G_1 + G_2} \left(\frac{RT}{F} \ln\frac{\tau_h \varepsilon I_o e^{-\varepsilon C^r l}}{k_o^t}\right)(1 - e^{-G_2 t/C_m})$$

$$+ \frac{G_2}{G_1 + G_2}\left\{\frac{RT}{F} \ln\frac{C^r k_1 k_2 \tau_e \tau_h D_1 \tau_h \varepsilon I_o e^{-\varepsilon C^r l}}{(1 + k_1 \tau_e)(1 + \tau_h D_1)(1 + k_2 \tau_h) D_2 A C_{d,bulk}^{o,H}}\right\}$$

$$x\ (1 - e^{-G_2 t/C_m}) \qquad (61)$$

for $0 \leq t \leq t_o$

and

$$V_c^{total} - V_o = - \frac{G_1}{G_1 + G_2} \left(\frac{RT}{F} \ln\frac{\tau_h \varepsilon I_o e^{-\varepsilon C^r l}}{k_o^t}\right)(1 - e^{-G_1 t_o/C_m})$$

$$x\ e^{-G_1 (t - t_o)/C_m}$$

$$+ \frac{G_2}{G_1 + G_2}\left\{\frac{RT}{F} \ln\frac{C^r k_1 k_2 \tau_e \tau_h D_1 \tau_h \varepsilon I_o e^{-\varepsilon C^r l}}{(1 + k_1 \tau_e)(1 + \tau_h D_1)(1 + k_2 \tau_h) D_2 A C_{d,bulk}^{o,H}}\right\}$$

$$x\ (1 - e^{-G_2 t_o/C_m}) e^{-G_2 (t - t_o)/C_m} \qquad (62)$$

for $t_o \leq t$.

The Effect of Temperature on the Biphasic Photopotentials

The Effect of Temperature on the Dark Membrane Potentials

For the purpose of analyzing the effect of temperature on the biphasic photopotentials using the theory developed in the previous section, it is necessary to understand the relationship between temperature and protonic membrane conduction. In order to accomplish this purpose, naturally, it is very helpful to establish the effect of temperature on the mobility of protons, which control both dark membrane potentials and mode of biphasic photopotentials.

According to Equation (2), the dark membrane potentials are the function of temperature, ionic transference numbers, and ionic activities. If we assume the last two terms are independent of temperature, the membrane potentials (E_m) should be linearly dependent on temperature (T). Shown in Figure 24, however, the membrane potentials have the following relationship with temperature:

$$E_m(T) = E_{m,o} e^{-E_a/RT} \tag{63}$$

where $E_{m,o}$ is a constant and E_a is the thermal coefficient of the development of the dark membrane potential and R is the gas constant. Thus, Equation (2) requires corrections to account for temperature variations, indicating either one or both of the following relationships may exist:

$$t^{+}(T) = t_{o}^{+} e^{- E_{t^{+}}/RT} \tag{64}$$

and

$$a_{MCl}(T) = a_{MCl,o} e^{- E_{a_{MCl}}/RT} \tag{65}$$

The dissociation constant of HCl is very high and its temperature dependence is fairly low. Although the partition coefficient of protons at the edge of the membrane may depend on temperature, the membrane and aqueous phases are symmetric except for the HCl concentration gradient. Thus, the substitution of Equation (65) into Equation (2) for the inside and outside HCl activities results in the cancellation of the thermal effect on the partition coefficient if we assume the effect of temperature on the partition coefficient is constant for the dilute salt concentrations such as those used in the present work. Furthermore, taking logarithm of the Equation (65) as indicated by Equation (2) eliminates the possible exponential relationship with temperature as expressed in Equation (63), if activity is temperature dependent. Thus, the possibility of the contribution of the thermal effect on the HCl activity and proton partition coefficient at the surfaces of BLM can be safely eliminated.

The transference numbers of ionic species must depend on the permeability through electric double layers and the hydrocarbon layer or:

$$t^{+} = f\ (t_{d}^{+},\ t_{h}^{+}) \tag{66}$$

where t_{d}^{+} is the transference number through the electric double layers and t_{h}^{+} is that through the hydrocarbon layer. As mentioned in the above paragraph, the partition coefficients at the inner and outer surfaces of the membrane can be assumed to be equally influenced by temperature under the present experimental condition: there is no electrical or adsorptive asymmetry across the membrane except the HCl concentration gradient, within the concentration range of $10^{-5} - 10^{-4}$ N. Thus, the effect of temperature on the partition coefficient, hence, t_{d}^{+}, is negligible.

Equation (66) may now be written as:

$$t^{+} = f\ (t_{h}^{+}) = t_{h}^{+} \tag{67}$$

Now, we should recall that t^{+} is the transference number of protons with respect to chloride ions. Thus, in addition to Equation (67) we have:

$$t^{-} = f\ (t_{h}^{-}) = t_{h}^{-} \tag{68}$$

for chloride ions, and also expressing the effect of temperature we have:

$$t_h^+(T) = t_{h,o}^+ e^{-E_{t_h^+}/RT} \tag{69}$$

and

$$t_h^-(T) = t_{h,o}^- e^{-E_{t_h^-}/RT} \tag{70}$$

where $t_{h,o}^+$ and $t_{h,o}^-$ are constants.

In dilute salt solutions the effect of increasing temperature, generally, is to bring the values of transference numbers from both ends of the extremes (1 and 0) to the value of midpoint or 0.5 (Taylor, 1938). If this is the case in the present model membrane, the transference number should decrease from unity as temperature is elevated beyond room temperature. On the contrary, the membrane potential increases, hence, the "effective transference number" increases beyond unity violating the requirement that $t^+ + t^- = 1$. This anomaly may be explained in the following way. Unlike the bulk salt solutions, the effect of temperature is not to influence the ionic selectivity. This is reasonable since the ionic selectivity is mainly determined by the electric double layers which do not contribute to the present transference number, meaning the electric double layer is not influenced by temperature so as to result in an increase in the partition coefficient of chloride ions. Then, an increase in the membrane potential must be due to an increase in the "effective proton transference number" caused by an increase in protonic mobility in the hydrocarbon layer. This can be expressed by the relationship:

$$t_h^+ = u^+/(u^+ + u^-) \tag{71}$$

where u's are mobilities in the hydrocarbon layer.

Furthermore, there is a relationship between the mobility and the diffusion coefficient of charged particles as pointed out by Einstein in the form:

$$u = DF/RT \tag{72}$$

where D is the diffusion coefficient and expressed in the form:

$$D = D_o e^{-E_D/RT} \tag{73}$$

where E_D is the activation energy for diffusion. Thus, if the above reasoning is correct, the activation energy of the development of the dark membrane potentials should agree with the activation energy of the diffusion of protons in the hydrocarbon layer or in bulk hydrocarbon saturated with water. There is substantial evidence in support of the above interpretation. The diffusion constant of water in hydrocarbon medium has exponential temperature dependence (Schatzberg, 1965). Indeed, the activation energy of the diffusion of water in hydrocarbon is about 4 kcal/mole (Schatzberg, 1965) and the activation energy of hydronium ions in water and ice is around 2 - 3 kcal/mole (Eigen and De Maeyer, 1958). The agreement between these values and the value obtained in the present work for the development of the dark membrane potentials (5 kcal/mole) can be considered as remarkable, taking into account the solid-like structure of the hydrocarbon layer of BLM. Thus, we may conclude:

$$E_a = E_u \approx 5 \text{ kcal/mole} \qquad (74)$$

The Effect of Temperature on the Membrane Conductivity

The concept that the effect of temperature is to influence protonic mobility through water channels in the hydrocarbon layer of BLM is also supported by the observations made on the temperature dependence of the dark membrane conduction. The conductivity, σ, is given by the sum of the contributions of the negative charge carriers and positive charge carriers:

$$\sigma = Gl/S = n^- e u^- + n^+ e u^+ \qquad (75)$$

where n^- and n^+ are the densities of the negative and positive charge carriers respectively, u^- and u^+ are the respective mobilities, e is the magnitude of the electronic charge, l is the thickness of the BLM, S is the area of

BLM, and G is the conductance. Due to the unity of the proton transference number, Equation (75) can be rewritten as:

$$\sigma = n_H e u_H \tag{76}$$

Temperature dependence of membrane conduction shows the exponential temperature dependence given by:

$$\sigma(T) = \sigma_o e^{-E_\sigma/RT} \tag{77}$$

where E_σ is an activation energy for conduction. Since σ as given in Equation (75) is the product of the charge density and the mobility, the exponential temperature dependence of σ could be due to the following two possibilities:

$$n(T) = n_o e^{-E_n/RT} \tag{78}$$

and

$$u(T) = u_o e^{-E_u/RT} \tag{79}$$

where E_n is the activation energy of charge generation. Hence, the conductivity can be expressed as:

$$\sigma(T) = n_o e u_o e^{-(E_n + E_u)/RT} \tag{80}$$

Since the experimental determination of E_a under the same experimental condition (the constant n_H) indicates that the E_u has about 5 kcal/mole, Equation (74). Since this is in good agreement with the value of E_σ, we may conclude:

$$E_\sigma = E_u \approx 7 \text{ kcal/mole} \tag{81}$$

Thus, the conductivity is determined by the charge mobility, which, in turn, is determined by the state of water channels in the hydrocarbon layer as mentioned in the previous section, and which will be discussed in detail shortly.

It is possible to evaluate permeability constant (P) from the knowledge of the transference number (t) and the bulk concentration of the ionic species (C) with the following equation (Miyamoto, 1966):

$$P = tRTG/F^2CS \tag{82}$$

Since the specific conductance of the membrane for 10^{-1} M KCl and 10^{-1} M NaCl and 4×10^{-5} N HCl was 10^{-7} mhos/cm^2, and the transference numbers were 0.5 for the first two salts and 1 for HCl at room temperature (23°C), from Equation (82) we get:

$$P_K = P_{Na} = P_{Cl} = 3 \times 10^{-8} \text{ cm/sec} \tag{83}$$

and

$$P_H = 2 \times 10^{-4} \text{ cm/sec} \tag{84}$$

where P's stand for the permeability coefficients of corresponding ions. Thus, the proton selectivity of this model membrane is now well established.

The concentration of all-<u>trans</u>-retinal may be assumed to be equal to bulk lipid concentration, which is 10^{-4} M/cm^3. This value is reasonable in view of the surface excess of the surfactants studied by Tien (1968c): the value was of the order of 10^{-4} M/cm^3, and retinal can be considered as a surface active component in the present model membrane. The maximum value of the hole concentration is not likely to be more than 10^{-7} M/cm^3, since the quantum efficiency of the most photosensitive organic materials is less than 10^{-3} charge carriers/photon. Taking the limiting concentration of holes as 10^{-7} M/cm^3, and the transference number of holes as unity, the conductance of the same order of protonic conductance can be obtained with the hole permeability coefficient:

$$P_h = 2 \times 10^{-1} \text{ cm/sec} \tag{85}$$

Comparing Equation (85) with Equation (84) we get:

$$\frac{P_h}{P_H} = 10^3 \tag{86}$$

This is the main reason why it is plausible to assume that the effect of temperature on the conductance of the hole is negligible compared with that of protons: it is known that the larger the permeability coefficient, the smaller the thermal coefficient of conductance (Taylor, 1938). At this point, it is appropriate to state that from Equation (81) we get:

$$E_{G_2} = E_u \approx 7 \text{ kcal/mole} \tag{87}$$

and

$$E_{G_1} \approx 0 \tag{88}$$

It may be asked whether high proton mobility is due to the presence of water channels in the hydrocarbon layer or not. In order to answer the question, first let us suppose that there are enough water molecules in the hydrocarbon layer so that the mechanism of proton transfer is essentially similar to the diffusion of proton in the bulk water; then, let us examine whether this assumption can lead to the feasible explanation of the experimental observations.

Since there is a close relationship between the permeability coefficient and partition coefficient given by:

$$P = uART/lF \tag{89}$$

knowing proton mobility in water is 3×10^{-3} cm^2/V sec (Eigen, De Maeyer, 1958), and assuming that the partition coefficient is unity and l is 45 A, we can evaluate the permeability coefficient of proton in the water layer with comparable thickness of the hydrocarbon layer of BLM as:

$$P_H = 4 \times 10^2 \text{ cm/sec} \tag{90}$$

Comparing Equation (90) with Equation (84) it becomes quite clear that the hydrocarbon layer of BLM is not quite similar to the water layer. In order to obtain the proton permeability coefficient of the present BLM, keeping the same diffusion mechanism, the partition coefficient must be less than unity or:

$$\begin{aligned} A &= P_{H,\ BLM}/P_{H,\ H_2O} \\ &= 5 \times 10^{-7} \end{aligned} \tag{91}$$

If there is protonic transfer through water chains in the BLM hydrocarbon layer, this partition coefficient also should exist in the present BLM.

The partition coefficient can be defined as:

$$A \equiv C_H^h / C_H^w = e^{-\,U/RT} \tag{92}$$

where C_H^h is the proton concentration in the hydrocarbon layer, C_H^w is the proton concentration in water, and U is the energy required for the proton transfer across the interface. U can be estimated as the energy of the transfer of a charge (q) as a function of the dielectric constants of water ($\varepsilon_w = 80$) and hydrocarbon layer (ε_h), taking the radius (r) of hydrated protons as 9 A by the following equation (Gurney, 1962):

$$U = \frac{q^2}{2r} \left(\frac{1}{\varepsilon_h} - \frac{1}{\varepsilon_w}\right) \tag{93}$$

At room temperature, T ≈ 300°K, the substitution of Equation (91) into Equation (92) gives:

$$U \approx 4 \text{ kcal/mole} \tag{94}$$

The resubstitution of Equation (94) into Equation (93) gives:

$$\varepsilon_h \approx 3.6 \tag{95}$$

The question now is whether Equation (95) is acceptable or not.

The dielectric constant of the hydrocarbon layer of BLM can be evaluated from the experimental value of membrane capacitance (C_m) by the relation (Tien and Diana, 1968):

$$C_m = \frac{\varepsilon_h S}{4\pi l} \tag{96}$$

where S is the membrane area and l is the thickness of the hydrocarbon layer. The value of the capacitance of all-*trans*-retinal BLM was reported by Tien and Kobamoto (1969) as 0.6 x 10^{-6} F/cm^2. Taking this value and l = 45 A as before, we obtain from Equation (96):

$$\varepsilon_h \approx 3.5 \tag{97}$$

A good agreement between Equation (95) and Equation (97) may be taken as an indication of the validity of the assumption made in deriving Equation (95), i.e. there may exist water channels, through which proton transfer can take place in almost crystalline structure of the hydrocarbon layer of all-*trans*-retinal BLM.

The Effect of Temperature on the Mode of Photoresponses

In the previous two sections it was concluded that an increase in temperature increased the proton mobility; thus leading to an increase in the membrane conductance, mainly the G_2 or protonic component. In this section we will examine how this variation of membrane conductance can modify the mode of biphasic photoresponses of all-*trans*-retinal BLM.

First let us consider the extreme cases of R2 = 0 or R1 = 0. In these cases there were only monophasic photoresponses, and upon light-off the decay of the potential started instantaneously. R2 = 0 was seen at lower temperatures while R1 = 0 at higher temperatures. These observations can be easily predicted from Equation (61) and (62) by putting $G_1 \gg G_2$ for the case of lower temperature and $G_2 \gg G_1$ for the case of higher temperature. Then, the time course of the photoresponses must follow an almost similar one to $V_c^1 - V_o$ for the lower temperatures and $V_c^2 - V_o$ for higher temperatures.

At the temperature range at which R1 and R2 can be seen in a single recording, G_1 and G_2 are about the same magnitude, indicating that both of the components, $V_c^1 - V_o$ and $V_c^2 - V_o$, can impose an influence on the other component. Now, let us examine how various aspects of R1 and R2 can be predicted from this theory, and how these predictions fit with the observations made in this work.

From Equation (61) we get:

$$\Delta V = V_c^{total} - V_o = - A(1 - e^{-at}) + B(1 - e^{-bt}) \tag{98}$$

Then, $d\Delta V/dt = -aAe^{-at} + bBe^{-bt}$ (99)

At the minimum $d\Delta V/dt = 0$, and Equation (99) becomes:

$$aAe^{-at} = bBe^{-bt} \tag{100}$$

By solving Equation (100) for t and noting that t is now the time required to reach the minimum of the photopotential we get:

$$t = t_{min} = \frac{1}{b - a} (\ln bB - \ln aA) \tag{101}$$

Since t_{min} must be greater than zero, we get:

$$bB > aA \tag{102}$$

The substitution of Equations (19) - (22) into Equation (102) gives:

$$G_2 > G_1 \left(\frac{V_1 - V_o}{V_2 - V_o}\right)^{1/2} \tag{103}$$

Equation (103) gives the conditions at which the minimum potential can be observed. These conditions are:

$$G_2 > G_1 \text{ if } V_1 - V_o = V_2 - V_o \tag{104}$$

$$V_2 - V_o > V_1 - V_o \text{ if } G_1 = G_2 \tag{105}$$

or

$$G_2 > G_1 \text{ and } V_2 - V_o > V_1 - V_o \tag{106}$$

The substitution of Equation (101) into Equation (61) gives:

$$\begin{aligned} V_1^{min} - V_o = & - \frac{G_1}{G_1+G_2}(V_1-V_o)\{1-\exp[- \frac{G_1}{G_2-G_1} \ln \frac{G_2^2(V_2-V_o)}{G_1^2(V_1-V_o)}]\} \\ & + \frac{G_2}{G_1+G_2}(V_2-V_o)\{1-\exp[- \frac{G_2}{G_2-G_1} \ln \frac{G_2^2(V_2-V_o)}{G_1^2(V_1-V_o)}]\} \end{aligned} \tag{107}$$

This potential was defined as the R1 component in the present work by Equation (3) (see also Figure 39). Equation (107) predicts that the R1 component decreases with an increase in G_2 exactly as observed in the present experiments of the temperature dependence of the photoresponses. It can also be seen that Equation (101) predicts that the time required for reaching the minimum potential (the peak time of R1) shortens with an increase of G_2. This point was repeatedly confirmed in the present work.

Next, let us examine the R2 component and the post effect. The post effect may be defined as the potential development beyond the light-off time. From Equation (62) we have:

$$\Delta V = - ACe^{- a(t - t_o)} + BDe^{- b(t - t_o)} \quad (108)$$

where

$$C = 1 - e^{-G_1 t_o / C_m} \quad (109)$$

and

$$D = 1 - e^{-G_2 t_o / C_m} \quad (110)$$

Then:

$$d\Delta V/dt = aACe^{-a(t - t_o)} - bBDe^{-b(t - t_o)} \quad (111)$$

At the maximum:

$$d\Delta V/dt = 0 \quad (112)$$

Figure 39.--The analysis of the time course of the biphasic photoresponses of all-*trans*-retinal BLM. See the text for the details.

Substituting Equation (112) into Equation (111), we get:

$$aACe^{-a(t - t_o)} = bBDe^{-b(t - t_o)} \tag{113}$$

Solving the Equation (113) for t and designating $t = t_{max}$, the time required for reaching the maximum potential, we get:

$$t_{max} = t_o + \frac{1}{a - b} (\ln aAC - \ln bBD) \tag{114}$$

Furthermore, by defining:

$$\Delta t = t_{max} - t_o \tag{115}$$

we get:

$$\Delta t = \frac{1}{a - b} (\ln aAC - \ln bBD) \tag{116}$$

Since the post effect, by its definition, must occur at:

$$\Delta t > 0 \tag{117}$$

Equation (116) becomes:

$$aAC > bBD \tag{118}$$

The substitution of Equations (19) - (22), (109) and (110) into Equation (118) gives:

$$\Delta V_2 < G_1^2(1 - e^{-G_1 t_o/C_m})\Delta V_1/G_2^2(1 - e^{-G_2 t_o/C_m}) \tag{119}$$

Equation (119) specifies the conditions at which the post effect can be seen. These are:

$$\Delta V_2 < V_1 \quad \text{if } G_1 = G_2 \tag{120}$$

$$G_2 < G_1 \quad \text{if } \Delta V_1 = \Delta V_2 \tag{121}$$

or

$$G_2 < G_1 \text{ and } \Delta V_2 < \Delta V_1 \tag{122}$$

The substitution of Equations (19) - (22), (109), and (110) into Equation (116) gives:

$$\Delta t = \frac{C_m}{G_1 - G_2} \ln \frac{G_1^2 (V_1 - V_o)(1 - e^{-G_1 t_o / C_m})}{G_2^2 (V_2 - V_o)(1 - e^{-G_2 t_o / C_m})} \tag{123}$$

Noting the conditions (120) - (122) for the existence of the post effect, the time required for reaching the maximum potential after the light-off increases with elongation of the illumination period, reaching the maximum value given by:

$$\Delta t = \frac{C_m}{G_1 - G_2} \ln \frac{G_1^2 (V_1 - V_o)}{G_2^2 (V_2 - V_o)} \tag{124}$$

Substituting Equation (123) into Equation (62) and subtracting Equation (61) after putting $t = t_o$, we get:

$$V_{max} - V_{t_o} = - \frac{G_1}{G_1 + G_2} (V_1 - V_o)(1 - e^{-G_1 t_o/C_m})$$

$$\times[\exp (- \frac{G_1}{G_1 + G_2} \ln \frac{G_1^2(V_1 - V_o)(1 - e^{-G_1 t_o/C_m})}{G_2^2(V_2 - V_o)(1 - e^{-G_2 t_o/C_m})}) - 1]$$

$$+ \frac{G_2}{G_1 + G_2} (V_2 - V_o)(1 - e^{-G_2 t_o/C_m})$$

$$\times[\exp (- \frac{G_2}{G_1 + G_2} \ln \frac{G_1^2(V_1 - V_o)(1 - e^{-G_1 t_o/C_m})}{G_2^2(V_2 - V_o)(1 - e^{-G_2 t_o/C_m})}) - 1]$$

(125)

From Equation (125) and the conditions given in Equations (120) - (122) it can be said that $V_{max} - V_{t_o}$ increases with an increase in G_2. This characteristic was clearly demonstrated in Figures 22 and 23, where the post effect increases with increasing temperature, which, in turn, increases the value of G_2. Not only Δt but also $V_{max} - V_{t_o}$ increase with an increase in t_o. This point was supported by the observation that keeping other experimental parameters constant, the elongation of the illumination time alone resulted in the appearance of the R2 component, which was not observed during the shorter time of illumination.

Finally, let us examine the temperature dependence of R2/R1. The substitution of Equation (124) for $t - t_o$ of Equation (62) gives R2 or:

$$R2 = -\frac{G_1}{G_1 + G_2}(V_1 - V_o)(1 - e^{-G_1 t_o/C_m}) \exp\left(- \frac{G_1}{G_1 - G_2} \ln \frac{G_1^2(V_1 - V_o)(1 - e^{-G_1 t_o \cdot C_m})}{G_2^2(V_2 - V_1)(1 - e^{-G_2 t_o/C_m})}\right)$$

$$+\frac{G_2}{G_1 + G_2}(V_2 - V_1)(1 - e^{-G_2 t_o/C_m})$$

$$\mathrm{x} \exp\left(- \frac{G_2}{G_1 - G_2} \ln \frac{G_1^2(V_1 - V_o)(1 - e^{-G_1 t_o/C_m})}{G_2^2(V_2 - V_o)(1 - e^{-G_2 t_o/C_m})}\right) \tag{126}$$

Dividing Equation (126) with R1 given by Equation (107), and rearranging, we get:

$$
\begin{aligned}
R2/R1 = &\{G_1\Delta V_1(1 - e^{-G_1 t_o/C_m})\}^{G_1/(G_2 - G_1)} \\
&\times \{G_2\Delta V_2(1 - e^{-G_2 t_o/C_m})\}^{-G_1/(G_2 - G_1)} \\
&\times \{(G_1/G_2)^{G_2/(G_2 - G_1)} - (G_1/G_2)^{G_1/(G_2 - G_1)}\}/ \\
&[G_1\Delta V_1 - G_2\Delta V_2 - (G_1\Delta V_1)^{G_2/(G_2-G_1)}(G_2\Delta V_2)^{-G_1/(G_2-G_1)} \\
&\times \{(G_1/G_2)^{G_2/(G_2 - G_1)} - (G_1/G_2)^{G_1/(G_2 - G_1)}\}]
\end{aligned}
\tag{127}
$$

Since biphasic responses must satisfy the conditions shown in Equations (104) - (105) and (120) - (122), it is permissible to assume:

$$G_1\Delta V_1 = G_2\Delta V_2 \tag{128}$$

The substitution of Equation (128) into Equation (127) gives:

$$- R2/R1 = (1-e^{-G_1 t_o/C_m})^{(G_1/G_2-1)}(1-e^{-G_2 t_o/C_m})^{(G_2/G_1-1)} \tag{129}$$

Now, it is possible to predict the variation of the ratio as a function of changes in G_2.

When $G_2 = G_1$, we get:

$$- R2/R1 = 1 \tag{130}$$

At this point we introduce the factor of temperature. It was found in the present work that the following relationship can hold:

$$G_2(T) = G_2^o e^{- E_{G_2}/RT} \tag{131}$$

where G_2^o is constant and E_{G_2} is the activation energy for protonic conduction. Assuming that G_1 is temperature insensitive or that $E_{G_1} \ll E_{G_2}$, the substitution of Equation (131) into Equation (129) gives:

$$- R2/R1 = (1 - e^{-G_1 t_o/C_m}) (\frac{G_1}{G_2^o} e^{E_{G_2}/RT} - 1)$$

$$\times (1 - e^{\frac{-t_o G_2^o}{C_m} e^{-E_{G_2}/RT}}) (\frac{G_2^o}{G_1} e^{-E_{G_2}/RT} -1) \tag{132}$$

As T increases the equation (132) behaves differently, depending on the relative values of G_1 and G_2^o. When $G_1 > G_2$: (133)

$$- R2/R1 \Rightarrow 0 \tag{134}$$

When $G_2^o > G_1$: (135)

$$-R2/R1 = (1-e^{-G_1 t_o/C_m})^{-1}\,(1-e^{-(G_2^o t_o/C_m)e^{-E_{G_2}/RT}})\,(\frac{G_2^o}{G_1}e^{-E_{G_2}/RT}-1)$$

(136)

Equation (134) does not agree with the observations made in the present work, while Equation (136) shows rapidly increasing function with respect to an increase in temperature, like the curve shown in Figure 21. Thus, it can be concluded that at the condition given by Equation (135), i.e., relatively higher protonic conduction than hole conduction, the theory developed in this work predicts rather satisfactorily. Since the generation of the R2 phase required higher protonic conductance, the requirement given by the Equation (135) should be considered as a reasonable prediction agreeing with experimental observations.

It is clear that the theory developed in the present work predicts and explains all the observations made as a function of temperature.

The Thermal Coefficients of Photoresponses

The thermal coefficient of R1 was far smaller than that of R2 and in the presence of sufficient electron acceptors was reduced to almost zero. If R1 would show temperature dependence, it was always a negative type: R1 decreased with increasing temperature. Because of the high hole permeability coefficient as shown in Equation (86), it can be assumed that the hole potential ΔV_1 is almost temperature independent. Thus, the negative temperature dependence of R1 may mean either the presence of the essential effect of temperature on the generation of holes, or the effect of increasing protonic potential or ΔV_2. The first possibility is not likely to be feasible with negative temperature dependence. Rather, since the photoresponses, R1 and R2, can be seen at the temperature above 40°C under proper experimental conditions, and the magnitude of this negative thermal coefficient was almost equal to that of the protonic resistance change as shown in Table 4, it is more likely that the second possibility is the case in the present model BLM. Thus, the analysis of thermal coefficient of R2 will provide the information valuable in understanding that of R1: R2 is mainly determined by the temperature dependence of the protonic potential.

The thermal coefficient of R2 was larger than both that of R1 and of the dark membrane potential development, and hence, also larger than that of the membrane conductance as shown in Table 4. Our task here is to understand why this is so. Examining Equation (126) shows that in order to get a single thermal coefficient we must have:

$$R2\ (T) \propto \frac{G_2(T)}{G_1(T) + G_2(T)} \qquad (137)$$

and other terms of Equation (126) must be negligible.

Let $E_{G_1} \ll E_{G_2}$ (138)

and $G_1(T) = G_1$ (139)

then, since at elevated temperatures:

$$G_2^2(T) \gg G_1^2 \qquad (140)$$

we get:

$$R_2(T)\ \alpha\ 1 - \frac{G_1}{G_2(T)} \qquad (141)$$

From Equation (81) we have:

$$G_2(T) = \frac{F^2 u_H(T) C_H^*(T) S}{l} \qquad (142)$$

Since $C_H^*(T) = AC_H = C_H e^{-U/RT} = C_H(T) e^{-U/RT}$ (143)

where $C_H(T)$ is the concentration of the protons including the photogenerated protons, and U is the energy required for the transfer of proton across the water/hydrocarbon interface. Thus, Equation (142) should have three exponentially temperature dependent terms which may appear as a single temperature coefficient as:

$$E_{R2} = E_u + U + E_{C_H} \tag{144}$$

where E_u is the thermal coefficient of proton mobility in the hydrocarbon layer and E_{C_H} is the thermal coefficient of the proton generation by the oxidation of water molecules. Since $E_{R2} \approx 16$ kcal/mole, the substitution of Equations (74) and (94) into Equation (144) gives:

$$E_{C_H} \approx 7 \text{ kcal/mole} \tag{145}$$

There are two points of interest:

1. Why cannot U be recognized in the thermal coefficient of the development of membrane potential and that of dark membrane conductance?
2. What is the significance of the value of E_{C_H} given in Equation (145)?

The first problem can best be understood by considering the effect of buffering. The dark parameters were measured at steady states at which it is possible to have equilibrium between the transfer of proton into the hydrocarbon layer and the proton transfer into the bulk aqueous solution by buffering capacity:

$$dC_U^H/dt - dC_b^H/dt = 0 \tag{146}$$

at the dark state. It is reasonable to assume that this equilibrium can exist at all temperatures if we allow the system to stay at a stable state. Hence, the positive effect of temperature on the proton transfer into hydrocarbon can be unobservable: this does not necessarily require:

$$U = E_b \qquad (147)$$

where E_b is the thermal coefficient for buffering. On the other hand, at illumination where the rate of the generation of protons is greater than the rate of buffering into the bulk aqueous solution, instead of Equation (146), we have:

$$dC_U^H/dt - dC_b^H/dt > 0 \qquad (148)$$

at the illuminated state. Under this condition it is obvious:

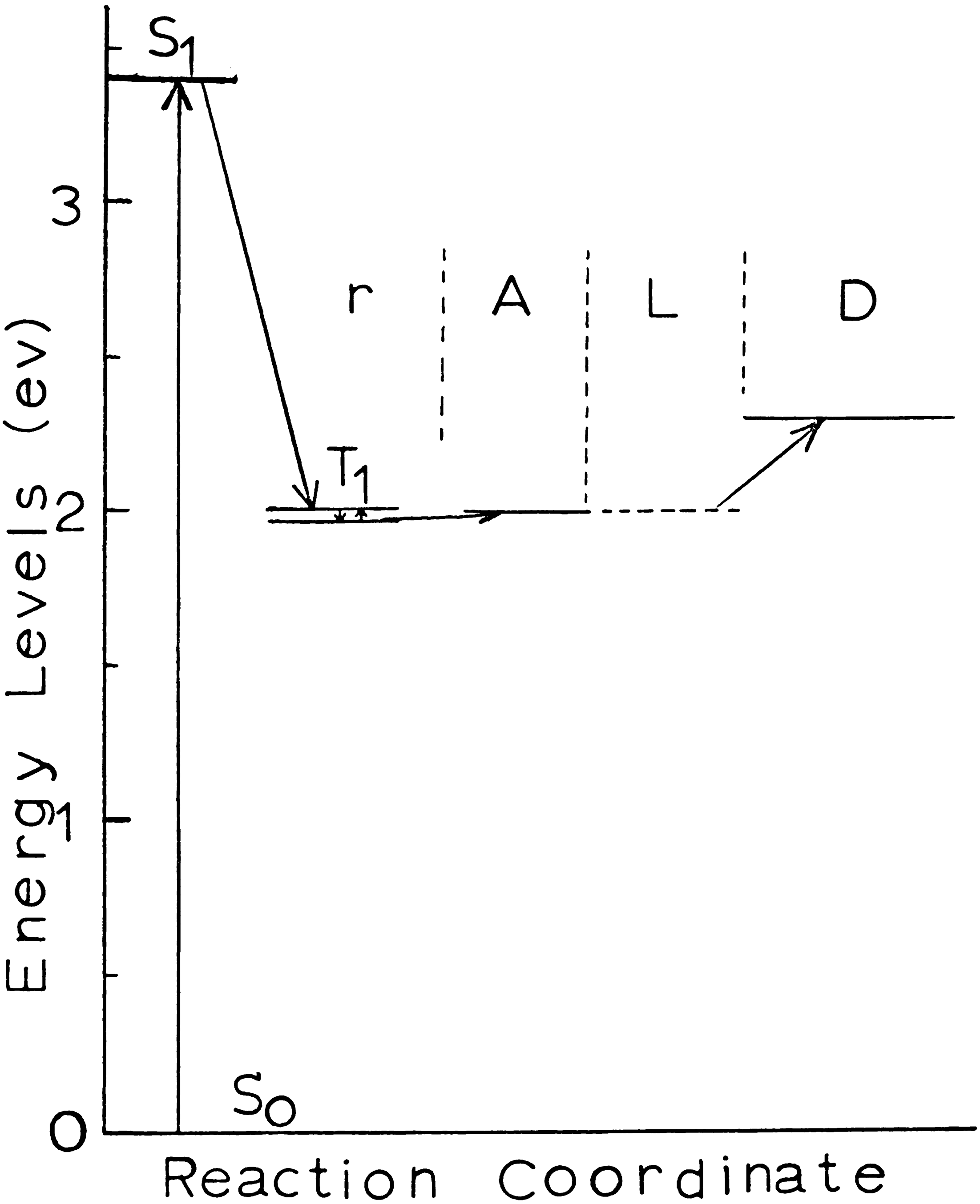
$$U > E_b \qquad (149)$$

since the former process requires the transfer of charged species across an interface, while the latter process is simple diffusion in the same aqueous phase. Thus, the thermal coefficient of the partition of ions can become observable in the case of photoresponse. The above discussion is feasible, since the buffer capacity closely controls generation ΔV_2 and, hence, R2 as discussed in a previous section. The second question may be answered in the following manner. Since the hydrogen bond of a water-carbonyl group is about 3 kcal/mole (Karyakin, Muradova, Sayenko, 1969), E_{C_H} can be considered as a reasonable estimation of the activation energy of proton generation. This lower oxidation energy may be due to the fact that the interface in the presence of excited retinal can function as a catalytic site for the generation of protons and possibly oxygen.

The energetics of generation of the charge carriers (holes and protons), discussed above, are schematically shown in Figure 40. Photoexcitation is considered to occur only via ground state singlet-1st excited singlet excitation, then intersystem transfer to the 1st excited triplet occurs. This is due to the fact that since there is good agreement between the action spectrum of photovoltaic and photo conductive effects of the all-*trans*-retinal BLM with the absorption spectrum of bulk lipid solution (Tien and Verma, 1970), we may conclude that the ground state singlet-1st excited singlet is the major, if not the only, excitation pathway, and the ground state singlet-1st excited triplet transition may not exist in a significant probability. Secondly, this is due to the fact that the triplet state is not directly involved in the process of charge generation, as can be seen in Figure

Figure 40.--The electronic energy levels of all-*trans*-retinal bimolecular lipid membrane. The values of 1st excited triplet was from Rosenberg and Harder (1967).

A - electron acceptors (ferric ions)
D - electron donors (water molecules)
L - hydrocarbon layer
r - all-*trans*-retinal molecules
S_o - ground state singlet
S_1 - 1st excited singlet
T_1 - 1st excited triplet


S1
T1
S0
r
A
L
D
Energy Levels (ev)
3
2
1
0
Reaction Coordinate

40. Triplet excitons are trapped in the surface sites as charge transfer complexes. It was assumed in deriving the kinetic equation that the concentration of this complex and triplet excitons can exist in equilibrium due to the trap depth of approximately kT. The charge generation step is the charge separation of this complex which can be activated by the thermal energy and not the direct generation of the 1st excited singlet or 1st excited triplet, since even the activation energy of semiconduction of the present BLM was lower than the energy levels involved in these two processes. The thermal coefficient of R1 is always less than that of R2; in the presence of high Fe^{3+} concentrations zero activation energy was observed. Thus, not only the thermal coefficient of hole diffusion but also that of hole generation is so low as to be insignificant. At lower ferric ion concentrations the thermal coefficient of R1 showed the negative of that of R2, and at higher ferric ion concentrations, the thermal coefficient was almost zero. This may be interpreted to mean that the negative activation energy is due to the negative contribution of the R2 component. The degree of their contribution may depend on the relative concentration of the two charge carriers. That is, if ferric ion concentration is low, the R2 effect is greater while at a higher ferric concentration the effect of R2 is negligible. From this it

is possible to regard that the activation energy of true R1 is far smaller than that of R2, or less than kT (10^{-2} ev). After charge generation, the triplet excited state, which has approximately the same energy level as the triplet state, diffuses across the hydrocarbon layer. Upon reaching the outer surface, the thermal activation of about 0.3 eV leads to generation of protons. The energy levels of the 1st excited singlet and 1st excited triplet may be considered the same as those in crystalline all-*trans*-retinals or 3.4 ev and 2.0 ev, respectively (Rosenberg and Harder, 1967). However, this triplet state has a considerably higher value than that of an all-*trans*-retinal solution in non-hydroxyl solvent, which is 0.8 ev (Abrahamson and Ostroy, 1967).

The Further Experimental Verification of the Theory

The experimental verification of the theory can be seen in the following four points, in addition to the various aspects of temperature dependence discussed in the previous section.

The Effect of Light Intensity: Logarithmic Dependence

In order to find the relationship between the R1 and R2 components and light intensity, first, let us obtain R1 by rearranging Equation (93):

$$R1 = \frac{1}{G_1 + G_2} [G_2(V_2 - V_o) - G_1(V_1 - V_o)$$

$$+ \{G_2(V_2 - V_o)\}^{-G_1/(G_2 - G_1)} \{G_1(V_2 - V_o)\}^{G_2/(G_2 - G_1)}$$

$$\{(G_1/G_2)^{G_1/(G_2 - G_1)} - (G_1/G_2)^{G_2/(G_2 - G_1)}\}] \qquad (150)$$

and R2 by rearranging Equation (126):

$$R2 = \frac{1}{G_1 + G_2} \{G_1(V_1 - V_o)(1 - e^{-G_1 t_o/C_m})\}^{G_2/(G_2 - G_1)}$$

$$\{G_2(V_2 - V_o)(1 - e^{-G_2 t_o/C_m})\}^{-G_1/(G_2 - G_1)}$$

$$\{(G_1/G_2)^{G_2/(G_2 - G_1)} - (G_1/G_2)^{G_1/(G_2 - G_1)}\} \qquad (151)$$

From Equation (150) we obtain:

$$dR1/d\ln I_o = \{RT/F(G_1 + G_2)\}[G_2 - G_1$$

$$+\{(G_1/G_2^{G_1/(G_2 - G_1)} - (G_1/G_2)^{G_2/(G_2 - G_1)}\}$$

$$G_2^{- G_1/(G_2 - G_1)} G_1^{G_2/(G_2 - G_1)}$$

$$x\{(V_2-V_o)^{-G_1/(G_2-G_1)}\{G_2/(G_2-G_1)\}(V_1-V_o)^{G_1/(G_2-G_1)}$$

$$-(V_1-V_o)^{G_2/(G_2-G_1)} G_1/(G_2-G_1)(V_2-V_o)^{-G_2/(G_2-G_1)}\}]$$

(152)

If we assume $(V_1 - V_o) = (V_2 - V_o)$, we get:

$$dR1/d\ln I_o = \{RT/F(G_1 + G_2)\}G_1^{G_2/(G_2 - G_1)} G_2^{-G_1/(G_2 - G_1)}$$

$$\{(G_1/G_2)^{G_1/(G_2 - G_1)} - (G_1/G_2)^{G_2/(G_2 - G_1)}\}$$

(153)

Similarly from Equation (151) we get:

$$
\begin{aligned}
dR2/d\ln I_o = {} & \frac{1}{G_1 + G_2}\,\frac{RT}{F}\{G_1(1 - e^{-G_1t_o/C_m})\}^{G_2/(G_2 - G_1)} \\
& \{G_2(1 - e^{-G_2t_o/C_m})\}^{-G_1/(G_2 - G_1)} \\
& \{(G_1/G_2)^{G_2/(G_2 - G_1)} - (G_1/G_2)^{G_1/(G_2 - G_1)}\} \\
& [(V_2-V_o)^{-G_1/(G_2-G_1)}\{G_2/(G_2-G_1)\}(V_1-V_o)^{G_1/(G_2-G_1)} \\
& -(V_1-V_o)^{G_2/(G_2-G_1)}\{G_1/(G_2-G_1)\}(V_2-V_o)^{-G_2/(G_2-G_1)}]
\end{aligned}
\tag{154}
$$

If we assume $V_1 - V_o = V_2 - V_o$, we get:

$$
\begin{aligned}
dR2/d\ln I_o = {} & \{RT/F(G_1 + G_2)\}\{G_1(1-e^{-G_1t_o/C_m})\}^{G_2/(G_2 - G_1)} \\
& \{G_2(1 - e^{-G_2t_o/C_m})\}^{-\frac{G_1}{G_2-G_1}} \\
& \{(G_1/G_2)^{G_2/(G_2 - G_1)} - (G_1/G_2)^{G_1/(G_2-G_1)}\}
\end{aligned}
\tag{155}
$$

The Equations (153) and (155) indicate the slope of the straight line which can be obtained by plotting either R1 or R2 against the logarithm of light intensity. It is shown in Figure 19 that such a linear relationship exists, suggesting the validity of the theory.

If we define:

$$\alpha = \frac{dR2/d\ln I_o}{dR1/d\ln I_o} \tag{156}$$

then the substitution of Equations (153) and (155) into Equation (156) gives:

$$\alpha = \{1 - \exp(-G_1 t_o/C_m)\}^{G_2/(G_2 - G_1)}$$

$$\times \{1 - \exp(-G_2 t_o/C_m)\}^{-G_2/(G_2 - G_1)} \tag{157}$$

Solving for G_1, we get:

$$G_1 = (2t_o/C_m \ln\alpha + 1/G_2)^{-1} \tag{158}$$

At the conditions specified in the legend, Figure 19 gives:

$$\alpha = 1.5 \tag{159}$$

with the substitution of Equation (159) into Equation (158), noting that t_o is one second, C_m is 0.6 $\mu F/cm^2$, and G_2 is 5×10^{-8} mhos cm^{-2}, we get:

$$G_1 = 3.7 \times 10^{-8} \text{ mhos cm}^{-2} \tag{160}$$

This prediction is reasonable since the steeper slope of R2 must arise from slightly greater G_2 with respect to G_1. From the above discussion it is clear that the light intensity dependence of photo-responses can be satisfactorily explained by the present theory.

The Effect of the Outside pH

From Equations (150) and (151) we get:

$$dR1/d\ln C_H^o = -\{RT/F(G_1 + G_2)\}$$

$$[G_2 - \frac{G_1}{G_2 - G_1}\{(G_1/G_2)^{G_1/G_2 - G_1)} - (G_1/G_2)^{G_2/(G_2 - G_1)}\}$$

$$\times \{G_1(V_1 - V_o)\}^{G_2/(G_2 - G_1)}\{G_2(V_2 - V_o)\}^{-G_2/(G_2 - G_1)}] \tag{161}$$

and

$$dR2/d\ln C_H^o = \{RT/F(G_1+G_2)\}\{G_1(V_1-V_o)(1-e^{-G_1t_o/C_m}\}^{G_2/(G_2-G_1)}$$

$$\{(G_1/G_2)^{G_2/(G_2-G_1)} - (G_1/G_2)^{G_1/(G_2-G_1)}\}$$

$$\{G_2(1-e^{-G_2t_o/C_m})\}^{-G_1/(G_2-G_1)}$$

$$[\{G_1/(G_2-G_1)\}(V_2-V_o)^{-G_2/(G_2-G_1)}]$$

(162)

If we put $V_1 - V_o = V_2 - V_o$, the Equations (161) and (162) become:

$$dR1/d\ln C_H^o = -RT/F(G_1+G_2)\ [G_2 - G_1/(G_1+G_2)$$

$$\{(G_1/G_2)^{G_1/(G_2-G_1)} - (G_1/G_2)^{G_2/(G_2-G_1)}\}$$

$$G_1^{G_2/(G_2-G_1)}G_2^{-G_2/(G_2-G_1)}]$$

(163)

and

$$dR2/d\ln C_H^o = \{RT/F(G_1 + G_2)\}\{G_1(1 - e^{-G_1 t_o/C_m})\}^{G_2/(G_2 - G_1)}$$

$$\{(G_1/G_2)^{G_2/(G_2-G_1)} - (G_1/G_2)^{G_1/(G_2-G_1)}\}\{G_1/(G_2-G_1)\}$$

$$\{G_2(1 - e^{-G_2 t_o/C_m})\}^{-G_2/(G_2 - G_1)} \qquad (164)$$

This linear relationship between the photoresponses and the logarithm of outside pH is clearly demonstrated in Figure 29: an increase in the outside pH induces an increase in R2 and a decrease in R1, both in a linear relationship. Thus, this point again shows good agreement between theory and the experimental observations.

The Effect of Buffer Capacity

Buffer capacity influences the photoresponses dramatically. This effect may be best understood by defining a coupling coefficient, g, between the rate of photogeneration of protons at the outer surface to the accumulation at the inner surface. By referring to Figure 36 and neglecting D_4 and k_5 as before we get:

$$g = (D_2 - k_8 - k_5 - k_9)/k_2 \qquad (165)$$

If the buffer capacities of two aqueous phases are the same, as they were in the present work, $k_8 = k_9$ and Equation (165) becomes:

$$g = (D_2 - 2k_8 - k_3)/k_2 \qquad (166)$$

Hence, the greater the buffer capacity, the smaller the coupling coefficient, meaning that R2 decreases in spite of the occurrence of the equally developed R1 component.

The Effect of Light Intensity: Threshold Requirement

Pure R1 responses could be transformed to biphasic responses by simply increasing light intensity without changing other experimental parameters (Figure 19). Can this fact be explained with the theory presented in this work?

There are two possibilities for the explanation of the requirement of threshold intensity for generation of the R2 component. One is threshold requirement to accumulate the photoproduct, or protons in the present case, and the other is the threshold requirement for a completely independent secondary process. The latter possibility can be eliminated by the following consideration. The only secondary process possible in the present system is photoinduced proton diffusion. However, the magnitude of R2 declined, after reaching a maximum under the effect of increasing temperatures. If this component is the proton diffusion potential, independent from the initial hole potential, such a decline should not be observed since the protonic conductivity increased monotonously with an increase in temperature. The presence of a maximum in R2 can be satisfactorily explained if we consider that R2 is the resulting potential of the initial hole potential. That is, an increase in protonic conduction, which requires an increase in hydration of the hydrocarbon layer, acts unfavorably for the development of R1 by decreasing the lifetime of holes in the hydrocarbon layer. This reasoning leads to the concept that there must be a threshold intensity for the initiation of the two components and that R2 must have a higher threshold intensity, since the accumulation of a certain concentration of photoproduct, protons, may be required for the generation of R2. There is another consequence of this reasoning: the maximum photopotential can be observed at a proper protonic conductivity where the positive effect of proton conductivity equals the negative effect on the hole conductivity. Thus, the presence of threshold intensity for the generation of R2 is in support of the present theory, indicating that R2 is the resulting diffusion potential of the accumulated protons, generated by the holes which have diffused through the membrane, which in turn have generated R1.

"Hole-Proton Diffusion" Mechanism and Its Alternatives

The formulation of the present theory was based on the fact that a pair of calomel electrodes can detect only the potentials developed by the diffusion of charge carriers. In order to use this concept the transference number of protons was experimentally determined as unity to chloride ions and the transference number of holes was also assumed to be unity. The assumption made concerning the hole transference number can be well justified by the observation that the presence of a strong electron acceptor (ferric ions) can have a definite effect on determining not only the direction but also the magnitude of R1, without exception, and agrees with the sign of the potentials predicted by the assumption of the hole diffusion from the side onto which ferric ions had adsorbed. This explanation is equivalent to the concept of hole injection into the hydrocarbon layer at the side of ferric ions after the excitation of retinal incorporated into the hydrocarbon layer. Such a possibility was also proposed to occur at semiconductor/water interfaces (Kallmann and Pope, 1960). The present work does not prove the presence of electronic (electron and hole) conduction in the all-<u>trans</u>-retinal BLM. Nevertheless, the present work strongly favors the presence of hole conduction by establishing the presence of protonic potential, which is free from the effect of other ionic species, and by demonstrating the presence of the non-protonic potential, which was proposed to be the hole potential. The fact that the non-protonic potential could not be leakage potential of other anions or cations was clearly demonstrated by the observation that the ten-fold concentration gradient of KCl and NaCl did not have any effect on the R1 and R2 components. Thus, the success of the present theory in explaining all observed facts indicates strongly that the assumption of the presence of the hole conduction in all-*trans*-retinal BLM is well justified.

CHAPTER VII

TOWARD A FURTHER UNDERSTANDING OF EARLY RECEPTOR POTENTIALS

Most of the observations made in the present work strongly suggest the basic similarities between the photoresponses of all-*trans*-retinal BLM and the early receptor potentials of biological photoreceptors. In this chapter, these relevant observations are fully discussed, 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.

The basic idea to be examined is that the mechanism proposed in this work for the explanation of the biphasic photoresponses of all-*trans*-retinal BLM may have a biological role as the primary process leading to generation of the receptor potentials. The proposed mechanism may be summarized in short: the photoinduced 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.

The Membranous Origin of the Early Receptor Potentials

This investigation was undertaken in order to examine the possibility as to whether the photoresponses similar to the early receptor potentials could be generated with the model membrane system like all-*trans*-retinal BLM. It is remarkable that the observations made in this work showed the striking similarities between the photoresponses of all-*trans*-retinal BLM and the early receptor potentials. This fact indicates that accessory structures may not be necessary for the production of the early receptor potentials and, hence, that the pigmented membrane of rods and cones can generate the early receptor potentials. The mechanism of the generation of the early receptor potentials of the pigment epithelium and in the plant cells may be different from that of the rods and cones to a certain degree.

It is well established that the wave form of the early receptor potentials is dependent on the amount and kinds of rhodopsin and its derivatives in the rods and cones (Cone, 1967; Pak and Boez, 1967; Hagins and McGaughy, 1967). The point of special interest is that the regeneration of rhodopsin derivatives can reverse the wave form completely. The result was explained as the recovering of the original charge distribution in and around the pigment molecules. This point may lessen the possibility of the generation of photochemical products such as protons and may lead to the conclusion that the early receptor potentials do not satisfy the condition of the visual receptor excitation, since rhodopsin alone, and not the derivatives of rhodopsin, determines the excitation (Pak, 1968).

On the other hand, it is possible to introduce the active role of the membranous structure into the interpretation of the above observation. The generation of rhodopsin derivatives necessarily produces changes in the membrane structure so that the membrane surfaces are changed from the original "rhodopsin state," causing proton generation and transport to act in the reverse direction. The structural changes of rhodopsin and its derivatives would change the charge distribution at two surfaces in a drastic manner. However, the salt concentration across the disc membrane is constant, regardless of the changes of the surface charge distribution due to the formation of the rhodopsin derivatives. If so, the generation of protons by the rhodopsin derivatives may not act as efficiently as the rhodopsin itself since the salt concentration gradient must be in an ideal condition in the latter case. Thus, although the generation of the early receptor potentials can be perfect, since protons can be generated and transported to the same extent as in the case of "rhodopsin state," the following cation transport may not be induced if the salt concentration gradient is not at the proper conditions in view of the energy coupling or the characteristics of the pores.

Lettvin (1965) proposed that the shift of dipoles with respect to the retinal surface or disc membrane could serve as the mechanism of the generation of the early receptor potentials. However, there seems to be no supporting evidence for this type of molecular arrangement of the pigment in the retinal disc membranes (Brindley and Gardner-Medwin, 1966). Furthermore, in the pigment epithelium and plant cell the pigment granules are in random orientation with respect to the surface of the tissues and the cell membranes (Figure 1). It is very important to note that in the cell wall of the pigment epithelium and the plant cell, pigment concentrations are usually low. It is at this point that the rhodopsin derivative hypothesis and dipole hypothesis encounter immense difficulty: the "excitation" has to be transferred to the non-pigmented cell wall from the pigment granules, since the simple charge distribution along the disc membrane due to the intramolecular charge redistribution is not applicable to the other pigmented tissues. In the mechanism developed in the present work it was proposed that photogenerated protons could perform the function of the mediator of the early receptor potentials. Thus, there is no difficulty of this type.

The possibility that electronic conduction along a conjugated carotenoid chain could be involved in visual excitation was first suggested by Dartnall (1948). The mechanism of such an electronic process was speculated as solid state photoconduction in photorecptor lamellar structures by Chynoweth and Schneider (1954) and Reid (1957). The first evidence of the existence of photoconduction in beta-carotene glasses was reported by Rosenberg (1958). The same worker has established the existence of photoconduction in the glasses of retinals (Rosenberg and Harder, 1967) and dried rods (Rosenberg, Orlando, and Orlando, 1961). Brown and Murakami (1964a) and Crawford, Gage, and Brown (1967) suggested that photoconductivity of the pigmented tissue could be the mechanism of the generation of early receptor potentials. Recently, Rosenberg and Postow (1970) pointed out a similarity between the biphasic mode of the early receptor potentials and the photoresponses of beta-carotene glasses, and suggested that the two phases could be due to the photovoltaic and photoconductivity effects. This analysis is similar to a certain degree to that of the present work, although the charge carriers in the former case are electronic for both phases, while those in the latter case are electronic for the first phase and protonic for the second phase.

The Coupling of Electronic Transport with Ionic Transport in Visual Excitation

The possibility of coupling electron movement across the biological membrane to ionic movement was first proposed by Lillie (1915, 1916) in way of making an analogy of the hypothetical metal electrode reversible to the ionic species which would be responsible for generation of membrane potential. About one decade later Lund (1928) extended the theory to the development of a continuous membrane potential with the mechanism of electron movement through respiratory enzymes. Along with the support of Lund's theory (Marsh, 1935), an ionic coupling theory was developed to account for the accumulation of salts across the biological membranes (Stiehler and Flexner, 1938; Lundegardh, 1939; Ussing, 1947; Leve and Ussing, 1948). In the 1940's Lund's theory was vigorously supported by many workers such as Lundegardh (1945; 1946), Robertson and Wilkins (1948a, b), and Crane, Davies and Longmuir (1948a, b). Quantitative approaches to the Lund theory have also been carried out by Conway and Davis and Ogston (1950). The coupling of electron transport to ionic transport and, then, to mitochondrial and photosynthetic phosphorylation was proposed by Mitchell (1961, 1966). It was Jahn (1962) who included non-enzymic electron pathways (such as beta-carotene and vitamin A derivatives) which could be coupled to ion transport. Further, Jahn

(1963) extended the possibility of the similar mechanism to visual excitation. Jahn's (1962, 1963) 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. Judging from the observations made in the present work, it is more than likely that Jahn's theory can be applied to the explanation of the generation of the early receptor potentials. Furthermore, a three-step excitation mechanism 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. Therefore, our next question is whether or not proton can be generated by light in photoreceptors. This point will be discussed in the following section. Section D discusses the possibility of coupling between the protonic transport and the sodium and potassium transport.

The Photogeneration of Protons in Photoreceptors

There is ample evidence to indicate changes in the proton concentrations of the aqueous media of the retina, the suspensions of the outer segment of the photoreceptors, and rhodopsin solutions upon illumination.

Frog retina, both in vitro and in vivo, became more acid when exposed to light (Kobakova, 1946). A rapid fall of pH occurred at the first 5-10 minutes of illumination falling from pH 7.8 to 7.7 reversibly. Ostrovskii, Fedorovich, and Polyak (1968) could not observe a significant pH change after illumination of the frog retina at the initial pH of 6.3-6.4, while the suspension of the outer segments of the photoreceptor showed an increase in pH ranging from 0.06 - 0.10 after a 3 minute illumination at the initial pH of about 6.15. The discrepancy between the observation of Kobakova (1946) and that of Ostrovskii, Fedorovich, and Polyak (1968) may be due to the effect of the initial pH of the bathing solutions since it is known that this factor influences the mode of the pH changes of rhodopsin solutions significantly, as mentioned below. Nevertheless, it is evident that the pH of the retina can be changed by exposure to light. Further understanding of this aspect may occur if we know the internal pH of the retina and the relationship between pH change and the photoresponses.

pH of a rhodopsin solution could be also changed by illumination. Radding and Wald (1955-1956a, b) observed that the illumination with flashes of intense light induced not only slow acid-base changes in rhodopsin solution, but also an immediate rise in pH. This immediate rise in pH was observed between pH 2 and pH 8 with a decrease in the magnitude at the neutral pH, and was followed by the slow changes which were always in the direction of neutrality. Studying the pH range between 4 and 10 Hara (1958) and Hara and Hara (1960) observed similar results reported by the above workers. They also observed that at an alkaline pH the immediate change of pH was toward the neutral region. The slower component stayed at a constant value of pH following an immediate rise at the acid pH; on the other hand, it decreased continuously toward the neutral pH at the alkaline region. Hara (1958) also observed an immediate increase in the resistance of the rhodopsin solution after exposure to the flashes of intense light at alkaline pH as well as acid pH. Recently, Fujishita (1968, 1969) observed the various modes of changes in the pH of rhodopsin solution. With dim green light a pH change could be induced without bleaching rhodopsin. The direction of a pH change is dependent on the temperature and the initial pH of the solution. Fujishita defined an "isoionic point," decreasing with increasing temperatures. The values were somewhat higher than those of the isoelectric point of the frog rhodopsin (pH 4.47) and those of the bleached product (4.57), determined by Broda and Victor (1940). McConnell, Rafferty, and Dilley (1968) reported that proton uptake and extrusion by bovine retinal outer segment fragments required the presence of unbleached rhodopsin while the stoichiometric ratio between proton uptake and rhodopsin bleached was a function of experimental conditions.

Most of the workers mentioned above tend to consider the mechanism of pH changes as the exposure of the acid-base groups to the aqueous phase due to the removal of retinal and/or the conformation changes of opsin. Due to the complex mode of the changes in pH on illumination, however, further studies are needed to confirm this interpretation. Nevertheless, an important question at the present time is whether, regardless of the mechanism of generation of photoinduced pH changes, pH can influence the mode of generation of the early receptor potentials, since if protons can function as the mediator there may be an interaction between protons and the mode of the photopotential generation via product inhibition.

The effect of pH on the early receptor potential was first studied by Brindley and Gardner-Medwin (1966). Thirty minute soaking of frog retinas in 120 mM phosphate buffers between pH 8.5 and 2.8 at 24°C showed a rather small effect on the mode of the early receptor potential. The biphasic response could be observed over this pH range. The magnitude of the two phases decreased and the time required for the development of R2 became longer as pH decreased. Pak, Rozzi, and Ebrey (1967), using the frog retina and a 50 mM buffer system between pH 3 and pH 11, observed, in general, a similar pH effect to that noted by Brindley and Gardner-Medwin (1966). Furthermore, Pak, Rozzi, and Ebrey (1967) observed that the amplitude of R2 had its optimum value near the neutral pH and reversibly decreased at high and low values of pH. The reversibility of responses decreased at the extreme ends of pH's in such a manner that the early receptor potential could be abolished after a 30 minute immersion. At the higher pH the dominant component was R2 while at the lower pH the response was dominated by R1. From these observations they concluded that lowering pH not only reduced R2 but also enhanced R1. Indeed, when the sample was cooled to 2°C to eliminate R2, R1 was extremely enhanced and prolonged at a pH lower than 4. Using the eyes of albino rats and a 110 mM sodium phosphate buffer, Arden, Bridges, Ikeda, and Siegel (1968) observed that the variation of pH between 4.4 and 9.0 had little effect on the early receptor potential. With the pigment epithelium of the frog, Crawford, Gage, and Brown (1967) observed a decrease in R2 and an increase in R1 as pH was lowered. From these studies it may be concluded that pH of the bathing solution has definite effects on the mode of generation of the early receptor potential.

At the higher pH R2 tends to increase and at the lower pH Rl tends to increase. At the neutral pH the early receptor potential is optimum while the pH deviation to both ends decreases the magnitude of the potential.

It was determined that proton is the medium of generation of the early receptor potential because of the following reasons:

1. Only H^+, OH^-, and H_3O^+ ions influence the early receptor potential.
2. Protons can modify the wave form and magnitude of the potentials drastically.
3. If indeed the transition from metarhodopsin I to metarhosopsin II is responsible for R2 generation, then, the ion generated in the transition is proton (Cone, 1967).
4. Falk and Flat (1966) reached the conclusion, after studying the conductance change of retina and rhodopsin solutions on illumination, that the faster component of the photoresponses in their system was possibly protonic conduction.

In summary, it is very likely that the finding, made in the present model system, that protons function as mediators of generation of the R2 component of photoresponses, can be successfully extended to the early receptor potential of the visual photoreceptors.

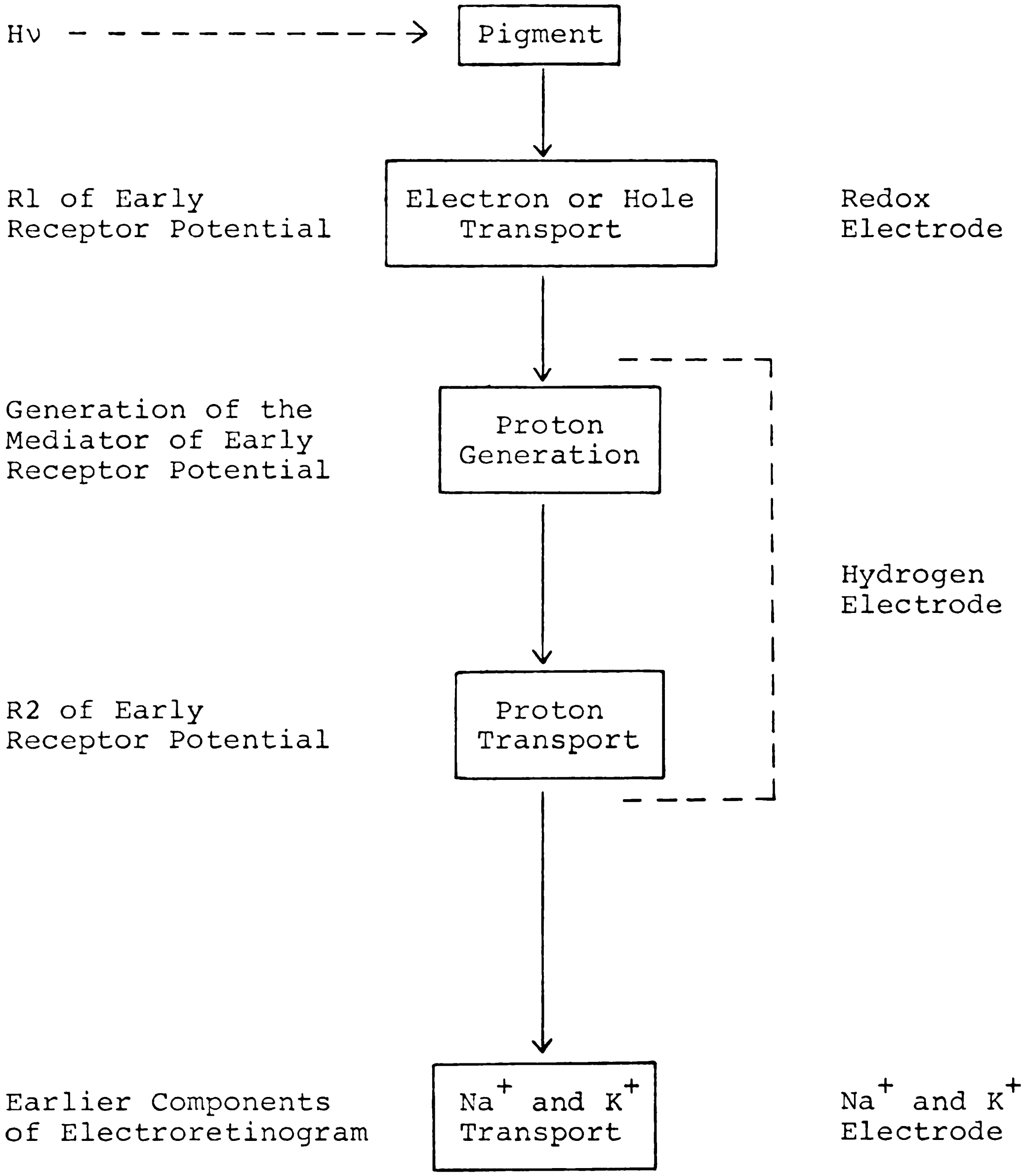
The Protonic Induction of Ionic Membrane Excitation

The mechanism of the generation of early receptor potentials, presented in this work, is independent of the organization of the membranous structure if there exist the pigmented organelle which can generate protons and the proton sensitive membrane electrode which has a proper proton permeability.

Since the early receptor potentials are most likely to be membrane phenomena, it is possible to consider 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 sodium, potassium, and chloride ions by the initial rapid protonic potentials (early receptor potentials) as shown in Figure 41.

In spite of enormous works on the determination of the internal pH of excitable cells, there is some controversy over not only the value of pH but also the effect of pH gradient across the cell membrane on the induction of excitation (Caldwell, 1956; Waddell and Bates, 1969). Nevertheless, the intracellular pH is about 7 and the pH gradient across the cell membrane is far less than 1 pH unit (Caldwell, 1958, 1960). With an improved technique Carter, Rector, Campion, and Seldin (1967) observed a Donnan distribution of protons with instantaneous response to an applied membrane potential. Furthermore,

Figure 41.--The schematic diagram of photoelectric transduction showing how hydrogen ion 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.


Hν
Pigment
R1 of Early Receptor Potential
Electron or Hole Transport
Redox Electrode
Generation of the Mediator of Early Receptor Potential
Proton Generation
Hydrogen Electrode
R2 of Early Receptor Potential
Proton Transport
Earlier Components of Electroretinogram
Na^+ and K^+ Transport
Na^+ and K^+ Electrode

Tasaki, Singer, and Takenaka (1965) observed that pH of the perfusion solution was essential for the production of normal electrical discharges of squid giant axons: the conduction was restored by changing the pH of the bathing solution of the same salt composition but with more alkaline pH, and at a pH greater than 7.5 the spontaneous repetitive discharges were observed. Thus, at the present it is not possible to eliminate the importance of proton concentration in the induction of the excitable membranes.

Recently, Stephens (1969) proposed that permeability changes of nerve membranes could be initiated by the rapid transport of protons, which control the chemical and structural properties of channels for the transport of cations. The influence of proton concentration upon the ionic permeability of mitochondrial and chloroplast membranes have been vigorously developed by Mitchell (1961, 1966). Mitchell's chemiosomotic hypothesis is not based on the structural changes of the membrane due to changes in proton concentrations at the vicinity of the membrane as is Stephens' hypothesis, but it is essentially the coupling of the charge flow across the membrane as previously suggested in terms of ionic coupling by Lund (1928), Lundegardh (1945), and Ussing (1947). It is clear from the above hypothesis that, given the local disturbance on proton concentration at the vicinity of the excitable membrane, the diffusion of protons due to this concentration gradient can cause the secondary flows of cations or anions across the membrane to neutralize the charges by utilizing metabolic energy (Mitchell's hypothesis) or by altering the structure of the channels (Stephens' hypothesis).

The Effect of Temperature on Proton Activity in the Induction of Photopotentials

A successful demonstration of the biphasic photoresponse in an all-*trans*-retinal BLM clearly indicates that this model membrane can be used for the study of the early receptor potential. It was further demonstrated that 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 BLM as a function of temperature can be seen in the following two points:

1. At the lower temperature the fast component (R1) dominates the photoresponse while at the higher temperature the slower component (R2) dominates.
2. The shift from R1 to R2 can occur within the temperature range from 0°C to 50°C under proper experimental conditions.

It is appropriate to consider the agreement in the above two points as the principal similarity between the two kinds of the photoresponses.

Unlike the present membrane model, the effect of temperature on the mode of the early receptor potential can be the effect of temperature via two mechanisms. The first mechanism is an increase in proton mobility as demonstrated in the present work. The second one is the direct effect of pH gradient across the receptor membrane, since the effect of temperature on the pH of the aqueous phases is a well established phenomenon in biological samples. Lovelock (1957) points out that when dilute aqueous phases are frozen, ice separates as a pure substance and the solutes are concentrated in the spaces remaining between the ice crystals. The increase in electrolyte concentration may appear as a change in proton concentration. Furthermore, sparingly soluble buffering salts may come out of solutions as the temperature falls and as water is removed from the solution.

The actual determination of pH changes due to temperature changes have been carried out by Finn (1932) and Harvey (1918) in muscle and cabbage juice respectively. More recently, Van den Berg (1961a, b) observed changes in the pH of milk, green beans, cauliflower, tomatoes, cod, haddock, and beef. Most of these works were carried out at subzero temperatures and the mode of the pH changes is complex. Van den Berg felt that these changes in pH were likely to be due to the precipitation of salts as demonstrated with pure buffer systems (Van den Berg and Rose, 1959a, b). Both such a precipitation of salts and the conformation changes or denaturation of proteins due to changes in pH would cause changes in the buffering capacity of the intracellular and intercellular liquid (Harvey, 1918). At the present time it is almost certain that temperature influences the pH and buffer capacity of the cellular liquid phases.

Finn (1932) determined changes in pH of muscle juice at temperatures above freezing. Again, pH decreased as temperatures were decreased. Since there are no comparable data on photoreceptors or axons, a definite statement cannot be made. It is likely, however, that the type of pH changes which generate the proton concentration gradient across the photoreceptor membrane can influence the mode of generation of the early receptor potential, as demonstrated clearly in the present work in the case of the photoresponse of all-*trans*-retinal BLM separating two aqueous phases which are different in their ionic compositions.

Finally, the present work demonstrates that it is possible in principle and feasible in view of available evidence about 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 in the main pathway of the visual excitation (Pak, 1968). 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. Future works may demonstrate that this approach will eventually elucidate the linkage between the early receptor potential and the generation of the electroretinogram as coupled consecutive membrane phenomena.

CHAPTER VIII

SUMMARY

1. Ionic selectivity of all-*trans*-retinal BLM was established: proton transport can satisfactorily account for the development of the observed dark membrane potentials and no other ionic species or mechanisms are required.

2. The time course of photopotentials showed a biphasic mode with the temperature sensitivity which, under appropriate experimental conditions, was quite similar to that of early receptor potentials.

3. The initial fast photoresponse (R1) could be best explained as a redox potential due to the photoexcited electrons and holes which interact with water molecules and/or other electron acceptors or donors. This potential was not influenced by the concentration gradient of K^+, Na^+, and Cl^- to any extent, indicating the non-ionic origin of this photoresponse.

4. The second slow photoresponse (R2) was identified as the diffusion potential of hydrogen ions.

5. The effect of temperature on the mode of biphasic photoresponses was due to an increase in hydrogen ion mobility with increasing temperature. It was discussed

that in vivo there could be another important factor of the generation of the pH gradient across receptor cell membranes as indicated by an increase in the R2 component with increasing pH gradient, since there do exist reports about the changes in pH of biological fluids as temperature was changed.

6. One of the future problems is to investigate the detailed mechanism of generation of the R1 component, as guided by the kinetics of the Langmuir adsorption isotherm as developed in the present work.

7. From a biological viewpoint, an important problem may be whether or not this hydrogen ion movement induced by the hole movement can induce, in turn, the movement of Na^+, K^+, or Cl^- ions across the membrane. Since all-*trans*-retinal BLM was permeable only to protons, this point could not be investigated. If ionic (e.g., Na^+, K^+, or Cl^-) selectivity can be incorporated into this membrane system without lowering the membrane resistance unduly, this three-step photoexcitation potential may be observable.

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