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Effect of Temperature on the Fluidity and Ordering of the Rod Outer Segment Membrane of *Gekko Gekko*†

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The lateral diffusion of rhodopsin molecules in *Gekko gekko* rod outer segment (ROS) membranes was measured by microspectrophotometry (MSP) after the retinas were pretreated at different temperatures. It was found that the fluidity of ROS membrane was the smallest after it had been pretreated at 45°C. Correspondingly, the ordering of ROS lipids, as measured by their birefringence under a polarizing microscope, was observed after the ROS were pretreated at various temperatures. The birefringence turned from positive to negative when the temperature of the treatment was higher than 46.5°C. These observations suggest that some sort of irreversible phase transition in the ROS membrane at about 45°C takes place.

INTRODUCTION

It is well known that there are various liquid crystal-like structures in biological cells and tissues. The relationship between the liquid crystal states of the different cell components and their specific biological functions is unclear.

Metabolism, energy transformation and information flow in biological systems are all closely related to the structural states of biological membranes. It is now generally accepted that the basic structure of biological membranes is a lipid bilayer. The lipid molecules organize themselves into a bilayer structure in which the hydrophilic, polar end shields the hydrophobic, hydorcarbon end

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from the acqueous environment surrounding the membrane. The functional protein molecules are inserted as independent entities in the lipid bilayer (Figure 1),[1] so that they can undergo either rotational or lateral diffusion in the lipid bilayer. Different membranes contain different kinds of proteins and lipids to perform their different functions. The function and activity of many proteins are closely related to the physical state of molecules surrounding them. In particular, the interactions between proteins and lipids in the membrane is one of the most important problems concerning the structure and function of biological membranes. The role of liquid crystals and phase transitions in biological membranes have been discussed in special reviews.[2-5]

One of the best understood and studied membranes is the photoreceptor membrane of the vertebrate rod.[6] The rod cells transform the absorbed light into electric signals. The vertebrate rod is divided into outer and inner segments (see review by Cohen;[7] Figure 2). About 90–95% of the membrane area in the rod outer segment (ROS) is composed of pinched-off disk membranes. The disks are organized in stacks of from 500 to 2000 depending on the species. The chemical composition[8,9] of vertebrate rod membranes is about 40% lipid (dry weight) and 60% protein, of which over 80% in rhodopsin. For each rhodopsin there are about 60–90 lipid molecules, mostly phospholipid.

The lipid and rhodopsin molecules are arranged in a well-defined manner in the disk membrane. Schmidt[10] and Liebman *et al.*[11] measured the birefrin-

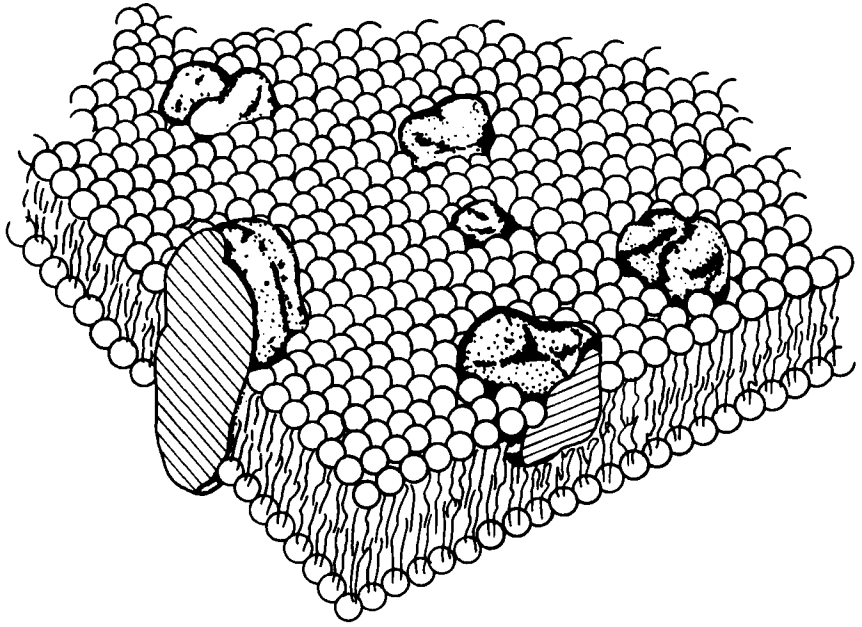

FIGURE 1 Fluid mosaic model of biomembrane.

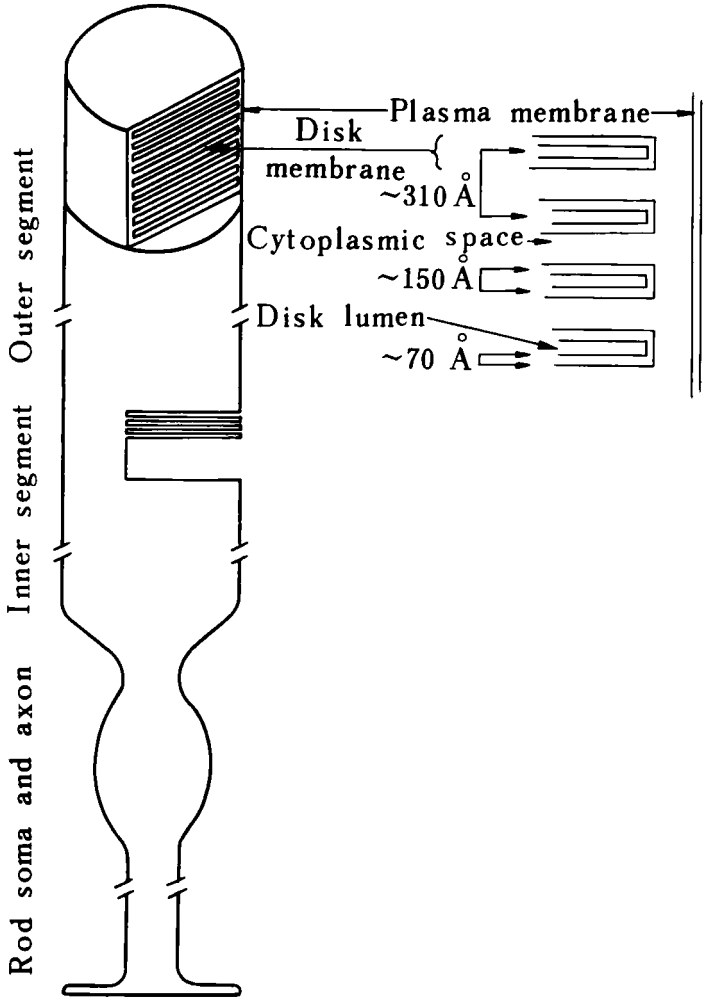

FIGURE 2 Schematic drawing of vertebrate rod outer segments with the dimensions and spacing of the disk membranes.

gence shown by ROS when examined under a polarizing microscope and showed that the lipid molecules in the rod membrane are organized in a bilayer. It is well-known that birefrigence is produced when light passes through a substance having an ordered structure. The birefringence produced by a ROS may be divided into two parts,[12] i.e. (a) intrinsic birefringence or positive birefringence, (b) form birefringence or negative birefringence due to the orientation of thousands of flat ROS disk membranes. The positive birefringence would be expected if there were a large number of molecules lined up so

that their long axes were parallel to the axis of the rod and these molecules were ordered in the disk membrane. Under normal conditions, the positive birefringence of a dark-adapted ROS is larger than the negative birefringence, so the net birefringence appears positive. Liebman *et al.* found the positive intrinsic birefringence in rods was disrupted after extraction with organic solvents, due to the removal of the lipids.

Studies of the dichroism[13] of ROS has shown that the orientation of the rhodopsin chromophore is perpendicular to the long axis of the rod, but in the plane of the disk membrane it is randomly oriented. Rhodopsin and the lipid molecules are in continuous movement. Brown[14] and Cone[15] showed that frog rhodopsin could rotate around an axis perpendicular to the plane of the membrane and the rotational relaxation time is about 20 μsec at 20° C. Furthermore Poo and Cone[16] showed that rhodopsin could also undergo lateral diffusion in the plane of the disk membrane. Liebman and Entine[17] obtained similar results in their experiments. Although the figures are not precise, close agreement was found between the membrane viscosities determined by either the rotational or the lateral diffusion times. This suggests that each rhodopsin molecule can be thought of as freely floating in the lipid bilayer.

The function of the visual pigment, rhodopsin, in ROS is to convert the ab-

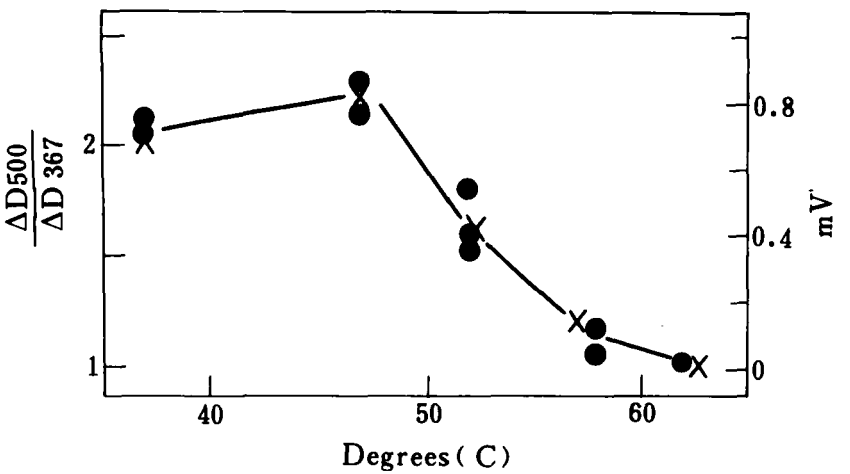

FIGURE 3 Effects of heating on the orientation of rhodopsin in the rods and on the early receptor potential (ERP). Pairs of albino rat eyes were held for 10 min. in the dark at the temperatures shown. Then the maximum ERP was determined in one eye, and the orientation of rhodopsin monitored in the retina of the mate eye, be measuring the difference spectrum in situ after bleaching in the presence of hydroxylamine. In the difference spectrum of randomly oriented rhodopsin the ratio of the rhodopsin peak at 500 nm to the retinal oxime minimum at 367 nm is 1. Owing to the orientation of rhodopsin in the rod disk membranes, this ratio for light passing down the axes of the rod outer segments normally is about 2.27. These experiments show that between 48 and 60°C the ERP falls to zero, and the rhodopsin, though still unbleached, becomes completely disoriented.[21] $\Delta D_{500}/\Delta D_{367}$; maximum amplitude of ERP.

sorbed light energy into an electrical signal. The mechanism of the light-electric transduction is unknown. Wald[18] and co-workers have shown that the action of light on rhodopsin is to photoisomerize the 11-cis chromophore; this initiates the complex chain of event leading to visual excitation.

The Early Receptor Potential (ERP) is a rapid electrical response from the rod. The ERP probably arises from charge displacements associated with transitions between various steps in the chain of events initiated by the absorption of light by rhodopsin.[19,20] A number of observations suggest that the orientation of the rhodopsin in the membrane is necessary for the generation of an ERP, and that the amplitude of the R_1 and R_2 components of the ERP is closely related to the ordering of rhodopsin molecules (see Figure 3).[21] Some of our colleagues at the Institute of Biophysics have observed that the amplitude of the R_2 component of the ERP of *Gekko gekko* becomes maximal, if the eyeball is first warmed to ca. 48° C before measuring (at room temperature).[22] The present investigation is trying to understand the effect of temperature on the fluidity and ordering of *Gekko gekko* ROS membrane, and it's relation with the ERP.

MATERIALS AND METHODS

Gekko gekko specimens were caught near Nanning in Kwangsi Autonomous Region. *Gekko gekko* is peculiar[23] in that the retina contains almost only rod cells and these cells are comparatively large, with a diameter of 6–10 μ and a length of about 40 μ.

Preparation of freshly isolated Rod Outer Segments. After a *Gekko gekko* had been dark-adapted for more than 10 hours, the eyeball was removed, and hemisected to remove the lens. In experiments where the temperature was varied, Ringer's solution was pre-warmed to equilibration in a water bath at various temperatures, the retina was removed with a cotton swab and placed in Ringer's solution for about 2 minutes. Then the cotton swab was squeezed in order to drop some rod outer segments on a microscopic slide, and a coverglass dropped on top. The freshly isolated rods were used to measure the fluidity of the ROS membrane by microspectrophotometry (MSP). They were also used for birefringence measurements, using a polarizing microscope.

Since the rhodopsin in the isolated rods, once bleached, could not regenerate, the entire experimental procedure including selection, focusing and alignment in microspectrophotometer carried on under dim red light.

Microspectrophotometry. Single rods which appeared intact and laid flat were selected for measurement. Samples were observed with 650 nm light on the stage of type SMP-05 microspectrophotometer (OPTON W. Germany) fitted with a high quantum efficiency photomultiplier RCA-1P28. The meas-

uring beam ($2 - 3\,\mu \times 20\,\mu$) was parallel to the bleaching beam. After a rod was located, the stage was rotated in order to put the long axes of the bleaching and the measuring light beams parallel to the long axis of the rod. The bleaching light illuminated $\frac{1}{3}$–$\frac{1}{2}$ of the width of the rod outer segment (i.e. 2–5 μ wide). The change of transmission at 520 nm in the bleached and unbleached sides of the ROS was measured for a period of 2 minutes after bleaching. 520 nm is the absorption maximum of *Gekko gekko's* rhodopsin (Figure 4). The sample was first bleached with a slit width of 0.5 for 2 seconds, and the slit width was then adjusted to 0.015. The results were recorded by an automatic recorder.

Polarizing microscope. Single intact rods selected from freshly isolated ROS samples were examined with a polarizing microscope using illumination wavelengths longer than 580 nm.[24] However, with the extremely small path length of a single rod, the specimen retardance and the residual instrument retardance are in about the same order of magnitude. To correct for this, we measured the retardance of rods with a 0.03λ mica rotary compensator and used the method of matching the specimen with the background to eliminate the instrument birefringence.[25]

The specimen and compensator were placed between polarizer and analyzer. From the symmetry of the rod, it was assumed that it behaves an an uniaxial crystal with its optic axis along the axis of the rod. The optic axis was

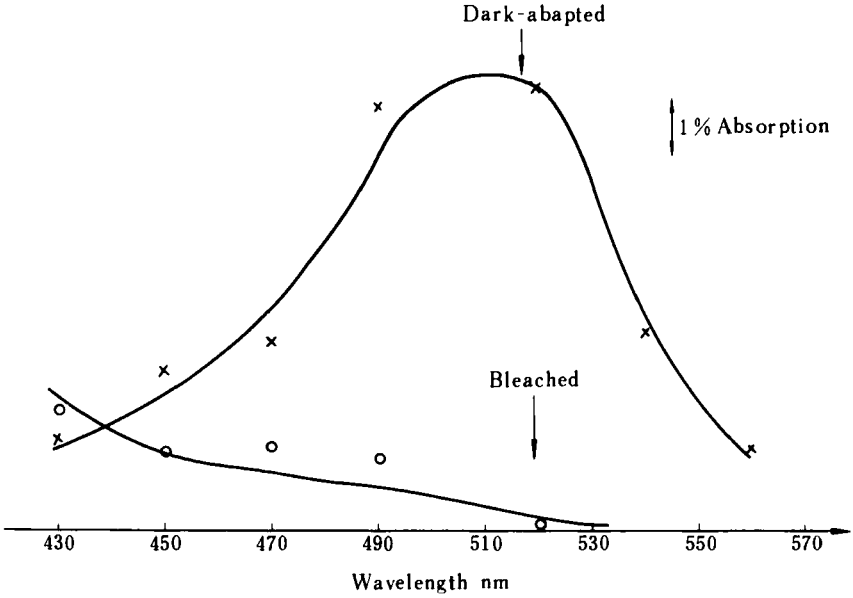

FIGURE 4 Absorption spectrum of *Gekko gekko* ROS in visible region.

arranged to lie at 45° C to the polarizer direction. The compensator was then rotated until the rod had the same intensity as the background. From the angle we calculated the birefringence.[26]

RESULTS

The Changes in Fluidity. Rhodopsin molecules can undergo lateral diffusion in the ROS membrane. The fluidity of membrane may be calculated from the speed of diffusion.[14-16] To obtain a measure of the fluidity we followed the procedures of Poo and Cone and Liebman *et al.* The rhodopsin molecules in the disk membrane themselves are good naturally labelled molecules to measure lateral diffusion. The absorption peak of dark-adapted ROS shifts to much shorter wavelengths after bleaching (Figure 4). Lateral diffusion between the bleached and unbleached sides of the rod would eliminate the absorption difference. The rate that the absorption difference disappears is thus related to the lateral mobility.

We found that immediately after partial bleaching of a ROS, the transmission at 520 nm in the bleached side increased as compared to the transmission of the unbleached side. But it then gradually decreased (Figure 5 right). As is shown in the left side of Figure 5, the transmission gradually increased in the unbleached side. It showed no further change after about 2 minutes. It has been shown that these changes are due to the rapid lateral diffusion of the bleached and unbleached rhodopsin molecules in the disk membranes.[16]

After treatment of ROS of *Gekko gekko* by incubating their retinas at fixed temperatures between 26–50° C for 2 min, the retina was returned to room temperature and bleached, and the change in transmission at 520 nm in the bleached and unbleached sides of the ROS was measured at room temperature. The results are shown in Figure 6. Each point is the mean from measurements in 5 cells. It appears that the smallest fluidity was found in ROS membrane pretreated at 45° C. For these rods there was smallest change of transmission for either the partially bleached or the unbleached sides. This suggests that the rhodopsin molecules could not diffuse laterally in the disk membrane. The ROS which were pretreated at 46.5° C and 48° C had a fluidity smaller than those ROS at room temperature, but larger than those pretreated at 45° C. This experiment was repeated one year later, and the results were the same.

The change of birefringence. We observed the birefringence of *Gekko gekko* ROS pretreated at different temperatures. When the mica rotary compensator was turned to a point where the light intensity of the ROS matched that of the background, the angle was recorded. The diameter of the ROS was also meas-

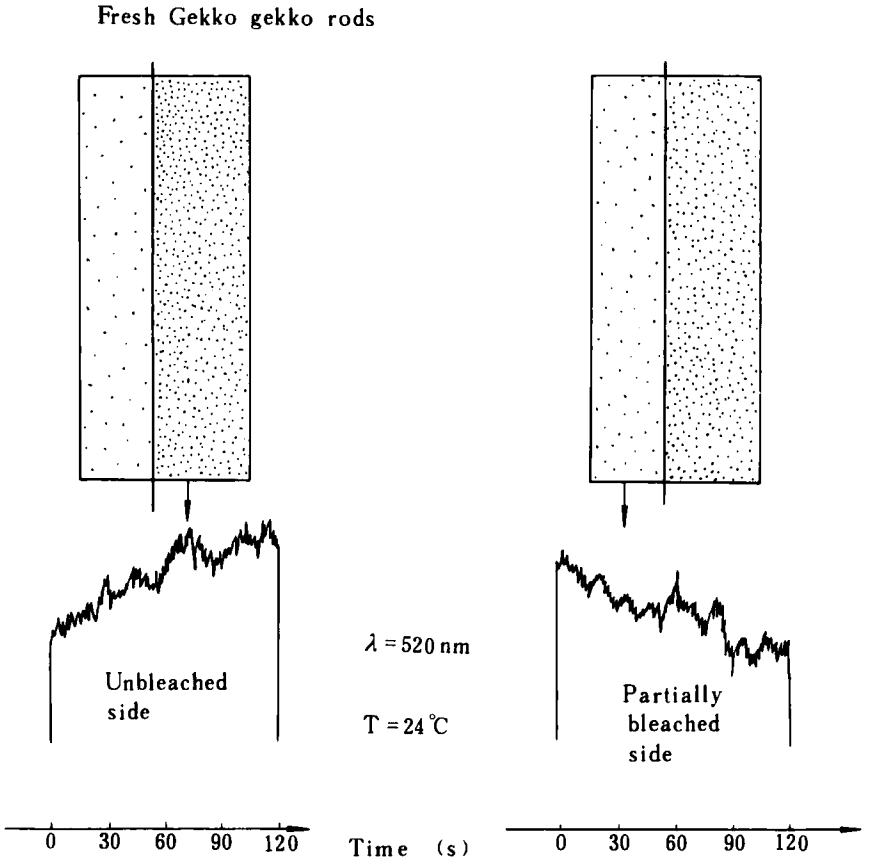

FIGURE 5 Change of transmittance after partial bleaching in *Gekko gekko* ROS.

ured and the birefringence of the ROS can be calculated from

$$n = 2R \text{ Sin } 2\epsilon d$$

Where n is the ROS birefringence, R is the retardation of the compensator crystal (in nm), ϵ is the angle between the polarizer axis and the slow axis of the compensator, and d is the diameter (in nm) of the ROS. We calculated the birefringence of *Gekko gekko* ROS which were pretreated at different temperatures; each measurement was the average obtained from three measurements of each ROS. The birefringence value at each temperature, which is the mean value of 10 ROS, is plotted versus the temperature (Figure 7). The birefringence of the ROS was positive when the rod was pretreated at temperatures lower than 45°C. The range is 0.492×10^{-3}—0.453×10^{-3}. After treatment at 46.5°C, half of the 10 ROS had a positive birefringence and the

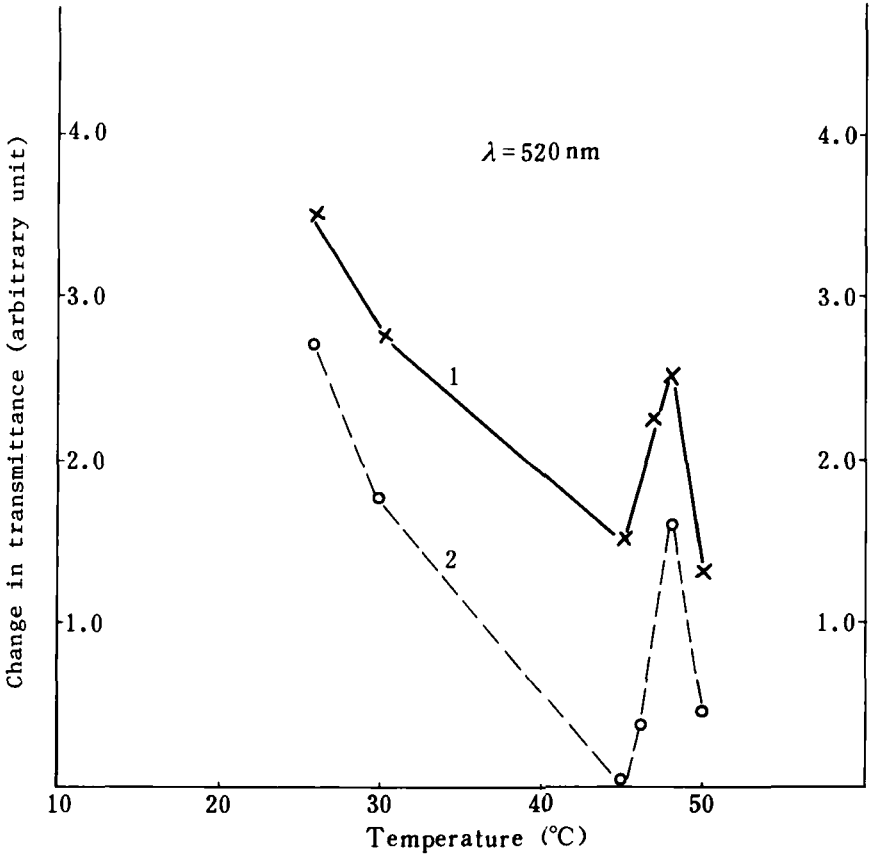

FIGURE 6 Effect of temperature on the fluidity of rhodopsin molecules in *Gekko gekko* ROS membrane. Curve 1. Increase in transmittance of unbleached side 2 minutes after bleaching. Curve 2. Decrease in transmittance of partially bleached side 2 minutes after bleaching.

other half appeared to have a negative birefringence. The birefringence of a ROS was negative when it was pretreated at a temperature over 48° C. This change was irreversible. This suggests that the ordered orientation of lipid bilayer was destroyed by heating. The negative birefringence is probably due to the intrinsic birefringence decreasing while the form birefringence remain unchanged producing a net negative birefringence.

DISCUSSION

After pretreatment at 45–48° C, the amplitude for the R_2 peak of the ERP of *Gekko gekko* is increased, the fluidity of its ROS membrane was the smallest, and the birefringence changed from positive to negative. These results suggest

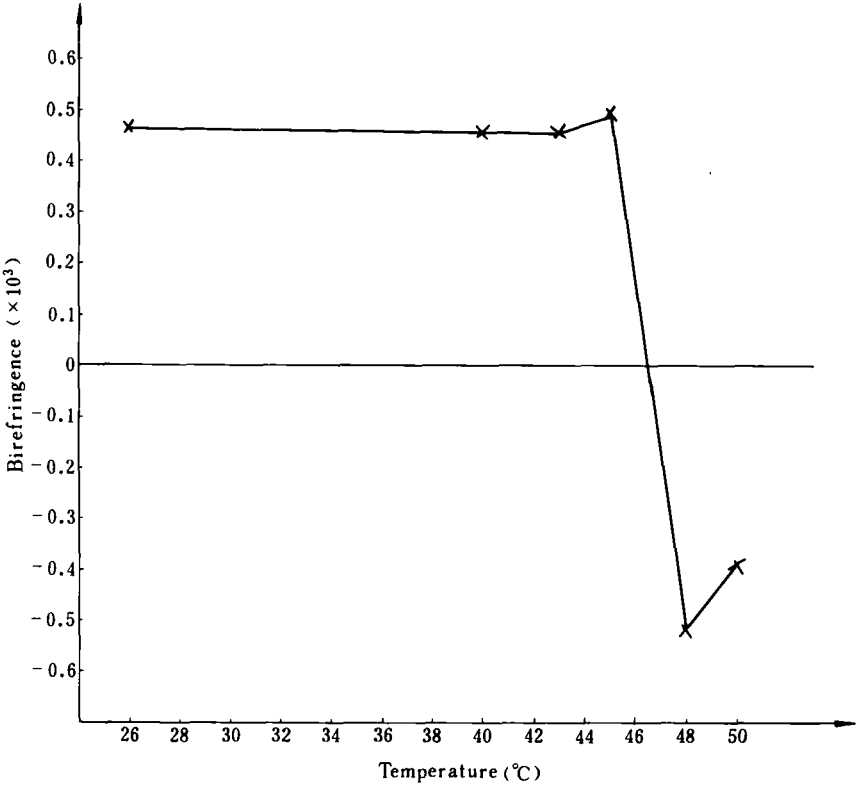

FIGURE 7 Effect of temperature on the Birefringence of *Gekko gekko* ROS.

that there is an irreversible transition in the state of the ROS membrane in the temperature range of 45°–48° C. In this new state, perhaps owing to the new arrangement of the lipid molecules, the rhodopsin molecules may be forced to re-orient themselves. The fluidity of rhodopsin was very low in this condition. It is known that the amplitude of the R_2 peak of the ERP depends upon the temperature and the ordering of rhodopsin molecules in the disk membranes.[20,21] In our Institute Tsai Hao-jan *et al.*[22] also measured the ERP from the isolated eyes of *Gekko gekko.* After treatment at different temperatures, the amplitude of the R_2 peak of the ERP was measured at room temperature. The R_2 peak amplitude was significantly increased when the eyes were pretreated at 45–48°C. The largest R_2 peak amplitude (about 6 times that of the control) was observed after pretreatment at 47°C. The R_2 peak amplitude decreased rapidly when the temperature of treatment was higher than 50°C. It disappeared after treatment at 56°C.

It seems that the large increase of the ERP R_2 peak of isolated *Gekko gekko's* eyeballs pretreated at 45° C–48° C is not due an increase of fluidity of the ROS membrane. After treatment in this temperature range, the physical state

of the molecules of the lipid bilayer membrane changes to prevent the lateral diffusion of rhodopsin molecules. This kind of change is irreversible because after treatment at the various temperatures, the measurements were performed at room temperature. The whole process, including the measurement takes about $\frac{1}{2}$–1 hour. Furthermore, in this temperature range, the rhodopsin molecules are not denatured. A control experiment showed that rhodopsin in ROS pretreated at 50° C still retained its color and still could be bleached. It is possible that a change in the orientation state of the lipid molecules may influence the fluidity of the rhodopsin. On the other hand, the orientation of the lipid molecules was destroyed when treated at 48° C, giving a negative birefringence due to the disappearance of positive intrinsic birefringence. At even higher temperatures, the rhodopsin molecules became disordered,[21] so the amplitude of ERP R_2 peak dropped rapidly.

From a physiological point of view, it seems as if there is no particular significance in the temperature range 45°–48° C. But since there is an effect on the ERP, the phenomena we have described here may have an important bearing in further studies of the mechanism of photoelectric response of biological membranes.

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