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KINETIC STUDY OF TRANSFER OF 11-*cis*-RETINAL BETWEEN ROD OUTER SEGMENT MEMBRANES USING REGENERATION OF RHODOPSIN

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The present study demonstrates some important facts on the regeneration of rhodopsin in rod outer segment membranes. 11-*cis*-Retinal added to a rod outer segment membrane suspension did not react directly with opsin but was rapidly solubilized into membranes and then recombined with opsin in the membrane. It was also revealed that the regeneration of rhodopsin was perturbed by the formation of retinylidene Schiff base with phosphatidylethanolamine in rod outer segment membranes, which decreased with increasing temperature. The activation energy of rhodopsin regeneration in rod outer segment membranes was 18.7 kcal/mol, being smaller than the value of 22 kcal/mol in 1% digitonin solution. 11-*cis*-Retinal could be found to transfer relatively fast (τ-$1/k_1 \sim 10^3$ s) between rod outer segment membranes by using the regeneration of rhodopsin. It was demonstrated that the kinetic measurement for the transport of membrane-soluble molecules such as retinal between membranes could be performed with ease and precisely by the method described in this paper.

1. Introduction

Bovine rhodopsin is decomposed into all-*trans*-retinal and opsin by the action of light and is regenerated by recombination of opsin with 11-*cis*-retinal [1]. all-*trans*-Retinal generated by the bleaching of rhodopsin in the eye flows into the pigment epithelium after conversion to all-*trans*- or 11-*cis*-retinol. The interstitial retinol-binding protein may be involved in this transport process [2,3]. The regeneration of rhodopsin can be supported if the retinoid lost to the pigment epithelium during light adaptation is returned to opsin in the rod outer segment as 11-*cis*-retinal by a reverse flow in darkness. However, it was suggested that 11-*cis*-retinal returns to the rod outer segment without the aid of 11-*cis*-retinal-binding protein [2]. Also, no retinoid-binding proteins have been detected inside the rod outer segment [2,3].

We have found that 11-*cis*-retinal could be exchanged between rod outer segment membranes. As this phenomenon is important for relation to the mechanism of transport of retinal between and in rod outer segments during light and dark adaptation, kinetic measurements were performed using the regeneration of rhodopsin. Since retinal is able to form Schiff bases with phosphatidylethanolamine in rod outer segment membranes, its effect on the regeneration of rhodopsin was also investigated.

2. Materials and methods

2.1. Preparation of samples

Procedures involving rod outer segment membranes were carried out under dim red light at

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4°C, unless otherwise stated. Fresh retinas were removed from bovine eyes purchased from a local slaughterhouse. Rod outer segments were detached by shaking in Ringer's solution (pH 7.0). The composition (in g) of Ringer's solution is 0.9 NaCl, 0.042 KCl, 0.024 $CaCl_2$ and 0.01 $NaHCO_3$ per 100 ml water. The suspension was filtered through a double-layered gauze. The filtrate was adjusted to 40% (w/v) with respect to sucrose and centrifuged at $16000 \times g$ for 40 min. The crude membranes floating on top were collected and diluted with an equal volume of 0.1 M Tris-HCl buffer (pH 7.0), and centrifuged at $16000 \times g$ for 40 min. The flotation and sedimentation procedure was repeated four to six times.

The purified rod outer segments were suspended in the Ringer's solution containing 20% (w/v) sucrose and sonicated with a Kubota 10 kHz sonicator (KMS-100) at 4°C for 3 h. The sonicator was operated at as low a power as possible to prevent denaturation of membrane proteins. The Ringer's solution was previously deoxygenated by bubbling with nitrogen gas. The suspension of rod outer segment membranes became optically clear after sonication and no sediment was observed on centrifugation at $6000 \times g$ for 30 min. An electron micrograph showed that the diameter of sonicated rod outer segment vesicles ranged from 50 to 100 nm.

Digitonin-solubilized rhodopsin was prepared as follows. Purified rod outer segment membranes were solubilized in 2% cholic acid solution containing 20% saturated ammonium sulfate and then rhodopsin was fractionated from other components by salting-out with ammonium sulfate. Details of the fractionation procedure have been previously described [4]. The purified rhodopsin pellet was dissolved in 1% digitonin solution after the removal of cholic acid and residual ammonium sulfate by dialysis against buffer solution. The solution of rhodopsin thus obtained had an A_{280}/A_{498} ratio of 1.6 and contained 5 mol phospholipid/mol rhodopsin [4].

Samples were bleached by exposure to light filtered through a Toshiba V-058 ($\lambda > 580$ nm) at 25°C. The concentration of opsin was determined from the difference in absorbance at 498 nm before and after bleaching of the sample in the presence of NH_2OH. The concentration was calculated using a molar extinction coefficient at 498 nm of 42000 $M^{-1} cm^{-1}$ [5].

2.2. Regeneration of rhodopsin in rod outer segment membranes

Experiments on the regeneration of rhodopsin in rod outer segment membranes were carried out in an optical cell placed in a recording spectrophotometer (Union SM401). The light path of the sample was 0.3 cm. The reaction was started by adding 750 μl of rod outer segment membrane suspension, equivalent to 3.8×10^{-5} M opsin, to 20 μl of 11-*cis*-retinal in ethanol (1.1×10^{-3} M) in the optical cell. The mixture was stirred gently during measurement. The time course of rhodopsin regeneration was monitored by following the increase in absorbance at 540 nm. The absorbance by a protonated retinylidene Schiff base (λ_{max} = 455 nm [6]) was negligible at 540 nm. No significant change in absorbance at 650 nm was observed during measurements. This result suggested that aggregation or sedimentation of rod outer segment membranes did not occur during the experiment.

2.3. Transfer of 11-cis-retinal between rod outer segment membranes

A set of two bleached specimens was prepared. One was incubated at pH 1.5 and 30°C for 30 min, the pH then being changed to 7.0. The pH was adjusted with HCl and NaOH. By this acid treatment, opsin lost the ability to recombine with 11-*cis*-retinal at pH 7.0 [7]. An ethanolic solution of 11-*cis*-retinal was added to this specimen. These membranes are henceforth referred to as donor membranes. The other bleached specimen, not exposed to acid, is denoted acceptor membranes. After mixing in an optical cell, the absorbance at 540 nm was measured as a function of time. The ratio of 11-*cis*-retinal in the donor specimen to opsin in the acceptor was 3.0×10^{-5} M/3.8×10^{-5} M = 0.8.

3. Results and discussion

3.1. Solubility of 11-cis-retinal in rod outer segment membranes

Firstly, the solubility of 11-*cis*-retinal in rod outer segment membranes was investigated. 11-*cis*-Retinal was added to suspensions containing various concentrations of rod outer segment membranes, to a final concentration of 4.8×10^{-5} M. After incubation at 30°C for 30 min, the mixture was centrifuged at $80\,000 \times g$ for 3 h at 15°C and the absorption spectra of the supernatant were recorded. Fig. 1 shows the concentration of 11-*cis*-retinal in the supernatant plotted vs. the concentration of rhodopsin in the suspension. The straight line intersects the abscissa at 2.6×10^{-6} M rhodopsin. This result shows that rod outer segment membranes could solubilize up to 18 mol 11-*cis*-retinal/mol rhodopsin. Furthermore, the absorption spectra did not indicate the presence of a retinylidene Schiff base and/or rhodopsin in the supernatant. This implies that the rod outer segment membranes were not destroyed by the dissolution of 11-*cis*-retinal as in the erythrocyte membrane [8].

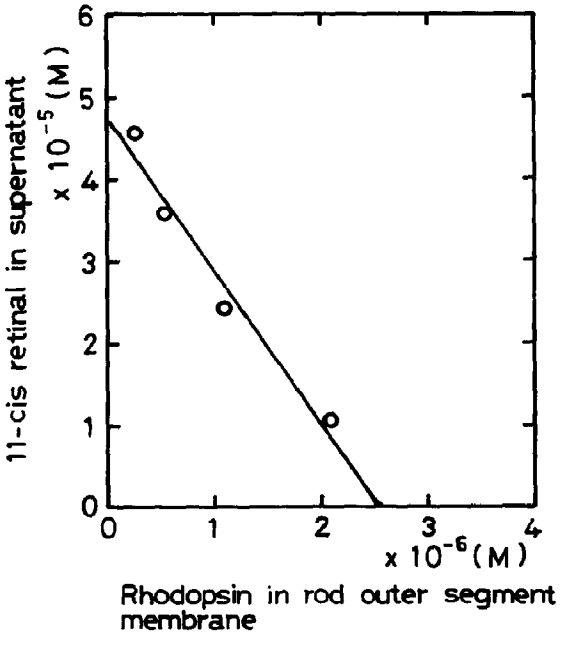


Fig. 1. Solubility of 11-*cis*-retinal in rod outer segment membranes. Rod outer segment membranes can solubilize 18 mol 11-*cis*-retinal/mol rhodopsin maximally.

3.2. Regeneration of rhodopsin in rod outer segment membranes

Fig. 2 shows the time courses of regeneration of rhodopsin in rod outer segment membranes which were measured at various concentrations of 11-*cis*-retinal and opsin at 30.2°C. Curve a follows pseudo-first-order kinetics due to the excess of 11-*cis*-retinal. The reaction depicted by curves b–e followed second-order kinetics, since the molar ratio of 11-*cis*-retinal to opsin was 2.3 in curves b–d and 1.2 in curve e, respectively. Curves b–d in fig. 2 indicate that the time course of the regeneration reaction did not depend on the concentration of membranes provided the molar ratio of 11-*cis*-retinal to opsin was the same. It is quite different from the reaction in detergent solution and, at first sight, seems inconsistent with second-order kinetics. However, it can be accounted for if the following is assumed. That is, 11-*cis*-retinal in the aqueous region did not combine directly with opsin but the reaction proceeded within rod outer segment membranes in which 11-*cis*-retinal was rapidly solubilized. In that case, the concentrations of reactants should be considered only within a rod outer segment membrane and were constant even though the suspension was diluted.

Fig. 3 illustrates the temperature dependence of the regeneration reaction in rod outer segment membranes at a low concentration of 11-*cis*-retinal. An Arrhenius plot for the second-order rate constants (k_2), obtained from the slopes of the straight lines in fig. 3, is given in fig. 4. The plot is not linear and k_2 shows a large decrease at lower temperatures. The result suggested that this phenomenon was due to the phase change in the rod outer segment membrane [9] or the presence of inhibitor which affected the regeneration reaction depending on temperature. The following study was performed to elucidate the reason for this unexpected result.

Firstly, the recombination reaction was ob-

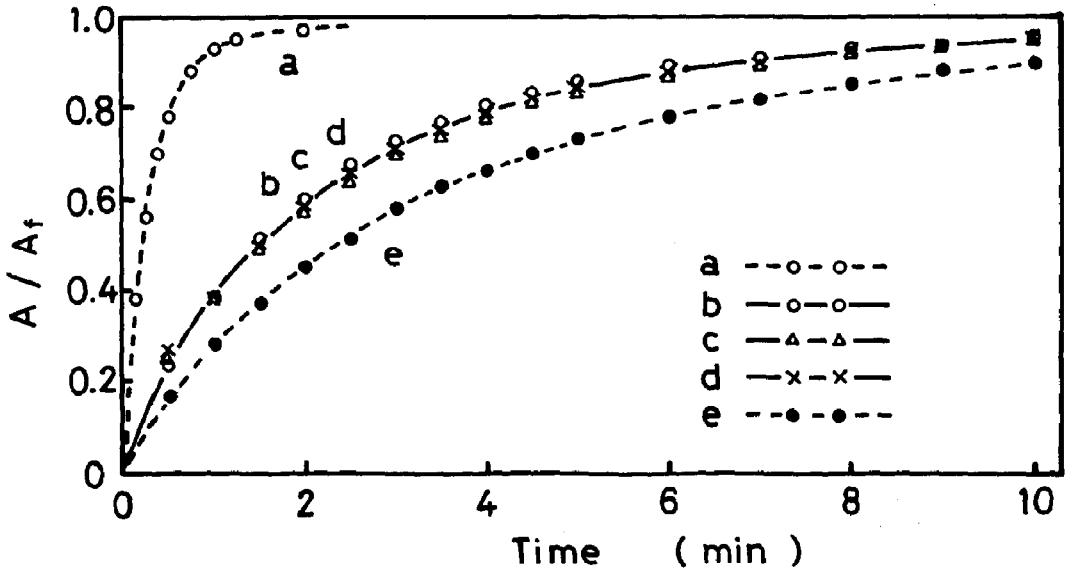


Fig. 2. Time course of regeneration of rhodopsin in rod outer segment membranes at 30.2°C. A, concentration of rhodopsin calculated from the absorbance at 540 nm; A_f, final concentration. Respective concentrations (M) of 11-*cis*-retinal and opsin in the rod outer segment membrane suspension: (a) 3.9×10^{-5}, 4.3×10^{-6}; (b) 3.9×10^{-5}, 1.7×10^{-5}; (c) 2.0×10^{-5}, 8.6×10^{-6}; (d) 9.8×10^{-6}, 4.3×10^{-6}; (e) 2.0×10^{-5}, 1.7×10^{-5}. The molar ratio of 11-*cis*-retinal to opsin is the same in curves b–d.

served at a high concentration of 11-*cis*-retinal and also with digitonin-solubilized rhodopsin, the result of which is shown in fig. 5. The abnormal decrease in reaction rate at low temperature was not observed in either case. A similar result with detergent-solubilized rhodopsin has been reported

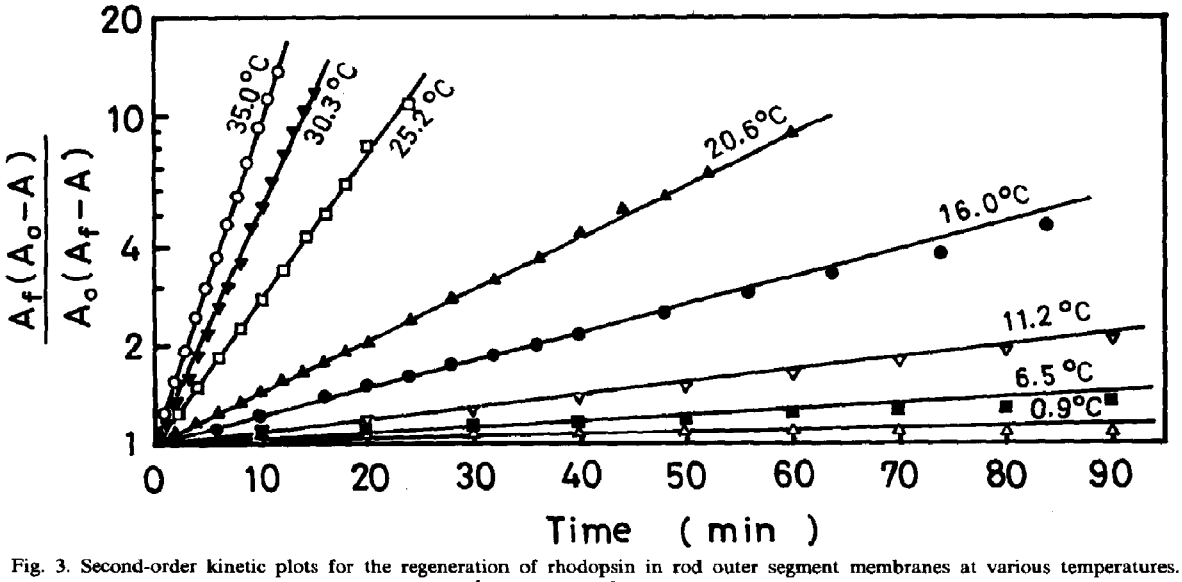


Fig. 3. Second-order kinetic plots for the regeneration of rhodopsin in rod outer segment membranes at various temperatures. Concentrations of 11-*cis*-retinal and opsin; 3.0×10^{-5} and 3.8×10^{-5} M, respectively. A and A_f as in fig. 2, A_o corresponding to the initial concentration of opsin.

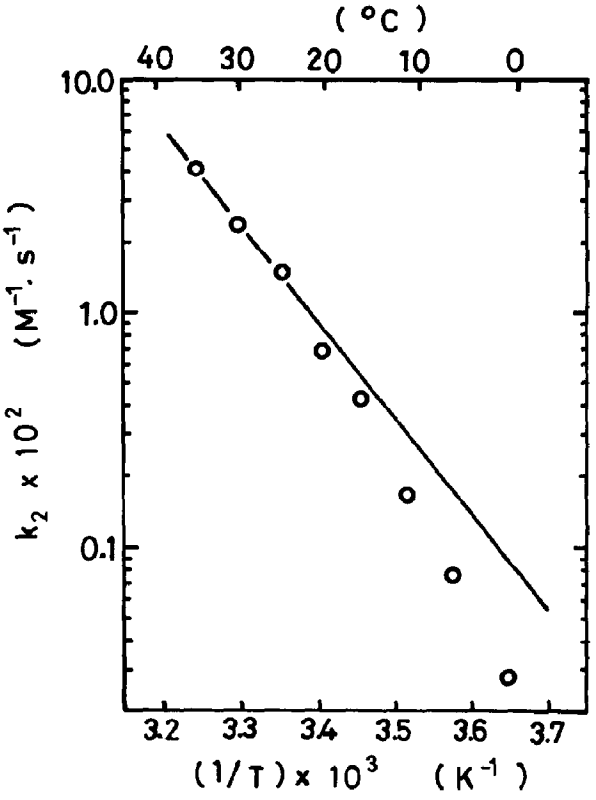
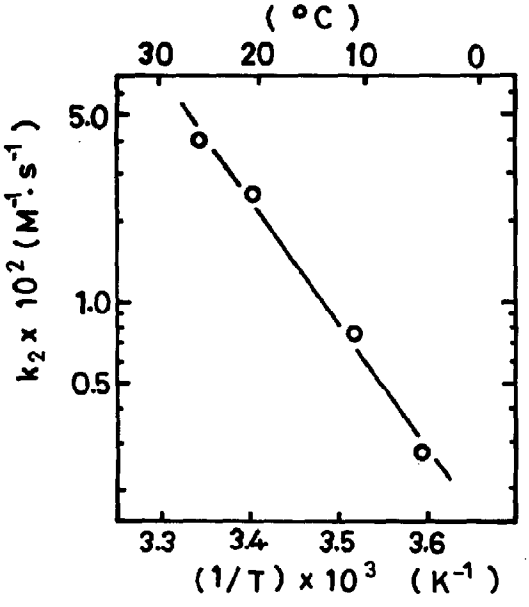


Fig. 4. Arrhenius plot for the second-order rate constants which were obtained from the slope of the straight lines in fig. 3.



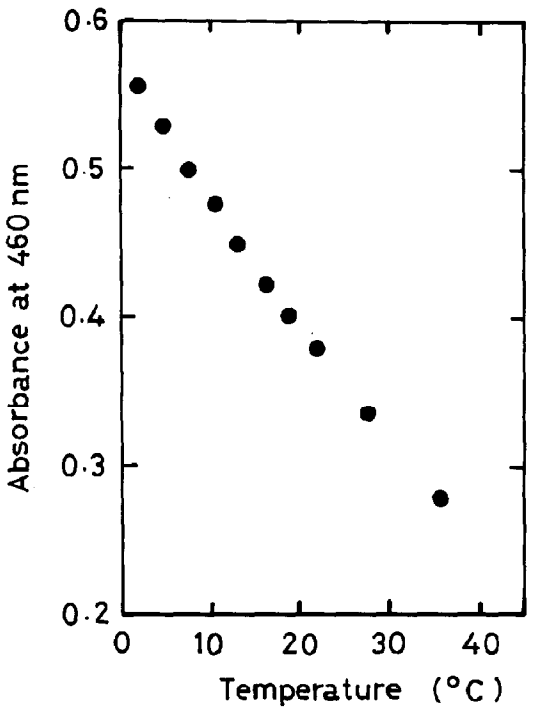


Fig. 6. Effect of temperature on the formation of a Schiff base between all-*trans*-retinal and phosphatidylethanolamine in rod outer segment membranes, which was observed by following the absorbance at 460 nm. The membrane suspension contained 3.0×10^{-5} M all-*trans*-retinal and 3.8×10^{-5} M rhodopsin. Rod outer segment membranes were added to the reference optical cell to the same concentration as that of the sample.

by Henselman and Cusanovich [10] and Matsumoto et al. [11]. This evidence suggested that the recombination reaction suffered from inhibition by phosphatidylethanolamine in rod outer segment membranes and that the formation of retinylidene Schiff base [6,12] was strongly dependent on temperature. Therefore, the temperature dependence of retinylidene Schiff base formation in rod outer segment membranes was investigated. all-*trans*-Retinal was used in this experiment to avoid the complexity which might be caused by the thermal isomerization of 11-*cis*-retinal. Difference spectra were measured at various temperatures with reference to the same concentration of

Fig. 5. Arrhenius plot for the second-order rate constants for regeneration of rhodopsin in 1% digitonin solution.

rod outer segment membranes as that of the sample. Since the wavelength of maximum absorbance of unprotonated Schiff base (~ 370 nm) was close to that of all-*trans*-retinal (~ 380 nm), the absorbance at 460 nm was measured and the amount of protonated Schiff base was estimated. The result is presented in fig. 6 and indicates the decrease in protonated Schiff base with increasing temperature. It cannot be decided whether the retinylidene Schiff base tends to decompose or if the equilibrium between protonated and unprotonated Schiff bases shifts to the unprotonated form, with increasing temperature. However, it may be true that the shift of equilibrium to free retinal occurred with increasing temperature, since the decrease in absorbance at 460 nm with increasing temperature (fig. 6) is complementary to the increase in rate of regeneration of rhodopsin.

3.3. *Transfer of 11-cis-retinal between rod outer segment membranes*

The plots shown in fig. 7 illustrate the time course of rhodopsin regeneration in the mixture of donor containing 11-*cis*-retinal and acceptor at various temperatures. The experiment was carried out over the range of temperature within which the recombination reaction was not affected by Schiff base formation or thermal denaturation of opsin. This plot is more in agreement with first-order than second-order kinetics. Thus, it is suggested that the transfer of 11-*cis*-retinal between rod outer segment membranes follows first-order kinetics and is the rate-determining step in the recombination reaction as illustrated by the following kinetic model;

$$\text{11-}cis\text{-retinal (donor)} \xrightarrow{k_1} \text{11-}cis\text{-retinal (acceptor)} + \text{opsin} \xrightarrow{k_2} \text{rhodopsin} \qquad (1)$$

The time course of the reaction was calculated numerically using k_2 obtained in section 3.2 and listed in table 1, by essentially the same equation as presented by Chien [13]. The rate constant k_1

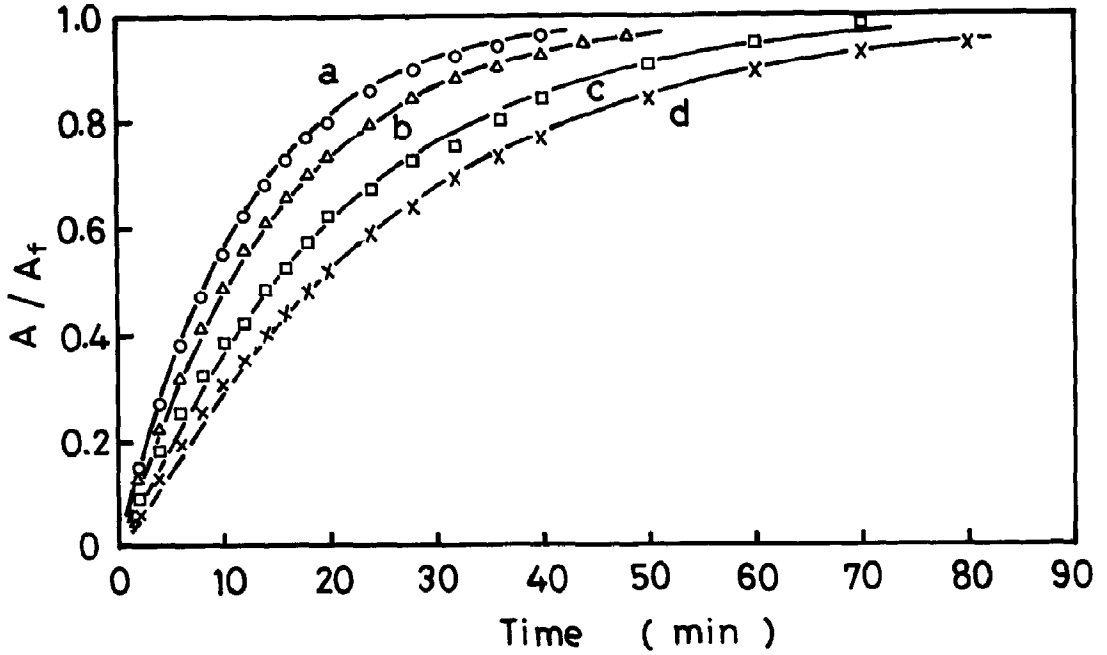


Fig. 7. Time courses of regeneration of rhodopsin in an acceptor. (a) 35.1°C, (b) 30.1°C, (c) 25.4°C, (d) 20.6°C. Solid lines show the kinetics of the reaction determined by numerical calculation assuming that the transfer of 11-*cis*-retinal between rod outer segment membranes follows first-order kinetics.

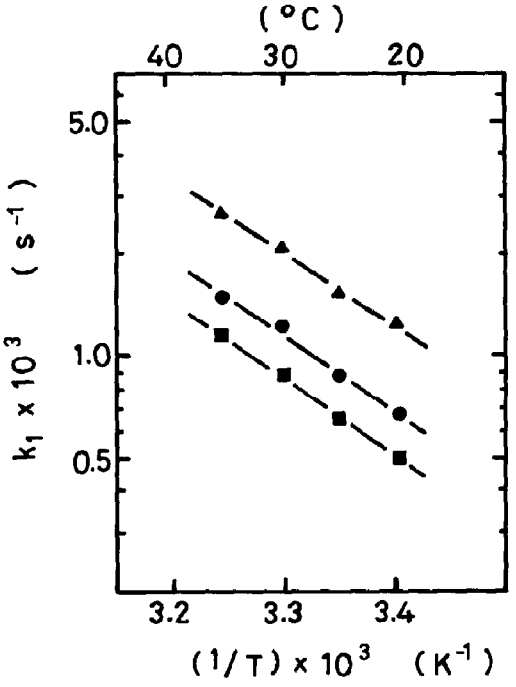


Fig. 8. Arrhenius plots for k_1 presented in table 1. The reaction mixture contained 20% sucrose and 75 mM Tris-HCl (pH 7.0). (●) 2.2 mM $CaCl_2$, (■) 19 mM $CaCl_2$, (▲) 3.8 mM EDTA.

was determined from curve-fitting analysis of the observed data. The resulting curves are shown by solid lines in fig. 7. The rate constants obtained above are listed in table 1 which also presents the values for the reactions in the presence of 19 mM $CaCl_2$ and 3.8 mM EDTA, respectively.

Arrhenius plots for the k_1 values in table 1 are shown in fig. 8. The activation energy for the transfer of 11-*cis*-retinal between rod outer segment membranes could be estimated from fig. 8. The obtained value was 11.4 kcal/mol, which is independent of the concentration of $CaCl_2$. The activation entropy decreased with increasing $CaCl_2$ concentration.

The following mechanism may be considered for the transfer of 11-*cis*-retinal between rod outer segment membranes: (i) fusion of membranes, (ii) exchange of 11-*cis*-retinal on collision of rod outer segment membranes and (iii) transport of 11-*cis*-retinal via the aqueous phase. The mechanism of

Table 1

Rate constants

Samples were suspended in Ringer's solution containing 20% sucrose, 75 mM Tris-HCl (pH 7.0) and (a) 2.2 mM $CaCl_2$, (b) 19 mM $CaCl_2$ and (c) 3.8 mM EDTA. k_2, rate constant for the regeneration of rhodopsin in rod outer segment membranes; k_1, rate constant for the transfer of 11-*cis*-retinal between rod outer segment membranes.

Sample	Temperature (°C)	k_2 ($M^{-1} s^{-1}$)	k_1 ($\times 10^4$) (s^{-1})
a	35.1	419	15.1
	30.1	231	12.3
	25.4	148	8.83
	20.6	70.2	6.83
b	35.1	416	11.5
	30.1	230	8.83
	25.4	150	6.50
	20.6	76.5	5.00
c	35.1	412	26.5
	30.1	232	21.7
	25.4	142	15.2
	20.6	72.0	12.6

fusion appears to be ruled out by the evidence that the light scattering at 650 nm did not change during the recombination reaction. The first-order kinetics can be expected in the second case if the frequency of collision between rod outer segment membranes is constant during reaction; then the rate of transfer depends only on the concentration of 11-*cis*-retinal in the donor. At present, work is in progress to elucidate the mechanism of transfer of 11-*cis*-retinal between rod outer segment membranes.

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