THE THERMAL STABILITY OF RHODOPSIN AND OPSIN

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ABSTRACT

Rhodopsin, the red photosensitive pigment of rod vision, is composed of a specific *cis* isomer of retinene, neo-*b* (11-*cis*), joined as chromophore to a colorless protein, opsin. We have investigated the thermal denaturation of cattle rhodopsin and opsin in aqueous digitonin solution, and in isolated rod outer limbs. Both rhodopsin and opsin are more stable in rods than in solution. In solution as well as in rods, moreover, rhodopsin is considerably more stable than opsin. The chromophore therefore protects opsin against denaturation. This is true whether rhodopsin is extracted from dark-adapted retinas, or synthesized *in vitro* from neo-*b* retinene and opsin. Excess neo-*b* retinene does not protect rhodopsin against denaturation. The protection involves the specific relationship between the chromophore and opsin. Similar, though somewhat less, protection is afforded opsin by the stereo-isomeric iso-*a* (9-*cis*) chromophore in isorhodopsin.

The Arrhenius activation energies (E_a) and entropies of activation ($\Delta S\ddagger$) are much greater for thermal denaturation of rhodopsin and isorhodopsin than of opsin. Furthermore, these values differ considerably for rhodopsins from different species —frog, squid, cattle—presumably due to species differences in the opsins.

Heat or light bleaches rhodopsin by different mechanisms, yielding different products. Light stereoisomerizes the retinene chromophore; heat denatures the opsin. Photochemical bleaching therefore yields all-*trans* retinene and native opsin; thermal bleaching, neo-*b* retinene and denatured opsin.

INTRODUCTION

Rhodopsin, the red photosensitive pigment of rod vision, consists of a chromophore derived from neo-*b* (11-*cis*) retinene, joined to a colorless protein, rod opsin or scotopsin. Light bleaches rhodopsin by causing it to dissociate into retinene and opsin. Radding and Wald (1955–56) have shown that cattle opsin is denatured by acids and alkalis much more readily than rhodopsin. Albrecht (1957) has recently found that acetylation lowers the stability of cattle rhodopsin only slightly, but induces a very rapid denaturation of opsin. As will be shown below, heat also denatures cattle opsin more readily than

259

rhodopsin. In all these situations, attachment of the chromophore therefore protects opsin against denaturation.

Rhodopsin and opsin are insoluble ("structural") proteins, and solubilizers are required to bring them into aqueous solution. The observations mentioned above were made upon rhodopsin and opsin dissolved in aqueous digitonin, the conventional solubilizer for these proteins. However, as we shall see, cattle opsin in digitonin solution exhibits a thermolability which is inconsistent with its functional status in a warm blooded animal. We have therefore examined the thermal stabilities of cattle rhodopsin and opsin in suspensions of rod outer limbs. In rods, opsin still is considerably more labile than rhodopsin, but both molecules are more stable in the rod than in digitonin solutions.

Methods

Rhodopsin and Opsin.—Cattle retinas are ground with 40 per cent sucrose in neutral M/15 phosphate buffer, and the suspension of retinal material layered under the same buffer in a centrifuge tube. Following centrifugation for 15 minutes at about 80,000 times gravity (Spinco preparative centrifuge with No. 30 rotor), the rod outer segments are collected from the sucrose-buffer interface. The residue is reground with 36 per cent sucrose in phosphate buffer, and the flotation repeated. The two batches of outer segments are pooled, sedimented from the sucrose solution following dilution with about two volumes of buffer, and washed several times with 10 to 20 times their volume of distilled water. The outer segments are then hardened in 4 per cent alum (potassium aluminum sulfate) for about 10 minutes with continuous stirring, again washed thoroughly with distilled water, followed by neutral phosphate buffer, and lyophilized. The dry rod powder is extracted with low boiling petroleum ether (b.p. 20-40°C.), and the petroleum ether evaporated off. Rhodopsin or opsin is extracted by stirring the rod powder vigorously with 2 per cent aqueous digitonin.

When preparing rhodopsin, all operations are carried out in dim red light. To prepare opsin, retinas are dissected in the light, and allowed to stand in diffuse daylight for an hour to complete the reduction of retinene to vitamin A (Wald and Hubbard, 1948-49). The fractionation and extraction are carried out in white light, at or near 5°C.

Rod Particles.—Rod outer segments are isolated by flotation in buffered sucrose, as described above. The outer segments from two flotations are again pooled and sedimented from buffer. They are then dispersed evenly with a teflon glass homogenizer into 34 per cent sucrose in phosphate buffer (M/15, pH 6.1).

The outer segments from 50 retinas are usually suspended in a final volume of about 50 ml., and the stock suspension is divided into 1 ml. aliquots, which are stored at −15°C. These aliquots are stable for long periods; before use they are thawed and shaken briefly. Microscopic examination of these suspensions reveals no intact outer segments, but only fragments and thin fibrils, some of which have the superficial appearance of myelin threads. This material is referred to as "rod particles."

Regenerated Rhodopsin and Isorhodopsin.—These pigments are synthesized by

mixing cattle opsin, prepared as described above, with neo-*b* (11-*cis*) or iso-*a* (9-*cis*) retinene,[1] in proportions such that opsin is always in excess. The retinene is dissolved in 1 per cent digitonin in M/15 phosphate buffer, pH 6.1 (*cf*. Hubbard, 1955–56), and incubated with opsin at room temperature for about 4 hours in the dark. The absorption spectra of the synthetic rhodopsin or isorhodopsin are then measured and the pigments stored in dark containers in the refrigerator.

Denaturation of Rhodopsin and Isorhodopsin in Aqueous Digitonin.—Heat bleaches rhodopsin or isorhodopsin to retinene and denatured opsin. The absorption band of retinene (λ_{max} about 385 mμ) is displaced sufficiently from that of cattle rhodopsin (λ_{max} 498 mμ) or isorhodopsin (λ_{max} 487 mμ) so that the thermal bleaching of these pigments can be followed spectrophotometrically. Extinctions were measured at 500 mμ, where rhodopsin and isorhodopsin absorb strongly and retinene only slightly, and at 625 mμ, where none of the three substances absorbs appreciably. Non-specific changes in absorption, caused by clouding, etc., are thus monitored at 625 mμ, and applied as corrections to the extinctions measured concurrently at 500 mμ.

In the present experiments these corrections were always small. The initial extinctions of rhodopsin or isorhodopsin at 500 mμ were always about 0.55 to 0.75; the final extinctions, following thermal bleaching, about 0.05 to 0.08. The extinctions at 625 mμ meanwhile rose from about 0.005 to about 0.011. That is, a loss of extinction of 0.5 to 0.7 at 500 mμ was accompanied by a rise of extinction of about 0.006 at 625 mμ.

All experiments were performed at pH 6.1 (about M/30 phosphate buffer), since rhodopsin and opsin both are most stable at this pH (Radding and Wald, 1955–56).

The rates of denaturation (thermal bleaching) were measured with a Beckman DU spectrophotometer, by heating the samples in place in the cell compartment. The temperature of the cell compartment was regulated by circulating water through Beckman thermospacers from a reservoir, the temperature of which was controlled to within 0.1°C. with a bimetallic thermoregulator (American Instrument Company). The temperatures of the reservoir and cell compartment were related as shown in Fig. 1. The temperature of the cell compartment was measured before and after each experiment (solid circles) and during two calibration runs (7.3.56 and 7.6.56). The three sets of data agree to within 0.5°, which represents the accuracy of this method.

Before each experiment, the cell holder and absorption cells containing all ingredients but rhodopsin or isorhodopsin were equilibrated in the prewarmed cell compartment for about half an hour. The samples, warmed to about 35°C., were introduced rapidly through a small opening in the cell compartment, and the changes in extinction were followed. The stock solutions of rhodopsin and isorhodopsin were sufficiently concentrated so that only 0.1 or 0.2 ml. needed to be added to 0.35 or

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[1] Crystalline neo-*b* retinene was prepared by Mr. Paul K. Brown in this laboratory (*cf*. Brown and Wald, 1956); crystalline iso-*a* retinene was a gift from Dr. Embree and Dr. Baxter of Distillation Products Industries, in Rochester, New York, and had been recrystallized by Mr. Brown.

0.25 ml. of digitonin, which was already at the temperature of the experiment. Nevertheless, the rates of thermal bleaching increased for the first minute or two, apparently while the samples were coming into temperature equilibrium. From then on, thermal bleaching followed the kinetics of a monomolecular reaction until all rhodopsin or isorhodopsin had been bleached (*cf*. Fig. 3).

Denaturation of Opsin in Aqueous Digitonin.—Native opsin combines with neo-*b* retinene to form rhodopsin. The loss of this capacity was used as the criterion for thermal denaturation.

Buffered digitonin (pH 6.1; 1.7 ml.) was preincubated in a constant tempera-
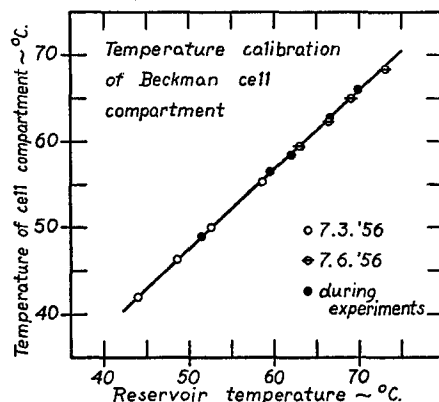

Fig. 1. Temperature calibration of Beckman cell compartment. The cell compartment was heated by circulating water from a constant temperature reservoir through Beckman thermospacers. Due to imperfect insulation, the cell compartment is somewhat cooler than the reservoir. The two temperatures are related as shown. Measurements were made in the course of experiments on the denaturation of rhodopsin and isorhodopsin (solid circles) and during two calibration runs (7.3.56 and 7.6.56).

ture bath, at one of a number of temperatures. Then 0.3 ml. of opsin was added, and at various times thereafter 0.3 ml. aliquots were removed, chilled, and incubated in the dark for 3 hours with excess neo-*b* retinene, to convert the residue of undenatured opsin to rhodopsin. The latter was measured by determining the loss of extinction at 500 mμ on bleaching in the presence of 0.2 M hydroxylamine (*cf*. Brown and Wald, 1956).

Denaturation of Rhodopsin in Rod Particles.—The thermal bleaching of rhodopsin in rod particles was followed by measuring the extinction of a rod suspension at 510 and 620 mμ initially, and after 10 and 20 minutes of incubation at a number of temperatures. For spectrophotometry, a thermally bleached suspension of the same initial extinction was used as blank (*cf*. Fig. 5). At the end of each experiment, the sample itself was completely bleached thermally, to check for small non-specific differences in extinction between it and the blank.

Denaturation of Opsin in Rod Particles.—To measure the denaturation of opsin in rod particles, 1 ml. aliquots of a suspension, milky white in color, were incubated for 10 minutes at a number of temperatures. They were then chilled and incubated at room temperature with excess neo-*b* retinene, added in 0.01 ml. acetone. As shown in Fig. 7, the course of regeneration in such a heterogeneous system is no different from that previously observed in solution (*cf.* Hubbard and Wald, 1952–53). At the end of 4 hours, 0.1 ml. M hydroxylamine was added and the difference in extinction before and after irradiation was measured at 510 and 620 mμ. The amount of opsin present in the suspension prior to thermal denaturation was determined similarly on an unheated aliquot of the same suspension. The spectrophotometry was performed against a portion of the suspension to which no retinene had been added, as blank.

RESULTS

Denaturation of Opsin, Rhodopsin, and Isorhodopsin in Aqueous Digitonin.— The course of denaturation of opsin and rhodopsin at five temperatures is illustrated in Figs. 2 and 3. In both figures, the data are plotted in terms of the equation for a monomolecular reaction in the form,

$$k = \frac{2.303}{t_2 - t_1} \log \frac{(a - x)_1}{(a - x)_2},$$

(1)

in which k is the velocity constant, and $(a - x)_1$ and $(a - x)_2$ are the concentrations of opsin or rhodopsin at times t_1 and t_2. The points fall on straight lines indicating that the thermal denaturation of opsin or rhodopsin follows the course of a monomolecular reaction.

As can be seen from equation (1), the velocity constant at each temperature is equal to 2.303 times the slope of the appropriate line in Fig. 2 or Fig. 3. Similar data have been obtained for the thermal denaturation of isorhodopsin. The velocity constants for thermal denaturation of the three molecules are shown in Table I (columns I and II).

As can be seen from Table I, opsin is denatured at temperatures about 20° below those that denature rhodopsin. To rule out the possibility that this lability of opsin was caused by some change introduced into the molecule during its purification and extraction, some of the same opsin preparation was converted to rhodopsin by incubation with neo-*b* retinene (*cf.* Methods). As shown in Fig. 3 and Table I, the thermal stability of this synthetic (regenerated) rhodopsin was the same as that of rhodopsin extracted from dark-adapted cattle retinas. The different thermostabilities of rhodopsin and opsin are therefore intrinsic properties of the two molecules.

The protection involves the specific attachment of the neo-*b* chromophore to opsin to form rhodopsin. The presence of excess neo-*b* retinene exerts no further stabilizing effect on rhodopsin. This was shown by incubating regenerated rhodopsin (same preparation as above) at room temperature for 1¾ hours
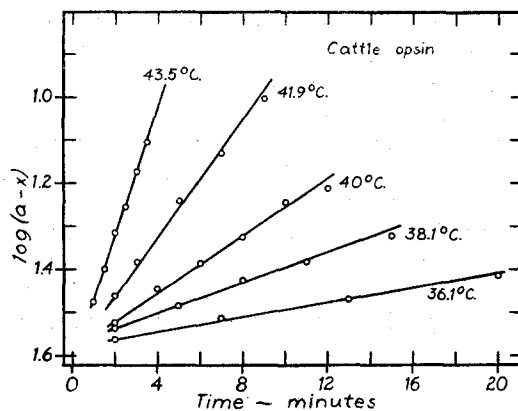
FIG. 2. Kinetics of thermal denaturation of opsin in aqueous digitonin (pH 6.1). The logarithm of the concentration of opsin remaining after incubation at various temperatures (log $(a$-$x)$) is plotted as ordinate against the time of incubation as abscissa. At each temperature, the points fall on a straight line, showing that the denaturation follows the course of a monomolecular reaction.
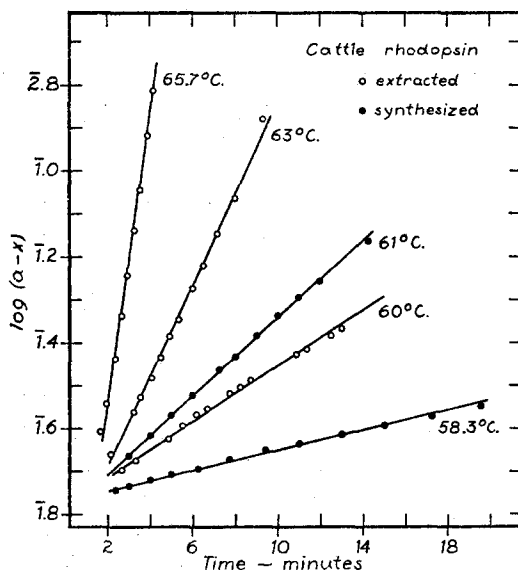
FIG. 3. Kinetics of thermal denaturation of rhodopsin in aqueous digitonin (pH 6.1). Data presented as in Fig. 2. Open circles show data obtained with rhodopsin extracted from dark-adapted retinas. Solid circles show measurements made with rhodopsin which had been synthesized *in vitro* by mixing neo-*b* retinene with an aliquot of the same opsin preparation which yielded the data shown in Fig. 2. The course of denaturation in both cases is that of a monomolecular reaction.

TABLE I

Thermal Denaturation of Rhodopsin, Isorhodopsin, and Opsin in 2 Per Cent Aqueous Digitonin, pH 6.1

Summary of data presented in Figs. 2, 3, and 4. The heats ($\Delta H\ddagger$), free energies ($\Delta F\ddagger$), and entropies ($\Delta S\ddagger$) of activation are derived from the Arrhenius activation energies (E_a; *viz.* Fig. 4), and from the velocity constants, k, at absolute temperatures, T, as described in the text.

I T	II k	III $\Delta H\ddagger$	IV $\Delta F\ddagger$	V $\Delta S\ddagger$
		Extracted rhodopsin		
°K.	sec.$^{-1}$	*cal. per mole*	*cal. per mole*	*cal. per mole degree*
329.5	0.000245	99,300	25,000	225.5
331.5	0.000448	99,300	24,750	224.9
333.0	0.00124	99,300	24,200	225.6
336.0	0.00407	99,300	23,600	225.3
338.7	0.0136	99,300	23,000	225.3
		Regenerated rhodopsin		
331.3	0.000455	99,300	24,700	225.2
334.0	0.00173	99,300	24,000	225.4
334.0*	0.00180	99,300	24,000	225.5
336.6	0.00516	99,300	23,500	225.3
Rhodopsin averages		99,300	24,100	225.3
		Isorhodopsin		
330.2	0.000587	95,300	24,400	214.6
331.3	0.000850	95,300	24,300	214.4
332.9	0.00172	95,300	23,900	214.4
334.6	0.00355	95,300	23,600	214.4
336.7	0.00858	95,300	23,100	214.3
Isorhodopsin averages		95,300	23,900	214.4
		Opsin		
309.1	0.000322	72,400	23,200	159.1
311.1	0.000670	72,400	23,000	159.1
313.0	0.00128	72,400	22,700	158.9
314.9	0.00263	72,400	22,400	158.9
316.5	0.00575	72,400	22,000	159.4
Opsin averages		72,400	22,700	159.1

* This sample was heated in the presence of a sixfold excess of neo-*b* retinene; the other samples had been regenerated with a limiting amount of neo-*b* retinene, so that none was present during the denaturation experiments.

with a sixfold excess of neo-*b* retinene. The rate of thermal denaturation, measured at 61.0°C., was the same as in the absence of excess retinene (*viz.* Table I and Fig. 4). This experiment shows further that the thermal denaturation of rhodopsin is irreversible, since the preparation regenerated no rhodopsin in the presence of a large excess of neo-*b* retinene.

It must be noted that we are using different criteria to judge the thermo-
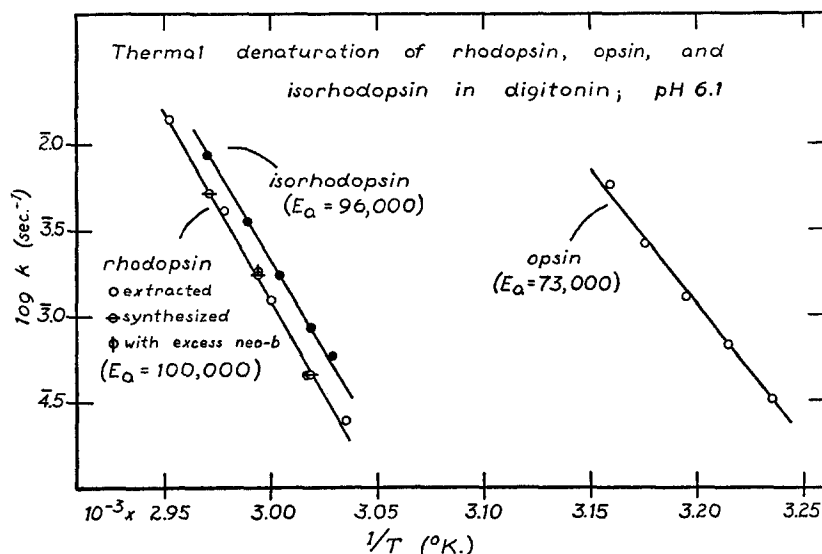

FIG. 4. Arrhenius plots for the thermal denaturation of opsin, rhodopsin, and isorhodopsin in aqueous digitonin. The data are plotted in terms of the Arrhenius equation as described in the text. The values of the velocity constants (k) at absolute temperatures T are given in Table I. The Arrhenius activation energies (E_a), arrived at by multiplying the slopes of the lines by 2.303 R (*cf.* Equation 2), are shown in the figure.

stabilities of rhodopsin and opsin. We use regenerability—the capacity to couple with neo-*b* retinene—as criterion for opsin, and maintenance of the absorption spectrum as criterion for rhodopsin. This is permissible only if rhodopsin on heating maintains its regenerability along with its absorption spectrum. Two instances have recently been reported from this laboratory—ageing (Radding and Wald, 1955-56) and acetylation (Albrecht, 1957)—in which the absorption spectrum of rhodopsin remains intact, but its regenerability is lost. As shown in the following experiment, however, regenerability and the absorption spectrum do not become dissociated from one another upon *heating* rhodopsin.

A rhodopsin solution was divided into halves, and one portion kept on ice as control, while the other was heated 10 minutes at 45.2°C. At this temperature rhodopsin, in terms of its absorption spectrum, has a half-life of about a week; whereas opsin has a half-life of 1.04 minutes, in terms of regenerability. If, therefore, the regenerability of rhodopsin were as labile as that of opsin, this treatment ought to abolish it altogether; whereas, if the regenerability of rhodopsin went with the integrity of its absorption spectrum, it should be unaffected by the incubation. At the end of the incubation, the heated sample was chilled. Both it and the control were bleached with orange (non-isomerizing) light and incubated with excess neo-*b* retinene to test their regenerability. After 3 hours, hydroxylamine was added to a final concentration of 0.3 M, and the amount of rhodopsin in each was determined by measuring the absorption spectrum before and after bleaching. The control regenerated 61 per cent of the initial rhodopsin, the heated sample, 59 per cent, which is well within experimental error. Heating rhodopsin at a temperature at which it does not bleach, therefore, does not impair its regenerability even when the same treatment would have completely abolished the regenerability of opsin.

Attachment of the neo-*b* (11-*cis*) chromophore therefore protects opsin against thermal denaturation. A similar, though slightly smaller stabilization is effected by the iso-*a* (9-*cis*) chromophore of isorhodopsin. This is shown in Table I and Fig. 4.

In these experiments, the same opsin preparation was used to make isorhodopsin and regenerated rhodopsin as had been examined for its own thermal stability. The opsin portion of all three molecules was therefore the same. Yet their thermal stabilities differed as shown in Fig. 4.

In Fig. 4, the data from columns I and II of Table I are plotted in terms of the Arrhenius equation in the form,

$$\log \frac{k_1}{k_2} = \frac{E_a}{2.303R} \left(\frac{1}{T_2} - \frac{1}{T_1} \right), \tag{2}$$

in which E_a is the Arrhenius activation energy, R the gas constant (1.986 cal. per mole degree), and k_1 and k_2 are the velocity constants of thermal denaturation at absolute temperatures T_1 and T_2. The plot of log k against $1/T$ yields straight lines for all three molecules. The Arrhenius energies are derived from the slopes of the lines, multiplied by 2.303 R (*cf.* equation (2)).

The temperature threshold and the Arrhenius energy for thermal denaturation are both highest for rhodopsin, slightly lower for isorhodopsin, and considerably lower for opsin. These differences in thermostability are interpreted more readily if one compares the entropies and free energies of activation ($\Delta S\ddagger$ and $\Delta F\ddagger$; *cf.* Glasstone, Laidler, and Eyring, 1941, chapter 1) for the denaturation of the three molecules.

These functions are derived from the velocity constant, k, on the assumption that the activation of a molecule which precedes its reaction is a reversible process

to which the laws of thermodynamics are applicable. The rate of reaction, k, depends upon the free energy of activation, according to the equation,

$$\Delta F\ddagger = 2.303RT \log \frac{k_B T}{kh}, \tag{3}$$

in which k_B is Boltzman's constant (1.38×10^{-16} erg degrees) and h Planck's constant (6.57×10^{-27} erg sec.). The entropy of activation is related to k by the equation,

$$\Delta S\ddagger = 2.303R \log \frac{kh}{k_B T} + \frac{\Delta H\ddagger}{T}. \tag{4}$$

$\Delta H\ddagger$, the heat of activation, is derived from the Arrhenius energy by the equation,

$$\Delta H\ddagger = E_a - RT. \tag{5}$$

The rate of reaction is therefore determined by the *free energy* of activation, and not by the Arrhenius energy which is related to the sum of the free energy and an entropy term.

The values of these functions for the thermal denaturation of rhodopsin, isorhodopsin, and opsin are shown in Table I. Their probable significance will be discussed below.

Denaturation of Rhodopsin in Rod Particles.—The difference spectrum of cattle rhodopsin in rod particles is shown in Fig. 5. It was obtained by measuring the absorption spectrum of a suspension of rod particles, prepared as described in Methods, against a previously bleached aliquot of the same suspension as blank. The difference spectrum has a maximum at 515 mμ, due to absorption by rhodopsin, and a minimum at 387 mμ, due to absorption by retinene. Thermal denaturation was followed spectrophotometrically, as described above. The data are shown in Figs. 6 and 8, and summarized in Tables II and III.

It should be noted that we are using *difference spectra* rather than *absorption spectra* to determine the rates of thermal denaturation of rhodopsin in solution and in rod particles. This introduces a small error, since retinene, produced by the denaturation of rhodopsin, absorbs slightly at the wavelengths used in these measurements (500 mμ with solutions, 510 mμ with rod particles). This error is easier to evaluate when rhodopsin is bleached with heat than with light. When rhodopsin is bleached with light, the spectroscopic picture is complicated by the presence of orange-red intermediates—predominantly metarhodopsin (*cf.* Wald, Durell, and St. George, 1950; Hubbard and Kropf, 1958)—which themselves have appreciable extinctions at 500 or 510 mμ. When rhodopsin is bleached with heat, there is no evidence of such intermediates: it apparently bleaches directly to retinene.

Under the conditions of the present experiments, the extinction at 500 mμ due to retinene is at most about 7 per cent that of rhodopsin. In a number of experiments, for example, thermal bleaching of rhodopsin in solution was accompanied
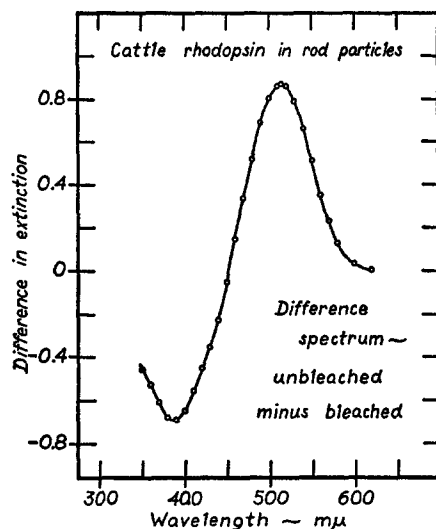
FIG. 5. Difference spectrum of cattle rhodopsin in rod particles. The spectrum is measured by comparing the extinction of a suspension of rod particles prepared from dark-adapted eyes, with a bleached aliquot of the same suspension as blank. Suspension medium: 34 per cent sucrose in M/15 phosphate buffer, pH 6.1 (for details, see Methods).
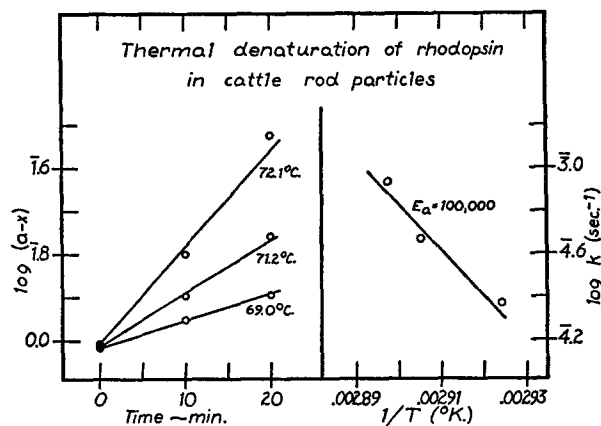
FIG. 6. Thermal denaturation of rhodopsin in rod particles. On the left, the data are plotted as in Figs. 2 and 3; on the right, as in Fig. 4. Values of k and T are given in Table II.

by a fall in extinction at 500 mμ from an initial value of 0.540 (due to rhodopsin plus any colored impurities) to a final value of 0.036 (due to retinene and the same impurities). If no impurities were present, a 6.6 per cent error would therefore be introduced by neglecting the absorption at 500 mμ due to retinene. If the absorp-

tion were due in part to impurities, the error would be correspondingly smaller. Since it is difficult to estimate the error accurately, we have used our measurements uncorrected.

TABLE II

Denaturation of Rhodopsin in Cattle Rod Particles

The velocity constants, k, at absolute temperatures, T, are derived from the data shown in Fig. 6 (left). The heats ($\Delta H\ddagger$), free energies ($\Delta F\ddagger$), and entropies ($\Delta S\ddagger$) of activation are calculated as described in the text.

I T	II k	III $\Delta H\ddagger$	IV $\Delta F\ddagger$	V $\Delta S\ddagger$
°K.	sec.⁻¹	cal. per mole	cal. per mole	cal. per mole degree
342.0	0.000233	99,300	26,000	214.4
344.2	0.000468	99,300	25,700	213.9
345.1	0.000860	99,300	25,300	214.4
Averages		99,300	25,700	214.2

TABLE III

Thermal Denaturation of Rhodopsin and Opsin in Cattle Rod Particles

Denaturation of rhodopsin measured as loss of extinction at 510 mμ (*viz.* Fig. 5); denaturation of opsin, as loss of capacity to form rhodopsin upon addition of neo-*b* retinene (*viz.* Fig. 7).

Rhodopsin		Opsin	
Temperature	Extent of denaturation in 10 min.	Temperature	Extent of denaturation in 10 min.
°C.	per cent	°C.	per cent
65.6	0	50.0	0
69.0	13.5	53.5	5.1
71.2	22.8	55.0	22.6
72.1	34.6	56.0	25.0
73.0	64.1	57.0	36.9
74.0	89.7	58.1	62.6
76.1	100	59.1	77.6
		60.1	90.7
		61.1	88.5
		63.3	94.5
		65.1	100

Data on the thermal bleaching of rhodopsin in rod particles are shown in Fig. 6. Neither the rate constants nor the estimate of the Arrhenius energy is as reliable as the values obtained with rhodopsin in solution, since both are derived from fewer experimental points. However, to a first approximation, the denaturation of rhodopsin is a first-order reaction in rod particles as well as in solution. The Arrhenius activation energies in both situations also are approxi-

mately the same, about 100,000 cal. per mole. The *threshold* for thermal bleach-
ing, however, is about 10° higher in rod particles than in solution.

The heats, free energies, and entropies of activation, calculated from the
data in Fig. 6, are shown in Table II and are discussed below.

Denaturation of Opsin in Rod Particles.—The thermal denaturation of opsin
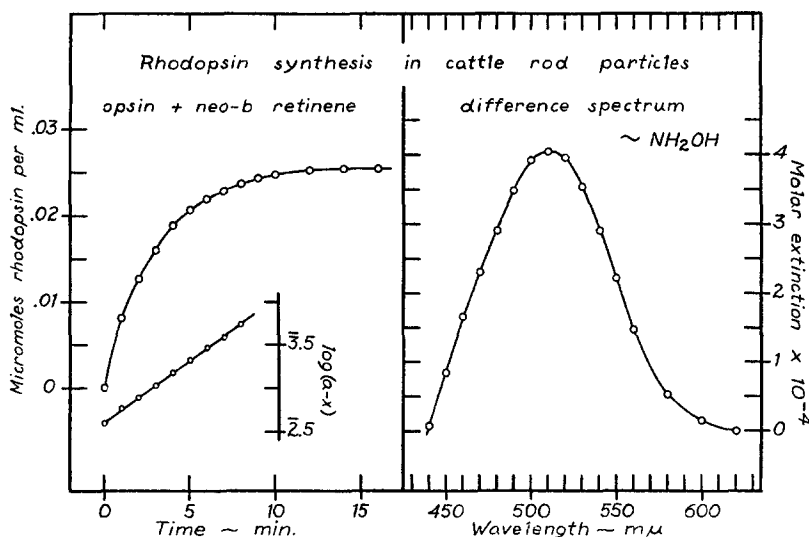
FIG. 7. Rhodopsin synthesis in cattle rod particles. Rod particles prepared from
bleached retinas are suspended in sucrose solution, as described in Methods. To
1 ml. of the suspension, containing 0.0254 micromole opsin, neo-*b* retinene (0.135
micromole) was added in 0.01 ml. acetone. The suspension was agitated and the
synthesis of rhodopsin followed by measuring the increase in extinction at 510 mμ.
As blank, a thermally denatured aliquot of the same rod suspension was used, to
which the same amount of neo-*b* retinene had been added. In the presence of such
a large excess of neo-*b* retinene, rhodopsin synthesis follows the course of a first-
order reaction (left). The difference spectrum of the synthesized rhodopsin is shown
on the right. It was obtained by measuring the extinction before and after bleach-
ing in the presence of 0.1 M hydroxylamine.

in rod particles was measured by comparing its capacity to form rhodopsin
before and after 10 minutes of incubation at various temperatures, as described
in Methods. No kinetic measurements were made.

As shown in Fig. 7, prior to heating, the stock suspension had the capacity
to form 0.0254 micromole rhodopsin per ml., upon incubation with excess
(0.135 micromole) neo-*b* retinene. In the presence of such a large excess of
neo-*b* retinene, rhodopsin synthesis follows the course of a first-order reaction,
as shown in Fig. 7 (left). When the synthesis was complete, the difference

spectrum was measured in the presence of 0.1 M hydroxylamine (Fig. 7, right). Its λ_{max} is at about 510 mμ, compared with 498 mμ for cattle rhodopsin in digitonin solution. Similar differences in λ_{max} of rhodopsin in rod particles and aqueous digitonin have been noted with rhodopsins from other species (cf. Wald and Brown, 1958).

The loss of the capacity to form rhodopsin was used as the criterion of thermal denaturation of opsin. Measurements made at eleven temperatures are summarized in Table III.
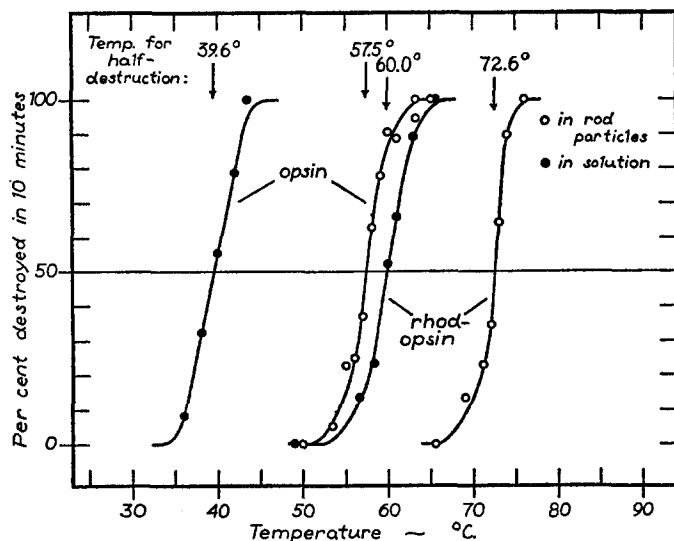
FIG. 8. Comparison of stabilities of rhodopsin and opsin in digitonin solution (solid circles) and in rod particles (open circles). The percentage denaturation during a 10 minute incubation at various temperatures is plotted as ordinate against the temperature as abscissa.

Temperature Thresholds for Denaturation of Rhodopsin and Opsin, in Solution and in Rod Particles.—A comparison of the thermal stabilities of rhodopsin and opsin, in solution and in rod particles, is presented in Fig. 8. The data for solutions are obtained from the rate constants listed in Table I, the data for rod particles, from Table III. It is clear from Fig. 8 that both rhodopsin and opsin are denatured more readily in solution than in suspensions of rod particles, and that in both situations, rhodopsin is much more stable than opsin.

Products of Thermal Bleaching of Rhodopsin and Isorhodopsin.—Denatured opsin is one product of thermal bleaching of rhodopsin or isorhodopsin. The other product is respectively neo-*b* or iso-*a* retinene, as shown in Figs. 9 and 10. However, during and immediately following thermal bleaching, the neo-*b*

or iso-*a* retinene liberated from the chromophore is isomerized rapidly, primarily to the all-*trans* configuration. This isomerization proceeds further during the thermal bleaching of rhodopsin (Fig. 9), but occurs to some extent also with isorhodopsin (Fig. 10).

If rhodopsin is denatured by placing a preparation, previously kept at room temperature, at 75.5°C., thermal bleaching begins within about 15 seconds and
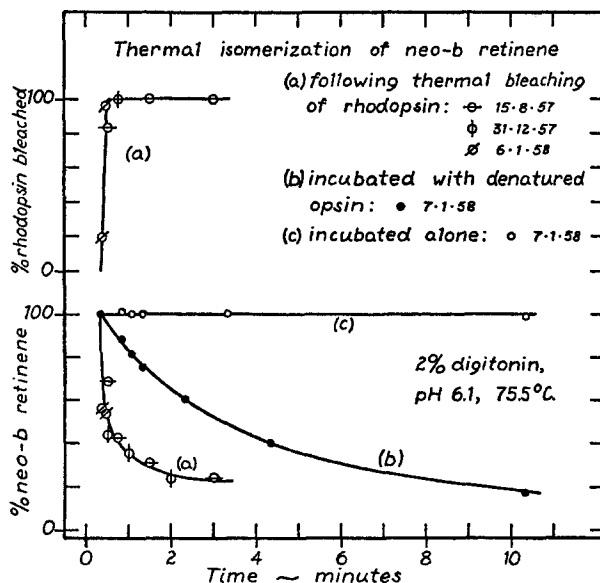
Fig. 9. Thermal isomerization of neo-*b* retinene (*a*) liberated by thermally bleaching rhodopsin, (*b*) during incubation with an equivalent amount of denatured opsin, and (*c*) during incubation in aqueous digitonin. All experiments performed at 75.5°C., pH 6.1. At this temperature, rhodopsin is bleached within 30 seconds (upper curve (*a*)), and the neo-*b* retinene it releases is rapidly isomerized (lower curve (*a*)), predominantly to the all-*trans* configuration. Denatured opsin also isomerizes neo-*b* retinene (curve (*b*)), but does so more slowly. Incubated by itself, neo-*b* retinene is not isomerized under these conditions (curve (*c*)).

is complete about 15 seconds later (upper half of Fig. 9). If during this period aliquots are chilled and assayed for neo-*b* retinene by incubation with excess opsin (*cf.* Brown and Wald, 1956), one finds that about two-thirds of the retinene liberated upon thermal bleaching is in the neo-*b* (11-*cis*) configuration (lower half of Fig. 9, curve (*a*)). If the samples are left at 75.5° a number of minutes, their neo-*b* content declines to a final level of about 20 per cent (curve (*a*)).

In similar experiments with isorhodopsin one finds that after 30 seconds at 75.3°C., all the isorhodopsin has been bleached and about three-fourths of the

retinene is in the iso-*a* (*9-cis*) configuration (Fig. 10). After another 15 seconds at this temperature, the iso-*a* content has dropped to about two-thirds, and thereafter remains at this level.

Thermal bleaching of rhodopsin and isorhodopsin therefore releases retinene predominantly, if not completely, in the same configurations which it has in the chromophores of the two molecules, 11-*cis* in the case of rhodopsin, 9-*cis* in the case of isorhodopsin. However, both *cis* isomers are rapidly isomerized during or immediately subsequent to their release from the chromoproteins.
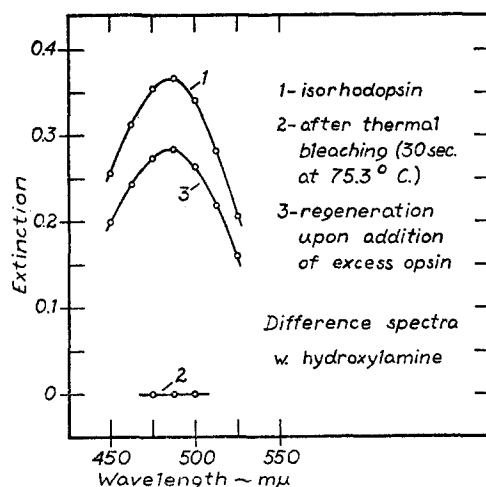
Fig. 10. Product of thermal bleaching of isorhodopsin. Isorhodopsin (curve 1) is bleached completely (curve 2) by incubation for 30 seconds at 75.3°C. (pH 6.1). Upon incubating the products of bleaching with excess opsin, 78 per cent of the initial isorhodopsin is regenerated (curve 3). This indicates that at least 78 per cent of the retinene liberated by thermal bleaching was in the iso-*a* (*9-cis*) configuration. All spectra shown are difference spectra, measured in the presence of about 0.2 M hydroxylamine.

We are investigating the mechanism of this thermal isomerization. Preliminary results are shown in Fig. 9 (curves (*b*) and (*c*)). Curve (*a*) in Fig. 9 contains data from three experiments, one performed with rhodopsin extracted from dark-adapted retinas (15.8.57), the other two (31.12.57 and 6.1.58) with rhodopsin synthesized from neo-*b* retinene and opsin. Another sample of the same opsin preparation was thermally denatured and then incubated with neo-*b* retinene at 75.5° (curve (*b*)), keeping the proportions of retinene and opsin the same as in the experiments of 31.12.57 and 6.1.58. In the presence of denatured opsin, neo-*b* retinene was isomerized again predominantly to the all-*trans* configuration (curve (*b*)). However, the isomerization was much faster

during the denaturation of rhodopsin than during incubation of neo-*b* retinene with an equivalent amount of denatured opsin (compare curves (*a*) and (*b*)). The heat treatment itself did not isomerize neo-*b* retinene in the absence of denatured opsin (curve (*c*)).

The 11-*cis* configuration therefore is sterically labile in the presence of denatured opsin. The labilization is greater during the denaturation of rhodopsin than can be accounted for by the mere coexistence in solution of equivalent concentrations of neo-*b* retinene and denatured opsin. It is difficult to evaluate the latter result, since one cannot know the effective concentrations of neo-*b* retinene and opsin during the denaturation of rhodopsin. When retinene leaves the chromophoric site on opsin, it is possible that the two molecules remain for a short time sufficiently close to raise their effective concentrations, and hence their probabilities of reacting with one another, considerably above those which obtain in free solution. These effects are being investigated further, with opsin as well as with other proteins.

<div align="center">DISCUSSION</div>

Thermal Stabilities of Opsin, Rhodopsin, and Isorhodopsin in Solution.—The experiments we have reported show that attachment of the chromophore, whether neo-*b* (11-*cis*) or iso-*a* (9-*cis*), protects opsin against thermal denaturation. The physiological (neo-*b*) chromophore is somewhat more effective in this regard than the iso-*a* chromophore.[2]

The differences in the rates of denaturation of the three molecules are reflected in the values of $\Delta F\ddagger$. In addition, the entropies of activation are about 70 entropy units greater for the denaturation of the chromoproteins than for that of opsin.

Protein denaturations, thermal or otherwise, entail a randomization of the protein fabric. In general, they are associated with relatively small free energies of activation—which is the reason they occur at or near room temperature—but involve large, positive entropies of activation (*cf*. Glasstone *et al.*, 1941, especially pp. 442–447; Stearn, 1949). $\Delta S\ddagger$ is a measure of the configurational change which accompanies the activation process. Most simple chemical reactions entail small configurational changes, and for these $\Delta S\ddagger$ is usually small, and positive or negative depending on whether the configuration of the activated complex is more or less probable (random) than that of the reacting molecules. Protein denaturations, however, generally involve large positive entropies of activation—of the order of 100 to 300 entropy units (Glasstone *et al.*, 1949, pp. 196, 442–447; Stearn, 1949, pp. 31–34). This suggests that the activation process itself is accompanied by considerable structural randomization. In summary,

[2] Stabilization of the protein by its chromophore has also been observed with hemoglobin. At 20°C., for example, globin is rapidly denatured, whereas hemoglobin is stable (*cf*. Riggs and Wolbach, 1955–56).

therefore, it does not require much work (free energy) to denature a protein. The large Arrhenius energies commonly associated with protein denaturations are due to the large structural (entropy) effects.

The differences in $\Delta S\ddagger$ for the denaturation of opsin and of the chromoproteins, may be brought about in at least two different ways. The configuration of opsin may be less ordered than that of the chromoproteins, so that its denaturation involves a smaller randomization. In this case, it would be legitimate to regard opsin as a partially "denatured" molecule (*cf.* Mirsky, 1936; Wald and Brown, 1951–52; Wald, 1956). Alternatively, opsin may be just as structured ("native") as the chromoproteins; but removal of the chromophore may expose sites on opsin which are more vulnerable to denaturation, and involve less randomization than those which are attacked when the chromoproteins are denatured. The first of these interpretations implies that denaturation proceeds by the same pathway with opsin as with the chromoproteins, but that opsin has a head start. The second implies that the pathways of denaturation are different for opsin and the chromoproteins, and that opsin takes a short cut, which is blocked by the chromophores in rhodopsin and isorhodopsin. There is no way of deciding between these possibilities until more is known about the thermodynamics of interconversions between opsin and the chromoproteins. It should then be possible to tell whether opsin is a less tightly structured molecule than rhodopsin.

The difference in the Arrhenius energies for denaturing rhodopsin and isorhodopsin is also largely a configurational matter. The free energies of activation differ by only about 0.2 kcal., whereas the entropies of activation differ by about 11 entropy units.

The Increased Stability of Rhodopsin and Opsin in Rod Particles.—Both rhodopsin and opsin are considerably more stable in rod particles than in solution (Fig. 8). The temperature thresholds differ by about 13° for rhodopsin, and by about 18° for opsin.

The free energies and entropies of activation have been calculated for rhodopsin (Fig. 6 and Table II), but we do not have sufficient data to calculate them for opsin.

A difference in $\Delta F\ddagger$ of about 1 kcal. (*viz.* Tables I and II) accounts for the observed differences in the temperature thresholds for denaturation of rhodopsin in rod particles as compared with solutions. $\Delta S\ddagger$ is, however, somewhat *smaller* for denaturing rhodopsin in rod particles than in solution. The Arrhenius energy, being related to the sum of $\Delta F\ddagger$ and $\Delta S\ddagger$, is approximately the same in the two situations (*cf.* Figs. 4 and 6).

The difference in $\Delta S\ddagger$ between solutions and rod particles no doubt reflects the fact that in rod particles rhodopsin is oriented in a highly organized structure, approaching to this degree the solid state. It may be that this situation does not permit as extensive a randomization as accompanies the denaturation of the molecule in solution.

Thermal Denaturation of Rhodopsins from Various Species.—The data on the denaturation of cattle rhodopsin in solution are compared in Table IV with similar observations made with rhodopsins from frog (Lythgoe and Quilliam, 1938) and squid (Hubbard and St. George, 1957–58). Table IV shows that rhodopsins from various species differ widely in their thermostabilities. The Arrhenius energies range from about 45 to 100 kcal., due primarily to large differences in $\Delta S\ddagger$, which is almost 4 times as large for the denaturation of cattle rhodopsin as for that of frog rhodopsin. Squid rhodopsin is least stable in terms of $\Delta F\ddagger$—hence has the lowest temperature threshold—but owing to compensating differences in $\Delta S\ddagger$, the Arrhenius energy for its denaturation is greater than that observed with frog rhodopsin.

The chromophore is the same—neo-*b* retinene—in all the rhodopsins examined so far. The opsins, however, differ from one species to another, as do most proteins. The differences in thermal stability summarized in Table IV constitute one aspect of the species specificity of opsins.

The Bleaching of Rhodopsin by Light and by Heat.—Light bleaches rhodopsin by isomerizing its chromophore from the neo-*b* to the all-*trans* configuration (Hubbard and Kropf, 1958). We have just seen that heat bleaches rhodopsin by denaturing opsin. Photochemically bleached rhodopsin therefore regenerates upon addition of neo-*b* retinene (Hubbard and Wald, 1952–53); thermally bleached rhodopsin, upon addition of native opsin (Figs. 9 and 10, above). The two ways of bleaching and regeneration can be formulated:

Rhodopsin $\xrightarrow{\text{light}}$ $\longrightarrow$ all-*trans* retinene + opsin

$\downarrow$ + neo-*b* retinene

Regenerated rhodopsin.

Rhodopsin $\xrightarrow{\text{heat}}$ neo-*b* retinene + denatured opsin

$\downarrow$ + native opsin

Regenerated rhodopsin.

There is by now considerable evidence that the color and structural integrity of rhodopsin depend upon a close steric fit between the retinene chromophore and the opsin surface (*cf.* Wald, 1956; Hubbard, 1958 *a*; Kropf and Hubbard, 1958). Light and heat both bleach rhodopsin by destroying this fit, light by changing the configuration of the chromophore, heat by changing the configuration of opsin.

A number of years ago, St. George (1951–52) showed that the energy required to bleach frog or cattle rhodopsin with light can be entirely supplied by the absorbed quanta provided the wavelength is shorter than 590 mμ; *i.e.*, provided the energy content of the quanta exceeds about 48 kcal. per mole.

At longer wavelengths, however, the quanta are too small to supply this energy, and bleaching by *light* involves a temperature coefficient. The latter continues to increase as the wavelength of light lengthens, so that the sum of the Arrhenius energy (obtained from the temperature coefficient) and the energy of the absorbed quanta remains roughly constant (44.4 to 48.5 kcal. per mole). It should be noted that in all St. George's experiments rhodopsin was bleached with *light*. The thermal contribution presumably increased the energy of the chromophore in its ground state, so that smaller quanta were able to raise it into the first excited state.

TABLE IV

Energetics of Thermal Denaturation of Rhodopsins from Cattle, Frog, and Squid, Measured in Aqueous Digitonin Solution

The data for frog rhodopsin (pH 6.8) are computed from Lythgoe and Quilliam (1938), Figs. 1 and 3; the data for squid rhodopsin (pH 6.1) taken from Hubbard and St. George (1957–58), Table IV. The temperatures at which rhodopsin is half-denatured in 10 minutes are computed with the aid of equation (2) (p. 267) from the rate constant for a monomolecular process with a half-time of 10 minutes (0.00115 sec.$^{-1}$), another rate constant, k, measured at temperature T, and the Arrhenius activation energies (E_a) shown in the table.

I Species	II Temperature for half-denaturation in 10 min.	III E_a	IV $\Delta F\ddagger$	V $\Delta S\ddagger$
	°C.	*cal. per mole*	*cal. per mole*	*cal. per mole degree*
Cattle.............	60.0	100,000	24,100	225.3
Frog..............	54.5	45,000	24,000	62.7
Squid.............	43.8	72,000	23,200	152.2

The minimum quantum energy thus determined for *photochemical* bleaching of rhodopsins from cattle and frogs was about the same as the Arrhenius activation energy (E_a) for the *thermal* bleaching of frog rhodopsin, the only species of rhodopsin on which it had then been measured (*cf.* Table IV). It was supposed at that time that the products of bleaching rhodopsin by light or by heat were identical (*cf.* Lythgoe and Quilliam, 1938). St. George therefore concluded that the energetics of the two processes were also the same.

There is no inconsistency between St. George's experiments and our own. Our experiments, however, show that the analogy previously drawn between bleaching by light and by heat is invalid as regards mechanism and energetics (*cf.* Hubbard, 1958 *b*).

Physiological Correlations.—There has been considerable speculation concerning the possibility that the spontaneous thermal decomposition of rhodopsin *in vivo* might trigger a nervous response and thus set a level of "thermal noise" in the retina (*cf.* Denton and Pirenne, 1954; Barlow, 1956, 1957). The

position has been summarized as follows (Denton and Pirenne, 1954): "Assume that a molecule changed by the dark reaction is capable of causing in the rod cell to which it belongs the same reaction as a molecule changed by the absorption of a light quantum. The dark reaction ('noise') will then determine a certain level of spontaneous nervous excitation in the living retina. If this random 'noise' exceeds a certain level, its continual fluctuations in time will make it impossible to detect a light stimulus ('signal') below a certain intensity."

All these speculations have rested on the assumption that the mechanism and energetics of bleaching by heat and by light are the same (viz. Barlow, 1956). The present experiments, however, show that the mechanism and products of bleaching by heat and by light are different; and that the energetics of bleaching by heat vary widely in different species. There is no present evidence that bleaching by heat can result in nervous excitation; and no basis for calculating the energetics of thermal bleaching in man from observations made with rhodopsins from other animals.

REFERENCES

Albrecht, G., Acetylrhodopsin, *Science*, 1957, **125**, 70.

Barlow, H. B., Retinal noise and absolute threshold, *J. Opt. Soc. America*, 1956, **46**, 634.

Barlow, H. B., Purkinje shift and retinal noise, *Nature*, 1957, **179**, 255.

Brown, P. K., and Wald, G., The neo-*b* isomer of vitamin A and retinene, *J. Biol. Chem.*, 1956, **222**, 865.

Denton, E. J., and Pirenne, M. H., The absolute sensitivity and functional stability of the human eye, *J. Physiol.*, 1954, **123**, 417.

Glasstone, S., Laidler, K. J., and Eyring, H., The Theory of Rate Processes, New York, McGraw-Hill Book Co., 1941.

Hubbard, R., Retinene isomerase, *J. Gen. Physiol.*, 1955-56, **39**, 935.

Hubbard, R., On the chromophores of the visual pigments, *Proc. Nat. Physic. Lab.*, *Symp. No. 8* (Visual problems of colour), H. M. Stationery Office, 1958 *a*, in press.

Hubbard, R., The bleaching of rhodopsin by light and by heat, *Nature*, 1958 *b*, **181**, 1126.

Hubbard, R., and Kropf, A., The action of light on rhodopsin, *Proc. Nat. Acad. Sc.*, 1958, **44**, 130.

Hubbard, R., and St. George, R. C. C., The rhodopsin system of the squid, *J. Gen. Physiol.*, 1957-58, **41**, 501.

Hubbard, R., and Wald, G., Cis-trans isomers of vitamin A and retinene in the rhodopsin system, *J. Gen. Physiol.*, 1952-53, **36**, 269.

Kropf, A., and Hubbard, R., The mechanism of bleaching rhodopsin, *Ann. New York Acad. Sc.*, 1958, in press.

Lythgoe, R. J., and Quilliam, J. P., The thermal decomposition of visual purple, *J. Physiol.*, 1938, **93**, 24.

Mirsky, A. E., The visual cycle and protein denaturation, *Proc. Nat. Acad. Sc.*, 1936, **22**, 147.

Radding, C. M., and Wald, G., The stability of rhodopsin and opsin. Effects of pH and aging, *J. Gen. Physiol.*, 1955-56, **39**, 923.

Riggs, A. F., and Wolbach, R. A., Sulfhydryl groups and the structure of hemoglobin, *J. Gen. Physiol.*, 1955-56, **39**, 585.

St. George, R. C. C., The interplay of light and heat in bleaching rhodopsin, *J. Gen. Physiol.*, 1951-52, **35**, 495.

Stearn, A. E., Kinetics of biological reactions with special reference to enzymic processes, *Advances Enzymol.*, 1949, **9**, 25.

Wald, G., The biochemistry of visual excitation, *in* Enzymes: Units of Biological Structure and Function, (O. H. Gaebler, editor), New York, Academic Press, Inc., 1956, 355.

Wald, G., and Brown, P. K., The role of sulfhydryl groups in the bleaching and synthesis of rhodopsin, *J. Gen. Physiol.*, 1951-52, **35**, 797.

Wald, G., and Brown, P. K., Human rhodopsin, *Science*, 1958, **127**, 222.

Wald, G., Durell, J., and St. George, R. C. C., The light reaction in the bleaching of rhodopsin, *Science*, 1950, **111**, 179.

Wald, G., and Hubbard, R., The reduction of retinene to vitamin A_1 *in vitro*, *J. Gen. Physiol.*, 1948-49, **32**, 367.