THE INTERPLAY OF LIGHT AND HEAT IN BLEACHING RHODOPSIN

By ROBERT C. C. St. GEORGE*

(*From the Biological Laboratories of Harvard University, Cambridge*)

(Received for publication, July 26, 1951)

I

INTRODUCTION

The absorption of light by rhodopsin in the retina activates the pigment molecules and leads eventually to the stimulation of rod vision. The initial light reaction is followed by a sequence of thermal reactions occurring equally in light or darkness, by which the rhodopsin, a conjugated carotenoid protein, ultimately bleaches to retinene_1 and protein (Wald, Durell, and St. George, 1950). The work described here deals with the initial activation of rhodopsin.

Rhodopsin can be bleached not only by light but by heat. At 50°C. or above it bleaches rapidly in the dark and at a rate that is very temperature-dependent. Lythgoe and Quilliam (1938) calculated an experimental activation energy of 44 kg. cal. per mole from their data on the temperature coefficient in neutral solutions. On the other hand, the rate of bleaching by white light is not temperature-dependent, showing that no thermal activation is needed (Hecht, 1920–21).

The purpose of the following research was to study the transition between photic and thermal activation. The argument runs as follows. On going from shorter wave lengths to red light a point should be reached beyond which the incident quanta cannot supply the entire energy needed to activate the rhodopsin molecule. If bleaching is to occur nonetheless, the deficit will have to be met by a contribution of thermal energy from the internal degrees of freedom of the molecule. There should therefore be a critical wave length beyond which the bleaching by light becomes temperature-dependent.

Measurement of the temperature coefficient of bleaching at intervals along the wave length scale showed that a temperature dependence does in fact appear in the orange at about 590 mμ. Moreover, the quantum energy at this wave length differs very little from the activation energy observed in heat bleaching. At wave lengths longer than 590 mμ, the temperature coefficient increases regularly so that the sum of the energy of the absorbed quanta and the thermal ac-

* The work was supported in part by a grant to Professor George Wald from the Medical Sciences Division of the Office of Naval Research.

tivation energy remains nearly constant. This is the expected result if light and heat can cooperate in bleaching a rhodopsin molecule.

One may inquire further into the temperature coefficient in red light. Since the rate of any photochemical reaction is the product of the number of quanta absorbed and the quantum efficiency, one or both of these factors must become temperature-dependent at 590 mμ. The effect of changing the temperature on the absorption spectrum of rhodopsin was measured and was shown to be small out to this wave length, but to become larger in the red.

Unfortunately, due to the low absorption of rhodopsin in the red, the measurements could not be carried beyond 620 mμ. One must therefore rely on an extrapolation to find the degree to which changes in the absorption spectrum govern the temperature coefficient of bleaching. It appears that changes in the absorption spectrum alone can account for only two-thirds of the change in the bleaching rate and that the quantum yield must also become temperature-dependent at long wave lengths.

II

Temperature Dependence of the Bleaching Rate

Experimental Method.—The rate of bleaching of rhodopsin in buffered aqueous digitonin (pH 6–7) was measured at $1\frac{1}{2}$–$2\frac{1}{2}$°C. and at 31–32°C. Light of various wave lengths between 400 and 750 mμ was used. The temperature coefficient was found by taking the ratio of the rates at the two temperatures and will be designated in what is to follow as the "Q_{30}."

The bleaching was followed by measuring at intervals the concentration of rhodopsin in the sample. Since this was done photometrically, the bleaching was made to proceed slowly enough so that the colored intermediates which eventually yield retinene$_1$ would not accumulate and interfere with the measurements. In runs at $1\frac{1}{2}$–$2\frac{1}{2}$°C., the sample was warmed up to 30° in the dark for about 15 minutes before taking a reading, to allow all intermediates to be converted to the final product. With this precaution, the measured rate was that of the light reaction alone.

The methods used to prepare rhodopsin solutions from cattle and frog retinas are given in the Appendix. The apparatus is described in Fig. 1.

A description of the spectrum of rhodopsin will help the reader to understand the experiments. The absorption spectrum has three bands in the visible and ultraviolet. The carotenoid prosthetic group gives rise to a broad α-band with its maximum at about 500 mμ and a small β-band at about 350 mμ. The high γ-band with a maximum at 278 mμ is due to absorption in the protein moiety of the molecule. On irradiation, light absorbed in the α-band or the β-band causes them both to disappear and the retinene$_1$ spectrum with a maximum at 385 mμ finally takes their place (see Fig. 1 in Wald, 1949). The γ-band does not change on irradiation, and there is some evidence that light absorbed in it is not available for bleaching (Goodeve, Lythgoe, and Schneider, 1941–42).

At 620 mμ and further into the red and infrared the absorption of rhodopsin be-

comes too small to be measured by spectrophotometry. On the other hand, at these long wave lengths the spectral sensitivity of human rod vision (Griffin, Hubbard, and Wald, 1947) should describe the bleaching spectrum of rhodopsin. The sensitivity of rod vision falls off evenly towards long wave lengths. At 1050 mμ it has dropped to $10^{-12.4}$ of its value at the maximum. Therefore, rhodopsin must still absorb light and bleach at 1050 mμ.

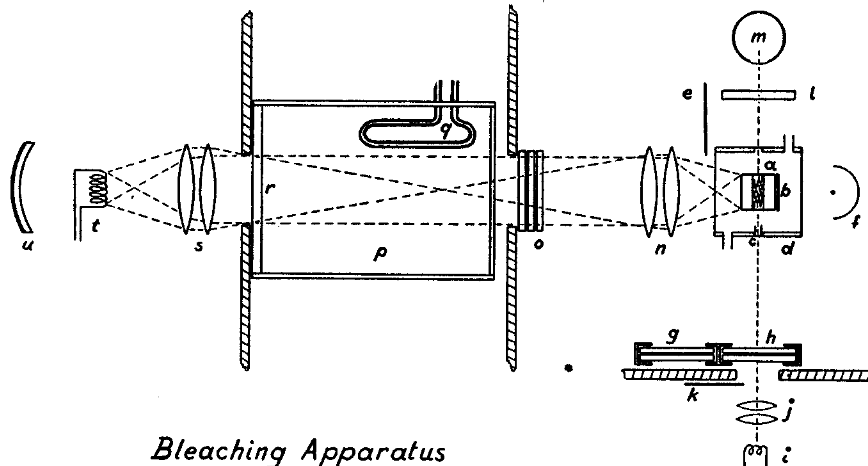

FIG. 1. (*a*) Quartz cell with trough 3 by 10 mm. (*b*) Aluminum mirror. (*c*) Window for the photometer beam. (*d*) Cell holder through which water at 1½ and 31°C. was pumped. The windows were double to prevent fogging. (*e*) Shutter. (*f*) Phototube. (*g*) Filter to obtain a 500 mμ photometer beam for measuring the bleaching rate of rhodopsin. (*h*) 390 mμ filter for measuring the formation of retinene_1. (*i*) Photometer lamp. (*j*) Lenses. (*k*) Shutter. (*l*) Corning No. 9780 filter to protect phototube. (*m*) Multiplier phototube RCA No. 981. (*n*) and (*s*) Lenses, $f = 5.2$ cm. (*o*) Light filters. (*p*) 15 cm. water filter. (*q*) Cooling coil to keep water at 25 to 30°C. (*r*) Sheet of pyrex. (*s*) Lenses, $f = 5.2$ cm. (*t*) Bleaching lamp (Mazda 1M/T-12P, 500/T-12P or 200/T-10P). (*u*) Spherical mirror, $f = 5.0$ cm.

The sensitivity of human rod vision in the spectrum should be equivalent to the bleaching spectrum of rhodopsin, provided that the visual sensitivity is not distorted by the selective absorption of light passing through the tissues of the eye. Actually such distortions due to filtering by ocular structures are present in the blue and ultraviolet, but above 500 mμ there is excellent agreement between the absorption spectrum of rhodopsin, its bleaching spectrum, and the spectral sensitivity of human rod vision (Wald, 1949). It is reasonable to assume that in the red and infrared the spectral sensitivity of the eye and of solutions of rhodopsin will continue to correspond, even though both may finally depart from the absorption spectrum due to some falling off in quantum efficiency.

Experimental difficulties were caused by the fact that the temperature coefficient of bleaching does not become measurably greater than one at wave lengths below

600 mμ. At 600 mμ the sensitivity of rhodopsin to light is only one-fiftieth of that at the 500 mμ maximum; and at 750 mμ, the longest wave length used, it is less than one-millionth. The chief problem was therefore how to throw enough red light into the rhodopsin sample to bleach it at a rate which could be measured accurately, and, at the same time, to keep out stray light of shorter wave length, a vanishingly

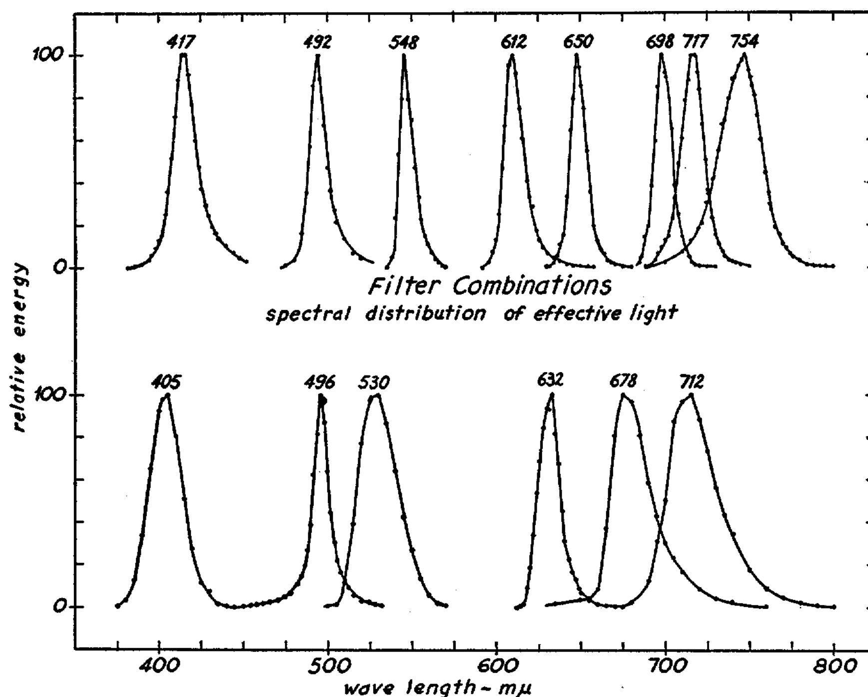

Fig. 2. Effective energy distribution of the spectral lights used to bleach rhodopsin. These were computed by multiplying together (*a*) the spectral energy distribution of the light source, (*b*) the spectral transmissions of the filters, and (*c*) the spectral sensitivity of rhodopsin. The energy is plotted as the per cent of its maximum value. Each spectral light is designated by the wave length of the center of gravity of the band. The filters used are listed in Table I.

small proportion of which might be as effective as an enormously greater amount of red light, and so might greatly distort the results.

The problem was solved by using an optical system which collected the light from a high-power projection lamp and, after converting it into a beam of nearly parallel rays, made it converge on a thin layer of rhodopsin solution at an angle of about 30°. To increase the bleaching rate further, the light was reflected back through the solution with a piece of polished aluminum cemented to the back of the container.

Sets of filters which had been tested for their opacity to unwanted wave lengths (*cf.* Wald, 1945) were placed in the parallel part of the beam. The filter sets were

made up of interference filters, which transmit wave length bands of 10 to 20 mμ half width, and of glass Corning and Jena filters, or Wratten gelatin filters, to remove stray light which passed through the interference filters.

The spectral distribution of the light passing through the filters and effective in bleaching the rhodopsin was calculated by multiplying together the following three quantities at suitable wave length intervals: (*a*) the spectral energy of the lamps, assuming that glowing tungsten emits black-body radiation; (*b*) the transmission of

TABLE I

Filter Combinations

Wave length (Center of gravity of bands in Fig. 2)	Interference filter* (Wave length of the transmission maximum)	Other filters
mμ	*mμ*	
405		Corning No. 3060; 4305; 4407; 5970‡
417	413	Corning No. 5113; 5850‡
492	496	Auxiliary blue-glass filter supplied with interference filter‡
496	496	Wratten No. 57A.‡ Auxiliary blue-glass filter supplied with interference filter
530		Corning No. 4407 Wratten No. 74‡
548	546	Corning No. 5120 Jena O.G. 2; B. G. 18‡
612	607	Corning No. 2418‡
632	630	Corning No. 2404‡
650	650	Corning No. 2403‡
678		Corning No. 2404 Jena R. G. 5; B. G. 17
698	700	Corning No. 2403 Jena R. G. 8
712		Corning No. 2404; 5850 Jena R. G. 5; B. G. 17
717	725	Corning No. 2403; 5850
754	754	Corning No. 2403; 2600; 5850

* Supplied by Baird Associates, Cambridge, Massachusetts.

‡ Wratten neutral filters were used to cut down the brightness.

the filters, measured in the Beckman spectrophotometer; (*c*) the bleaching spectrum of rhodopsin obtained from spectrophotometric data out to 600 mμ and from the spectral sensitivity of rod vision at longer wave lengths. The wave lengths by which the various filter combinations are designated (*cf.* Fig. 2) correspond to the centers of gravity of the spectral bands. The light filters used are listed in Table I.

In order to introduce as few variables as possible, the conditions of the experiments were kept as nearly as possible the same in all runs. The intensity of the light was adjusted to give about the same bleaching rate at all the wave lengths studied; *i. e.*, about 25 per cent drop in the rhodopsin concentration in 3 to 5 hours.

In all runs lasting 3 hours or longer at 32°C., control measurements were made of the dark rate of decomposition of rhodopsin. This dark decomposition caused an increase in the light transmission of 1½% every 10 hours in frog preparations, and of 2 per cent every 10 hours in cattle preparations. It is shown in Figs. 3 and 4 by broken lines.

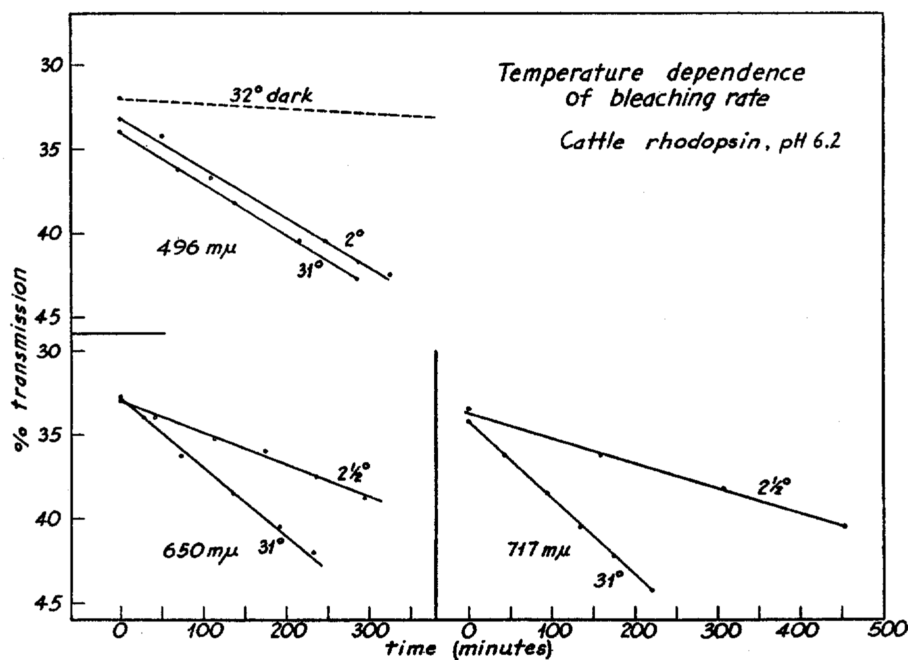

Fig. 3. Temperature dependence of the bleaching rate of cattle rhodopsin at various wave lengths. The lines show the change with time in the transmission of light of wave length 500 mμ by irradiated rhodopsin solutions. The bleaching rate is independent of temperature in light of wave length 496 mμ, but a high degree of temperature dependence is found at 650 and 717 mμ. The broken line shows the bleaching rate of a cattle rhodopsin solution kept in the dark at 32°C.

It was expected that the rhodopsin solution would become warmer than the surrounding water bath under the intense irradiation with red light; but it was found that the temperature of the sample never rose more than ½°C. at 750 mμ, and never more than ¼°C. at 717 mμ. These small effects called for no special correction.

Results.—It has been known for some time that the temperature coefficient of bleaching in white light is nearly one. Although white light contains red and infrared radiation, the rhodopsin is mainly bleached by the shorter wave lengths which it absorbs most strongly. The absence of a temperature dependence in white light shows that at these shorter wave lengths no thermal energy is re-

quired, the absorbed quanta supplying all the energy needed to activate the molecule.

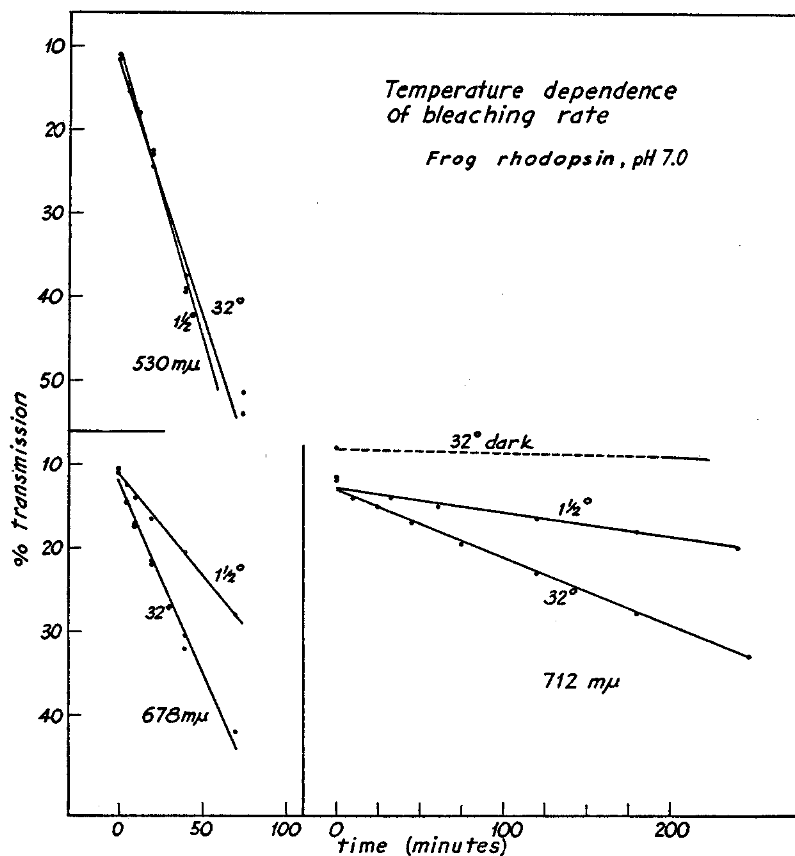

FIG. 4. Temperature dependence of the bleaching rate of frog rhodopsin at various wave lengths. The lines show the change in transmission of light of wave length 500 mμ by irradiated rhodopsin solutions. The bleaching rate is independent of temperature in light of wave length 530 mμ, but a high degree of temperature dependence is found at 678 and 712 mμ. The broken line shows the bleaching rate of a frog rhodopsin solution kept in the dark at 32°C.

This is not true in red light. Typical bleaching curves obtained in blue and green light and in red light are compared in Figs. 3 and 4 for cattle rhodopsin and frog rhodopsin respectively. At shorter wave lengths the rates of bleaching are the same at $1\frac{1}{2}$–$2\frac{1}{2}$° and at 31–32°C.; but in red light the rate is considerably greater at the higher temperature.

The results of a number of runs at different wave lengths are collected in

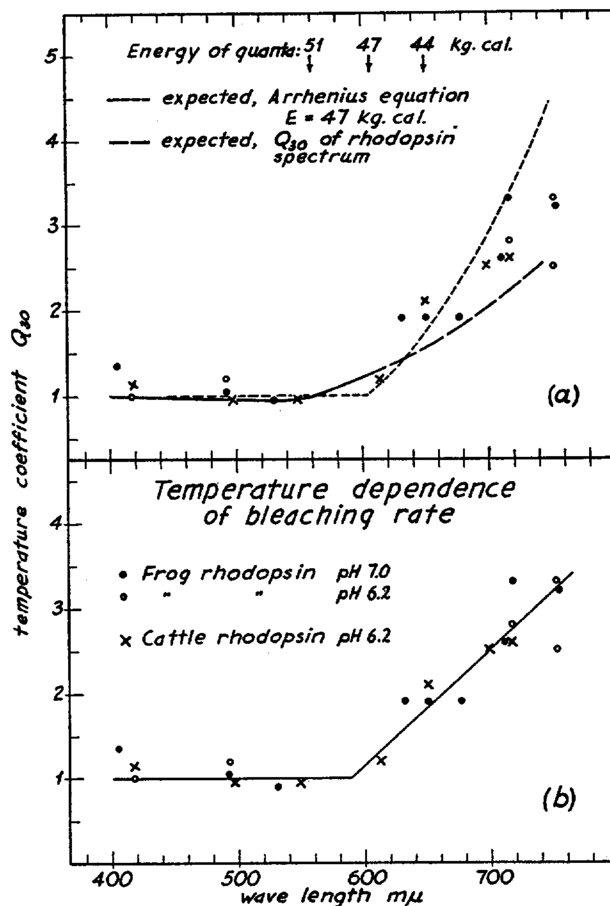

FIG. 5*a* and *b*. Temperature dependence of the bleaching rate of rhodopsin as a function of the wave length of light. The temperature coefficient of the bleaching rate is expressed as Q_{30}, the ratio of the rates at 1½–2½°C. and 31–32°C.

(*a*) The steep broken line gives the Q_{30} which would have been found if 47 kg. cal. is the minimum energy needed to bleach rhodopsin, and if the energy deficit of the quanta of red light is made up from a contribution of thermal energy related to the temperature coefficient by the Arrhenius equation. The lower line, solid out to 620 mμ and broken between 620 and 740 mμ, gives the temperature dependence of the absorption spectrum of rhodopsin, $Q_{30}^{\text{abs.}}$.

(*b*) The straight line drawn through the experimental points shows that the bleaching rate of rhodopsin becomes temperature-dependent at about 590 mμ. This corresponds to a minimum requirement of light energy to bleach rhodopsin equal to 48.5 kg. cal. At longer wave lengths the temperature coefficient, *i. e.* the thermal energy component, increases as the quantum energy falls off.

Fig. 5 *b*. The temperature dependence of the bleaching rate is expressed as Q_{30}, the ratio of rates at $1\frac{1}{2}$–$2\frac{1}{2}$° and 31–32°C. It is clear that Q_{30} is nearly one throughout most of the visible specturm. But at a wave length corresponding to orange light, Q_{30} rises above unity and becomes progressively larger in the red. The existence of a Q_{30} greater than one, *i.e.* of a thermal activation energy, shows that in the red the rhodopsin molecule draws upon its internal energy to bleach.

A line drawn through the experimental points in Fig. 5 *b* shows that the temperature dependence begins to appear at about 590 mμ. The energy of the absorbed quanta at this wave length is 48.5 kg. cal. per einstein (mole of quanta) a value to be compared with the 44 kg. cal. per mole activation energy found by Lythgoe and Quilliam for the heat bleaching of rhodopsin.

TABLE II

Activation Energy of Rhodopsin Bleaching

Wave length	Q_{30}	Arrhenius energy	Quantum energy	Total energy
mμ		*kg. cal. per mole*	*kg. cal. per mole*	*kg. cal. per mole*
Dark (Lythgoe and Quilliam)	*	44		44
590	1.0	0	48.5	48.5
650	1.8	3.3	43.9	47.2
700	2.5	5.1	40.8	45.9
750	3.2	6.4	38.0	44.4

* Q_{30} not applicable to Lythgoe and Quilliam's data.

The thermal energy component, as is evident from the changing temperature coefficient, increases with increasing wave length; *i.e.*, with decreasing size of quantum. One may calculate the apparent activation energy of photic bleaching at various wave lengths by means of the Arrhenius equation. When this is done the sum of quantum energy and activation energy is found to remain nearly constant from 590 mμ out into the far red (*cf.* Table II).[1]

[1] The relation between the temperature dependence of the rate and the activation energy is given by the Arrhenius equation:

$$\frac{d \ln k}{dT} = \frac{E}{RT^2} \tag{1}$$

in which k is the rate constant, T the absolute temperature, E the activation energy, and R the gas constant. To obtain the results in Table II this equation was used in the form

$$\ln \frac{k_1}{k_2} = \frac{E}{R} \cdot \left\{ \frac{1}{T_2} - \frac{1}{T_1} \right\}$$

in which the subscripts 1 and 2 refer to the higher and to the lower temperature. In this case the two are 30°C. apart.

Actually the calculated total energy falls to some extent between 590 and 750 mμ. If the total energy were to remain constant, one would expect the temperature dependence of the bleaching spectrum to be a steeper function of the wave length than the line drawn through the points in Fig. 5 *b*. In Fig. 5 *a* the steep dashed line shows the temperature coefficients expected for a total energy requirement of 47 kg. cal. This theoretical line, though clearly too steep, is nevertheless the best fit to the experimental data providing that the total energy is constant and that the Arrhenius equation holds. The possibility of getting a better fit by using a reasonable modification of the Arrhenius equation and not abandoning the idea of a constant energy requirement will be considered in the discussion.

III

Temperature Dependence of the Absorption Spectrum

Substances with continuous absorption commonly show a narrowing of the absorption bands on cooling. A pronounced narrowing and sharpening of the rhodopsin α-band on cooling to −73°C. was reported by Broda and Goodeve (1941–42). My own measurements over the same temperature range reveal a very much smaller effect of temperature on the absorption. The changes are in fact so small as to be evident only when spectra measured at widely different temperatures are superimposed. Yet this small effect accounts for a large part of the temperature dependence of the bleaching spectrum.

Experimental Method.—The solutions of rhodopsin were prepared in the same way as those used in the experiments of section II of this paper, except that they were initially more concentrated. The solutions were buffered at pH 7.0–7.4 and were diluted 2:1 with glycerol. Such mixtures do not freeze on cooling, but form a glass which is transparent even at −100°C.

The spectra were measured with a Beckman spectrophotometer using a specially constructed Dewar flask in the place usually occupied by the cell holder. The Dewar was silvered except for a window. A quartz cell containing the sample could be lowered into the photometer light beam which passed through the window. The temperature was controlled by adjusting the amount of dry ice or liquid nitrogen in the Dewar, and was measured with a thermocouple immersed in the sample.

The spectra of rhodopsin measured in this way must be corrected for the thermal contraction of the solution, which amounts to 5 per cent for a temperature drop of 100°C.; and for the absorption of digitonin in the glycerol-water mixture. This second correction is small and independent of temperature above 450 mμ but becomes increasingly larger and somewhat temperature-dependent at shorter wave lengths.

Results.—Figs. 6 and 7 show the spectra of cattle and of frog rhodopsin at temperatures down to −100°C. On cooling, the height of the α-band maximum

increases and the band moves toward the red, while to either side of the maximum the absorption drops. The effect of temperature on the β-band may be different in cattle and frog rhodopsins, rising in one and dropping in the other, but it is not sure that the measured differences are significant in view of the large blank correction in the ultraviolet.

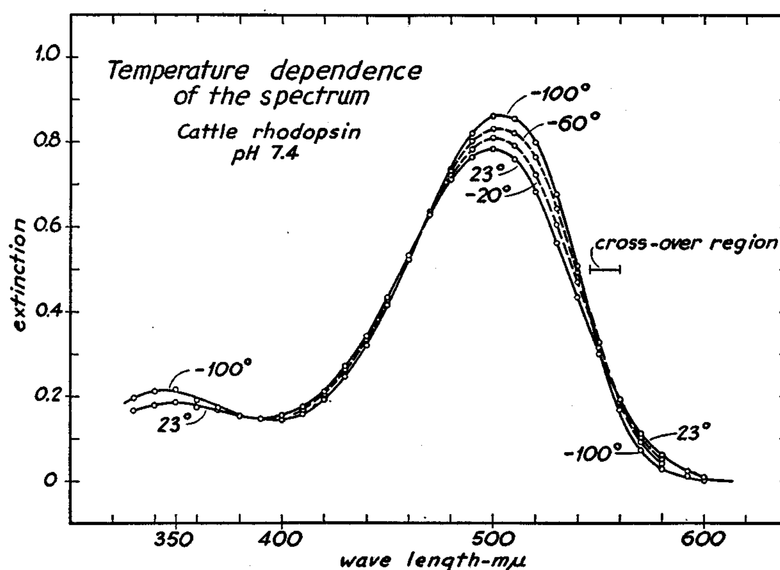

FIG. 6. Temperature dependence of the absorption spectrum of cattle rhodopsin in 2:1 glycerol-water mixture. The extinction was measured at 23, −20, −60, and −100°C. On *lowering* the temperature by 123°C. the maximum of the 500 mμ band *rises* and moves towards the red, while the band as a whole becomes narrower. At the "cross-over" region around 560 mμ light absorption is nearly independent of temperature. At longer wave lengths the absorption *rises* on *raising* the temperature. At 590 mμ the extinction changes by more than a factor of two over the 123°C. temperature interval. (The extinction is the common logarithm of the reciprocal of the fraction of light transmitted.)

The effect of temperature on absorption at different wave lengths is as follows. Between 400 and 570 mμ the absorption rises about 10 to 20 per cent on cooling some 125°C. Around 560 to 570 mμ there is a "cross-over" region where the absorption is nearly temperature-independent. At longer wave lengths a drop in the temperature causes the absorption to fall. Moreover, as the wave length increases the change in absorption for a given fall of temperature quickly becomes a much larger fraction of the total absorption than it is anywhere below 590 mμ. So it turns out that a temperature coefficient of absorption appears at the cross-over region and increases with the wave length in a way that is roughly parallel to the temperature coefficient of the bleaching rate.

In order to show this more precisely the temperature coefficients for the interval $-5°$ to 25°C. were measured in two cattle rhodopsin solutions, paying special attention to the longer wave lengths. One solution was that used to obtain the data of Fig. 6 and the other was made up to be about twice as concentrated. The experimental data were plotted as *absorption vs. temperature*

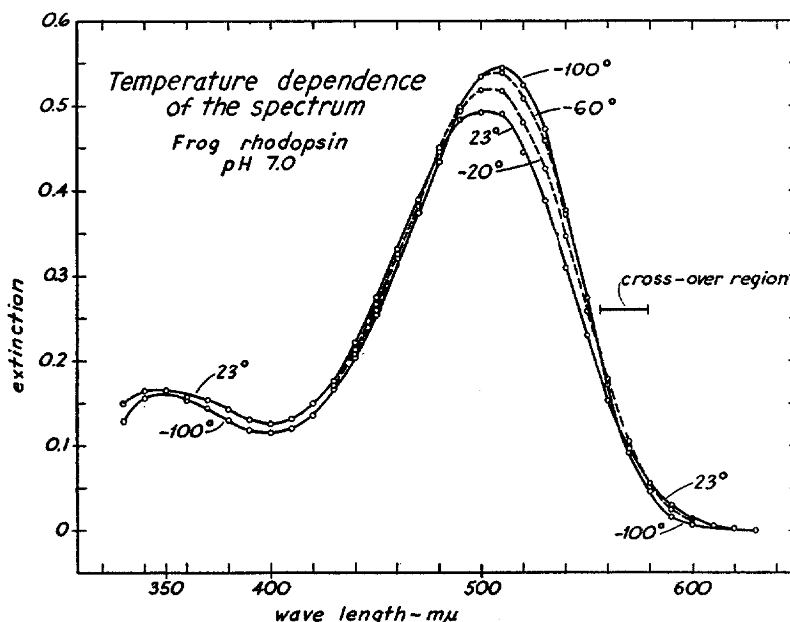

FIG. 7. Temperature dependence of the absorption spectrum of frog rhodopsin in in 2:1 glycerol-water mixture. The extinction was measured at 23, -20, -60, and -100°C. On *lowering* the temperature by 123°C., the maximum of the 500 mμ band *rises* and moves towards the red, while the band as a whole becomes narrower. At the "cross-over" region around 570 mμ light absorption is nearly independent of temperature. At longer wave lengths the absorption *rises* on *raising* the temperature. At 590 mμ the extinction changes by more than a factor of two over the 123°C. temperature interval.

over the range $-100°$ to 25°C. at wave lengths 10 mμ apart. From these plots temperature coefficients were determined for the fall of temperature of 30°C. ($Q_{30}^{\text{abs.}}$).

The effect of temperature on the absorption spectrum shows that the possession of thermal energy assists rhodopsin to absorb light of wave lengths longer than about 570 mμ. The effect of temperature on absorption is analogous to its effect on the bleaching rate, but there is some evidence that it is smaller in the case of absorption. When the values of the logarithm of $Q_{30}^{\text{abs.}}$ are plotted against the wave length, as in Fig. 8, a linear relation is found which may readily be

extrapolated to longer wave lengths. The values of $Q_{30}^{abs.}$ itself are replotted in Fig. 5 *a*, the measured values as a solid line joined at 620 mμ to a broken line which gives the extrapolated values. Apparently, the temperature dependence of absorption rises more slowly with increasing wave length than does the tem-

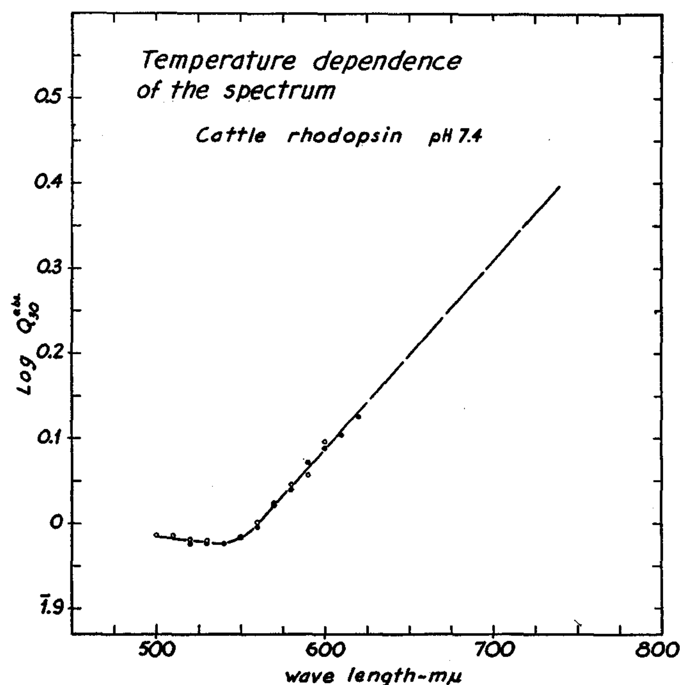

FIG. 8. Temperature dependence of the absorption spectrum of cattle rhodopsin in a 2:1 glycerol-water mixture. The temperature coefficient of absorption, $Q_{30}^{abs.}$, is the ratio of the per cent absorbed at 25°C., to that at −5°C. The common logarithm of $Q_{30}^{abs.}$ is plotted as a function of the wave length. The temperature dependence of absorption is small at shorter wave lengths but becomes increasingly larger in red light. Log $Q_{30}^{abs.}$ passes through the value zero corresponding to a temperature coefficient of 1 at 570 mμ. The points fall on a straight line between 560 and 620 mμ, and so may readily be extrapolated. The values of $Q_{30}^{abs.}$ are replotted in Fig. 5*a* in order to compare the temperature coefficients of absorption and of the bleaching rate.

perature dependence of the bleaching rate. At wave lengths longer than about 600 mμ Q_{30} is always about $1\frac{1}{2}$ times greater than $Q_{30}^{abs.}$.

Though we need experimental proof that the extrapolation is correct, it may be stated tentatively that the two processes which govern the bleaching rate, *i.e.* the absorption of light and the quantum efficiency, both become temperature-dependent in red light. If it is true that Q_{30} is greater than $Q_{30}^{abs.}$, then the

internal energy of the rhodopsin molecule is a more critical factor in bleaching than it is in the process of light absorption. Some of the molecules which absorbed a quantum of red light, but which had too little thermal energy before absorption to become fully activated by the quantum, do not bleach; and the quantum efficiency consequently falls.

IV

DISCUSSION

Energy Relations in Bleaching Rhodopsin.—The fact that the temperature coefficient increases as the quantum energy supplied decreases below 48.5 kg. cal. shows that rhodopsin can be bleached not only by light or heat alone, but also by a combination of both. At long wave lengths the photochemical reaction becomes partly thermal, but the total energy required for bleaching remains nearly the same (Table II). It is as if the photochemical reaction were merging into the thermal reaction in the red and infrared parts of the spectrum. At wave lengths shorter than 590 mμ, all the energy required for activation enters by the absorption of light in the chromophore. At longer wave lengths both the absorbed quantum and the thermal energy of the molecule are required for bleaching. Heat bleaching in the dark is the extreme case, in which the entire burden of providing activation energy falls on the internal energy of the molecule.

The rod sensitivity data of Griffin, Hubbard, and Wald fall into line with the idea that the minimum total energy needed for activation does not change in the transition from photochemical to heat bleaching. It has been shown that both the absorption of light by rhodopsin and its ability to react after absorption depend at long wave lengths on the thermal energy of the molecule. The low absorption and the low sensitivity to light at long wave lengths are due to the smallness of the population of molecules having a large enough energy.

At 1050 mμ the sensitivity of rod vision has fallen to $10^{-12.4}$ of its maximum value. $10^{12.4}$ times more light energy—or $10^{12.7}$ times more incident quanta—are needed than at 510 mμ in order to decompose enough rhodopsin to give a threshold stimulus in the dark-adapted eye. Evidently only one rhodopsin molecule in about 10^{13} is in a high enough state of internal energy to react at 1050 mμ.

According to the simplified form of the Maxwell distribution law

$$\frac{N_E}{N} = e^{-E/RT},$$

in which N_E/N is the fraction of molecules having energy greater than E in two degrees of freedom. 10^{-13} is the fraction of molecules having an internal energy of 16 kg. cal. per mole or more at room temperature. The energy of a mole of quanta at 1050 mμ is 27 kg. cal. This gives 43 kg. cal. as the minimum energy

needed for bleaching at 1050 mμ. This value is in good agreement with the minimum energy required for bleaching by heat alone or by light alone. Nevertheless, it should be pointed out that this argument and calculation rest on tentative assumptions.

It is clear that the protein portion of the rhodopsin molecule is involved in its thermal activation. In the first place, the temperature needed for rhodopsin to bleach thermally is about the same as that needed for the heat denaturation of other proteins in aqueous solution. In the second place, Lythgoe and Quilliam have shown that the bleaching rate and the activation energy of rhodopsin depend on pH except in the region of neutrality. This fact also points to an involvement of the protein because the absorption bands in the spectrum deriving from the prosthetic group do not vary with pH.[2]

These last two observations bear out the idea that the thermal energy component of bleaching in red light comes at least in part from the protein. If so, energy can move freely between the protein and the carotenoid prosthetic group. Similar energy transference is familiar elsewhere in chemistry, for example in the sensitization of photographic emulsions with dyes. How this comes about in rhodopsin will now be discussed.

Unimolecular Reactions and Predissociation.—On exposing rhodopsin to light in a glycerol-water glass at -100°C. or in a dry gelatin film at room temperature lumi-rhodopsin is formed instantaneously (Wald, Durell, and St. George, 1950). If this initial light reaction involved any other molecule in addition to rhodopsin it could not take place so rapidly under these conditions, which severely limit the motions of molecules. It seems reasonable to conclude that the light reaction of rhodopsin is always monomolecular, not only when extremely cold or dry, but in red light in solution at room temperature. If so, the thermal component of the activation energy needed in red light must come from the energy stored in the internal degrees of freedom of the molecule. This interpretation is in agreement with ideas that have gained some acceptance in the study of unimolecular reactions and predissociation.

The subject of unimolecular reactions in gases has been reviewed by Hinshelwood (1942). If the transfer of activation energy by collision were the rate-determining step in unimolecular decomposition, the rate constant at absolute temperature T would be equal to $k = Z\, e^{-E/RT}$, *i.e.* the product of the number of collisions in unit time, Z, and of the number of molecules having an activation energy of E or more. Moreover, the rate should depend on the pressure

[2] The activation energy of protein reactions calculated from temperature coefficient measurements is doubtful if the reaction rate is dependent on the pH, since temperature-dependent acid-base equilibria, by affecting the concentration of the reactive ionic species, may play a part in determining the temperature dependence of the rate (Steinhardt, 1937). In rhodopsin the pH dependence is not appreciable in the region of neutrality where the activation energies referred to here were found.

as does the collision number. However, the rate of unimolecular reactions is usually much faster than would be expected according to this equation and is independent of pressure over a wide range.

It seems that the transfer of energy between molecules cannot be rate-determining in unimolecular reactions. Instead it was suggested that intramolecular energy transfer limits the rate of activation. The energy so utilized would have been acquired in one or more collisions at an earlier time and stored in the internal degrees of freedom until, in the course of random fluctuations, a sufficient amount of energy was present in the reacting degrees of freedom for a chemical change to occur. The theory is consistent with the fact that most, if not all, molecules which react unimolecularly are complex.

Hinshelwood (1942, p. 81) gives the number of degrees of freedom which must be involved in the thermal decomposition of some organic molecules. He finds, for example, that 25 degrees of freedom must take part in the activation of azomethane and 45 to 50 in azoisopropane.

The effect of temperature on the thermal bleaching of rhodopsin has been described by Lythgoe and Quilliam (1938; *cf.* their Fig. 3). At pH 6.83 and $T = 320°$ K. the rate constant $k = 0.00013$ sec.$^{-1}$. Inserting these values in the expression for k, Z is found to be 10^{26} sec.$^{-1}$. This is much larger than the usual values one finds for the collision number; *i.e.*, around 10^{13} sec.$^{-1}$. One would therefore be justified in counting the thermal bleaching of rhodopsin among the unimolecular reactions in which internal energy from many degrees of freedom takes part.

The photochemical counterpart of unimolecular reactions is the phenomenon of predissociation. The study of this subject, reviewed by Burton and Rollefson (1938), has shown that it is possible for internal energy from many degrees of freedom to take part in a photochemical reaction.

The term predissociation was first used to describe regions in the absorption spectra of certain compounds where there is vibrational band structure, but no —or at most washed-out—rotational fine structure. The reason for this is believed to be that the excited molecule has a lifetime which is too short for rotational quantization to occur before it decomposes, but long enough for vibrational quantization to occur. Predissociation appears between a region of continuous absorption at shorter wave lengths, where decomposition follows directly upon absorption, and a region at longer wave lengths where the spectrum is fully quantized. In this intermediate region the quantum energy becomes a critical factor.

During the short lifetime of the predissociating molecule it may be possible for the internal energy to redistribute itself in such a way as to add itself to the absorbed light energy. In investigations on acetaldehyde and benzaldehyde vapor, Henri (1929) found that the minimum quantum energy required to bring about decomposition decreases with rising temperature. At the same time the

predissociation limits move to longer wave lengths. The decrease in the required quantum energy calculated from the difference in the wave length limits turns out to be equal to the increase in the thermal energy of the molecule as measured by the heat capacity at constant volume. This relationship led Henri to say: "There is an exact addition of thermal energy and light energy. . . . This temperature influence is especially high for molecules with great molecular specific heat where we have a large number of degrees of freedom."

Theory of Energy Transfer in Rhodopsin.—Predissociation occurs in the transition between quantum energies which bring about decomposition easily, and quantum energies which are ineffective, so that the molecules are as though in the dark, so far as chemical changes are concerned. In rhodopsin, it is tempting to think of the transition between photic and heat bleaching, which is found in red light, as a phenomenon closely related to predissociation.

There is no reason to suppose that any photosensitive molecule exists which does not show the utilization of thermal energy in photolysis at longer wave lengths. Even in Cl_2 the vibrational energy may contribute to photic activation.[3] The phenomenon in other simple molecules has escaped notice probably because it is not obvious. But in rhodopsin the role of internal energy becomes striking probably because, owing to the large number of degrees of freedom in the molecule, there is a relatively large amount of intramolecular thermal energy.

The very size and complexity of the rhodopsin molecule should bring about a certain facilitation of its energy exchanges compared with those of simpler molecules. In small molecules quantum mechanical rules limit the possibilities of gaining or losing energy to a few discontinuous, relatively big jumps. For this reason the spectra of simple gases at low pressures show sharp discrete spectral lines. But on liquefaction of such gases the discrete lines are replaced by continuous absorption bands because the molecules are brought close together and interact strongly. The existence of a large number of internal energy levels in larger molecules and the many possibilities for interaction within the molecule must also give rise to the continuous type of spectrum. Thus retinene$_1$, which is no more than a small part of the rhodopsin molecule, containing only 20 carbon atoms, already has a continuous and homogeneous spectrum.

In large molecules the many rotational and vibrational energy levels must be very close together, or they may be continuous with one another. The transference of thermal energy should be very easy because there is virtually no restriction on the smallness of the amount of energy which can be lost or gained.

[3] The temperature coefficient of the hydrogen-chlorine reaction has been discussed by Wulf (1930). The agreement which he finds between the observed and calculated values is fair. It should be pointed out that the agreement becomes excellent when the wave length of effective light is determined from the spectral transmission of the light filters and the "bleaching spectrum" of chlorine, rather than by visual observation through a spectroscope.

In rhodopsin, with thousands of atoms, it is probable that even a part of the molecule can gain or lose energy in a continuous way. Moreover, if the parts are closely coupled it should be possible for energy to flow freely from one part of the rhodopsin molecule to another. As a result, small contributions of energy from many parts could add up to give rise momentarily to a large collection of energy in one part, and eventually in the part which can utilize the energy to bring about bleaching.

The possibility of easy energy transfer within the rhodopsin molecule would mean that the limiting factor in the decomposition is the total energy available to the molecule. When a quantum of light is absorbed, the limiting factor is the total energy after absorption. The experiments indicate that this minimum total energy is 48.5 kg. cal. per mole, corresponding to 590 mμ, the wave length at which the temperature coefficient > 1 first appears. It is interesting in this connection that the work on the temperature dependence of absorption points to a temperature dependence of the quantum yield beginning at this wave length, indicating that some of the molecules which absorb at longer wave lengths than 590 mμ do not achieve a total energy content great enough for decomposition.

Although the temperature coefficients of the dark thermal bleaching and the bleaching at wave lengths longer than 590 mμ in Table II seem to indicate that less than 48.5 kg. cal. may cause bleaching, another interpretation is perhaps to be preferred. According to Hinshelwood (1942), the true activation energy of a unimolecular reaction may be greater than the value calculated from temperature dependence data by means of the Arrhenius equation (1). This occurs if more than two degrees of freedom are involved. It should be possible to extend this thought to photochemical reactions at long wave lengths where a thermal contribution is needed.

Let it be assumed that when red light of a given wave length is used to bleach rhodopsin, the molecules which react must have an internal energy equal to or greater than the difference between the energy of the quanta and the value 48.5 kg. cal. per mole. The fraction of molecules having an energy of E or more in n degrees of freedom is given by the following expression from Hinshelwood (1942 p. 80):

$$\frac{e^{-E/RT}\ (E/RT)^{1/2n-1}}{(1/2n - 1)!} \tag{2}$$

The temperature dependence of the rate constant should be equal to the temperature dependence of the population of reactive molecules. Taking the logarithm of expression (2) and differentiating with respect to temperature we find

$$\frac{d \ln k}{dT} = \frac{E - (1/2\ n - 1)RT}{RT^2} \tag{3}$$

Equation (3) shows that when n is large the energy actually needed is larger than the activation energy calculated by means of the Arrhenius equation by an amount equal to $(\frac{1}{2}n - 1)\ RT$.

The number of internal degrees of freedom which contribute thermal energy to bleaching rhodopsin could be calculated from equation (3) assuming that 48.5 kg. cal. per mole is the minimum energy needed for bleaching. The energies of activation in Table II calculated with the Arrhenius equation should be too small by an amount $\Delta = (\frac{1}{2}n - 1)\ RT$. Setting Δ equal to the difference between 48.5 kg. cal. per mole and the sum of the quantum energy and the Arrhenius energy one finds the values of n given in Table III.

The values of n given in Table III are small for a protein. They are of the size calculated by Hinshelwood for the thermal decomposition of much simpler

TABLE III

Degrees of Freedom in the Activation of Rhodopsin

Wave length	Δ	n
mμ	*kg.cal. per mole*	
590	0	2
650	1.3	6–7
700	2.6	11–12
750	4.1	15–16

molecules. However, these values were found by assuming that each degree of freedom contributes its entire energy, $\frac{RT}{2}$ calories per mole on the average. In view of the picture of rhodopsin energetics which is being developed here, it is more likely that each of a much larger number of degrees of freedom contributes a small fraction of its internal energy. Accordingly, the values of n in Table III are minimal values.

Moreover, one should not suppose that all the degrees of freedom in rhodopsin take part in the activation process. The measurements of Goodeve, Lythgoe, and Schneider (1941–42) imply that light of wave length 254 mμ has a very low efficiency in bleaching rhodopsin. This shows that the size of the quantum absorbed is less critical than that it be absorbed in an effective part of the molecule. As to the portion of the molecule which is involved in activation, the increase in n towards long wave lengths fits the idea that an increasingly larger part of the protein comes into play as the thermal component of the activation energy becomes greater.

It seems probable that the energy relations of rhodopsin are similar to those of other proteins. Energy transference between the prosthetic group and the protein, such as has been discussed in connection with rhodopsin, provides a reasonable explanation for the high efficiency with which quanta absorbed in

the protein moiety of carbon monoxide–myoglobin liberate CO from the heme group (Bücher and Kaspers, 1947). Another case in point is the carotenoid-sensitized fluorescence of chlorophyll in living diatoms; the absence of sensitization in acetone extracts of the pigments suggests that the protein of the photosynthetic complex is involved in the transference of energy (Dutton, Manning, and Duggar, 1943).

The great reactivity of proteins in general and the fact that as enzymes they confer reactivity on smaller molecules connected to them may depend on an ability to transfer activation energy from a considerable portion of the protein to the site of the reaction. Such energy transfer is foreshadowed in the phenomenon of predissociation, a process familiar in the chemistry of smaller molecules.

The writer wishes to express his gratitude to Professor Wald for having suggested this research and for help in carrying it out. Frequent conversations with him added clarity to the problem and enjoyment to the work. Particularly the discussion of energy relations in large molecules and their connection with the phenomenon of predissociation owes much to an unpublished essay on these matters written by Professor Wald while a graduate student in Selig Hecht's laboratory.

SUMMARY

Rhodopsin, the pigment of the retinal rods, can be bleached either by light or by high temperature. Earlier work had shown that when white light is used the bleaching rate does not depend on temperature, and so must be independent of the internal energy of the molecule. On the other hand thermal bleaching in the dark has a high temperature dependence from which one can calculate that the reaction has an apparent activation energy of 44 kg. cal. per mole.

It has now been shown that the bleaching rate of rhodopsin becomes temperature-dependent in red light, indicating that light and heat cooperate in activating the molecule. Apparently thermal energy is needed for bleaching at long wave lengths where the quanta are not sufficiently energy-rich to bring about bleaching by themselves. The temperature dependence appears at 590 mμ. This is the longest wave length at which bleaching by light proceeds without thermal activation, and corresponds to a quantum energy of 48.5 kg. cal. per mole. This value of the minimum energy to bleach rhodopsin by light alone is in agreement with the activation energy of thermal bleaching in the dark.

At wave lengths between 590 and 750 mμ, the longest wave length at which the bleaching rate was fast enough to study, the sum of the quantum energy and of the activation energy calculated from the temperature coefficients remains between 44 and 48.5 kg. cal. This result shows that in red light the energy deficit of the quanta can be made up by a contribution of thermal energy from the internal degrees of freedom of the rhodopsin molecule.

The absorption spectrum of rhodopsin, which is not markedly temperature-dependent at shorter wave lengths, also becomes temperature-dependent in

red light of wave lengths longer than about 570 to 590 mμ. The temperature dependence of the bleaching rate is at least partly accounted for by the temperature coefficient of absorption.

There is some evidence that the temperature coefficient of bleaching is somewhat greater than the temperature coefficient of absorption at wave lengths longer than 590 mμ. This means that the thermal energy of the molecule is a more critical factor in bleaching than in absorption. It shows that some of the molecules which absorb energy-deficient quanta of red light are unable to supply the thermal component of the activation energy needed for bleaching, so bringing about a fall in the quantum efficiency.

The experiments show that there is a gradual transition between the activation of rhodopsin by light and the activation by internal energy. It is suggested that energy can move freely between the prosthetic group and the protein moiety of the molecule. In this way a part of the large amount of energy in the internal degrees of freedom of rhodopsin could become available to assist in thermal activation.

Assuming that the minimum energy required for bleaching is 48.5 kg. cal., an equation familiar in the study of unimolecular reaction has been used to estimate the number of internal degrees of freedom, n, involved in supplying the thermal component of the activation energy when rhodopsin is bleached in red light. It was found that n increases from 2 at 590 mμ to a minimum value of 15 at 750 mμ. One wonders what value n has at 1050 mμ, where vision still persists, and where rhodopsin molecules may supply some 16 kg. cal. of thermal energy per mole in order to make up for the energy deficit of the quanta.

APPENDIX

Preparation of Rhodopsin Solutions

(*a*) *Preparation of Frog Rhodopsin from Whole Retinas.*—The following procedure, which is essentially that of earlier workers (Wald, 1937–38), was followed in preparing rhodopsin solutions.

After dark-adapting bullfrogs for 8 hours or more, the animals are killed and the eyeballs removed in the dark room under a ruby lamp. The optic nerve and the portion of the sclera surrounding it are sliced away with a razor blade so that the retina is no longer held to the optic cup by the nerve. A longitudinal slit along the periphery of the iris is made with the razor, and the anterior portion of the eye separated by extending the slit around the periphery with a pair of sharp scissors. When the front part of the eye is lifted off, the lens and the retina come with it. In this way retinas are obtained without any visible pigment epithelium attached to them. The retinas are then cut away from the lens and placed in 4 per cent aluminum alum.

After 4 hours the retinas are centrifuged down, and, after pouring off the alum, washed twice with distilled water, once with 0.2 molar phosphate buffer, pH 7.0, and again with water, centrifuging each time. Finally 2 per cent digitonin is added; the retinas are slurried with a stirring rod and allowed to stand at room tempera-

ture overnight. The retinas and the colloidal material are then separated from the rhodopsin solution by centrifuging at 12,000 to 14,000 R.P.M. for 10 minutes or more.

Solutions prepared in this way had a 400/500 ratio[4] of 0.30 to 0.38. The yield is roughly such as to give an extinction of about 1 at 500 mμ for six large retinas in 1 ml. of solution.

(*b*) *Cattle Rhodopsin from Isolated Rods.*—It is difficult to dissect retinas free of pigment epithelium from cattle eyes, so a different method was used to prepare clean rhodopsin solutions. The lower density of rod outer limbs as compared to the rest of the tissues was used to advantage. The rods were separated from the retinas by centrifuging in a sucrose solution of density such that the rods went to the top while most of the remaining tissue went to the bottom (Saito, 1938).

The retinas are scooped out of the eyes into a chilled mortar without making any attempt to remove the large amount of pigment epithelium adhering to them. The retinas are then ground gently, without pressure, for 3 to 5 minutes. After this a solution of 33.5 gm. of sucrose in 66.5 ml. of 0.1 M phosphate buffer is stirred up with the slurried tissue (about 4 ml. of solution for every five retinas). It is important that salts be present in the sucrose solution, since otherwise a stringy mass of protein is formed. The suspension is centrifuged at high speed (12,000 to 14,000 R.P.M.) for 10 minutes. The rods, which rise to form a red layer at the top of the liquid, are scooped off, suspended in alum solution, and collected 4 hours later by centrifuging. The tissue obtained in this way is almost free of pigment epithelium, and appears on microscopic examination to contain about 50 per cent damaged rods and 50 per cent other tissue debris. Rods are washed by the usual procedure, except that the high-speed centrifuge is used each time. Finally the rods are frozen-dried and extracted twice with petroleum ether for 4 hours in the cold. One hundred eyes yield about 0.3 gm. of dried tissue, which gives up all its rhodopsin to 2 per cent digitonin in an hour or less. The 400/500 ratio is usually 0.25 to 0.28. The total rhodopsin concentration obtained from 100 eyes may give an extinction of 1 in as much as 40 ml. of solution, but the batches which give a lower yield usually give a more stable preparation. The extraction with petroleum ether is necessary because of the high lipid content of the tissue. If fats are present, they quickly saturate the digitonin and diminish its capacity to extract rhodopsin.

REFERENCES

Broda, E. E., and Goodeve, C. F., 1941–42, The behaviour of visual purple at low temperature, *Proc. Roy. Soc. London, Series B*, **130,** 217.

Bücher, T., and Kaspers, J., 1947, Photochemische Spaltung des Kohlenoxydmyoglobins durch ultraviolette Strahlung (Wirksamkeit der durch die Proteinkomponente des Pigments absorbierten Quanten), *Biochim. et Biophysic. Acta,* **1,** 21.

Burton, M., and Rollefson, G. K., 1938, Primary processes in predissociation, *J. Chem. Physics*, **6,** 416.

[4] The ratio of the extinction at 400 mμ to that at 500 mμ. This is the criterion of optical purity suggested by Wald (1937–38). In the purest rhodopsin solutions this ratio has a value of about 0.25.

Dutton, H. J., Manning, W. M., and Duggar, B. M., 1943, Chlorophyll fluorescence and energy transfer in the diatom, *Nitzschia closterium*, *J. Physic. Chem.*, **47**, 308.

Goodeve, C. F., Lythgoe, R. J., and Schneider, E. E., 1941–42, The photosensitivity of visual purple solutions and the scotopic sensitivity of the eye in the ultraviolet, *Proc. Roy. Soc. London, Series B*, **130**, 380.

Griffin, D. R., Hubbard, R., and Wald, G., 1947, The sensitivity of the human eye to infra-red radiation, *J. Opt. Soc. America*, **37**, 546.

Hecht, S., 1920–21, Photochemistry of visual purple. II. The effect of temperature on the bleaching of visual purple by light, *J. Gen. Physiol.*, **3**, 285.

Henri, V., 1929, Absorption spectra of polyatomic molecules—Predissociation and dissociation of these molecules, *Tr. Faraday Soc.*, **25**, 756. (See also remarks of Herzberg and Henri's reply, p. 778.)

Hinshelwood, C. N., 1942, The Kinetics of Chemical Change, Oxford University Press.

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

Saito, Z., 1938, Isolierung der Stäbchenaussenglieder und spektrale Untersuchung des daraus hergestellten Sehpurpurextraktes, *Tohoku J. Exp. Med.*, **32**, 432.

Steinhardt, J., 1937, The stability of crystalline pepsin, *K. Danske Vidensk. Selsk. Mat.-fys. Medd.*, **14**, No. 11.

Wald, G., 1937–38, On rhodopsin in solution, *J. Gen. Physiol.*, **21**, 795.

Wald, G., 1945, The spectral sensitivity of the human eye. I. A spectral adaptometer, *J. Opt. Soc. America*, **35**, 187.

Wald, G., 1949, The photochemistry of vision, *Documenta Opthalmologica*, **3**, 94.

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

Wulf, O. R., 1930, The temperature coefficient of the photochemical formation of hydrogen chloride, *Proc. Nat. Acad. Sc.*, **16**, 27.