Tautomeric Forms of Metarhodopsin

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ABSTRACT Light isomerizes the chromophore of rhodopsin, 11-*cis* retinal (formerly retinene), to the all-*trans* configuration. This introduces a succession of unstable intermediates—pre-lumirhodopsin, lumirhodopsin, metarhodopsin —in which all-*trans* retinal is still attached to the chromophoric site on opsin. Finally, retinal is hydrolyzed from opsin. The present experiments show that metarhodopsin exists in two tautomeric forms, metarhodopsins I and II, with λ_{max} 478 and 380 mμ. Metarhodopsin I appears first, then enters into equilibrium with metarhodopsin II. In this equilibrium, the proportion of metarhodopsin II is favored by higher temperature or pH, neutral salts, and glycerol. The change from metarhodopsin I to II involves the binding of a proton by a group with pK 6.4 (imidazole?), and a large increase of entropy. Metarhodopsin II has been confused earlier with the final mixture of all-*trans* retinal and opsin (λ_{max} 387 mμ), which it resembles in spectrum. These two products are, however, readily distinguished experimentally.

On exposure to light, rhodopsin bleaches over transient intermediates to an eventual mixture of retinal[1] and opsin. If one begins with cattle rhodopsin, for example, at the temperature of liquid nitrogen (−195°C), the product of irradiation is pre-lumirhodopsin with λ_{max} 543 mμ. On warming above −140°C, this goes over in the dark to lumirhodopsin (λ_{max} 500 mμ), and above −40°C, to metarhodopsin (λ_{max} 478 mμ) (*cf.* Yoshizawa and Wald, 1963). Above −15°C, metarhodopsin fades to a yellow compound, and since this reaction occurs only in the presence of water, we have heretofore assumed that it corresponds to the hydrolysis of metarhodopsin to all-*trans* retinal (λ_{max} about 387 mμ) and opsin (*cf.* Wald, Durell, and St. George, 1950; Hubbard and Kropf, 1958).

This hydrolysis, however, is slow, requiring about an hour for completion

[1] We shall use the nomenclature proposed by the Commission on the Nomenclature of Biological Chemistry of the International Union of Pure and Applied Chemistry (1960), according to which vitamin A is now known as retinol, and retinene (vitamin A aldehyde), as retinal. We shall, however, refer to retinene oxime as retinaldehyde oxime, and on occasion to retinene as retinaldehyde, in order to avoid the confusion which might be engendered by the use of "retinal" in its adjectival form.

at room temperature, and many hours in the neighborhood of 0°C (Q_{10} = 2 to 3). Yet we observed some years ago that the irradiation of solutions of frog rhodopsin at 5°C yields almost immediately an absorption band with λ_{max} 380 mμ, resembling that of retinal, though displaying an abnormally high absorbance by comparison with the amount of rhodopsin that had gone into its formation. There is an obvious contradiction between the slow rate of hydrolysis of metarhodopsin and the rapid appearance of this yellow product resembling free retinal. Unable to resolve this anomaly at the time, we laid the experiments aside in the hope that later work might clarify them (Wald and Brown, unpublished observations).

A related observation was reported by Hagins (1956), working with excised rabbit eyes. He found that upon exposing the dark-adapted eye to a brilliant flash of light lasting 20 microseconds, there was a transient rise in absorption at 486 mμ, presumably owing to the formation of metarhodopsin, followed by an exponential decay of this product to one absorbing strongly at 400 mμ. The half-time of decay was 20 milliseconds at 12°C, and 1 millisecond at 26°C. This process looked like the hydrolysis of metarhodopsin to retinal and opsin, yet was thousands of times more rapid than the latter reaction in solution.

An accumulation of such unexplained observations in this and other laboratories led us to undertake the present investigation. Its main point is to describe an intermediate of bleaching that follows metarhodopsin at temperatures above about −15°C. Like metarhodopsin, it contains all-*trans* retinal attached to opsin at the chromophoric site of rhodopsin. However, whereas earlier intermediates are highly colored, having absorption maxima at longer or only slightly shorter wave lengths than rhodopsin, this pigment is pale yellow, with λ_{max} at 380 mμ. We shall call this intermediate *metarhodopsin II,* because of its special relationship to its immediate precursor, heretofore called simply "metarhodopsin," which we shall now rename *metarhodopsin I.*

On warming in the dark, metarhodopsin II is hydrolyzed to all-*trans* retinal and opsin. This reaction is accompanied by a shift of λ_{max} from 380 to 387 mμ, and a decrease in ϵ_{max} from 42,000 to 39,000. The spectroscopic properties of cattle rhodopsin, metarhodopsins I and II, and free retinal are summarized in Table I.

Metarhodopsins I and II are in thermal tautomeric equilibrium, which is not promoted in either direction by light:

$$\underset{\lambda_{max}\ 478\ m\mu}{\text{Metarhodopsin I}} \rightleftharpoons \underset{\lambda_{max}\ 380\ m\mu}{\text{metarhodopsin II.}} \qquad (1)$$

This equilibrium depends upon the temperature, pH, solvent, and the species of rhodopsin. Increase in temperature, in acidity (within the pH stability range of about 5 to 8 at 4°C), or in ionic strength, and replacement of water

by glycerol or methanol, all shift the equilibrium toward metarhodopsin II. Also, for a given setting of these conditions, the proportion of metarhodopsin II at equilibrium increases progressively in preparations from cattle, leopard frogs (*Rana pipiens*), bullfrogs (*Rana catesbiana*), and cusk.

On exposure to light, the all-*trans* chromophore of either metarhodopsin I or II is isomerized, in part to the 11-*cis* and 9-*cis* configurations. Since in the metarhodopsins retinal is still attached at the chromophoric site on opsin, these isomerizations yield directly rhodopsin and isorhodopsin:

$$\underset{\text{(all-}trans\text{)}}{\text{Metarhodopsin I or II}} \overset{\text{light}}{\rightleftharpoons} \underset{\text{(11-}cis\text{)}}{\text{rhodopsin}} + \underset{\text{(9-}cis\text{)}}{\text{isorhodopsin}} \qquad (2)$$

TABLE I

ABSORPTION MAXIMA (λ_{max}) AND MAXIMUM MOLAR ABSORBANCES (ϵ_{max}) AT 4°C OF CATTLE RHODOPSIN AND PRODUCTS OF ITS IRRADIATION IN AQUEOUS DIGITONIN

Compound	λ_{max}	ϵ_{max}	Result of irradiation with ultraviolet light at about 0°C
	mμ	*liter/mole cm*	
Rhodopsin	499	42,000	
Metarhodopsin I	478	45,000	
Metarhodopsin II	380	42,000	Formation of rhodopsin, isorhodopsin, and 465 mμ pigment, too rapid to measure the rate
All-*trans* retinal + opsin	387	39,000	Decrease in ϵ_{max}, and shift of λ_{max} to shorter wave length; formation of rhodopsin and isorhodopsin with half-time of about 9 hrs.
Alkaline indicator yellow	367	41,000	No change

In addition to the isomerization of the chromophore, this transformation involves some configurational rearrangement of opsin; but all of this goes rapidly, even at temperatures as low as −20°C. Prolonged irradiation of either tautomer of metarhodopsin therefore yields steady state mixtures of the metarhodopsins with rhodopsin and isorhodopsin (*cf.* Hubbard and Kropf, 1958; Kropf and Hubbard, 1958).

Of course such irradiations demand light that the metarhodopsins can absorb, blue-green light being most effective with metarhodopsin I, and near ultraviolet with metarhodopsin II. (The irradiation of metarhodopsin II with ultraviolet light yields also a new pigment with λ_{max} about 465 mμ, of which more below.)

Irradiation with near ultraviolet light provides a ready means of distinguishing metarhodopsin II from the mixture of all-*trans* retinal and opsin that results from its hydrolysis. As already said, such irradiation of metarho-

dopsin II yields a steady state mixture of that pigment with rhodopsin, isorhodopsin, and the 465 mμ pigment, even at low temperatures. A similar irradiation of all-*trans* retinal yields in part the free 11-*cis* and 9-*cis* isomers, which can combine with opsin to yield rhodopsin and isorhodopsin:

$$\text{All-}trans\text{ retinal} \xrightleftharpoons{\text{ultraviolet light}} 11\text{-}cis + 9\text{-}cis\text{ retinal} \xrightarrow{\text{opsin}} \text{rhodopsin} + \text{isorhodopsin} \quad (3)$$

The condensation of retinal with opsin, however, is a slow reaction, with a half-time of about 15 minutes at 20°C, and a high temperature coefficient (Q_{10} about 6). In summary, therefore, irradiation of metarhodopsin II with ultraviolet light near 0°C causes a fall in absorption in the ultraviolet and a simultaneous rise in the visible. Similar irradiation of the mixture of all-*trans* retinal and opsin shifts the spectrum toward slightly shorter wave lengths and depresses the absorbance, due to the formation of *cis* isomers of retinal, which at this temperature condense with opsin with a half-time of about 9 hours.

In a recent communication, Bridges (1962) has reported experiments with frog rhodopsin which overlap to a degree with some of those we are about to describe. His interpretation, however, differs substantially from ours, in that he identifies the yellow precursor of retinal with "alkaline indicator yellow," originally described by Lythgoe and Quilliam (1938).

We have already taken pains to distinguish two ultraviolet-absorbing products of bleaching rhodopsin which have sometimes been confused: metarhodopsin II and the mixture of retinal and opsin into which it hydrolyzes. We now must extend these distinctions to cover two further terms that enter this same province: alkaline indicator yellow and *N*-retinylidene-opsin.

All these terms apply to substances which absorb in overlapping regions of the ultraviolet. Their spectroscopic properties and behavior upon irradiation with ultraviolet light are summarized in Table I.

Ball *et al.* (1949) showed that alkaline indicator yellow is an indiscriminate collection of Schiff bases of retinal with all the available amino groups on opsin (and whatever other proteins happen to be present):

$$\underset{\substack{\text{retinal}\\(ca.\ 387\ m\mu)}}{C_{19}H_{27}C(H)=O} + H_2N\text{-opsin} \rightleftharpoons \underset{\substack{\text{Schiff base}\\(ca.\ 367\ m\mu)}}{C_{19}H_{27}C(H)=N\text{-opsin}} + H_2O \quad (4)$$

Later, Collins (1953) and Morton and Pitt (1955) showed that in rhodopsin itself, the retinal chromophore is attached to opsin in Schiff base linkage, in this case, however, involving a specific amino group in the chromophoric site.

Both these later investigations, though demonstrating this point clearly, involved experimental conditions which undoubtedly produced a denatured product, the Schiff base of retinal with denatured opsin in Collins's experiment (λ_{max} 367 mμ), and its conjugate acid ($C_{19}H_{27}CH{=}NH^+$–denatured opsin; λ_{max} 440 mμ) in the experiments of Morton and Pitt.

Pitt *et al.* (1955) and Morton and Pitt (1957) suggested that the specific Schiff base of retinal at the chromophoric site be called *N*-retinylidene-opsin, and that the term indicator yellow be reserved for the random collection of Schiff bases of retinal with all available amino groups.

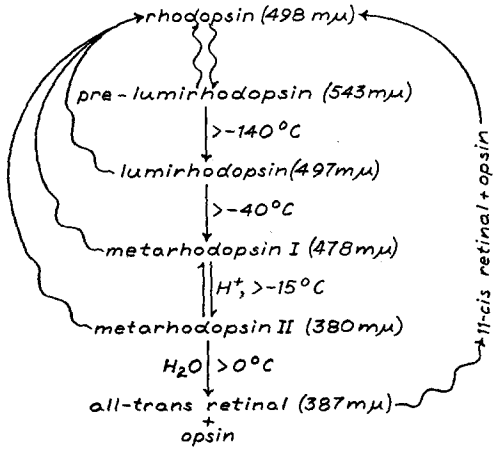

FIGURE 1. Stages in the bleaching of rhodopsin. Photochemical reactions are denoted by wavy lines, thermal (dark) reactions, by straight lines. The photoisomerization of the 11-*cis* chromophore of rhodopsin to the all-*trans* configuration, present in all subsequent compounds, is followed by a series of thermal reactions, which alter the configurational interplay between the chromophore and opsin, and terminate in the hydrolytic cleavage into opsin and all-*trans* retinal. Light can regenerate rhodopsin at each stage. The regeneration from pre-lumirhodopsin probably involves only the photoisomerization of the chromophore from all-*trans* to 11-*cis*. However, the photoconversion of all subsequent products back to rhodopsin must involve also thermal reactions, since they arise from rhodopsin by a combination of photochemical and thermal reactions. However, only when rhodopsin is photoregenerated from all-*trans* retinal + opsin are the thermal reactions slow enough for us to observe them.

Metarhodopsin II is readily distinguished from alkaline indicator yellow, so defined (*cf.* Table I), for it possesses a longer λ_{max} and a slightly higher molar absorbance than alkaline indicator yellow. Moreover, unlike metarhodopsin II, alkaline indicator yellow is *not* isomerized by light.

On the other hand, one could with some justice equate metarhodopsin II with *N*-retinylidene-opsin. Since, however, its absorption maximum lies at an appreciably longer wave length than that of a simple Schiff base of retinal, and because of its special relationship to metarhodopsin I, we prefer to call

it metarhodopsin II. It is, as we shall show, a true physiological intermediate in the bleaching of rhodopsin; and so far as we yet know, it is the immediate precursor of retinal and opsin. In these terms the stages in the bleaching of rhodopsin can be summarized as in Fig. 1.

EXPERIMENTAL RESULTS

Effects of pH and Composition of the Solvent on the Metarhodopsin Equilibrium

Fig. 2 shows equilibria between metarhodopsins I and II at various pH's.

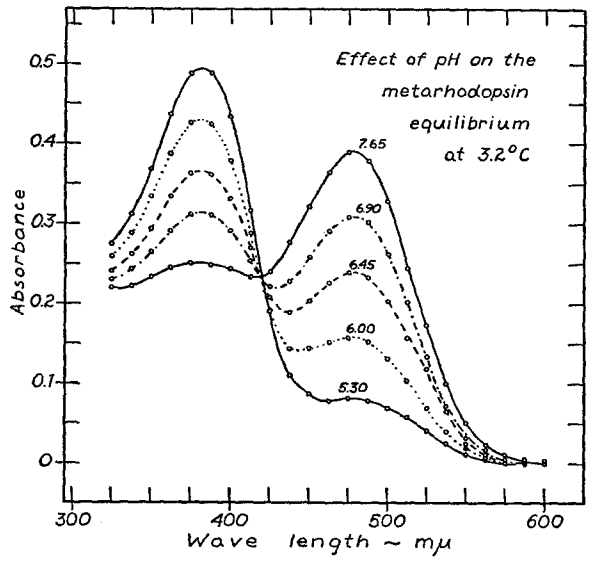

FIGURE 2. Effect of pH on the metarhodopsin equilibrium. Aliquots of rhodopsin at the pH's shown in the figure are cooled to 3.2°C in place in the Cary spectrophotometer (see Appendix for a description of the constant temperature cell). After recording the initial spectrum, not shown in the figure, each rhodopsin sample is irradiated 6 min. with light at 578 mμ, isolated from the emission spectrum of a General Electric H-3 mercury arc by means of a Jena OG 2 filter. The absorption spectrum is recorded again, and corrected as described in the text to yield the curves shown. Acidity favors metarhodopsin II; all curves pass through an isosbestic point at about 420 mμ.

Increase in H^+ concentration favors metarhodopsin II, the relationship following approximately the titration curve of one ionizable group with pK 6.4. The curve we observe departs from the theoretical curve for such a titration to the extent shown in Fig. 3, perhaps due to the ionic strength of the solvent in which these measurements were made (0.17 M phosphate buffer), since the departures are even greater at higher ionic strengths. Increase in ionic strength favors metarhodopsin II, but this effect varies with pH in a way that foreshortens the titration curve. However, ionic strength has little or no effect on the apparent pK of the titration curve.

We shall briefly describe the procedure by which the data in Fig. 2 were obtained, since it is used throughout the experiments which follow. Aliquots of a solution of cattle rhodopsin in 2 per cent aqueous digitonin were brought to the pH's shown in Fig. 2 by adding phosphate buffer, to a final concentration of 0.17 M. In each case the rhodopsin solution had a maximal absorbance (E_{max}) of 0.439. Each sample was irradiated with orange light as described in the legend to Fig. 2. Its spectrum was recorded 30 seconds after the light was turned off, and again 10 minutes later. Both these spectra were virtually identical, since at 3.2°C equilibrium is attained during the irradiation (*cf.* Fig. 6, below).

This type of experiment always yields photosteady state mixtures of metarhodopsin, rhodopsin, and isorhodopsin (Hubbard and Kropf, 1958; Kropf and Hubbard, 1958), which must be analyzed further to determine how much metarhodopsin I and II they

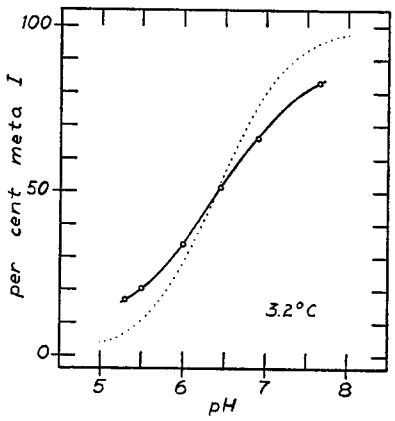

FIGURE 3. Effect of pH on the metarhodopsin equilibrium. Summary of data from Fig. 2 and similar experiments, plotted as a titration curve. The curve is roughly symmetrical about its midpoint (pK) at about pH 6.4. The theoretical titration curve for an ionizable group with pK 6.4 is dotted in for comparison.

contain. For this purpose, hydroxylamine was added at a final concentration of 0.1 M and the solution was warmed to room temperature in the dark. This converts all the metarhodopsin to all-*trans* retinaldehyde oxime + opsin. The concentration of rhodopsin + isorhodopsin is then estimated from the difference spectrum, the change of absorption spectrum caused by bleaching with white light. In the present experiment, this amounts to 10 to 20 per cent of the initial rhodopsin content. From the known spectra of rhodopsin and isorhodopsin, the spectrum of the photoregenerated mixture is constructed, and this small correction is then subtracted from the spectrum of the photosteady state mixture to yield the spectra of metarhodopsins I and II alone. These are shown in Fig. 2.

The effect of glycerol on the metarhodopsin equilibrium is shown in Figs. 4 and 5. Increasing glycerol concentrations favor metarhodopsin II (Fig. 4); and this effect is reversed, though not completely, upon dilution with buffer (Fig. 5). The same is true when changing pH: on raising the pH at 4°C, metarhodopsin II is converted back to metarhodopsin I, but never quite to the extent one would expect from Fig. 3. This is probably due to the fact

that prolonging the experiment, even at 4°C, allows a small amount of metarhodopsin II to be hydrolyzed (*cf.* Fig. 8, below). Furthermore undoubtedly small, transient rises of temperature occur during our dilutions of glycerol and changes of pH.

Methanol, in concentrations that do not denature metarhodopsin, acts qualitatively like glycerol. So also do such neutral salts as lithium bromide, or sodium and potassium phosphate. Sucrose, however, does not affect the metarhodopsin equilibrium.

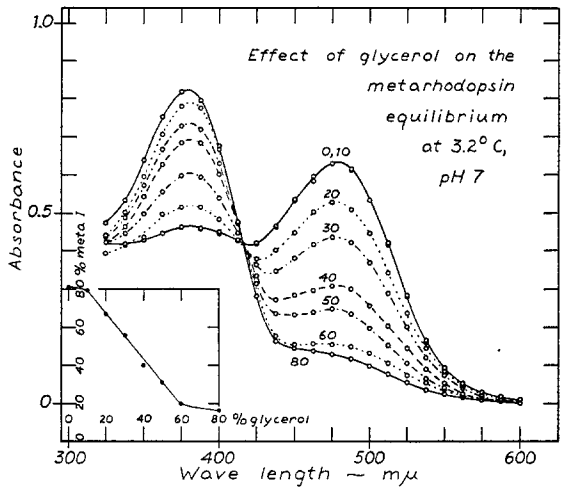

FIGURE 4. Effect of glycerol on the metarhodopsin equilibrium. Cattle rhodopsin in 1 per cent digitonin and M/10 neutral phosphate buffer was mixed with various dilutions of glycerol. All samples had E_{500} of 0.737; the glycerol concentrations are shown in the figure. After cooling to 3.2°C, each sample was irradiated with light at 578 mμ, and its absorption spectrum recorded and corrected as described for Fig. 2. The metarhodopsin equilibrium is the same in aqueous solution and 10 per cent glycerol; but as the glycerol concentration increases, the amount of metarhodopsin I at equilibrium decreases by about 12 per cent for each 10 per cent increase in glycerol (see insert at the lower left). The effect levels off between 60 and 80 per cent glycerol; and cannot be followed to pure glycerol, since rhodopsin denatures in this solvent. The absorption spectra are isosbestic at about 417 mμ.

Effect of Temperature The effect of temperature on the metarhodopsin equilibrium is summarized in Fig. 6. At −20°C, cattle metarhodopsin I is stable indefinitely. As the temperature is raised, successive equilibria are established which progressively favor metarhodopsin II. Lowering the temperature in the dark reverses these changes; again, however, with some apparent loss of total chromoprotein, probably by hydrolysis.

In Fig. 7, the constant, K, for the metarhodopsin equilibrium is plotted as a function of temperature. This yields a heat (ΔH) of +13,100 cal per mole

for the reaction, cattle metarhodopsin I → II. From these data one can calculate the free energies (ΔF) and entropies (ΔS) shown in Table II.

Temperature affects not only the position of equilibrium, but also the rate at which it is attained. This is in contrast to the effect of glycerol, which shifts the position of the equilibrium, but has little or no effect on the rate of achieving it.

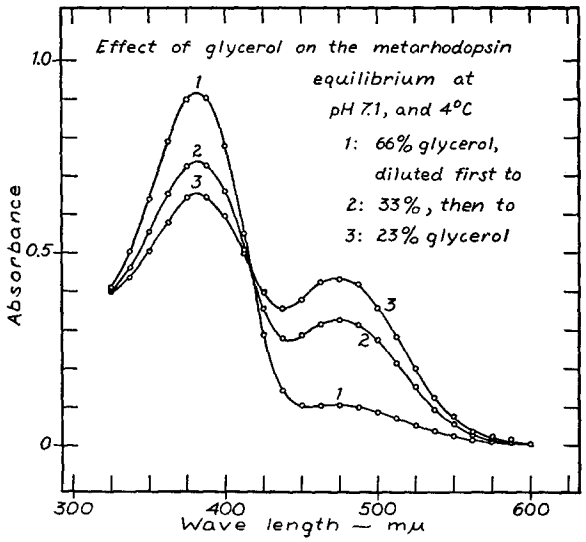

FIGURE 5. Reversibility of the glycerol effect. Cattle rhodopsin in neutral 66 per cent glycerol (E_{500} = 0.835) is cooled to 4°C, and bleached 7 min. with orange light from a 100 watt tungsten lamp shielded by a Jena OG 2 filter. Curve 1, recorded at this point, shows that the equilibrium strongly favors metarhodopsin II. The glycerol concentration is now reduced in the dark by dilution with ice cold phosphate buffer, first to 33 per cent, then to 23 per cent, and the spectrum recorded after each dilution. These spectra, corrected for dilution, are shown in curves 2 and 3. Each dilution shifts the equilibrium about ⅓ of the way to the value it would have, had the sample been irradiated at that concentration of glycerol. As in Fig. 4, the spectra are isosbestic at about 417 mμ.

The rates at which metarhodopsin I is converted to the equilibrium mixture at three temperatures are shown in the insert to Fig. 6. The half-times ($t_{1/2}$) decrease from about 100 minutes at −9° to about 1 minute at +1°, showing that the reaction, metarhodopsin I → II, has the very large temperature coefficient (Q_{10}) of approximately 100.

Recapitulation The transformation of metarhodopsin I to the hydrolyzed product, all-*trans* retinal + opsin, proceeds over an intermediate, metarhodopsin II, which has an absorption spectrum close to that of free retinal, but contains all-*trans* retinal still attached to opsin at the chromophoric site.

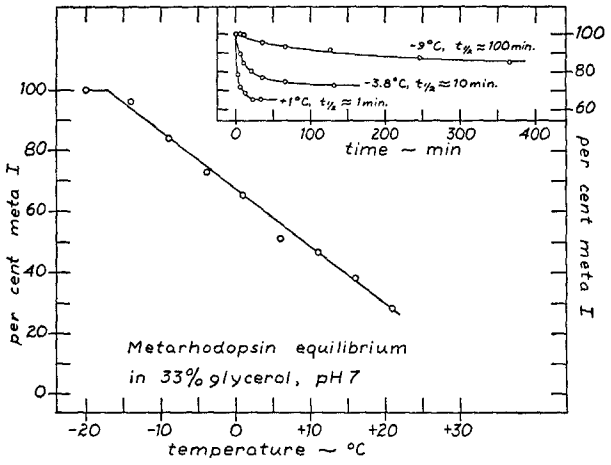

FIGURE 6. Effect of temperature on the metarhodopsin equilibrium, measured in neutral 33 per cent glycerol. Aliquots of cattle rhodopsin at various temperatures are irradiated 1.5 min. with light of 578 mμ, and the absorption spectra are subsequently recorded in the dark, to follow the reaction, metarhodopsin I → II, to equilibrium. Metarhodopsin I is stable below −17°C; but as the temperature increases, there is a proportional decrease in the amount of metarhodopsin I at equilibrium. The rates at which equilibria are established at −9°, −3.8°, and +1°C are shown in the insert; above +1°C, equilibrium is reached too rapidly for us to measure rates.

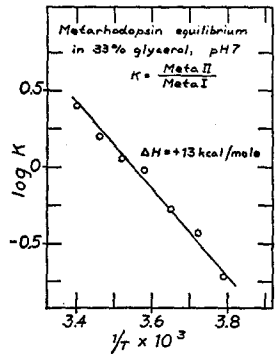

FIGURE 7. Heat (ΔH) of the reaction, metarhodopsin I → II. This is obtained from the type of experiment shown in Fig. 6, by the equation,

$$\log \frac{K_1}{K_2} = -\frac{\Delta H}{2.303R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right),$$

where K_1 and K_2 are the equilibrium constants at absolute temperatures, T_1 and T_2, and R is the gas constant. Plotting log K against $1/T$ yields the straight line shown. From its slope one arrives at a value of ΔH of +13,100 cal per mole.

Metarhodopsins I and II exist in a tautomeric equilibrium in which higher temperature or acidity, and glycerol, methanol, or neutral salts favor metarhodopsin II. This equilibrium also varies from species to species. Light has no effect upon it.

Other Reactions of Metarhodopsin II. The 465 mμ Pigment We have so far discussed metarhodopsin II primarily as an intermediate in the bleaching of rhodopsin—that is, in relation to metarhodopsin I, its precursor, and to all-*trans* retinal + opsin, its product. This pigment, however, takes part also in a

number of other reactions, which have an intrinsic interest, though they may not ordinarily enter the visual cycle.

As shown in Fig. 8, metarhodopsin II, on being held in the dark at 3°C, is slowly converted to a compound with λ_{max} about 465 mμ. This compound differs from metarhodopsin I not only in λ_{max}, but also in that it is converted back to metarhodopsin II *by light*, yet not in the dark. Nor should it be confused with the mixture of rhodopsin and isorhodopsin usually found in these

TABLE II

THERMODYNAMICS OF THE EQUILIBRIUM OF CATTLE METARHODOPSIN IN NEUTRAL 33 PER CENT GLYCEROL

Values of the equilibrium constant, K, are calculated from the concentrations of metarhodopsins I and II present at different absolute temperatures, T (*cf.* Fig. 6), by the equation, K = (metarhodopsin II)/(metarhodopsin I). The free energies (ΔF) are obtained by the equation, $\Delta F = RT \ln K$, where R is the gas constant. ΔF, together with ΔH, determined graphically in Fig. 7, yields the values of ΔS in entropy units (e.u.), by the familiar relationship, $\Delta F = \Delta H - T\Delta S$.

I T	II K	III ΔF	IV ΔS
°K		*cal/mole*	*e. u.*
264	0.193	+865	+46.3
269	0.374	+526	+46.7
274	0.532	+344	+46.6
279	0.953	+26.8	+46.9
284	1.14	−75.9	+46.4
289	1.60	−271	+46.3
294	2.49	−535	+46.4
Average			+46.5

experiments. It differs from them in λ_{max}, and in that 0.1 M hydroxylamine converts its chromophore to retinaldehyde oxime in the dark. Moreover the concentrations of rhodopsin and isorhodopsin remain constant throughout this experiment.

The 465 mμ compound is likely to be seen under all conditions which tilt the metarhodopsin equilibrium toward metarhodopsin II. It therefore often appears in experiments with rhodopsin extracted from scup or cusk, two marine fishes in which metarhodopsin II is favored considerably more than in cattle preparations (Brown and Brown, unpublished observations). The 465 mμ pigment is stable at 4°C, but at higher temperatures goes over to retinal and opsin.

Wald (1937–38) described the formation and subsequent disappearance

of what is probably the same compound at room temperature, when bleaching frog rhodopsin in mildly acid aqueous digitonin (*cf.* Wald's "thermal components I and II;" see also Bridges, 1962). The characteristic feature at room temperature is a *rise* in absorption in the visible region as a transient component of bleaching.

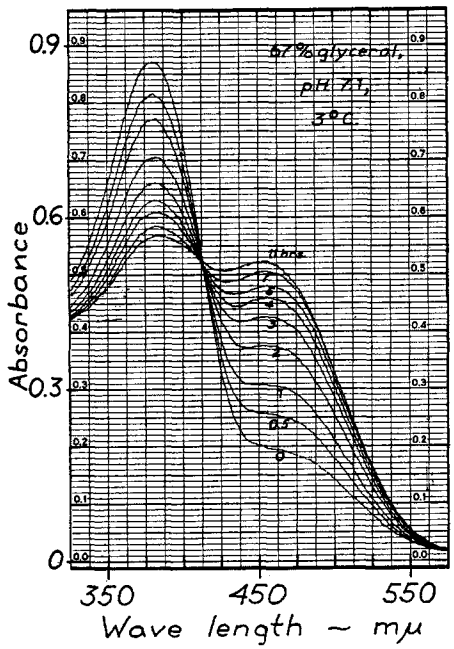

FIGURE 8. Dark reactions of metarhodopsin II. A rhodopsin solution with $E_{500} = 0.854$ (spectrum not shown) was cooled to 3°C, and bleached 15 min. with orange light from a 100 watt tungsten microscope lamp, transmitted through a Jena OG 2 filter. The spectrum, recorded immediately (0 time), exhibits the absorption band of metarhodopsin II with λ_{max} at 380 mμ, and a hump at longer wave lengths, due to a small amount of rhodopsin + isorhodopsin. Subsequent spectra were recorded at the times shown during a prolonged incubation in the dark. The absorption falls in the ultraviolet, and rises in the visible with an isosbestic point at 412.5 mμ, as metarhodopsin II is converted to a compound with λ_{max} about 464 mμ, as determined by the differences between successive spectra. Difference spectra, measured in the presence of 0.23 M hydroxylamine, show that the amount of rhodopsin + isorhodopsin does not change during the incubation (E_{500} is 0.043 initially and 0.045 after 11 hours). After 11 hours, considerable absorption remains in the ultraviolet. However, this is due to all-*trans* retinal, and not to metarhodopsin II, which hydrolyzes slowly even at 3°C.

This can be seen even more readily when rhodopsin is bleached in D_2O instead of H_2O, presumably due to the fact that heavy water hydrolyzes metarhodopsin II about four times more slowly than ordinary water. The bleaching of cattle rhodopsin in mildly acid D_2O is shown in Fig. 9 (right).

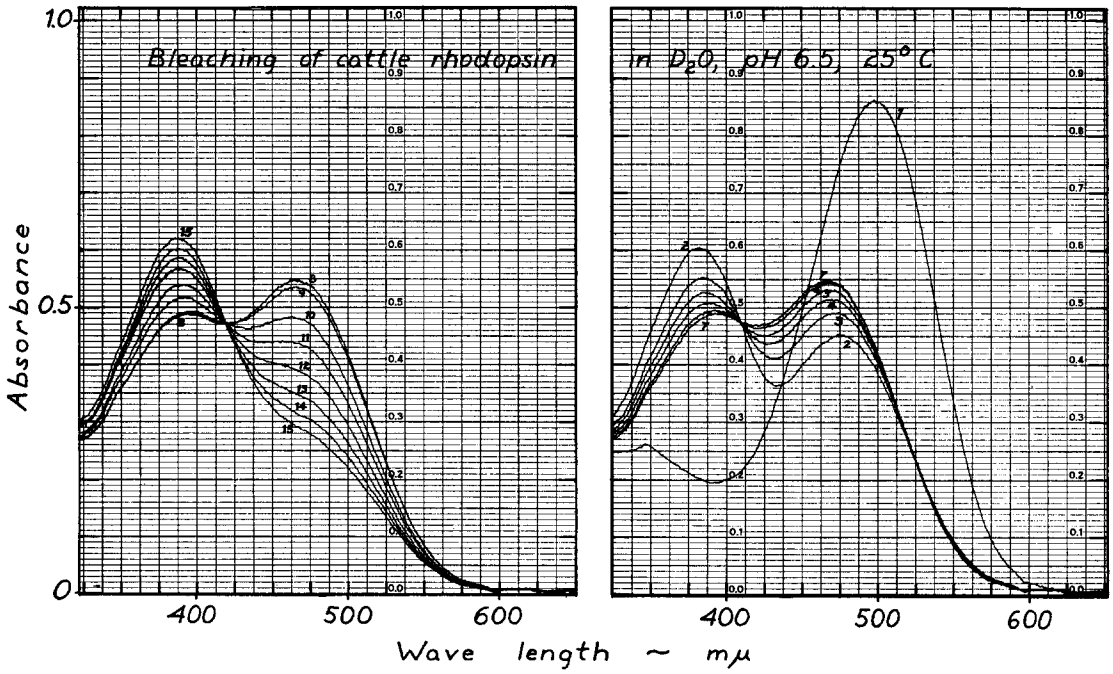

FIGURE 9. Bleaching of rhodopsin in D_2O. Cattle rhodopsin in 2 per cent aqueous digitonin was mixed with an equal volume of M/15 phosphate buffer (pH 6.5), and transferred to D_2O, as described in the Appendix. This solution (spectrum 1) was irradiated 1 min. with orange light from a tungsten source, transmitted through a Jena OG 2 filter, then kept at about 25°C. Subsequent spectra were scanned at a rate of 2.5 mμ per second, starting at 650 mμ at the following times after the light was turned off: spectrum 2 at 25 sec.; 3 at 4 min.; 4, 7 min.; 5, 10 min.; 6, 15 min.; 7, 20 min.; 8, 25 min.; 9, 35 min.; 10, 65 min.; 11, 90 min.; 12, 2 hrs.; 13, 2.5 hrs.; 14, 3 hrs.; and 15, 3.5 hrs. At this point hydroxylamine was added to a final concentration of 0.1 M, and the absorbance due to rhodopsin + isorhodopsin determined from the difference spectrum. This had $E_{max} = 0.122$, or about 14 per cent of the initial E_{max} of rhodopsin (*cf.* spectrum 1). For the first 20 minutes following irradiation, the absorption falls in the ultraviolet and at wave lengths longer than 525 mμ, and rises between two isosbestic points at 410 and 525 mμ (spectra 2 to 7). After 25 minutes, the absorption falls everywhere in the visible, simultaneously rising in the ultraviolet, with an isosbestic point at 417 mμ (spectra 8 to 15). Between spectra 2 and 15, λ_{max} of the absorption band in the ultraviolet shifts from its initial position at 380 mμ (due to metarhodopsin II), to λ_{max} of all-*trans* retinal at 387 mμ.

Upon irradiation with orange light, the absorption spectrum of rhodopsin (curve 1) is replaced by that of a mixture (curve 2), which in addition to the absorption band of metarhodopsin II at 380 mμ, displays a strong absorption maximum at 475 mμ. During the next 20 minutes in the dark, the absorption in the visible rises, meanwhile falling in the near ultraviolet, with an isosbestic point at about 410 mμ (spectra 2 to 7), due to the conversion of metarhodopsin II to a compound with λ_{max} at a longer wave length.

Difference spectra between curves 7 and 2 exhibit a maximum at about 450 mμ. However, close inspection of curves 2 to 7 reveals a second isosbestic point at about 525 mμ, due to the fact that while metarhodopsin II is converted to the new compound, metarhodopsin I is fading to metarhodopsin II. This distorts the difference spectra in such a way that the λ_{max} of the new compound appears to lie at a shorter wave length than is actually the case.

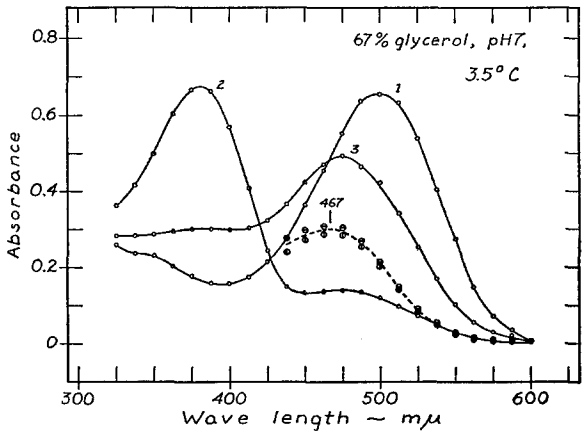

FIGURE 10. Irradiation of metarhodopsin II. Cattle rhodopsin in neutral 67 per cent glycerol is cooled to 3.5°C (spectrum 1, with E_{max} 0.653), and irradiated 5 min. with orange light from a tungsten lamp, transmitted by a Jena OG 2 filter. This yields spectrum 2, due mainly to metarhodopsin II, with a small admixture of rhodopsin + isorhodopsin. An aliquot, bleached in the presence of 0.1 M hydroxylamine, shows that E_{max} of the latter mixture is 0.058. The solution is now irradiated 14 min. with ultraviolet light at 365 mμ, isolated from the emission of a General Electric H-3 mercury arc by a Jena UG 1 filter. This yields spectrum 3, with λ_{max} 475 mμ and a hump in the ultraviolet, due to a residue of metarhodopsin II. To an aliquot of this sample at room temperature hydroxylamine is added to a final concentration of 0.1 M. This causes the absorption at 467 mμ to decrease in the dark to about ⅓ the value in spectrum 3, as a compound with λ_{max} 467 mμ is converted to retinaldehyde oxime. Its difference spectrum is shown by the vertically dashed circles. The thermally stable residue is now bleached with light, and identified by its difference spectrum as a mixture of rhodopsin + isorhodopsin, with E_{max} = 0.210. Subtracting this difference spectrum from spectrum 3, one obtains the horizontally dashed circles. The two sets of circles agree except for a small baseline correction, and define the absorption spectrum of the new compound, with λ_{max} 467 mμ (dashed line). The ultraviolet irradiation decreases the absorption at 380 mμ due to metarhodopsin II by about 0.45, and simultaneously generates the 467 mμ compound with E_{max} 0.30 and rhodopsin + isorhodopsin with E_{max} 0.15. All these compounds therefore have about the same molar absorbance at λ_{max}.

During the next phase (left, curves 8 to 15), the absorption falls in the visible and rises in the ultraviolet, with an isosbestic point at about 417 mμ. Now both the new compound and metarhodopsin I are fading; and by curve 15 have arrived at all-*trans* retinal and opsin (λ_{max} 387 mμ). The difference

spectrum, with λ_{max} about 473 mμ, therefore is again distorted, but in the opposite sense: it now appears to lie at longer wave lengths than is actually the case.

These experiments bracket the λ_{max} of the new compound between 450 and 473 mμ. They are therefore consistent with the conclusion, drawn from the other properties of the compound, that it is probably identical with the 465 mμ compound observed in aqueous glycerol (Fig. 8).

As mentioned earlier, a compound with λ_{max} at about 465 mμ also is obtained by irradiating metarhodopsin II with ultraviolet light. Fig. 10 shows that irradiation of metarhodopsin II (spectrum 2) results in an increased absorption in the visible, maximal at about 475 mμ (spectrum 3). If hydroxylamine is added and the temperature is raised to 25°C in the dark, some of this absorption disappears and is replaced by the absorption band of retinaldehyde oxime (not shown in Fig. 10). The difference spectrum of the pigment which disappears is shown by the circles with vertical bars; while the pigments which remain are readily identified as a mixture of rhodopsin and isorhodopsin. When the latter are bleached with light, and their difference spectrum subtracted from spectrum 3, one obtains the circles with horizontal bars. The two sets of circles differ only to the extent of a small baseline correction, and define the absorption spectrum of a new compound with λ_{max} about 467 mμ. This may contain a small admixture of metarhodopsin I, present prior to the irradiation (*cf.* spectrum 2).

In other words, irradiation of metarhodopsin II yields rhodopsin, isorhodopsin, and the new compound whose difference spectrum is defined by the dashed line. It resembles the 465 mμ dark product of metarhodopsin II (Figs. 8 and 9) in its absorption properties; in the fact that it is converted to metarhodopsin II in the light, but not in the dark; and in its sensitivity to hydroxylamine.

The nature of this compound has not been definitely established. However, the fact that it is readily interconvertible with metarhodopsin II by light, suggests that its chromophore may be a *cis* isomer of retinal other than 9- or 11-*cis*. In preliminary experiments, in which the chromophores of metarhodopsin II and of the 465 mμ compound were converted to retinaldehyde oxime in the dark, the oxime derived from the 465 mμ compound had an absorption spectrum displaying a lower absorbance and a shorter λ_{max} than that derived from metarhodopsin II. This is consistent with the thought that the 465 mμ compound contains a *cis* isomer of retinal.

Which *cis* isomer can it be? Since 11-*cis* and 9-*cis* retinal are accounted for by rhodopsin and isorhodopsin, the 465 mμ compound would have to contain the 13-*cis* isomer or one of the three possible di*cis* isomers (9,11; 11,13; 9,13). (The 9,11,13-tri*cis* isomer is too improbable to be considered.) Since the 465 mμ pigment forms spontaneously from metarhodopsin II in the dark, and

at temperatures near 0°C, it must represent a highly probable structure, and if this is to be accounted for by a spontaneous isomerization to a *cis* form of retinal, the 13-*cis* isomer is by far the most likely.

The Occurrence of Metarhodopsin II in the Retina Metarhodopsin II is an intermediate in the bleaching of rhodopsin in the retina as well as in solution.

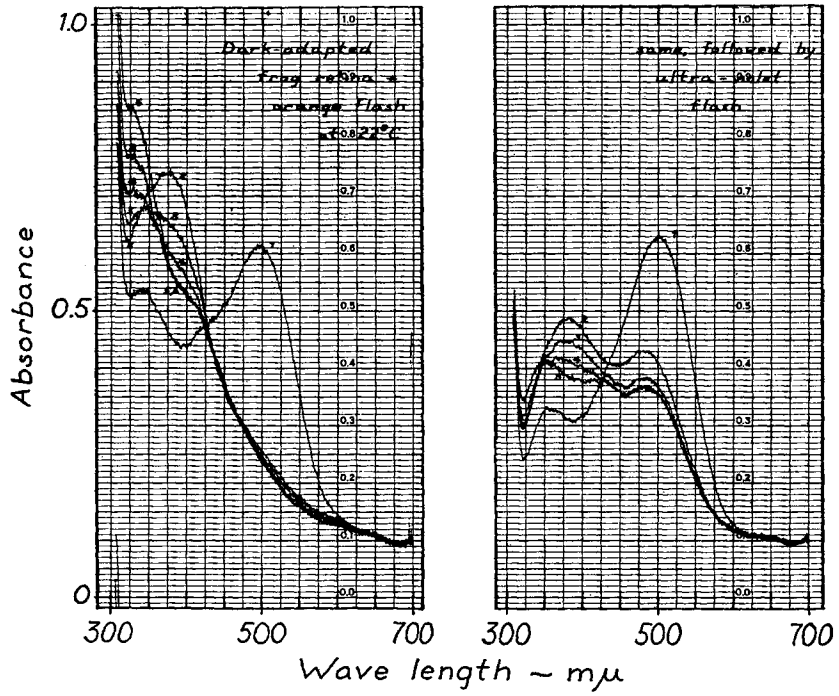

FIGURE 11. Occurrence of metarhodopsin II in the retina. All spectra are measured with the microspectrophotometer, which has previously been described (Brown, 1961). On the left, a patch of dark-adapted frog retina (spectrum 1) is exposed to two flashes of orange light obtained from M5 flash bulbs with Corning 3486 and Jena BG 18 filters. Spectrum 2 (recorded at a scanning speed of 2.5 mμ per second, starting at 700 mμ within 15 seconds of the last flash) exhibits an absorption band with λ_{max} at about 375 mμ. The retina is now left in the dark, and subsequent spectra are recorded at the same speed, starting at 700 mμ at the following times after the flash: 3, 6 min.; 4, 12 min.; 5, 20 min.; and 6, 30 min. During this time, the absorption at 375 mμ decreases, while rising at shorter wave lengths, as the 375 mμ compound is converted to retinol, with λ_{max} 325 mμ.[1] On the right, another patch of the same dark-adapted retina (spectrum 1) is exposed to two orange flashes as before; but now these are followed within 15 seconds by two flashes of ultraviolet light (M5 flash bulbs with a Jena UG 1 filter, which transmits maximally at about 355 mμ), and the retina then left in the dark. Spectrum 2 is recorded immediately after the ultraviolet flashes, and subsequent spectra at the following times: 3, 5 min.; 4, 11 min.; and 5, 20 min. A comparison of the left and right halves of the figure shows that ultraviolet irradiation converts a considerable fraction of the 375 mμ compound to compounds absorbing at longer wavelengths (*cf.* Fig. 10).

This is shown in Fig. 11 with microspectrophotometric measurements on the retina of the leopard frog (*Rana pipiens*) (*cf.* Brown, 1961). The bleaching of rhodopsin is shown at the left. Spectrum 2, obtained immediately after exposing a patch of the dark-adapted retina to two flashes of orange light, delivered within 15 seconds of one another, shows that the absorption band of rhodopsin (spectrum 1) has been replaced by a new band, with λ_{max} about 375 mμ. During the next half hour in the dark, this band gives way to one maximal at about 325 mμ (curves 3 to 6), due to retinol.[1]

Does spectrum 2 represent all-*trans* retinal + opsin or metarhodopsin II? It will be recalled that this question can be answered readily by irradiation with ultraviolet light (*cf.* Table I and Fig. 10).

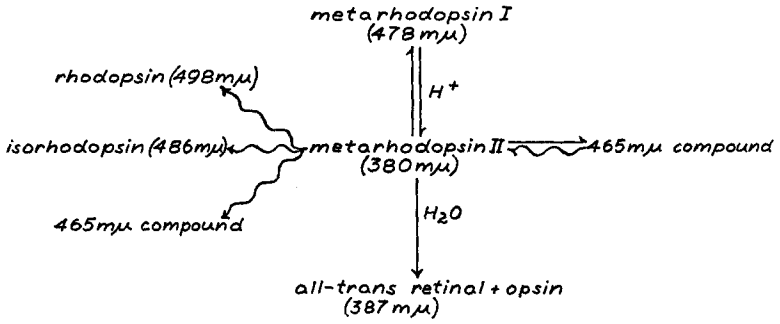

FIGURE 12. Summary of the reactions of metarhodopsin II. Thermal (dark) reactions are indicated by straight lines, photochemical reactions, by wavy ones (*cf.* Fig. 1). Metarhodopsin II participates in three dark reactions: (1) it reverts to metarhodopsin I, (2) forms a compound with λ_{max} about 465 mμ, and (3) hydrolyzes to all-*trans* retinal + opsin. In the light, it is converted to rhodopsin, isorhodopsin, and a compound with λ_{max} about 467 mμ, which is probably identical with the product of thermal reaction (2).

In the right half of Fig. 11 therefore another patch of the same dark-adapted retina (spectrum 1) is bleached with orange light as before. This time, however, instead of pausing to record the spectrum, it is reirradiated within 15 seconds with two flashes of ultraviolet light, and spectrum 2 recorded immediately thereafter. This spectrum exhibits considerably less absorption at 375 mμ than spectrum 2 on the left, and shows instead a new absorption band with λ_{max} about 480 mμ. The latter possesses two components: (*a*) a pigment which fades slowly in the dark, and judging from its difference spectrum (2 minus 3) has λ_{max} about 469 mμ; and (*b*) a component with λ_{max} about 485 mμ, which is stable in the dark, and can readily be identified as a mixture of rhodopsin and isorhodopsin. The former pigment (*a*) appears to be identical with the 465 mμ compound observed upon irradiation of metarhodopsin II in solution (Fig. 10).

It is significant that Fig. 11 shows essentially no metarhodopsin I. The

reasons for this are probably as follows: (*a*) In *Rana pipiens*, the metarhodopsin equilibrium favors metarhodopsin II more than in cattle. (*b*) The measurements were made at 22°C, whereas most of the preceding experiments were conducted at or below 4°C. (*c*) The excised retina, suspended in a closed space under a coverslip, probably is somewhat acidic. There is no need to assume therefore that the situation in the retina differs materially from that observed in solution.

The reactions of metarhodopsin II in light and darkness are summarized in Fig. 12.

TABLE III

A COMPARISON OF THE TAUTOMERIC PAIRS OF METARHODOPSINS FROM CATTLE AND SQUID

Data obtained in aqueous digitonin at about 4°C, except when stated otherwise. ΔH and ΔS are given for the reactions in the directions in which they occur when rhodopsin bleaches; that is, from the long wave length to the short wave length tautomer. Data on squid metarhodopsin are taken from Hubbard and St. George (1957–58), except for the effect of glycerol, which has been determined in the course of the present experiments.

Property	Cattle		Squid	
	Meta I	Meta II	Acid meta	Alkaline meta
λ_{max}, $m\mu$	478	380	500	380
Effect of pH	H^+ favors meta II		H^+ favors acid meta	
pK of change	6.4		7.7	
Effect of temperature	Warming favors meta II		Warming favors alkaline meta	
ΔH, *cal/mole*	+13,100		+7850	
ΔS, *e.u.*	+46.5		−8	
Effect of glycerol	Glycerol favors meta II		No effect	

DISCUSSION

Vertebrate vs. Squid Metarhodopsins The metarhodopsins of cattle and squid were characterized at about the same time (St. George and Wald, 1949; Wald, Durell, and St. George, 1950). It soon became evident that squid metarhodopsin exists in two tautomeric forms, acid and alkaline, with λ_{max} at 500 and 380 mμ (*cf.* Hubbard and St. George, 1957–58). This situation must now be compared with the tautomerism of vertebrate metarhodopsin described in the present investigation.

The characteristics of the tautomeric pairs of metarhodopsins from cattle and squid are summarized in Table III. Though similar in absorption properties and in their behavior as a function of temperature, they differ fundamentally in their response to changes of pH. Whereas acidity favors the *long* wave length tautomer of squid metarhodopsin (hence "acid metarhodopsin"), in cattle, leopard frogs, and bull frogs—perhaps vertebrate preparations in general—acidity favors the *short* wave length tautomer, metarhodopsin II.

The effect of temperature on the equilibrium of squid metarhodopsin, where warming favors the short wave length, alkaline tautomer, is readily explained as an effect on the acid dissociation. Nearly all acid groups have positive heats of dissociation; *i.e.*, warming favors the release of H^+. The value of ΔH for the acid dissociation of squid metarhodopsin is intermediate between the heats of dissociation of histidine imidazolium and amino acid ammonium ions (*cf.* Hubbard and St. George, 1957–58).

Cattle and frog metarhodopsins present a basically different situation. Here again, warming favors the short wave length tautomer (metarhodopsin II). But now this involves the *addition* of a proton, and if the large ΔH we have observed were ascribed simply to the group which adds the proton, that would imply a large *negative* heat of dissociation. Very few ionizable groups present in proteins have negative heats of dissociation, and for those that do, ΔH is about three orders of magnitude smaller than the value we observe for the reaction, cattle metarhodopsin I $\rightarrow$ II (that is, about 20 as against our 13,000 cal per mole; *cf.* Fig. 7, and Edsall and Wyman, 1958, chapter VIII, Table III). The ΔH we measure in Fig. 7 therefore cannot go with the addition of the proton which governs the pH-dependence of the cattle metarhodopsin equilibrium (*cf.* Figs. 2 and 3), but must characterize some other feature of the molecule that affects the color of the chromophore.

The most likely property of this sort is the configuration of opsin, upon which its steric relationships with the retinaldehyde chromophore depend. The conversion of cattle metarhodopsin I to II involves a very large increase in entropy, particularly for a reaction in which there is no increase in the number of molecules (*cf.* Table II). This reaction must therefore involve a considerable randomization of the configuration present in metarhodopsin I. The observed effect of temperature and the large ΔH therefore are associated primarily with the fact that metarhodopsin II is the tautomer of higher entropy.

By contrast, the conversion of squid acid to alkaline metarhodopsin involves a small *decrease* in entropy (Table III), as is expected if changes in temperature affect primarily the dissociation of the Schiff base ammonium ion (*cf.* Edsall and Wyman, 1958, chapter VIII, Tables III and VI).

To summarize this argument, in squid rise in temperature favors the short wave length metarhodopsin by dissociating an acid group; whereas in vertebrate metarhodopsins, the major effect of raising the temperature is to favor the more open configuration of metarhodopsin II.

The effect of solvents is in line with this interpretation, to the degree that neutral salts, glycerol, and methanol, all known to influence the tertiary structure of proteins, alter the metarhodopsin equilibrium in cattle and frog preparations (Figs. 4 and 5), but have no such effect in squid.

Nature of the Titrable Groups Involved in the Tautomerism of Metarhodopsin In

squid metarhodopsin, its pH indicator properties, as well as the thermodynamic properties of the equilibrium, suggest that the tautomerism involves the titration of the Schiff base linkage which attaches the retinaldehyde chromophore to opsin:

$$\underset{\text{alkaline metarhodopsin}}{C_{19}H_{27}C{=}N\ \text{opsin}} + H^+ \rightleftharpoons \underset{\text{acid metarhodopsin}}{C_{19}H_{27}\overset{H}{\overset{|}{C}}{=}\overset{+}{N}\ \text{opsin}.} \qquad (5)$$

It should be recalled, however, that the addition of a proton to the Schiff bases which retinal forms with aliphatic amino compounds involves a shift of λ_{max} from about 367 mμ for the unprotonated base to about 440 mμ for its conjugate acid (*cf.* Ball *et al.*, 1949). In squid metarhodopsin, both absorption maxima are displaced to longer wave lengths: 380 mμ for the alkaline form, and 500 mμ for the acid. This displacement depends on the integrity of the opsin moiety, for denaturation yields "indicator yellow," with λ_{max} 369 mμ in alkaline and 443 mμ in acid solution (*cf.* Brown and Brown, 1958). It seems clear therefore that in the native state both tautomers of squid metarhodopsin display spectra appreciably shifted toward the red by interactions between the retinal chromophore and opsin (*cf.* Hubbard, 1959).

We assume that similar interactions between the all-*trans* retinal chromophore and opsin account for the shift toward the red of the spectra of vertebrate metarhodopsins: metarhodopsin I, presumably a protonated form, lying at 478 mμ as compared with an ordinary protonated Schiff base at about 440 mμ; and metarhodopsin II, presumably a deprotonated form lying at 380 mμ as compared with a simple Schiff base at about 367 mμ.

The assumption, however, that the change from metarhodopsin I to II includes the deprotonation of the Schiff base linkage raises obvious problems. Increase of acidity favors this change, whereas it should be expected to run counter to a deprotonation. We have in fact to assume that in going from metarhodopsin I to II, in addition to losing a proton at the Schiff base linkage, a proton is added elsewhere in the molecule, and that primarily the latter event determines the pH-dependence of this tautomerism. The simplest assumption is that the observed titration curve (Fig. 3) is that of an amino acid residue in opsin, the acid dissociation of which enhances the color of the chromophore. The observed pK of 6.4 suggests that this might be an imidazole group of histidine, which would indeed be likely to be a more effective auxochrome in its uncharged than in its protonated state.

Radding and Wald (1955–56) showed that when cattle rhodopsin bleaches, an acid-binding group with pK about 6.6 (perhaps imidazole) appears within 30 seconds at 20°C. The pK, as well as the time relationships, suggests that they were observing the binding of the hydrogen ion which accompanies the

conversion of metarhodopsin I to II. They noted further that this reaction is followed by the much slower *release* of an H^+, bringing the product back to about its original pH. However, since this latter reaction takes 20 to 30 minutes to complete at 20°C, it probably is associated with the hydrolysis of metarhodopsin II, and does not concern us here.

To summarize this situation: We have definite acid-base evidence for the binding of a proton, perhaps by an imidazole residue, in going from metarhodopsin I to II. This accounts at once for the pH-dependence of this change, and for the observation by Radding and Wald of an immediate rise of pH accompanying the bleaching of rhodopsin. On the other hand, the present theory of the rhodopsin chromophore as a protonated Schiff base implies the loss of a proton at this same stage of bleaching, for which we have experimental evidence only in cephalopods. Either, therefore, the Schiff base formulation is misleading at this point; or we have to explain why the simultaneous loss of a proton at the Schiff base linkage and gain of a proton elsewhere in the molecule lead to a net rise of pH as though a single proton were being bound. An easy way out of this dilemma would be to assume that in fact *two* protons are bound, perhaps by imidazole residues, with the simultaneous loss of one proton at the Schiff base linkage. That would explain all our present observations, and perhaps is as well as can be done until further evidence is available. Finally a slow dissociation of a further proton, apparently accompanying the hydrolysis of retinal, brings the molecule back to its original pH.

The Metarhodopsin Equilibrium, and Flash Photolysis of Rhodopsin Wulff *et al.* (1958), analyzing the decay of metarhodopsin (the present metarhodopsin I), found that it was not a simple unimolecular reaction. Their data could be fitted by assuming a succession of three or four unimolecular processes, differing considerably in rate constants. They concluded that metarhodopsin I is composed of three or four fractions, each of which decays to retinal and opsin with its own first order rate constant (see also Abrahamson *et al.*, 1960).

It is clear from the present experiments, however, that one cannot expect the disappearance of metarhodopsin I to follow first order kinetics. Whereas the first stage of this transformation probably involves principally the unimolecular conversion of metarhodopsin I to II—perhaps identical with process 1 in the analysis of Wulff *et al.* (1958)—this is rapidly joined by the back reaction, from metarhodopsin II to I. At room temperature, one must further consider two other thermal reactions (*cf.* Fig. 12): (*a*) the formation of the 465 mμ pigment, which subsequently decays to retinal and opsin with an unknown rate constant; and (*b*) the hydrolysis of metarhodopsin II to retinal and opsin.

An analysis of data obtained at −9°C, at which metarhodopsin II undergoes only the back reaction to metarhodopsin I, shows that they fit the modi-

fied rate expression for equilibria between two unimolecular reactions.[2] The present experiments therefore do not support the suggestion that metarhodopsin I consists of more than one type of molecule.

Recently, Grellmann *et al.* (1962) have found that the intermediates produced by flash photolysis of rhodopsin at −195°C and above—that is, prelumirhodopsin and lumirhodopsin—also behave as though each was composed of two or three fractions, which decay at very different rates. We have confirmed this for the conversion of lumirhodopsin to metarhodopsin I. Both the reactions, pre-lumirhodopsin → lumirhodopsin, and lumirhodopsin → metarhodopsin I, appear to be irreversible (*cf.* Fig. 1), so that one cannot call upon the sort of argument advanced above to explain the apparent inhomogeneity of these intermediates. We are therefore led to conclude that pre-lumi- and lumirhodopsin decay as though they were not homogeneous, whereas metarhodopsin I acts like a single substance achieving equilibrium with its tautomer metarhodopsin II.

APPENDIX ON METHODS

Preparation of Rhodopsin Rhodopsin was prepared from cattle retinas obtained from the George A. Hormel Co. of Austin, Minnesota, by a modification of the methods previously used in this laboratory (*cf.* Wald and Brown, 1951–52; Hubbard, 1958–59). The retinas were dissected under red light at the slaughter house, frozen, and shipped in light-proof containers on dry ice. On arrival, they were stored at −15°C. The subsequent procedures were carried out under dim red light.

To prepare rhodopsin, 50 to 100 thawing retinas are ground in a mortar to a thick paste, which is subsequently diluted with an equal volume of sucrose solution (40 per cent w/v in neutral M/15 phosphate buffer) added in small portions, taking care to grind to a smooth suspension after each addition of sucrose. The final suspension is centrifuged in the preparative Spinco ultracentrifuge (model L) operated at about 30,000 × gravity (20,000 RPM) for 15 to 20 minutes, and the turbid supernatant is discarded. The residue is reground with sucrose, added in small portions up to a total volume of 35 ml per 50 retinas; the suspension is layered under neutral phosphate buffer and centrifuged as before. Rods and rod fragments aggregate at the interface, from which they are collected with a syringe, taking care to exclude the sucrose layer, which is discarded along with the supernatant. The rod suspension is diluted with about 2 volumes of buffer, and centrifuged to sediment the rods.

[2] This expression states that in an equilibrium of the type, $A \rightleftharpoons B$, the rate,

$$-\frac{dc}{dt} = (k_1 + k_{-1})(c - c_e),$$

where c is the concentration of A at time t; c_e, its concentration at equilibrium; and k_1 and k_{-1}, the rate constants for the forward and back reactions. A plot of $\log(c - c_e)$ against time should therefore fall on a straight line of slope, $\frac{k_1 + k_{-1}}{2.303}$ (*cf.* Kunitz, 1948–49).

These are reground into a smooth paste with about 10 ml sucrose, layered under buffer, and centrifuged. The rods are collected from the interface as before, diluted with buffer, and sedimented. This second flotation enhances the purity considerably.

From here on the procedure is essentially as described before (*cf.* Wald and Brown, 1951–52). Briefly, the rods are washed several times with distilled water, soaked 10 to 15 minutes in 4 per cent alum, again washed repeatedly with water, and once

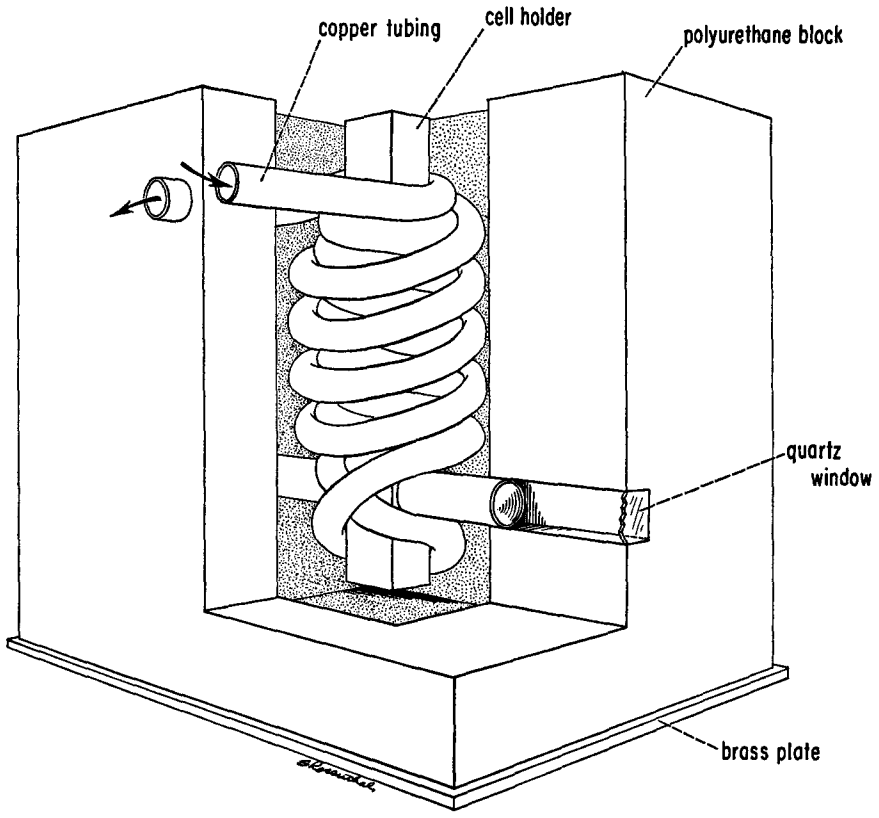

FIGURE 13. Diagram of the constant temperature cell.

with phosphate buffer. The rods are then lyophilized, and the dry rod powder is extracted with petroleum ether and dried. Finally, the rhodopsin is extracted with 2 per cent aqueous digitonin.

For transfer to D_2O, the extract is brought to the desired rhodopsin concentration and pH by dilution with water or phosphate buffer, and the mixture lyophilized. The rhodopsin powder is then dissolved in an appropriate volume of D_2O, and stored at least 24 hours at 5°C to allow time for deuterium to exchange for most of the "exchangeable hydrogen" in rhodopsin (*cf.* Hvidt and Linderstrøm-Lang, 1955). These solutions have the same absorption properties as aqueous solutions of rhodopsin; and upon bleaching with light, the opsin combines with 11-*cis* retinal at the same rate and to the same extent as in aqueous controls.

Description of the Constant Temperature Cell, Used with the Cary Model 11 Spectrophotometer A drawing of the cell is shown in Fig. 13.[3] Briefly, the cell consists of a copper T-tube of square cross-section, surrounded by a tightly wound double coil of ⅜ inch copper tubing, both inserted into a hollow block of polyurethane. To wind a copper coil of these dimensions sufficiently tightly for adequate temperature control, the tubing is initially filled with molten cerrobend (mp = 60°C). The finished coil is then immersed in boiling water, and the cerrobend blown out.

The space between T-tube, coils, and block is filled with cerrobend to increase the rate of heat transfer, and the entire block is cemented onto a brass plate, drilled to fit the pins in the Cary cell compartment, which ordinarily align the standard cell holder. The light path of the spectrophotometer passes through the horizontal limb of the T, which is closed off by quartz windows taped onto the outside of the polyurethane block. The vertical limb of the T serves as cell holder for standard absorption cells of 1 cm square cross-section. The sample can be irradiated through a window in the cover of the Cary cell compartment.

Coolant from an Aminco constant temperature bath is circulated through the coils, and the temperature of the sample is monitored with a "thermistemp" telethermometer (Yellow Springs Instrument Co., Yellow Springs, Ohio) inserted into the cell holder. The unit maintains temperatures down to −30° C and lower, without fogging of the optical surfaces.

Note Added in Proof We have observed two kinds of solute effects on the metarhodopsin equilibrium: specific changes effected by low concentrations of reagent; and much less specific changes associated with the replacement of a substantial fraction of water by a variety of solutes in high concentration. The latter include not only glycerol, methanol, and neutral salts, as discussed above, but also dimethyl sulfoxide. All these substances in high concentration displace the metarhodopsin equilibrium toward metarhodopsin II in proportion to their replacement of water. Yet simple replacement of water is not enough, since high concentrations of sucrose (41 volumes per cent) or urea do not have this effect at 4°C.

We have now investigated also a specific reagent, α-sulfopalmitic acid, which in a concentration of 10^{-3} M (about 40 molecules per molecule of rhodopsin) favors metarhodopsin II at 4°C.

The first class of reagents seems to have no effect on the regeneration of rhodopsin from 11-*cis* retinal and opsin, provided the conditions are such as not to denature opsin. Concentrations of glycerol or dimethyl sulfoxide high enough to favor metarhodopsin II strongly at 4°C do not appreciably inhibit regeneration.

The regeneration of rhodopsin is strongly blocked, however, by α-sulfopalmitic acid. Five minutes' exposure of opsin to this reagent in 10^{-3} M concentration at 30°C causes a 60 per cent decrease in the amount of rhodopsin formed.

This research was supported in part by grants to G. Wald from the National Science Foundation and the Office of Naval Research, and to R. Hubbard from the United States Public Health Service (N.I.N.D.B. Grants No. B-568 C 9 and 10). These experiments were described at the Federation Meetings in April, 1963 (*Fed. Proc.*, **22**, No. 2, pt. 1, 360).
Received for publication, May 2, 1963.

[3] We wish to thank Robert Chapman and Bernard Dillon for helping us design this cell, and for constructing it.

REFERENCES

ABRAHAMSON, E. W., MARQUISEE, J., GAVUZZI, P., and ROUBIE, J., Flash photolysis of visual pigments, *Z. Elektrochem.*, 1960, **64**, 177.

BALL, S., COLLINS, F. D., DALVI, P. D., and MORTON, R. A., Studies in vitamin A. 11. Reactions of retinene with amino compounds, *Biochem. J.*, 1949, **45**, 304.

BRIDGES, C. D. B., Studies on the flash-photolysis of visual pigments. III. Interpretation of the slow thermal reactions following flash-irradiation of frog rhodopsin solutions, *Vision Research*, 1962, **2**, 201.

BROWN, P. K., A system for microspectrophotometry employing a commercial recording spectrophotometer, *J. Opt. Soc. America*, 1961, **51**, 1000.

BROWN, P. K., and BROWN, P. S., Visual pigments of the octopus and cuttlefish, *Nature*, 1958, **182**, 1288.

Collins, F. D., Rhodopsin and indicator yellow, *Nature*, 1953, **171**, 469.

Commission on the Nomenclature of Biological Chemistry, Definitive Rules for the Nomenclature of Amino Acids, Steroids, Vitamins, and Carotenoids, *J. Am. Chem. Soc.*, 1960, **82**, 5575.

EDSALL, J. T., and WYMAN, J., Biophysical Chemistry, N. Y., Academic Press, Inc., 1958, **1**.

GRELLMANN, K-H., LIVINGSTON, R., and PRATT, D., A flash-photolytic investigation of rhodopsin at low temperatures, *Nature*, 1962, **193**, 1258.

HAGINS, W. A., Flash photolysis of rhodopsin in the retina, *Nature* 1956, **177**, 989.

HUBBARD, R., The thermal stability of rhodopsin and opsin, *J. Gen. Physiol.*, 1958–59, **42**, 259.

HUBBARD, R., On the chromophores of the visual pigments, *Proc. Nat. Physic. Lab., Symp. No.* **8** (Visual problems of colour), H. M. Stationery Office, 1959.

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.

HVIDT, A., and LINDERSTRØM-LANG, K., Exchange of deuterium and O^{18} between water and other substances. 3. Deuterium exchange of short peptides, Sanger's A chain and insulin, *Compt. rend. trav. Lab. Carlsberg, ser. chim.*, 1955, **29**, 385.

KROPF, A., and HUBBARD, R., The mechanism of bleaching rhodopsin, *Ann. New York Acad. Sc.*, 1958, **74**, 266.

KUNITZ, M., The kinetics and thermodynamics of reversible denaturation of crystalline soybean trypsin inhibitor, *J. Gen. Physiol.*, 1948–49, **32**, 241.

LYTHGOE, R. J., and QUILLIAM, J. P., The relation of transient orange to visual purple and indicator yellow, *J. Physiol.*, 1938, **94**, 399.

MORTON, R. A., and PITT, G. A. J., Studies on rhodopsin. 9. pH and the hydrolysis of indicator yellow, *Biochem. J.*, 1955, **59**, 128.

MORTON, R. A., and PITT, G. A. J., Visual pigments, *Fortschr. Chem. org. Naturstoffe*, 1957, **14**, 245.

PITT, G. A. J., COLLINS, F. D., MORTON, R. A., and STOK, P., Studies on rhodopsin. 8. Retinylidenemethylamine, an indicator yellow analogue, *Biochem. J.*, 1955, **59**, 122.

RADDING, C. M., and WALD, G., Acid-base properties of rhodopsin and opsin, *J. Gen. Physiol.*, 1955–56, **39**, 909.

ST. GEORGE, R. C. C., and WALD, G., The photosensitive pigment of the squid retina, *Biol. Bull.*, 1949, **97**, 248.

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

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., DURELL, J., and ST. GEORGE, R. C. C., The light reaction in the bleaching of rhodopsin, *Science*, 1950, **111**, 179.

WULFF, V. J., ADAMS, R. G., LINSCHITZ, H., and ABRAHAMSON, E. W., Effect of flash illumination on rhodopsin in solution, *Ann. New York Acad. Sc.*, 1958, **74**, 281.

YOSHIZAWA, T., and WALD, G., Pre-lumirhodopsin and the bleaching of visual pigments, *Nature*, 1963, **197**, 1279.