Energetics of Primary Processes in Visual Excitation: Photocalorimetry of Rhodopsin in Rod Outer Segment Membranes†

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ABSTRACT: A sensitive technique for the direct calorimetric determination of the energetics of photochemical reactions under low levels of illumination, and its application to the study of primary processes in visual excitation, are described. Enthalpies are reported for various steps in the bleaching of rhodopsin in intact rod outer segment membranes, together with the heats of appropriate model reactions. Protonation changes are also determined calorimetrically by use of buffers with differing heats of proton ionization. Bleaching of rhodopsin is accompanied by significant uptake of heat energy, vastly in excess of the energy required for simple isomerization of the retinal chromophore. Metarhodopsin I formation involves the uptake of about 17 kcal/mol and no net change in proton ionization of the system. Formation of metarhodopsin II requires an additional energy of about 10 kcal/mol and involves the uptake of one hydrogen ion from solution. The energetics of the overall photolysis reaction, rhodopsin → opsin + *all-trans*-retinal, are pH dependent and involve the exposure of an additional titrating group on opsin. This group has a heat of proton ionization of about 12 kcal/mol, characteristic of a primary amine, but a pK_a in the region of neutrality. We suggest that this group is the Schiff base lysine of the chromophore binding site of rhodopsin which becomes exposed on photolysis. The low pK_a for this active lysine would result in a more stable retinal–opsin linkage, and might be induced by a nearby positively charged group on the protein (either arginine or a second lysine residue). This leads to a model involving intramolecular protonation of the Schiff base nitrogen in the retinal–opsin linkage of rhodopsin, which is consistent with the thermodynamic and spectroscopic properties of the system. We further propose that the metarhodopsin I → metarhodopsin II step in the bleaching sequence involves reversible hydrolysis of the Schiff base linkage in the chromophore binding site, and that subsequent steps are the result of migration of the chromophore from this site.

Spectroscopic studies of the bleaching of the vertebrate visual pigment rhodopsin have demonstrated that the overall reaction is complex, involving a sequence of intermediate photoproducts (Figure 1) recognizable by their different spectral properties. Apart from their color, however, little is known concerning the physical or chemical properties of these intermediates, nor of their relevance in the mechanism of visual excitation.

Rhodopsin is an intrinsic membrane glycoprotein, of molecular weight about 35 000 (Papermaster and Dreyer, 1974), associated with lipid bilayer discs in the outer segments of vertebrate rod photoreceptor cells. Each rhodopsin molecule contains 11-*cis*-retinal as chromophore, which is thought to be attached to the ϵ-amino group of a lysine residue on the protein via an aldimine (Schiff base) linkage (Bownds, 1967; Akhtar et al., 1968; Fager et al., 1972). The Schiff base may be protonated (Oseroff and Callender, 1974). The bleaching process involves, among other effects, the isomerization of the chromophore to the all-trans configuration with subsequent hydrolysis and release of the chromophore (Wald, 1968). Photoisomerization must, presumably, be accompanied by changes, albeit transitory, in the physical and/or chemical properties of the protein and/or membrane which initiate the generation and amplification of the visual response. Kinetic arguments suggest that this takes place at, or before, the metarhodopsin stage (Figure 1). Subsequent intermediates may be associated with the regeneration process.

The photosensitivity and insolubility in normal aqueous buffers of rhodopsin and its photoproducts, together with the thermal instability of many of the intermediates, have hampered study of the bleaching process in the native state, and many studies are confined to detergent-solubilized preparations of uncertain biological relevance.

The energetics of this system are of fundamental importance to the mechanism of visual excitation but have received little attention (Wald, 1973). The heat of isomerization of free retinal is, perhaps surprisingly, very small (11-*cis* → *all-trans*-retinal, 25 °C in heptane, ΔH = +0.15 kcal/mol; Hubbard, 1966), and tentative experiments have suggested that rhodopsin (frog) converts at least 90% of the absorbed light energy into heat (W. A. Hagins, unpublished observation, reported in Hagins and McGaughy, 1967). Falk and Fatt (1968) reached a similar conclusion from indirect observations.

Bleaching of rhodopsin, after initial photon absorption, proceeds by a sequence of spontaneous and, in most cases, irreversible steps (Figure 1) which indicates a progressive decrease in thermodynamic free energy in the system. In the dark, however, rhodopsin is thermally stable and will spontaneously regenerate in mixtures of opsin and free 11-*cis*-retinal, suggesting that, of all the available states, native rhodopsin has the lowest free energy of all. This implies that, in the initial photic event, some fraction of the energy of the absorbed photon (equivalent to about 57 kcal/mol at 500 nm) must be utilized to generate a state of higher free energy, which may then be dissipated in subsequent dark reactions. Immediate dissipation of all the photon energy as heat would imply a completely entropic photic process, with intriguing consequences for the mechanism of the overall photochemical re-

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action. Studies of the effect of temperature on the meta I[1] ⇄ meta II quasi-equilibrium in digitonin-solubilized rhodopsin solutions (Matthews et al., 1963; Ostroy et al., 1966) have shown an energy uptake of 10–13 kcal/mol for this reaction. Current techniques in microcalorimetry are sufficiently developed that enthalpies of this order are readily observable in biological samples (Sturtevant, 1972; Wadsö, 1968) and this has encouraged us to attempt a more precise determination of the energetic changes induced in rhodopsin by photon absorption.

We report here direct calorimetric data for various steps in the bleaching of rhodopsin in cattle rod outer segment (ROS) membrane suspensions, determined by a new technique of photocalorimetry. Because of temperature limitations we are, as yet, unable to detect the very earliest intermediates (prelumi- and lumirhodopsin). Protonation changes, also determined by calorimetry, together with the energetics of relevant model reactions, are also given. These data lead to a plausible model for some of the protein–retinal interactions in the chromophore binding site of rhodopsin and for the meta I → meta II reaction.

Experimental Section

Materials. Rod outer segments were prepared from frozen cattle retinas (Hormel) by the method of Papermaster and Dreyer (1974), and were either used immediately or stored frozen in 27% (w/w) sucrose, 10 mM Tris-acetate, pH 7.4, until use. Sodium dodecyl sulfate gel electrophoresis showed only bands corresponding to opsin and the associated high-molecular-weight protein (Papermaster and Dreyer, 1974), with minor contaminant bands apparent only on heavily loaded gels. Spectra of detergent-solubilized samples indicated that the ROS contained between 10 and 20% bleached rhodopsin.

Thin-section electron micrographs of isolated ROS, fixed in the dark, show predominantly intact outer segments with occasional vesicular material apparently derived by disruption of disc membranes.

Concentrations of rhodopsin and extent of bleaching in the photocalorimeter were determined from the absorbance at 500 nm of ROS aliquots solubilized in 2% CTAB, 0.2 M Tris-acetate, pH 7.4, 25 °C, containing 10 mM hydroxylamine, using ϵ_{500} 41 000. Spectra were determined in a Cary 14 spectrophotometer fitted with thermostated cells and flushed with nitrogen.

ROS samples for photocalorimetry were prepared by the addition of concentrated buffer (usually 0.6 M), to give a final buffer concentration of 0.1 M and a sucrose concentration of about 22%. Rhodopsin concentrations were such as to give 500-nm absorbances between 1.0 and 2.0. Unless otherwise specified, all pH values refer to 3 °C, and all manipulations of photosensitive material were performed in the dark or under dim red light.

Photocalorimetry. A schematic of the photocalorimeter system devised for this work is shown in Figure 2. The instrument is based on the LKB isothermal heat conduction batch microcalorimeter, described by Wadsö (1968). In instruments of this type, heat evolved in the reaction vessel is conducted through a thermopile to a comparatively large surrounding heat sink. The voltage generated by the thermopile is proportional to the rate of heat transfer between the reaction vessel and the heat sink so that, after appropriate calibration, integration of the output voltage over the period of the reaction gives the total heat of the reaction. Dual reaction vessels with the thermopiles connected in opposition are used so as to minimize the effects of thermal fluctuations within the system.

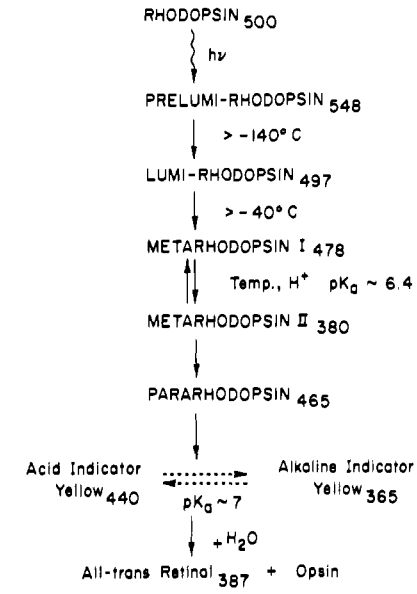

FIGURE 1: The generally accepted sequence of intermediates in the bleaching of rhodopsin (after Ostroy et al., 1966). Subscripts indicate absorbance maxima in nanometers. The status of a very early photoproduct, hypsorhodopsin (Yoshizawa, 1972), observable only at liquid helium temperatures, is unclear; the actual sequence of events after meta II is still subject to some dispute. Alternative names frequently used are: bathorhodopsin (prelumirhodopsin); metarhodopsin III (pararhodopsin); NRO, *N*-retinylidenopsin (indicator yellow).

In the photocalorimeter, reactions are initiated by irradiation of the sample by light of an appropriate wavelength from an external source, directed into the calorimeter via flexible fiber optics light guides. All light entering the calorimeter cell is absorbed either by the sample or by the opaque walls of the vessel. In the case of an inert sample, all the light energy is converted into heat, and the calorimeter measures merely the total light energy flux into the cell. If, however, the light induces a photochemical reaction in the sample, one observes an additional heat of reaction, over and above the energy of the incident light itself. Thus, by comparison of the photochemically active sample with some suitable inert reference, and by determining the extent of the light-induced reaction, it is possible to measure directly the absolute enthalpy of the reaction.

The active volume of each calorimeter cell is about 2.5 ml, the cell consisting of a rectangular gold compartment about 1-cm square in cross-section by 3 cm in height. The fiber optics (Corning No. 5010) enter from the top, through a vapor-tight silicon rubber collar on the loading port of the calorimeter cell, and are positioned such that their ends lie just below the surface of the sample or reference solution. These ends of the fiber bundles are stripped of their PVC cladding and embedded in clear epoxy, for mechanical stability. Both sample and reference cells are fitted with identical light guides which are led, through appropriate holes drilled in the calorimeter heat sink, insulation, and thermostatic bath, to an external light source consisting of a stabilized 200-W mercury–xenon arc lamp, grating monochromator, and electrically timed shutter. The entrance faces of the light guides are mounted on a moveable platform at the exit slit of the monochromator so that either

[1] Abbreviations used: ROS, rod outer segment membranes; CTAB, cetyltrimethylammonium bromide; meta I, metarhodopsin I; meta II, metarhodopsin II.

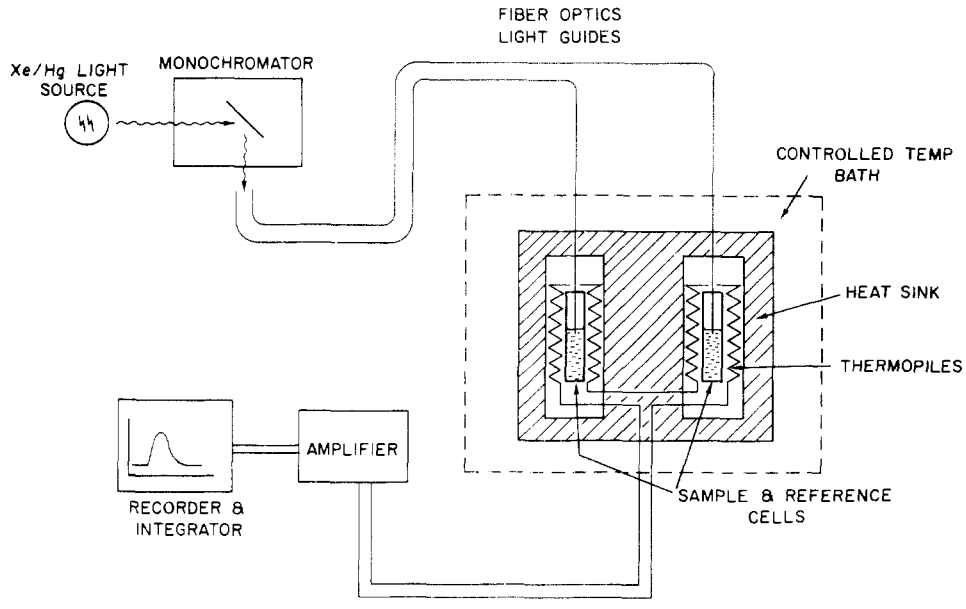

FIGURE 2: Photocalorimeter schematic. Not shown are the shutter and the moveable fiber optics mount at the exit slit of the monochromator, which allow either cell to be illuminated independently.

cell may be illuminated at will. The calorimetric unit and optical system are mounted in a temperature controlled dark room, with electronics and control system mounted outside so that, after sample loading and equilibration, all operations may be performed with minimal fluctuations in ambient temperature.

The output voltage of the calorimeter thermopiles is connected to a Keithley Model 149 microvoltmeter (usually used at 10 μV full scale) and thence to a chart recorder fitted with a disc integrator. The instrument is calibrated electrically and checked using the potassium ferrioxalate actinometry system (Hatchard and Parker, 1956, see Results).

The total energy flux in the reaction vessels of this instrument can be measured with a precision of 1–2%, and overall heat effects as low as 0.05 mcal (2×10^{-4} J; 1 cal = 4.184 J) can be detected. With the present light source, illumination in the reaction vessels corresponds to energy fluxes in the range 0.01–0.3 mW, depending on wavelength. This means that, for samples with a reasonably high quantum efficiency, irradiation times of a minute or so are generally sufficient to give a significant amount of photochemical reaction, and that enthalpies of these reactions may be determined with a precision of about ± 1 kcal/mol. Transmission properties of the fiber optics limit illumination to the visible region (>380 nm) and the current instrument cannot be operated below 0 °C.

In a typical experiment, the sample and reference cells of the calorimeter are loaded with the sample and with a suitably inert reference solution, respectively, and, after thermal equilibration, are illuminated for identical lengths of time. Reference cell irradiation gives merely the total light energy flux so that the difference in the integrated thermal response of the two cells gives the heat of the photochemical reaction induced in the sample. In practice, multiple irradiations of the reference, before and after sample irradiation, were performed and averaged, and frequent checks were made with inert solutions in both cells in order to compensate for small differences in thermal sensitivity of the cells. For the rhodopsin experiments, the reference solution usually consisted of a solution of methylene red in 0.1 N HCl, diluted so as to approximate the spectral properties of rhodopsin in the visible region, although plain water was found to be an equally effective reference.

Flow Calorimetry. The heats of various secondary dark reactions (retinal oxime and Schiff base formation, etc.) were determined at 3 °C using the flow modification of the Beckman Model 190 microcalorimeter (Sturtevant and Lyons, 1969; Sturtevant, 1969; Velick et al., 1971). Appropriate corrections were made for viscous heating and heats of dilution of the various reagents, and the calorimeter was calibrated using the heat of protonation of Tris (National Bureau of Standards solution calorimetry standard) and heat of ionization of water (Grenthe et al., 1970).

The enthalpy of retinal oxime formation was determined by mixing detergent-solubilized *all-trans*-retinal (~1.25 mM, Sigma) with excess hydroxylamine (15–50 mM) in the flow calorimeter, in the appropriate buffers. Detergents used were either 1% (v/v) Emulphogene BC 720 (General Aniline Film Corp.) or a mixed micelle system of 0.5% Emulphogene–0.5% sodium dodecyl sulfate (sodium lauryl sulfate, Fisher). No significant differences were observed in the different detergent systems, under otherwise identical conditions.

The heat of formation of the Schiff base complex between *all-trans*-retinal and *n*-butylamine (as an analogue of the lysine side chain) was determined in the flow calorimeter at 3 °C, using either absolute ethanol or 1% aqueous Emulphogene (unbuffered) as solvents. Retinal (~1.3 mM) was mixed with butylamine solutions (10–200 mM), and the resultant heat was found to be independent of butylamine concentration over this range. Separate spectral studies showed that the dissociation constant for this reaction is less than 1 mM, under these conditions, thus ensuring completion of the reaction. Heat of protonation of the Schiff base complex was determined under similar conditions by mixing ethanol, or detergent, solubilized retinal–butylamine complex with acidified ethanol, or 0.01 N HCl–detergent, as appropriate. Schiff base solutions were prepared either by treatment of retinal with a slight excess of butylamine or, alternatively, by formation of the complex in ethanol using excess amine, followed by evaporation of the excess butylamine and solvent under a stream of nitrogen, and re-solution. Correction was made for the heat of protonation of any excess butylamine where necessary.

Results

Potassium Ferrioxalate. Before attempting measurements on the rhodopsin system, it was felt necessary to characterize the photocalorimeter with some relatively simple standard photochemical reaction. The potassium ferrioxalate photoredox reaction described by Hatchard and Parker (1956) is ideal for this, having a high quantum yield and good absorbance in the visible region. The overall reaction may be written:

$$2Fe^{3+} + C_2O_4^{2-} \xrightarrow{h\nu} 2Fe^{2+} + 2CO_2$$

This is an exothermic reaction with an enthalpy, calculated from standard heats of formation of reactants and products, of about -11.3 kcal/mol of Fe^{2+}.

Figure 3 shows the result of a 15-min irradiation at 436 nm of 0.15 M potassium ferrioxalate in 0.1 N H_2SO_4, in the photocalorimeter. As can be seen, the heat effect on irradiation of ferrioxalate is at all times greater than that due to the light energy alone, and corresponds to an additional light-induced exothermic reaction. Determination of the yield of Fe^{2+} in the reaction, using 1:10 phenanthroline (Hatchard and Parker, 1956), leads to a mean enthalpy for the reaction, from a series of experiments at both 3 and 25 °C, of -12.9 (± 1.9) kcal/mol of Fe^{2+}, with no discernible dependence on temperature. To our knowledge, no direct determinations of the heats of photochemical reactions have been previously reported, but this result is in reasonable agreement with the value estimated from standard heats of formation.

The potassium ferrioxalate system can, in addition, be used to check the photic efficiency of the photocalorimeter since the quantum yield of the reaction is accurately known (Hatchard and Parker, 1956). These measurements show that, for an optically dense sample, about 70% of the light entering the photocalorimeter is available for photochemistry. The remaining 30% appears to be dissipated as heat in the cell walls and in the ends of the optical fibers. Slight improvements can be obtained by careful grinding and polishing of the fiber ends after embedding in epoxy, but this deteriorates fairly rapidly with use and was of no great advantage in our experiments.

Photoproducts of ROS. Interpretation of energetic data from the photocalorimeter requires an analysis of the photoproducts resulting from irradiation of ROS suspensions. Furthermore, since the thermal response of the calorimeter is relatively slow (in the order of minutes), it is necessary to obtain conditions under which stable and interesting photoproducts are produced. Spectral studies of ROS suspensions are hampered by the intrinsic turbidity of such samples, but must be attempted. Detergent solubilized rhodopsin is of little value here because the nature and kinetics of photolysis products are very dependent on environment (Lamola et al., 1974; Applebury et al., 1974), and can be misleading. We have been able to make reasonable correction in the spectrophotometer for light scattering by use of a combination of ground glass screens and synthetic turbid solutions as reference. The most satisfactory references we have found consist of a standard 1-cm quartz cuvette, rotated 90° about a vertical axis from the conventional orientation so that the reference light beam passes through the ground faces of the cuvette, and filled with a turbid suspension of commercial "nondairy creamer" in water. In this manner it is possible to obtain spectra of whole ROS suspensions which remain on scale over the 260–650-nm range, and which show well-resolved peaks in the appropriate positions. Naturally, the absolute absorbances obtained this way have little meaning, but the spectra are qualitatively as good as those for detergent-solubilized rhodopsin, and allow identification of major photoproducts after bleaching of the ROS under various conditions.

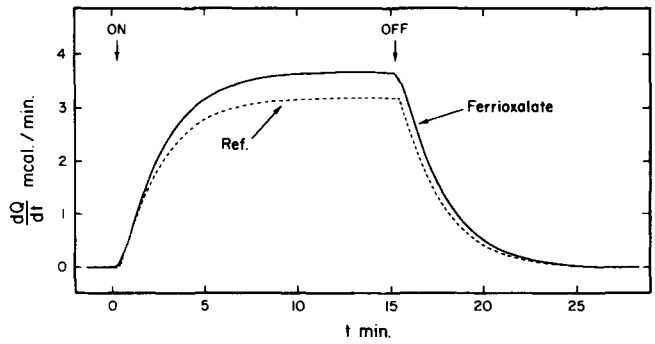

FIGURE 3: Photocalorimeter voltage traces, converted to heat output rate, of 15-min irradiations of a potassium ferrioxalate solution and of an inert reference, at 436 nm, 25 °C. Two separate experiments are shown, superimposed. The traces are not smoothed and show the actual noise level. The difference in area of the two curves, obtained by integration, gives the total photochemical heat of reaction, which is exothermic in this case.

Spectra of intact ROS, after irradiation at 546 nm, were obtained under a variety of conditions. At 25 °C bleaching resulted in a major shift of the absorbance maximum from 500 nm (unbleached rhodopsin) to the region of 465 nm (pararhodopsin), but this peak was, in most cases, quite broad and decayed fairly rapidly (~30 min) to a complex mixture of later photoproducts (indicator yellow, opsin + retinal?). Occasionally a rapidly decaying shoulder at about 380 nm (meta II?) could be observed in the spectra immediately after illumination.

Bleaching at 3 °C, however, resulted in much more stable products, which were pH dependent. At pH 8, 546-nm irradiation gave a peak at 480 nm, of the same intensity as the original 500-nm absorbance, which we identify as metarhodopsin I. No change in this spectrum was observed for over 1 h at 3 °C, but warming to 25 °C resulted in a decrease in magnitude and shift to lower wavelengths indicative of formation of a mixture of pararhodopsin and indicator yellow. Bleaching at pH 5.4, 3 °C, gave metarhodopsin II (380 nm), which showed only a slight decrease in intensity after standing for 1 h at 3 °C and gave, once again, a complex mixture of later products on warming to 25 °C. The photoproducts at 3 °C seem to consist of greater than 90% meta I or meta II at pH 8.0 or 5.4, respectively. Intermediate pH's gave stable mixtures of these products. No intermediates earlier than meta I were observable in these experiments. We have obtained similar results using sonicated ROS suspensions, in which problems due to light scattering are less extreme, in agreement with the observations of Lamola et al. (1974). The behavior on bleaching of intact ROS at temperatures in the region of 3 °C is, therefore, similar to that of rhodopsin solubilized in digitonin (Matthews et al., 1963) but not, apparently, to the behavior of rhodopsin in most other detergents.

Photocalorimetry of ROS. Illumination in the visible region of suspensions of rod outer segments at 3 °C gives a thermal response in the photocalorimeter markedly, and reproducibly, lower than the reference. An example of the calorimetric response to a 1-min irradiation at 546 nm of ROS at pH 8.0 is given in Figure 4. This illustrates that formation of photoproduct (meta I in this case) involves a significant uptake of heat; i.e., the photoinduced reaction is endothermic. A series of experiments using different periods of illumination show that the heat effect is proportional to the extent of bleaching, up to about 30% bleach of the total rhodopsin in the sample (Figure 5). Longer periods of illumination were found to result in

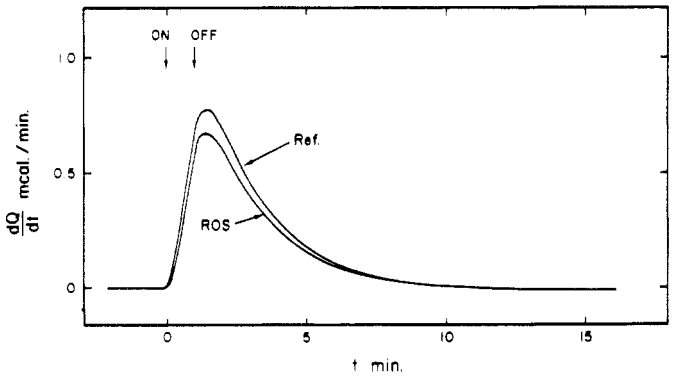

FIGURE 4: Superimposed photocalorimeter response for 1-min irradiations at 546 nm of rod outer segment membranes (ROS, 0.1 M Tris-acetate, pH 8.0, 3 °C) and of an inert dye solution. The reaction is rhodopsin → meta I, and is endothermic. The noise level in this case is about twice the line width of the traces. Note that the shape of the curves is determined solely by the thermal response time of the calorimeter and does not reflect the kinetics of the reaction, which is essentially instantaneous on this time scale.

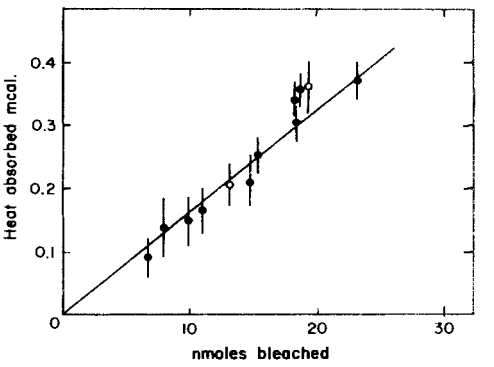

FIGURE 5: Collected data for rhodopsin → meta I at pH 8.0, 3 °C. Light induced heat uptake is plotted as a function of the extent of reaction, up to about 30% bleach. Slope of the line gives ΔH (rhodopsin → meta I) = 16.8 (±1.4) kcal/mol. Open circles: 0.1 M phosphate. Filled circles: 0.1 M Tris-acetate. Error bars indicate ±1 SD determined from reference irradiations.

marked deviation from linearity, which was more pronounced when 500-nm irradiation was used, and which was correlated with formation of isorhodpsin by secondary photochemical reactions in the photoproduct mixtures. This is a particular problem when meta I is the stable end product since its absorbance band partially overlaps the irradiation wavelength, but the effects seem to be relatively small for limited bleaching at 546 nm. No heat effect is seen with previously bleached samples, nor upon irradiation at 650 nm where no bleaching takes place. Approximate quantum yields for bleaching of the ROS can be estimated from the extent of reaction in any one experiment using the total light input given by the reference cell measurements. After correction for light absorbed by the fiber optics and the walls of the vessel, we obtain values in the region of 0.5–0.6 for the quantum efficiency of bleaching. This is in good agreement with more accurate determinations (Dartnall, 1972) and, together with the other controls, confirms that the heat effects we observe are directly related to photochemical processes in rhodopsin.

Table I summarizes the various results. The reaction rhodopsin → meta I, at pH 8, goes with an overall uptake of about 16.8 kcal/mol, and the heat effect is the same in either Tris-acetate or phosphate buffer. The use of buffers with differing heats of proton ionization, under otherwise identical conditions, allows us to determine any change in protonation from differences in the heats of reaction in the various buffers (Sturtevant, 1962). This follows because any H^+ ion released from or taken up by the reactants is, in a well-buffered system, taken to or from the appropriate buffering ion. Calorimetry measures the totality of heat effects in any reaction and, thus, includes any heat due to uptake, or release of H^+ ions by the buffer, in addition to the heat of reaction itself. The heat of protonation of phosphate (near neutral pH) is close to zero, whereas protonation of Tris is exothermic ($\Delta H_i \approx -11.5$ kcal/mol; Grenthe et al., 1970). This implies that no change in protonation takes place in the reaction rhodopsin → meta I, at pH 8. For rhodopsin → meta II, on the other hand, a significant buffer effect is seen (Table I). Observed at pH 5.4, the reaction is even more endothermic than meta I formation, and shows a difference of about 7 kcal/mol between acetate and piperazine buffer. The heat of protonation of acetate is essentially zero (Christensen et al., 1967), whereas ΔH_i for piperazine (second ionization) is about −7.1 kcal/mol (Paoletti et al., 1963). It follows that the reaction rhodopsin → meta II, at pH 5.4, involves the uptake of one proton and has an enthalpy, corrected for buffer ionization, of about +27 kcal/mol. Since meta I formation, at pH 8, involves no change in protonation and is about 17 kcal/mol endothermic, this suggests, but does not yet prove (see Discussion), that the meta I → meta II transition involves the uptake of one H^+ ion and about 10 kcal/mol of energy. This is in good agreement with the values determined from the temperature and pH dependence of the meta I ⇄ meta II equilibrium in digitonin-solubilized rhodopsin (Matthews et al., 1963; Ostroy et al., 1966).

The overall reaction, rhodopsin → opsin + *all-trans*-retinal, is much too slow to be observable directly in the photocalorimeter, but an indirect route is available to obtain energetic data for this. Hydroxylamine reacts readily with the retinal chromophore of rhodopsin after illumination, probably at the metarhodopsin stage, to give the apoprotein, opsin, plus free retinal oxime. Thus, the energetics of the overall reaction may be determined from separate measurements of the two reactions:

$$\text{rhodopsin} + NH_2OH \xrightarrow{h\nu} \text{opsin} + \text{retinal oxime} + H_2O$$

$$\text{retinal} + NH_2OH \rightarrow \text{retinal oxime} + H_2O$$

which, by subtraction, gives:

$$\text{rhodopsin} \xrightarrow{h\nu} \text{opsin} + \text{retinal}$$

The first reaction is readily observable in the photocalorimeter at 3 °C and gives essentially zero heat of reaction in various buffers at pH 8 or 5.4 (Table I). The second reaction was studied by flow calorimetry of mixtures of *all-trans*-retinal and hydroxylamine at 3 °C in various detergents (Table II). This reaction is complicated by protonation of hydroxylamine. At pH 5.4 the reaction may be written

$$\text{Ret-CHO} + NH_3{}^+OH \rightarrow \text{Ret-CH=NOH} + H_2O + H^+$$

and the heat of reaction will include an additional effect due to uptake of the H^+ ion by buffer. This is seen in the approximately 7 kcal/mol difference for the heat of this reaction in acetate or piperazine buffers. No protonation effect is expected at pH 8 (hydroxylamine $pK_a \sim 6.4$, at 3 °C), and the measured enthalpy of oxime formation is about −24 kcal/mol. Unfortunately, there is a fairly large uncertainty in this measurement arising from the slow decomposition of hydroxylamine in solution at alkali pH. This gives rise to a significant heat effect in the flow calorimeter which partially obscures the heat of retinal oxime formation and which is difficult to correct for

TABLE I: Enthalpy and Proton Uptake during Bleaching of Rhodopsin in ROS Membranes at 3 °C.[a]

Reaction	pH	Buffer[b]	ΔH (kcal/mol)[h]	δH^+
Rhodopsin → meta I	8.0	Tris-Acetate or phosphate	16.8 (±1.4)	0
Rhodopsin → meta II	5.4	Acetate	27.4 (±1.3)	+1
	5.4	Piperazine	34.0 (±0.8)	
Rhodopsin + NH_2OH → opsin + Ret-CH=NOH + H_2O	8.0	Tris-Acetate or phosphate	1.0 (±1.8)	0
Rhodopsin + NH_3OH^+ → opsin + Ret-CH=NOH + H_3O^+	5.4	Acetate	0.3 (±1.1)	0
	5.4	Piperazine	−0.8 (±2.7)	
Rhodopsin → opsin + *all-trans*-retinal[c]	8.0	Tris-Acetate or phosphate	25.6 (±5)[d] 22.1 (±2)[e]	0
	5.4	Acetate	11.7 (±2)	+1
	5.4	Piperazine	18.0 (±2)	
Meta I → meta II[f]	(7)	(2% digitonin)	10	+1
	(7)	(2% digitonin + 33% glycerol)	13	
11-*cis*-Retinal → *all-trans*-retinal[g]		(*n*-Heptane)	0.15	

[a] Determined here by direct calorimetry, unless otherwise indicated; 1 cal = 4.184 J; protonation changes (δH^+) to nearest integer. [b] 0.1 M. [c] Calculated from hydroxylamine reactions, as described in the text. [d] Using experimental value for retinal oxime formation at pH 8. [e] Using the heat of retinal oxime formation calculated from pH 5.4 data (see text and Table II). [f] From Matthews et al. (1963) and Ostroy et al. (1966), noncalorimetric. [g] From Hubbard (1966), noncalorimetric. [h] Mean and standard deviation of at least three separate determinations.

TABLE II: Heats of Retinal Oxime Formation and Model Reactions Determined by Flow Calorimetry, 3 °C.

Reaction	pH	Solvent	ΔH (kcal/mol)
Ret-CHO + NH_2OH → Ret-CH=NOH + H_2O	8.0	0.1 M phosphate[a]	−24.6 (±3)[b] −21.1 (±1)[c]
Ret-CHO + NH_3OH^+ → Ret-CH=NOH + H_3O^+	5.4	0.1 M acetate[a]	−11.4 (±0.3)
	5.4	0.1 M piperazine[a]	−18.8 (±0.8)
Ret-CHO + $CH_3(CH_2)_3NH_2$ → Ret-CH=N$(CH_2)_3CH_3$ + H_2O		Ethanol	−12.9 (±1.1)
		1% Emulphogene	−7.4 (±0.3)
Ret-CH=N$(CH_2)_3CH_3$ + H^+ → Ret-CH=N$(CH_2)_3CH_3$ H^+		Ethanol	−8.25 (±0.8)
		1% Emulphogene	−9.9 (±0.9)

[a] In detergent, either 1% Emulphogene or Emulphogene–sodium dodecyl sulfate mixed micelles. [b] Experimental value. [c] Value calculated from pH 5.4 results, using ΔH_i = −9.7 kcal/mol for protonation of hydroxylamine.

with any precision. Hydroxylamine decomposition appears to be platinum catalyzed (Falk and Fatt, 1968) and is a particular problem with the platinum flow tubing of the flow calorimeter used here. No difficulties were encountered with hydroxylamine in the photocalorimeter (gold cells). It is possible, however, to calculate ΔH from the results at pH 5.4 using the known heat of protonation of hydroxylamine (ΔH_i = −9.7 kcal/mol, 3 °C; estimated from Lumme et al., 1965). This gives a value for oxime formation at pH 8 of about −21 kcal/mol which, we feel, is possibly a more realistic value than the experimental measurement under these circumstances. The combination of results for retinal oxime formation and for bleaching in the presence of hydroxylamine gives the enthalpy of the overall reaction shown in Table I.

Pararhodopsin and indicator yellow also form too slowly at 3 °C for us to detect. Preliminary experiments at 25 °C have given heat uptakes in the region of 5 to 15 kcal/mol but are not very reproducible, apparently because of variation in the composition of the final photoproduct mixture. We do not yet fully understand the causes of this variability.

Model Reactions. The heats of formation of aldimine (Schiff base) complexes of *all-trans*-retinal with *n*-butylamine, as a model for at least part of the interaction of retinal with opsin, are presented in Table II. Formation of the unprotonated Schiff base from an unprotonated primary amine is exothermic and quite sensitive to environment since significantly different enthalpies are observed in anhydrous ethanol or in detergent solutions. Protonation of the aldimine linkage is also exothermic but relatively insensitive to environment, as one might expect for a protonation reaction involving no charge separation (Edsall and Wyman, 1958).

The heats of these model reactions give some idea of the possible energetics of the acid and alkali indicator yellow intermediates, which are thought to be the result of nonspecific Schiff base formation between retinal and available amine groups in the protein or lipid of ROS (Morton and Pitt, 1955).

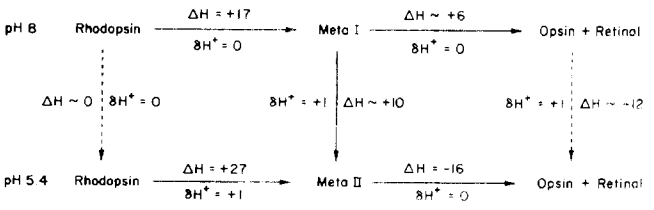

FIGURE 6: Overview of the changes in enthalpy (ΔH, kcal/mol) and protonation (δH^+) during bleaching of ROS. Solid lines refer to experimentally determined quantities, while the dashed lines are calculated to fulfill the condition of zero net change in any thermodynamic cycle.

Discussion

We are able, with the use of the photocalorimeter, to measure directly the relative energies of rhodopsin, meta I, meta II, and the hydrolysis products, opsin + *all-trans*-retinal, in intact ROS membranes at 3 °C. In addition, using buffers with different heats of proton ionization, we have obtained the protonation changes for the various steps. All the energy changes observed are endothermic; i.e., the observable photoproducts are all of higher energy than the parent rhodopsin. Metarhodopsin I formation, at pH 8, involves the uptake of about 17 kcal/mol and, in agreement with previous observations (Wald et al., 1950), results in no change in protonation of the system. Hydrolysis at pH 8 of the retinal–opsin linkage involves an uptake of a further 6 kcal/mol and, once again, no change in protonation.

ΔH for the generation of metarhodopsin II, at pH 5.4, is about +27 kcal/mol and, in this case, the reaction involves the uptake of one hydrogen ion. The overall reaction at this pH has an enthalpy of about +11 kcal/mol and also involves the uptake of a single proton. This implies that the reaction meta II → opsin + retinal is exothermic, at pH 5.4, with $\Delta H \approx -16$ kcal/mol and involves no net change in protonation since we know that protonation has already occurred at the meta II stage.

The overall energy and protonation scheme is depicted in Figure 6. Unfortunately we have no way, from our data alone, of relating the results at the two different pH's. This is because all the energy and protonation changes are measured with respect to the parent rhodopsin at the appropriate pH, and it is conceivable that the absolute energy and protonation state of the rhodopsin might change in going from pH 8 to pH 5.4. Fortunately independent studies of the meta I → meta II reaction are available (Matthews et al., 1963; Ostroy et al., 1966) which allow us to connect up the two sets of data. These studies of the temperature and pH dependence of the meta I ⇄ meta II equilibrium in digitonin-solubilized bovine rhodopsin show that the meta I → meta II reaction involves the uptake of one proton and an enthalpy increase, at constant pH, of about 10 kcal/mol. Although this work was done using rhodopsin solubilized in the detergent, digitonin, separate studies (Lamola et al., 1974) and our own unpublished observations on intact or sonicated rod outer segment membranes suggest, for the metarhodopsin stage at least, that the behavior of rhodopsin in digitonin closely parallels that in the membrane. Assuming that the data for meta I → meta II in digitonin are applicable to the intact membrane system, we can complete the various thermodynamic cycles (Figure 6) and compute the energy and protonation changes upon change of pH for rhodopsin and for the photolysis products.

Thus, it appears that taking rhodopsin from pH 8 to 5.4 involves no change in protonation and, within experimental error, no change in enthalpy. This is in marked contrast to the behavior of the apoprotein, opsin. Noting that retinal does not protonate and is unaffected by pH, we see (Figure 6) that taking opsin from pH 8 to 5.4 involves the exothermic protonation of the protein, with ΔH about −12 kcal/mol.

However, these statements should be qualified somewhat. Since the thermodynamic data for the meta I → meta II transition were determined from spectral changes induced by temperature and pH (Matthews et al., 1963; Ostroy et al., 1966), these data refer only to effects which are coupled to the chromophore absorbance of the rhodopsin. In other words, the values of ΔH and δH^+ upon change of pH for opsin or rhodopsin (Figure 6) refer only to changes associated with the spectral properties of the molecule, and we cannot rule out the possibility that other groups on the protein, not coupled to its photochemical properties, might protonate in this pH range with an associated enthalpy change. However, only those protonation changes detected here have any influence on the spectral properties of rhodopsin and its intermediates.

The protonation data compare well to previous studies on pH changes induced during bleaching of ROS membranes (McConnell et al., 1968; McConnell, 1975) and confirm that only single protonation changes take place during bleaching of rhodopsin in the neutral pH range and in its native membrane environment. It is worth noting here that the calorimetric determination of proton ionization changes (Sturtevant, 1962) requires that the system be well buffered and is, therefore, free of the long-term drift and sensitivity problems associated with studies of pH changes in unbuffered media.

The demonstration of a marked difference in behavior with pH of rhodopsin and its apoprotein, opsin, is perhaps the most significant observation so far. It is clear that in opsin there is an additional group which titrates with an enthalpy of about −12 kcal/mol in going from pH 8.0 to 5.4, and that this group is not available for titration in native rhodopsin in this pH range. The pK_a of this group might suggest that it is either an N-terminal amino group of the protein, which is unlikely since the N terminus of opsin is apparently blocked (Albrecht, 1957; DeGrip et al., 1973), or a histidine residue. However, the heat of protonation is more characteristic of primary amine groups ($\Delta H_i \sim -10$ to −14 kcal/mol, Christensen et al., 1969) than of the histidine imidazole group ($\Delta H_i \approx -7$ kcal/mol), and would indicate that the titrating group is the amino group of a lysine residue which has been exposed by photolysis. Bearing in mind that hydrolysis of the retinal–opsin Schiff base in rhodopsin would generate a free lysyl ϵ-amino group (Bownds, 1967; Akhtar et al., 1968) available for protonation, a simple interpretation of the data is immediately apparent: Schiff base photolysis is the source of the additional titrating group in opsin. In other words, the observations suggest that the active lysine in the retinal binding site of opsin titrates anomalously, with a pK_a in the region of 7. Bownds (1967) made a similar suggestion based on the apparent hydrophobicity of the active site peptide.

Hydrogen ion titration of primary amino groups, involving no net charge separation, is relatively insensitive to environmental dielectric constant effects (Edsall and Wyman, 1958). However, pK_a's are very sensitive to charge proximity effects and, as may be seen from a comparison of pK_a's in diamino compounds (Christensen et al., 1969), the presence of a nearby positively charged group readily results in amine pK_a's of the order 6 to 7. Furthermore, the effect is mainly entropic and the characteristically high heats of amine protonation are relatively unaffected. Thus we suggest that a plausible explanation for a low pK_a of the active lysine in opsin is the presence of a nearby positively charged perturbing group (Figure 7). This group could either be the ϵ-amino function of another, normally

titrating, lysine residue or, possibly, the guanido function of an arginine side chain. (The amino group of phosphatidylethanolamine seems an unlikely candidate since completely delipidated rhodopsin retains its spectral integrity (Hong and Hubbell, 1973).) In either case the presence of the nearby positively charged group would inhibit protonation of the active lysine. This would, furthermore, suggest a mechanism for intramolecular protonation of the Schiff base nitrogen in rhodopsin (Figure 7), possibly involving protonation via hydrogen bonding of the general type -NH- -N- (Pimentel and McClellan, 1960), a form of which has been observed in association with aldimine-like linkages in crystals (Hall and Llewellyn, 1956).

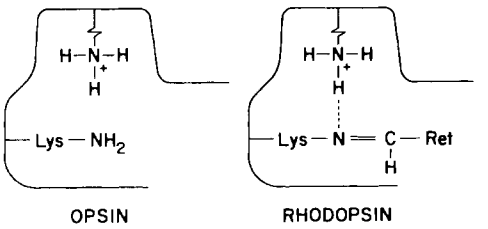

FIGURE 7: Proposed interactions in the retinal binding site of rhodopsin. A nearby protonated group inhibits titration of the active lysine residue in opsin and provides a source of intramolecular protonation of the aldimine nitrogen in rhodopsin. The protonating group has been depicted as the ϵ-NH_2 of another lysine, but could equally well be an arginine residue.

The proposal that the Schiff base linkage in rhodopsin might be protonated originated from the need to explain the large red shifts of the spectra of visual pigments with respect to free retinal and unprotonated model Schiff bases. The only direct evidence for the protonated Schiff base comes from the observation of a particular band in resonance enhanced Raman spectra of rhodopsin (Oseroff and Callender, 1974; Mathies et al., 1976). This band at about 1655 cm^{-1}, assigned to the C=N stretch of the aldimine linkage, is characteristic of protonated model Schiff base complexes and is sensitive to deuteration, suggesting the presence of an exchangeable hydrogen associated with the Schiff base nitrogen (Oseroff and Callender, 1974). The intramolecular protonation scheme shown in Figure 7 is qualitatively consistent with these observations and would seem to provide the required electron delocalization in the chromophore necessary to explain at least part of the red shift in native rhodopsin. Moreover, without elaborating on various alternative models, it is the simplest scheme consistent with the protonation and energetic differences between rhodopsin and its photolysis products determined above. In addition, and in contrast to simple model Schiff base complexes (Morton and Pitt, 1955), the reduced pK_a of the active lysine, together with intramolecular protonation of the aldimine linkage, provides a pigment which should be insensitive to pH below about pH 9, and less susceptible to hydrolysis in the native state—both of which are features of rhodopsin.

The meta I → meta II step in the bleaching process has received considerable attention since it is relatively easy to study and is the last step in the sequence which could conceivably be related to the primary visual excitation mechanism. The molecular processes involved in the reaction remain, however, unclear. As we have seen, the reaction proceeds with the uptake of one proton and an enthalpy increase of about 10 kcal/mol. The reaction requires the presence of water (Wald et al., 1950) and is accompanied by an increase in volume of the system (Lamola et al., 1974). As pointed out in the original description of this reaction (Matthews et al., 1963), metarhodopsin II has an absorption spectrum close to that of free retinal but, because uv irradiation readily regenerates rhodopsin or isorhodopsin, contains *all-trans*-retinal still attached to opsin at the chromophoric site. By analogy with model Schiff base complexes, Matthews et al. (1963) proposed that the meta I → meta II reaction involved the deprotonation of the isomerized retinal–opsin aldimine linkage but, realizing that the reaction actually involves the *uptake* of one proton, were forced to suggest that two additional protein groups become protonated in the process. This seems an inelegant solution and is, indeed, difficult to justify in any thermodynamically consistent model.

If we now note that proton uptake at the metarhodopsin stage occurs in a similar pH range to the proton uptake already described for opsin alone, we are encouraged to suggest, in the absence of any evidence for rearrangement of protonation sites in the intermediate steps, that the protonating group is identical in the two situations; i.e., it is the active lysine of the chromophore binding site. This presupposes that this lysine actually exists in an accessible form in meta II and implies that the Schiff base has hydrolyzed. Thus, we suggest that the meta I → meta II reaction actually involves the hydrolysis of the specific retinal–opsin Schiff base linkage, with consequent exposure and protonation of the ϵ-amino group of the reactive lysine residue, giving *all-trans*-retinal, in the free aldehyde form, still bound by noncovalent interactions in the region of the active site of opsin. This would give a product with spectral properties similar to free retinal and, as long as the retinal resides in the active site, the reaction would be readily reversed by appropriate adjustment of pH and temperature. After hydrolysis to form metarhodopsin II, the retinal could subsequently migrate from the active site forming, on its way, nonspecific Schiff base complexes (indicator yellow?) with any available primary amino groups on the same, or adjacent, proteins or on ethanolamine in the lipid moiety, terminating eventually in opsin plus completely free *all-trans*-retinal under appropriate conditions.

In support of this hypothesis we note: (i) that meta I → meta II will not proceed in the absence of water (Wald et al., 1950), an obvious requirement for hydrolysis; (ii) hydroxylamine, a reagent which forms oximes with free retinal aldehyde groups, is reactive at the metarhodopsin stage, apparently with meta II and not meta I (Falk and Fatt, 1968; Johnson, 1970); (iii) the enthalpy of the meta I → meta II reaction is, at least, consistent with hydrolysis of an aldimine linkage as determined from appropriate model reactions (Table II). The energetics of hydrolysis are not decisive, however, since we have seen that they are sensitive to environment even in model systems. Furthermore, the actual metarhodopsin reaction may well include additional energetic factors arising from conformational changes in the protein and/or lipid components of the system.

We have, so far, given little consideration to the absolute values of the enthalpy changes induced in rhodopsin by bleaching. Since the cis–trans isomerization of free retinal is essentially isoenergetic (Hubbard, 1966), it follows that the enthalpies of regeneration of rhodopsin from opsin + 11-*cis*-retinal, whatever the mechanism, are simply the reverse of the heats of overall photolysis (Table I). These energies must be interpreted with caution, but it does appear that the energies are somewhat higher than might be expected from Schiff bases alone. The additional energy could reflect contributions to the stability of the complex arising from noncovalent interactions or protein conformational changes.

Our current aim is to extend these measurements to much lower temperatures in order to render accessible the very

earliest steps in the bleaching process and thus determine the fate of the photon energy after absorption by the chromophore. The earliest intermediate we can observe with our present instrument is metarhodopsin I, but it is clear even at this stage, presumably prior to any hydrolysis of the chromophore, that significant energy changes, vastly in excess of the energy of retinal isomerization alone, have taken place. We have no way of telling whether this energy increase represents energy stored in the system from the initial photon absorption or whether it is the result of subsequent thermal reactions, but it apparently reflects significant structural and/or chemical changes in the protein and/or membrane system related to the overall visual excitation process.

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