THE MOLAR EXTINCTION OF RHODOPSIN

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Recent progress in the chemistry of rhodopsin has stimulated interest in its molecular properties and has at the same time provided improved means for determining them. One of the most useful properties is the molar extinction. This can serve as a measure of concentration, a criterion of purity, and a basis for establishing stoichiometric relations among rhodopsin, its precursors, and the products of its bleaching.

The purpose of the present paper is to determine the molar extinction of rhodopsin in terms of its retinene equivalent—the extinction of rhodopsin which arises from, or yields on bleaching, a molar solution of retinene. This is the ***minimum molar extinction*** of rhodopsin. Its true molar extinction is some simple multiple of this value, depending upon the number of retinene residues which one rhodopsin molecule contains. This information is applied to calculating the quantum efficiency of the bleaching of rhodopsin, and the number of sulfhydryl groups exposed in this process.

General Considerations

Rhodopsin is formed by the combination of the cis isomer of retinene, neoretinene *b*, with opsin; and it bleaches to a mixture of all-trans retinene and opsin. The molar extinction of all-trans retinene has been determined accurately, that of neoretinene *b*—for want of material—somewhat less accurately. One might therefore base the estimation of the molar extinction of rhodopsin on the amount of all-trans retinene liberated in its bleaching.

The difficulty with this procedure, however, is that all-trans retinene emerges from the bleaching of rhodopsin, not as the free molecule, but bound to amino groups of opsin in the labile complex called "indicator yellow." The absorption spectrum of this complex changes markedly with pH, in shape, height, and position of the maximum. At all pH values it probably differs significantly from the spectrum of free retinene (Collins and Morton, 1950 *a*; Collins, 1953).

For this reason we have preferred to base our determination of the molar extinction of rhodopsin upon the all-trans retinene oxime formed when rhodopsin is bleached in the presence of hydroxylamine. Unlike "indicator yellow,"

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this is a single, well defined product which replaces all the fortuitous combinations that retinene might otherwise form with whatever amino groups happen to be present. Its absorption spectrum is also relatively independent of pH, remaining virtually unchanged between pH 4 and 9.

Measurements

The net results of our measurements are summarized in Table I. The relevant operations are described below.

TABLE I

Extinctions of rhodopsin; of neoretinene *b*, the isomer of retinene from which it is formed; of all-trans retinene, the isomer which it yields on bleaching; and of all-trans retinene oxime, the product of its bleaching in the presence of hydroxylamine. Each extinction is measured at the appropriate absorption maximum ($\lambda_{max.}$); and is presented as E (1 per cent, 1 cm.)—the extinction of a 1 cm. layer of a 1 per cent solution; or as the molar extinction, ϵ.

Substance	Solvent	$\lambda_{max.}$	E (1 per cent, 1 cm.)	ϵ (cm.)^2 per mole
		mμ		
All-trans retinene oxime	Ethanol	355	2020	60,400
	Digitonin	367	1730	51,600
All-trans retinene	Ethanol	383	1510	42,900
	Digitonin	389	1350	38,300
Neoretinene *b*	Ethanol	377.5	(950)	(27,000)
	Digitonin	384	(825)	(23,500)
Rhodopsin (per retinene equivalent)	Digitonin	500	—	40,600

Molar Extinction.—This quantity, otherwise called the molar or molecular extinction coefficient, is defined by the equation:

$$\log_{10} I_0/I = \epsilon \cdot c \cdot l,$$

in which I_0 is the intensity of light incident on the solution, I the intensity transmitted, ϵ the molar extinction, c the concentration in moles per liter, and l is the depth of the solution in centimeters. Since the ratio I_0/I is a pure number, and the product $c \cdot l$ has the dimensions:

$$\frac{\text{Moles} \times \text{centimeters}}{(\text{Centimeters})^3} = \frac{\text{moles}}{\text{cm.}^2}$$

the molar extinction, ϵ, has the dimensions cm.^{2} per mole.

It has become customary in carotenoid chemistry, particularly with relation to the vitamins A and retinenes, to express the extinction in terms of E (1 per cent, 1 cm.), the extinction of a solution containing 1 gm. of solute per 100 ml., measured in a layer 1 cm. in depth. This quantity, even in dealing with a

single substance, has no advantage over the molar extinction; and it is useless in specifying equivalence relations among a variety of interacting molecules. The time has probably come to replace it altogether by the molar extinction. In the present paper it is retained along with the molar extinction only because it has had such wide currency in the past.

Since E (1 per cent, 1 cm.) is the extinction of a solution containing 10 gm. of solute per liter, the molar extinction is this quantity multiplied by one-tenth the molecular weight:

$$\epsilon = E(1 \text{ per cent, } 1 \text{ cm.}) \times \frac{\text{molecular weight}}{10}$$

All-Trans Retinene Oxime.—This compound was prepared by treating all-trans retinene with a large excess of hydroxylamine in ethanol solution. It was purified by chromatographic adsorption on calcium carbonate or on powdered

TABLE II

Extinction of All-Trans Retinene Oxime in Solution in Ethyl Alcohol

Preparation	Crystallized from	Melting point	E(1 per cent, 1 cm.)
		°C.	
17.IV.53	Petroleum ether, then methanol		2025
23.IV.53	Twice from methanol		2065
29.V.53	Twice from methanol, twice from petroleum ether	153–154.4	1980
Average			2020

alumina which had been "weakened" by stirring in 5 per cent by weight of water. In each of three independent preparations, the oxime was crystallized 2 to 4 times from petroleum ether or methyl alcohol. The results are shown in Table II.

The average E (1 per cent, 1 cm.) is 2020; and since the molecular weight of retinene oxime is 299, the corresponding value of the molar extinction, ϵ, is $29.9 \times 2020 = 60{,}400$.

The extinction of all-trans retinene oxime in digitonin was determined by diluting a solution of the pigment in ethanol with an equal volume of 2 per cent aqueous digitonin; and comparing the result with that of diluting a solution of the pigment in digitonin with an equal volume of ethanol. In this way, through the common medium of the 1:1 mixture of solvents, the ratio of extinctions in ethanol and aqueous digitonin, each at its own $\lambda_{max.}$, was determined. This proved to be:

$$\frac{\text{Extinction of all-trans retinene oxime in ethanol}}{\text{Extinction of all-trans retinene oxime in aqueous digitonin}} = 1.17$$

This ratio, applied to the extinctions in ethanol, yielded the extinctions in digitonin shown in Table I.

Rhodopsin.—The retinene which emerges from the bleaching of rhodopsin is predominantly the all-trans isomer. This, however, is isomerized by simple exposure to light, specifically by the blue, violet, and ultraviolet light which retinene absorbs, with consequent changes in the height and position of the absorption spectrum. Indeed a trace of isomerization appears to be inevitable; for even when, as in the present experiments, rhodopsin is bleached with orange light, which has scarcely any effect upon retinene itself, it still seems to accomplish some isomerization of one or both intermediate products of bleaching —lumi-rhodopsin and meta-rhodopsin. These intermediates have absorption spectra close to that of rhodopsin, and so absorb any quality of light which can be used for bleaching (Wald, Durell, and St. George, 1950; *cf.* Hubbard and Wald, 1952–53).

Hydroxylamine improves this situation in two ways. In its presence, the intermediate stages of bleaching pass almost instantaneously, so that light has little opportunity to work upon them. In addition, the final product, retinene oxime, has its absorption spectrum displaced so far into the ultraviolet compared with free retinene, that it is easily protected from isomerization by the lights used to bleach rhodopsin. These circumstances tend to hold isomerization of the products of bleaching to a minimum.

Fig. 1 shows the absorption spectrum of a relatively pure solution of cattle rhodopsin, and the spectrum of the product of its bleaching in the presence of hydroxylamine. The ratio of the extinctions at 400 and 500 mμ ("400/500 ratio") of this rhodopsin preparation was 0.22; and this and the entire course of the absorption spectrum show it to have been relatively free from impurities absorbing in the ultraviolet.

The ratio of the extinction of rhodopsin to that of the resulting retinene oxime was:

$$\frac{\text{Extinction of rhodopsin at 500 m}\mu}{\text{Extinction of retinene oxime at 367m}\mu} = 0.787$$

An added assurance that the retinene oxime was virtually entirely the all-trans isomer is given by the comparison with the absorption spectrum of crystalline all-trans retinene oxime in 2 per cent aqueous digitonin, shown in Fig. 1. It is plain therefore that the molar extinction of rhodopsin, in terms of its retinene equivalent, is:

$$\epsilon = 51{,}600 \times 0.787 = 40{,}600 \text{ cm.}^2 \text{ per mole}$$

All-Trans Retinene.—Earlier estimates in this and other laboratories of E (1 per cent, 1 cm.) for this substance in ethyl alcohol have ranged between 1400 and 1660 (Hubbard, Gregerman, and Wald, 1952–53). We have under-

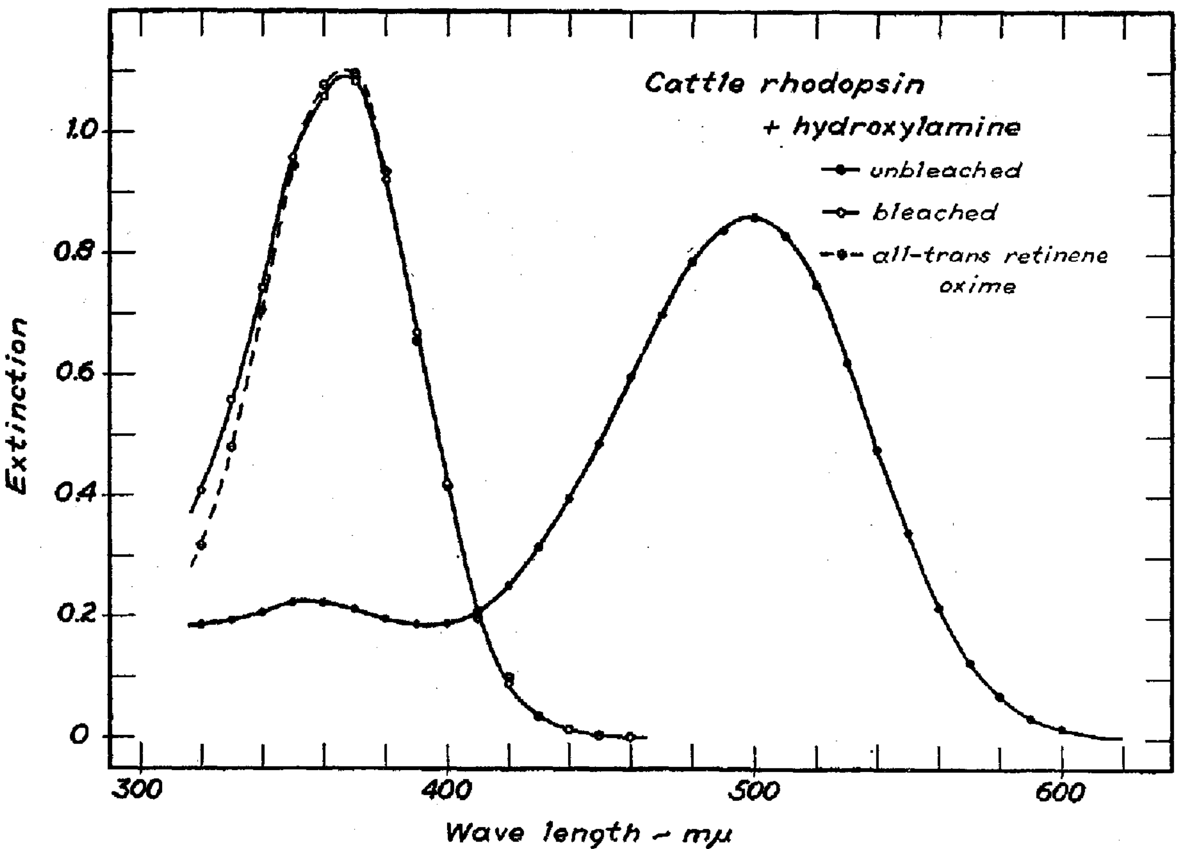

FIG. 1. Absorption spectra of rhodopsin and of the product of its bleaching in the presence of hydroxylamine (0.25 M). The rhodopsin was bleached in the orange light of a tungsten filament lamp passing through a Jena OG2 filter; such light is not absorbed by retinene oxime, and so does not isomerize it. Under such circumstances the products of bleaching are opsin and all-trans retinene oxime. The absorption spectrum of crystalline all-trans retinene oxime in aqueous digitonin solution is shown for comparison.

taken a new series of such measurements, using three independent preparations, each of which had been crystallized 2 to 5 times from methyl alcohol or petroleum ether. The results are shown in Table III.

The average E (1 per cent, 1 cm.) is 1510; and since the molecular weight of retinene is 284, the corresponding value of the molar extinction, ϵ, is $1510 \times 28.4 = 42{,}900$.

The extinction of all-trans retinene in 2 per cent aqueous digitonin was determined by the same procedure as was used above with retinene oxime. The ratio of extinctions was determined in ethanol, and in this diluted with an equal volume of aqueous digitonin; this was compared with the ratio of ex-

TABLE III

Extinction of All-Trans Retinene in Solution in Ethyl Alcohol

Preparation	Crystallized from	Melting point*	E(1 per cent, 1 cm.)
		°C.	
23.IV.53	Twice from methanol	67.1–68.4	1500
27.IV.53	Twice from methanol, twice more from petroleum ether	67.2–67.9	1516
28.V.53	Five times from petroleum ether	67.7–69.3	1500
Average			1510

* The melting points shown in this and Table II were measured with a micro melting point apparatus of the Kofler type. Single crystals were observed in groups of three. In the temperature ranges as given, the lower temperature is that at which the crystals began to liquefy, the upper that at which liquefaction was complete. Each range is the average of several such determinations. To check the procedure, a series of similar measurements was performed with p-dichlorobenzene. The melting point of this substance is given as 53°C.; the average of our low and high values was 52.7°C. This implies that the best single value for our melting points is the average of the low and high readings. For all-trans retinene this is 68°; for all-trans retinene oxime, 154°.

tinctions in aqueous digitonin alone, and diluted with an equal volume of ethanol. From these ratios, it was apparent that:

$$\frac{\text{Extinction of all-trans retinene at 383 m}\mu\text{ in ethyl alcohol}}{\text{Extinction of all-trans retinene at 389 m}\mu\text{ in aqueous digitonin}} = 1.12$$

The E (1 per cent, 1 cm.) of all-trans retinene in aqueous digitonin is therefore $1510/1.12 = 1350$; and its molar extinction is $42{,}900/1.12 = 38{,}300$.

From these measurements it appears that the ratio of the extinctions of all-trans retinene and its oxime in ethanol should be $42{,}900/60{,}400 = 0.71$. This ratio has been determined directly by adding hydroxylamine to a solution of all-trans retinene in ethanol, converting it to the oxime. The ratio observed under these conditions was 0.72.

From the foregoing data one can conclude also that the extinction of all-trans retinene in digitonin produced by bleaching unit extinction of rhodopsin is given by the ratio:

$$\frac{\text{Molar extinction of all-trans retinene at 389 m}\mu}{\text{Molar extinction of rhodopsin at 500 m}\mu} = 0.943$$

This is close to the ratio of extinctions observed when rhodopsin is bleached in approximately neutral solution with non-isomerizing light—light which lacks the violet and ultraviolet components which are absorbed by and hence isomerize retinene. As already remarked, however, the all-trans retinene produced under these circumstances is in large part loosely bound to amino groups of opsin, and therefore is not strictly comparable with free retinene.

Neoretinene b.—The extinction of neoretinene *b* is known only approximately, since only a small amount of this substance has been crystallized, and none recrystallized. The E (1 per cent, 1 cm.) has been estimated to be 900 to 1000 at 377.5 mμ in ethyl alcohol (Hubbard, Gregerman, and Wald, 1952–53). We may tentatively take 950 as an approach to the correct value. The E (1 per cent, 1 cm.) in aqueous digitonin may be taken to be about 950/1.15 = 825 (384 mμ). The corresponding molar extinctions are 27,000 in ethyl alcohol and 23,500 in digitonin. From these values it appears that the ratio between the extinction of neoretinene *b* in aqueous digitonin and the extinction of rhodopsin to which it gives rise on incubation with opsin should be:

$$\frac{\text{Extinction of neoretinene } b \text{ at 384 m}\mu}{\text{Extinction of rhodopsin at 500 m}\mu} = \frac{23{,}500}{40{,}600} = 0.58$$

This relation provides some basis for estimating the activity of preparations of neoretinene *b* in forming rhodopsin.

DISCUSSION

Retinene Equivalents of Rhodopsin and Its Chromophore.—As already noted, the molar extinction of rhodopsin per retinene equivalent is its ***minimum*** molar extinction. The true molar extinction is this value, multiplied by the number of retinene molecules involved in the formation or bleaching of a single molecule of rhodopsin. This number is not yet known.

A related problem is concerned with the extinction per chromophore of rhodopsin. Having originally suggested that each chromophore may be made of two molecules of retinene (Wald, 1949), a notion developed further by Collins and Morton (1950 *b*), we have recently considered it more probable that a single molecule of retinene constitutes each rhodopsin chromophore (Wald, 1953). In the latter case the molar extinction per retinene equivalent is also the molar extinction of the chromophore.

Quantum Efficiency of Bleaching.—As the result of a careful quantitative

study of the bleaching of frog rhodopsin in solution, Schneider, Goodeve, and Lythgoe (1939) evaluated the product of the absorption coefficient and the quantum efficiency to be:

$$\alpha\gamma = 9.1 \times 10^{-17}$$

in which α is the absorption coefficient ($\log_e I_0/I$) of a solution of rhodopsin which contains 1 chromophoric group per ml.; and γ is the quantum efficiency; *i.e.*, the number of chromophores of rhodopsin destroyed for each quantum of light absorbed.

This value of $\alpha\ \gamma$ is converted to the basis of molar extinction by multiplying by Avogadro's number, 6.0×10^{23}; by 0.434, the factor which converts natural to common logarithms; and by 0.001 to convert from milliliters to liters. The resulting product is

$$\epsilon'\gamma = 23{,}700$$

ϵ' here is the molar extinction in terms of chromophoric groups of rhodopsin.

We have already evaluated the molar extinction in terms of retinene equivalents of rhodopsin (ϵ). If, as we believe probable, $\epsilon' = \epsilon$, *i.e.* each chromophore of rhodopsin is equivalent to a single molecule of retinene, the quantum efficiency of bleaching is $23{,}700/40{,}600 = 0.58$; *i.e.*, it is a little more than one-half. If on the other hand it were true that each rhodopsin chromophore is made from two molecules of retinene, the quantum efficiency of bleaching would be a little more than one-quarter.

An altogether independent reason exists for believing that the quantum efficiency of bleaching in solution may in fact be about one-half. When rhodopsin is irradiated at very low temperatures or in the dry state, it undergoes at first the light reaction alone, converting it to lumi-rhodopsin. When this is allowed to complete its transformations in the dark, at room temperature and in the presence of water, about half of it is converted to retinene and opsin, while the other half reverts to a mixture of rhodopsin and isorhodopsin. Of all the molecules of rhodopsin which go through the light reaction under these conditions therefore, only about half bleach to retinene (Wald, Durell, and St. George, 1950; Collins and Morton, 1950 *b*).

We attach no special importance to the value one-half for this fraction, unlike Collins and Morton (1950 *b*), who suggest that it is the outcome of a dismutation. Recent unpublished experiments by St. George in our laboratory show that the fraction can vary widely, depending upon the conditions of irradiation and the nature of the preparation.

We would conclude that in all probability the chromophore of rhodopsin is derived from a single molecule of retinene; that the primary photochemical process which converts rhodopsin to lumi-rhodopsin has a quantum efficiency of one; and that subsequent dark reactions, by reverting some of the photo-

chemical product to rhodopsin and isorhodopsin, may reduce the over-all quantum efficiency of bleaching by various amounts, on occasion to as little as one-half.[1]

We do not know how closely these relations observed in solution hold also for the bleaching of rhodopsin in the rods. This question is of some interest, for it has been shown by direct measurement that 5 to 15 quanta of light absorbed by rhodopsin in the retina in a single flash can be seen. Since this small number of quanta was distributed over a retinal area containing some 500 rods, almost certainly the absorption of 1 quantum can stimulate a rod (Hecht, Shlaer, and Pirenne, 1941–42). This quantum requirement is already so surprisingly low that it is difficult to believe it to be reduced further by a low quantum efficiency of bleaching. Fortunately no such assumption seems necessary. The times required for visual excitation are so short as to make it almost certain that the excitation process cannot wait upon the relatively slow bleaching of rhodopsin to retinene, but must depend instead upon the light reaction itself or some process which follows instantly upon it (Wald, Durell, and St. George, 1950). As noted above, the quantum efficiency for these initial stages in the bleaching process seems to be one.

Liberation of Sulfhydryl Groups in Bleaching.—The bleaching of rhodopsin not only liberates retinene, but exposes new sulfhydryl groups on opsin. These have been measured by titration with silver nitrate. It was estimated that on the average two —SH groups appear for each retinene molecule that is liberated (Wald and Brown, 1951–52).

At the time of these observations, the role of stereoisomers in the formation and bleaching of rhodopsin was not yet appreciated, and we did not have as accurate an estimate of the molar extinction of rhodopsin as is now available. We have therefore recalculated our measurements on the basis of the present information.

The results are shown in Table IV. All the measurements agree in estimating the number of —SH groups liberated per retinene molecule as 2 to 3, with an over-all average near 2.5. The results obtained with cattle rhodopsin lie somewhat lower, averaging 2.18; while those with frog rhodopsin lie higher, averaging 2.72. The single determination on squid rhodopsin does little more than indicate that it lies within the same range.

Obviously in the rhodopsin molecule as a whole we must be dealing with

[1] Having assumed that each chromophore group of rhodopsin is made from two molecules of retinene, Collins and Morton (1950 *b*) concluded that the primary photochemical conversion of rhodopsin to lumi-rhodopsin has a quantum efficiency of one half, and that over-all bleaching has a quantum efficiency of about one fourth. They had calculated also that the molar extinction of rhodopsin is about 48,000 per retinene residue; and hence set 96,000 as the approximate molar extinction per chromophoric group.

whole numbers of —SH groups and retinene residues. Such a value of the —SH: retinene ratio as 2.5 must mean that each rhodopsin molecule contains at least 2 retinene residues, and exposes at least 5 —SH groups on bleaching. On the other hand there is no *a priori* reason why the rhodopsins from different animals should have the same molecular constitution or be of the same size. The results with cattle rhodopsin do not depart sufficiently from 2 to make

TABLE IV

Sulfhydryl: Retinene Ratio in the Bleaching of Rhodopsin

Comparison of the numbers of sulfhydryl (—SH) groups and retinene molecules liberated in the bleaching of rhodopsin. The molar retinene equivalent of rhodopsin is calculated from the molar extinction, $\epsilon = 40{,}600$. The moles of sulfhydryl exposed on bleaching are estimated by silver titration. Data from Wald and Brown, 1951–52.

Animal	Preparation	Moles × 10^{-9}		Mole ratio, —SH:retinene	Averages
		Retinene equivalent of rhodopsin	—SH groups		
Cattle	(1)	4.05	10.0	2.47	
		2.03	4.5	2.22	
		2.03	4.5	2.22	
	(2)	6.05	15.5	2.56	
	(3)	6.56	12.0	1.83	
		6.56	11.5	1.75	2.18
Frog	(1)	1.26	4.0	3.17	
		0.88	2.25	2.55	
	(2)	3.91	10.0	2.56	
		1.96	5.45	2.78	
		1.96	5.00	2.55	2.72
Squid	(1)	2.32	6.70	2.89	
Over-all average					2.46

this an improbable value; in this case the whole molecule may contain a single residue of retinene, and expose two —SH groups on bleaching. In frog rhodopsin the —SH: retinene ratio seems to be higher, perhaps 5:2 or even 3:1.

Some evidence exists that the sulfhydryl groups which appear when rhodopsin is bleached were engaged at least in part in binding retinene to opsin. It is easy to find place for one sulfhydryl group per retinene in this role; two pose a little difficulty, and more than two are well nigh impossible. Some sulfhydryl may therefore have had a different origin. The bleaching of rhodop-

sin presents many of the properties of a protein denaturation; and like many denaturations it may result in exposing sulfhydryl and other groups on the protein, which had previously been unavailable to reagents (Wald and Brown, 1951–52).

SUMMARY

The molar extinction of rhodopsin is 40,600 cm.[2] per mole equivalent of retinene; *i.e.*, this is the extinction of a solution of rhodopsin which is produced by, or yields on bleaching, a molar solution of retinene. The molar extinctions of all-trans retinene and all-trans retinene oxime have also been determined in ethyl alcohol and aqueous digitonin solutions.

On the assumption that each chromophoric group of rhodopsin is made from a single molecule of retinene, it is concluded that the primary photochemical conversion of rhodopsin to lumi-rhodopsin has a quantum efficiency of 1; though the over-all bleaching of rhodopsin in solution to retinene and opsin may have a quantum efficiency as low as one-half.

On bleaching cattle rhodopsin, about two sulfhydryl groups appear for each molecule of retinene liberated. In frog rhodopsin the —SH:retinene ratio appears to be higher, 5:2 or perhaps even 3:1. Some of this sulfhydryl appears to have been engaged in binding retinene to opsin; some may have been exposed as the result of changes in opsin which accompany bleaching, comparable with protein denaturation.

Note Added after Submission of Manuscript.—Since this paper was written, Hubbard (1953–54) has completed a study of the molecular weight of cattle rhodopsin in digitonin solution. She has found the molecular weight to be about 40,000; and that the molecule apparently contains a single chromophore group composed of one molecule of retinene. The minimum molar extinction which we have computed here on the basis of the retinene equivalent is therefore the true molar extinction of cattle rhodopsin.

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