THE MOLECULAR WEIGHT OF RHODOPSIN AND THE NATURE OF THE RHODOPSIN-DIGITONIN COMPLEX

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I

INTRODUCTION

Rhodopsin, the photosensitive pigment of rod vision, is a chromoprotein composed of the colorless protein, opsin, combined with the yellow carotenoid, retinene ($C_{19}H_{27}CHO$). The chemistry of rhodopsin has been explored for many years, but its molecular weight is still unknown. This is primarily due to the fact that rhodopsin, as also its protein moiety opsin, can be brought into aqueous solution only with the aid of such solubilizers as bile salts (Kühne, 1879) or digitonin[1] (Tansley, 1931). These solutions contain not free rhodopsin, but complexes of rhodopsin with the solubilizer (*cf.* Wald, 1944). A number of such protein-detergent complexes have recently been described (for a review, see Putnam, 1948).

Some years ago, Hecht and Pickels (1938) found that digitonin solutions of frog rhodopsin sediment in the ultracentrifuge as particles of uniform size, with a molecular weight of about 270,000, and they believed this to be the molecular weight of rhodopsin. But they were in fact observing the sedimentation behavior of a complex containing both rhodopsin and digitonin. From its weight one cannot draw any conclusions about the molecular weight of rhodopsin.

Smith and Pickels (1940) later showed that digitonin itself sediments in the ultracentrifuge in the form of monodisperse micelles with a particle weight of at least 75,000, and containing at least sixty molecules of digitonin. All the digitonin molecules engaged in micelle formation, so that practically none was left in the supernatant.

We have reexamined the sedimentation behavior of solutions of digitonin and rhodopsin in digitonin. From this and other analytic procedures it has

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[1] Digitonin ($C_{56}H_{92}O_{29}$) is a *non-ionic* detergent with a molecular weight of 1229.3 gm.

been possible to derive values for the molecular weight of rhodopsin, the number of chromophore groups per rhodopsin molecule, and the number of retinene molecules per chromophore.

II

Methods

Preparation of Rhodopsin Solutions.—Rhodopsin was prepared by a modification of the method described by Wald and Brown (1952). Dark-adapted cattle eyes were obtained fresh from the slaughter house, and all operations carried out in red light. The retinas were dissected immediately and stored in the dark at −12°C. until needed. Then the rod outer limbs were isolated from sixty to seventy retinas. For this, the retinas were thawed and ground with neutral M/15 phosphate buffer, and the suspension filtered through several layers of coarse cheese-cloth. The filtrate was centifuged at 3000 R.P.M. for about 25 minutes to spin down the rods and other tissue fragments, and these were stirred with 25 or 30 ml. of 36 per cent sucrose in neutral M/15 phosphate buffer. The suspension of particles in sucrose was centrifuged about 5 minutes at 1800 R.P.M. The outer limbs are isopycnotic with about 30 per cent sucrose (Collins, Love, and Morton, 1952 *a*), and remain in the supernatant. This was diluted with phosphate buffer to 30 per cent sucrose, and recentrifuged about 4 minutes at 1800 R.P.M. The sediment was again discarded, and the centrifugation repeated two or three times, always discarding the sediments, until no further separation was achieved at this speed. This procedure is more wasteful of outer limbs than the method described by Wald and Brown, but it consistently yields solutions of high purity by spectrophotometric criteria (*viz.* section III).

The sucrose suspension was now diluted with 3 volumes of buffer and centrifuged at 3000 R.P.M. to sediment the rod outer limbs. These were hardened in 4 per cent alum (potassium aluminum sulfate) for about 1 hour at 5°C., and washed once each with distilled water and neutral phosphate buffer. They were then dried by grinding with about 3 volumes of anhydrous sodium sulfate, and extracted with three successive portions of petroleum ether. The dry outer limb–sodium sulfate powder was extracted with distilled water until free of sodium sulfate, and the outer limbs washed with neutral phosphate buffer and stored frozen at −12°C. When needed, they were thawed and the rhodopsin extracted at 5°C. with a solution of 1.8 per cent digitonin (Hoffmann–La Roche, Inc.) in M/15 phosphate buffer, pH 6.3. For experiments at other pH's, the extraction was performed with aqueous digitonin, and the extracts diluted with buffer of the desired pH. Outer limbs from 20 retinas were extracted with 2.3 ml. digitonin solution, but they always retained a small amount of rhodopsin. The purity of these preparations is discussed in section III.

Ultracentrifuge Measurements.—The ultracentrifuge studies were performed with a Spinco ultracentrifuge, model E, with type A analytical rotor. Although the ultracentrifuge pattern of rhodopsin is not changed by bleaching (see section IV), we used unbleached rhodopsin in order to correlate the sedimentation of the rhodopsin color and refractive index boundary, particularly in mixtures exhibiting more than one boundary.

For this purpose, the ultracentrifuge cell was filled and mounted in red light. As

an added precaution, a shutter was attached to the collimating lens between the light source and rotor compartments, and opened only during a measurement. An orange filter (Ilford 808), transmitting light only at wave lengths longer than 555 mμ, was also inserted in the light path, and stray light from the lamp housing eliminated by a black cardboard baffle. With these precautions, less than 1 per cent of the rhodopsin bleached in the course of a sedimentation experiment.

Determination of Density and Viscosity.—To calculate the sedimentation constant (s_{20}) of a particle from its sedimentation velocity,[2] one must know the density and viscosity of the medium through which it moves.[3] These properties were therefore determined for phosphate buffer (M/15, pH 6.3) and buffered solutions of digitonin.

Densities were measured using a constriction pipette (Linderstrøm-Lang and Holter, 1940), calibrated to hold 0.7579 ml., as a pycnometer. The openings at the constriction and the tip of the pipette were sufficiently small to permit accurate weighing of the filled pipette. The viscosities were calculated for each solution from its density and the rate of flow relative to water in a modified Ostwald viscosimeter. The density and viscosity of the phosphate buffer at 20°C. were thus found to be respectively 1.0050 gm. per ml. and 1.0291 poises. The corresponding values for buffered 1.8 per cent digitonin were 1.0109 gm. per ml. and 1.1005 poises.

Determination of the Partial Specific Volumes[2] *(V_{20}) of Digitonin and Rhodopsin-Digitonin.*—The partial specific volume of a substance can be defined as the increase in volume caused by the addition of 1 gm. of the substance to a solution at infinite dilution. It is determined experimentally by dissolving a known weight of the substance in a measured (and relatively large) volume of solvent, and determining the densities of the solvent and solution. From these values, one can readily calculate the volume occupied by 1 gm. of the solute.

The densities of dilute aqueous solutions of digitonin and rhodopsin-digitonin were determined by the method described above. The concentration of the digitonin solution was determined by dissolving a known weight of the dry powder in a weighed amount of water. With rhodopsin-digitonin, a weighed sample of an aqueous solution was dried to a constant weight *in vacuo* over P_2O_5.

Electrophoresis.—An electrophoresis experiment was performed with the Tiselius-Svensson apparatus, type L.K.B. 3023 (Svensson, 1946), with a tungsten ribbon

[2] We are using the conventions with regard to nomenclature established by Svedberg and Pedersen (1940). s_{20} represents the reduced sedimentation constant, corrected for sedimentation in water at 20°C. Likewise, the symbols D_{20}, ρ_{20}, and V_{20} are used below to represent the reduced values of the diffusion constant, particle density, and partial specific volume, respectively.

[3] These calculations are based upon the mathematical formulations of Svedberg and Pedersen (1940), and on the nomographic charts prepared by Wyman and Ingalls (1943) to correlate some properties of proteins.

filament lamp as light source. To protect rhodopsin from bleaching, a red filter (Ilford 205) was mounted on the lamp housing, and stray light from the housing reduced by a baffle. Measurements were made in a darkened room, and at 0°C.[4]

Paper electrophoresis experiments were done in a darkened cold room at 5°C., using the hanging strip method (Durrum, 1950). The solutions were applied to strips of filter paper (Whatman No. 3), and the experiments performed at a D.C. potential of 220 volts, with a gradient of 3.8 volts per cm.[5]

Estimation of Digitonin Concentration.—The concentration of digitonin was determined by a method based on its strong hemolytic activity at very low concentrations (1 to 4 γ of digitonin per ml.; *cf.* Ponder and Cox, 1952). It can be applied even in the presence of rhodopsin. The digitonin solutions were incubated with a constant volume of washed human red blood corpuscles, and the hemolytic activity determined by measuring the amount of hemoglobin released into the solution. For this purpose, the hemoglobin was converted to carboxyhemoglobin by bubbling a slow stream of carbon monoxide through the solutions. The extinction was then measured at 565 mμ, a wave length maximum in the absorption spectrum of carboxyhemoglobin.

Ponder and Cox found a linear relationship between hemolytic activity and digitonin concentration up to concentrations which cause complete hemolysis. We were able to confirm this observation. Determinations on samples of unknown digitonin concentration were made in duplicate, parallel with serial dilutions of known concentration. These were prepared in 0.9 per cent saline and contained 1, 1.5, 2, 2.5, 3, 5, and 10 γ of digitonin per ml. Complete hemolysis is attained at a concentration of about 4 γ per ml.

Without further refinement, this method was accurate only to about 10 per cent; but this was adequate for the present purpose.

Estimation of Nitrogen Content.—The nitrogen content of the rhodopsin solutions was determined by the micro Kjeldahl method.[6] A control sample of aqueous digitonin contained no nitrogen. In order to correlate nitrogen content with the rhodopsin concentration, the amount of nitrogen in a rhodopsin solution was divided by its extinction at 500 mμ (K_{500}). The data are therefore presented as milligrams of nitrogen per milliliter of solution with a K_{500} of 1.0.

III

The Purity of Rhodopsin Solutions

Recent methods have yielded rhodopsin solutions of higher purity than any prepared before (Wald, 1951; Collins, Love, and Morton, 1952 *a*). Unfortunately, all criteria of purity are based on the shape of the absorption spectra, and as the spectrum of pure rhodopsin is not known, they provide information

[4] This experiment was performed in collaboration with Dr. M. Ottesen of the Chemical Department of the Carlsberg Laboratory.

[5] These experiments were performed jointly with Dr. E. E. B. Smith at the Institute for Cytophysiology of the University of Copenhagen.

[6] The determinations of nitrogen content were kindly performed by Mrs. B. M. Møller of the Chemical Department of the Carlsberg Laboratory.

only concerning relative purity. The most prevalent criteria have been the ratio of extinctions at the wave length minimum (400 mμ) and maximum (500 mμ) of the absorption band of the rhodopsin chromophore, and the ratio of extinctions at the maximum of the protein band (280 mμ) and the chromophore maximum. For convenience, these are designated $K_{400/500}$ and $K_{280/500}$, respectively. The lowest values reported for these ratios have been $K_{400/500}$ of 0.22 to 0.26, and $K_{280/500}$ of 2.05 to 2.2 (Wald, 1951; Collins, Love, and Morton, 1952 *a*). The photosensitivity of rhodopsin at 400 and 500 mμ corresponds to a 400/500 ratio of 0.22 (Goodeve, Lythgoe, and Schneider, 1942). It has also been estimated that the $K_{400/500}$ of pure rhodopsin cannot be less than 0.20 (Wald, 1938). Above 400 mμ, the absorption spectra of the best solutions are therefore nearly identical with the spectrum of pure rhodopsin.

TABLE I

Purity of Rhodopsin Solutions. Extinction Ratios and Nitrogen Contents of the Nine Rhodopsin Solutions Used in the Present Investigation

For each solution the ratio of extinctions has been determined at 400 and 500 mμ ($K_{400/500}$), and at 280 and 500 mμ ($K_{280/500}$). The nitrogen content of eight of the solutions has also been measured and is presented as milligrams of nitrogen per milliliter of solution with an extinction of 1.0 at 500 mμ.

Preparation	$K_{280/500}$	$K_{400/500}$	Nitrogen content (nitrogen per ml. rhodopsin with a K_{500} of 1.0)
			mg.
30-6-52	2.46	0.28	0.255
18-8-52	2.52	0.24	0.220
25-8-52	2.54	0.26	0.242
21-11-52	2.39	0.25	0.220
28-11-52	2.44	0.26	0.250
21-12-52	2.05	0.24	—
21-1-53	2.63	0.26	0.236
11-2-53	2.06	0.24	0.154
27-3-53	2.17	0.23	0.182
Mean	2.36	0.25	0.221
Standard deviation	0.20	0.014	0.033

Table I lists the extinction ratios and nitrogen contents of the nine rhodopsin solutions used in the present study. The ratios are similar to those cited above. The nitrogen contents of the different preparations, however, are considerably more variable than their extinction ratios. The purest solutions by optical criteria also have the lowest nitrogen content; but for somewhat less pure solutions, there is no correlation between these two quantities.

The purest rhodopsin solution which we have prepared, by all three criteria, had a $K_{400/500}$ of 0.24, a $K_{280/500}$ of 2.06, and a nitrogen content of 0.154 mg. per ml. of solution with an extinction of 1.0 at 500 mμ.

IV

Sedimentation of Digitonin and Rhodopsin-Digitonin in the Ultracentrifuge

We have found, in agreement with Smith and Pickels (1940), that solutions of digitonin sediment in the ultracentrifuge with a single boundary. Three experiments yielded sedimentation constants (s_{20}) of 6.32, 6.36, and 6.37 Svedberg units, respectively. These are somewhat higher than the average value cited by Smith and Pickels, but fall within the range of their individual measurements. Digitonin therefore sediments as a large micelle, which we shall refer to hereafter as D-1.

Solutions of cattle rhodopsin in digitonin were also homogeneous with respect to particle size, and sedimented with a single sharp boundary with an s_{20} of 9.77 Svedberg units. This value is the average of twelve determinations with a standard deviation of 0.22. It is about 12 per cent lower than the s_{20} determined by Hecht and Pickels, which may be due to a genuine difference between rhodopsin solutions from cattle and frog.

The s_{20} rises slightly on dilution, approaching a value of about 10.3 at infinite dilution, but the average is sufficiently accurate for our purposes. Its pH independence has been established between pH 6.3 and 9.6. The red rhodopsin color sediments completely with the refractive index boundary, leaving a clear, colorless supernatant. These solutions therefore contain only the rhodopsin-digitonin micelle which we shall designate RD-1.

If rhodopsin solutions are bleached before centrifugation, the s_{20} is unchanged and the yellow color of retinene sediments with the opsin-digitonin boundary. This co-sedimentation is not surprising, since retinene combines readily with the amino groups of opsin to form a retinene-opsin-digitonin micelle (*cf.* Ball, Collins, Dalvi, and Morton, 1949).

The stability of the RD-1 micelle was tested by varying the proportions of digitonin and rhodopsin over a tenfold range. We also tested the behavior of D-1 in the presence of ovalbumin to see whether the co-sedimentation of digitonin with protein is a more general phenomenon.

Sedimentation Pattern of a Mixture of D-1 with Ovalbumin.—We have observed the sedimentation behavior of a solution containing 0.7 per cent ovalbumin and 1.8 per cent digitonin in phosphate buffer, pH 6.3. This mixture sedimented with two boundaries: a slow one characteristic of ovalbumin (s_{20} of 3.34 in this experiment), and a faster one characteristic of D-1 (s_{20} of 6.29). No complex formation could be observed, nor was the D-1 boundary distorted to any measurable extent.

Electrophoretic measurements had previously shown that ovalbumin does not combine with a non-ionic detergent (Putnam, 1948, p. 108), which led to the conclusion that "ionic groups in the detergent are requisite for interaction with a protein." This clearly is not true for rhodopsin. It may be that soluble proteins are sufficiently polar to prevent association with a non-ionic detergent, whereas the very insolubility of rhodopsin favors such a combination. The polar character of a protein must be a function of its total acid- and base-binding capacity. But it is likely that its solubility as well as its affinity for non-ionic detergents depends on its over-all configuration more than on the actual number of titrable groups. The contrasting solubilities of ovalbumin and rhodopsin as well as their opposed behavior toward digitonin suggest a considerable difference in the relative distribution of polar and hydrocarbon components in the two proteins.

Sedimentation of Mixtures of RD-1 with D-1.—We have mixed solutions of D-1 and RD-1 in various proportions over a range between 0.5 and 5 volumes of 1.8 per cent digitonin per volume of RD-1 solution. These mixtures always sedimented with two boundaries: a fast one with the s_{20} characteristic of RD-1, and a slower boundary with a somewhat variable sedimentation constant. As far as can be judged by detailed visual inspection of the sedimenting boundaries, the rhodopsin color sediments completely with RD-1.

In 13 experiments, the average s_{20} of the fast component (RD-1) was 9.86 Svedberg units, with a standard deviation of 0.18. The average s_{20} of the slow component was 6.96 Svedberg units. This value is about 10 per cent higher than the sedimentation constant of D-1. Furthermore, it varied between 7.46 and 6.34, with a standard deviation of 0.31, a much wider variation than was observed for D-1 or RD-1. This variation was systematic, depending on the mixing ratios of the two components. For mixtures containing equal volumes of D-1 and RD-1, the slow component had an s_{20} of 7.08 Svedberg units, with a standard deviation of only 0.12. On the whole, the s_{20} of the slow component was high in mixtures containing predominantly RD-1, and fell off as the proportion of digitonin increased.

The addition of D-1 to solutions of RD-1 also caused a decrease in the area under the RD-1 sedimentation curve, indicating a fall in the concentration of the RD-1 component. This suggests that a certain amount of RD-1 may be dispersed among the D-1 micelles and increase their micelle weight. An increase in the concentration of a slow component in a mixture, however, provokes refractive index anomalies which tend to diminish the area under the sedimentation diagram of the fast component (Johnston and Ogston, 1946; Schachman, 1951). The apparent decrease in the concentration of RD-1 may therefore be an artefact induced by the optical properties of the system.

Consequently, we cannot be sure whether the slow components (referred to collectively as D-2) are identical with D-1, or represent a family of modified

digitonin micelles. It is certain that they do not contain visually detectable rhodopsin, nor affect the sedimentation constant of RD-1.

The average values of the sedimentation constants discussed so far are summarized in Table II, and the sedimentation diagrams of the four types of solution shown in Fig. 1.

Effect of Dialysis on the Sedimentation of RD-1.—Smith and Pickels suggested that the digitonin micelle (D-1) must be in equilibrium with a few molecules of digitonin, as digitonin passes through dialyzing membranes which do not permit the diffusion of particles one-tenth the probable size of D-1. This must

TABLE II

Sedimentation Constants (s_{20}) of Digitonin and Rhodopsin-Digitonin and Their Standard Deviations, under Various Conditions

All determinations were made in phosphate buffer, pH 6.3.

Description of solution	No. of experiments	Sedimentation constant (Svedberg units)		Standard deviation	
		Slow component	Fast component	Slow component	Fast component
Digitonin (D-1)	3	6.35	—	0.026	—
Cattle rhodopsin in digitonin (RD-1)	12	—	9.77	—	0.22
Rhodopsin in digitonin diluted with an equal volume of digitonin (RD-1 + D-2)	5	7.08	9.89	0.12	0.15
Rhodopsin in digitonin diluted with digitonin in various proportions (RD-1 + D-2)	13	6.96	9.86	0.31	0.18
Digitonin (Smith and Pickels)	6	5.88	—	0.45	—
Frog rhodopsin in digitonin (Hecht and Pickels)	4	—	11.1	—	0.25

apply equally to the digitonin portion of RD-1, as dialysis lowers also its digitonin content.

Solutions of RD-1 have been dialyzed in the cold until they retained only one-third to one-half their original digitonin content. We expected that this would lower the s_{20}, perhaps yielding a new upper limit for the sedimentation constant of rhodopsin. Instead, the sedimenting boundary became blurred, indicating a distribution of micelle sizes, while the mean sedimentation constant of the micelle population increased. In one experiment, for example, the mean s_{20} rose to 25. This aggregation is probably due to the insolubility of rhodopsin in buffer. Digitonin holds rhodopsin in aqueous solution by complexing with it. As it leaves the micelle, the complexes become unstable and condense with one another to form aggregates of varying size. We have called these RD-2, with the implication that they contain a higher ratio of rhodopsin

to digitonin than RD-1. The RD-2 micelles never seem to reach a stable configuration; they apparently continue to grow until they precipitate out of solution when their digitonin content becomes too low (*cf.* Broda, Goodeve, and Lythgoe, 1940).

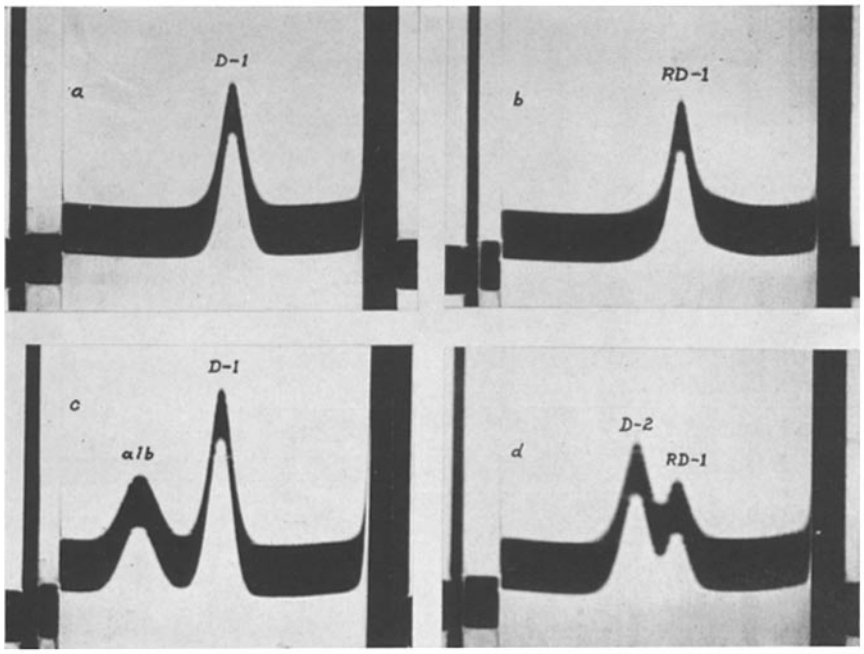

FIG. 1. Sedimentation diagrams of (*a*) digitonin (D-1), (*b*) rhodopsin-digitonin (RD-1), (*c*) a mixture of ovalbumin and digitonin (alb + D-1), and (*d*) a mixture of rhodopsin-digitonin and digitonin (RD-1 + D-2). The direction of sedimentation is from left to right; all solutions sedimenting in M/15 phosphate buffer, pH 6.3, at about 21°C. The photographs were taken (*a*) 76 minutes, (*b*) 51 minutes, (*c*) 71 minutes, and (*d*) 49 minutes after the beginning of centrifugation, so that the digitonin peaks are roughly comparable in (*a*) and (*c*), and the RD-1 peaks comparable in (*b*) and (*d*). Digitonin and rhodopsin-digitonin sediment with a single boundary. When digitonin is added to ovalbumin or rhodopsin-digitonin, it does not complex with either, but sediments with a separate boundary.

The aggregation of RD-1 into a population of RD-2 micelles is reversed by the addition of digitonin. The results of such an experiment are summarized in Table III and Fig. 2. The data show that dialysis causes the micelle size to increase, yielding an inhomogeneous population of RD-2 micelles. Digitonin redisperses the RD-2 aggregates into a mixture of RD-1 and D-2. The rhodopsin color again sediments with the RD-1 boundary.

Qualitative Comparison of the Various Micelles.—It is worth noting a qualitative difference among the four types of solution discussed so far, namely those containing D-1, RD-1, both RD-1 and D-2, or a variable population of RD-2 micelles. Digitonin itself (D-1) precipitates spontaneously a few days after being brought into aqueous solution, and redissolves only on heating. Our determinations of the s_{20} of D-1 were all made with such freshly heated solutions. Concentrated solutions of rhodopsin in digitonin (RD-1), however, are stable for months without showing any tendency to precipitate. In solutions containing extra digitonin (RD-1 plus D-2), a nearly colorless precipitate appears after a few days. The RD-2 micelles form a red gelatinous precipitate on standing.

TABLE III

Sedimentation Constants (s_{20}) of Rhodopsin and Dialyzed Rhodopsin before and after Addition of Digitonin

The sedimentation constant of RD-1 is not changed by the addition of digitonin, but is increased by dialysis. Addition of digitonin to dialyzed RD-1 (family of RD-2 complexes) reconstitutes the original RD-1 micelle.

Description of solution	Sedimentation constant (s_{20})	
	Slow component	Fast component
1. Rhodopsin-digitonin (RD-1)	—	9.80
2. Sample (1) + equal volume of digitonin (RD-1 + D-2)	7.24	9.64
3. Sample (1) after dialysis (RD-2)	—	10.82*
4. Sample (3) + equal volume of digitonin (RD-1 + D-2)	7.29	9.80

* Mean s_{20} of a micelle population sedimenting with a diffuse boundary. The solution included some components which sedimented much faster.

When D-1, RD-1, or mixtures of RD-1 and D-2 are spun down in the ultracentrifuge, the sediment can be resuspended by slight agitation. The RD-2 complexes, however, form a red insoluble gel of a consistency which suggests some agglutination or polymerization process.

Recapitulation.—RD-1 is a stoichiometric complex of rhodopsin with digitonin. Addition of digitonin in various proportions does not alter its sedimentation constant or, presumably, its composition. Removal of digitonin results in a condensation of partial RD-1 micelles to an inhomogeneous family of RD-2 complexes, some of which are very large. Digitonin disaggregates the RD-2 micelles by reconstituting the stoichiometric RD-1 complex. Digitonin and egg albumin sediment independently of each other; if a complex is formed, it contains too little digitonin to be detected.

Homogeneity of RD-1 during Electrophoresis.—We have also briefly examined

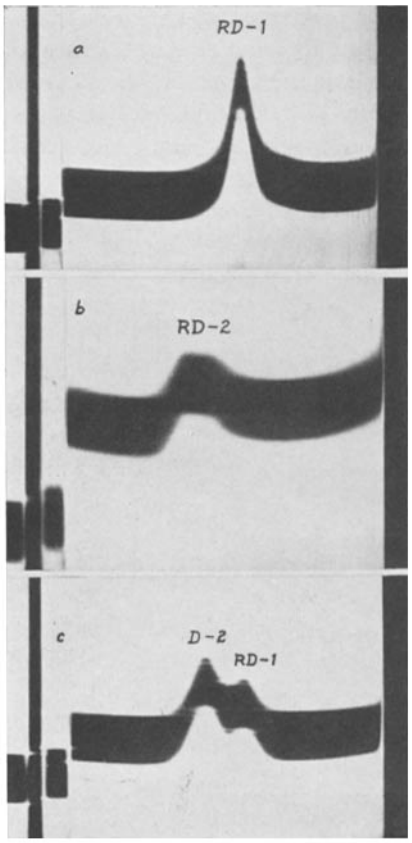

FIG. 2. Sedimentation diagrams of (*a*) rhodopsin-digitonin (RD-1), (*b*) the same after dialysis (RD-2 complexes), and (*c*) dialyzed rhodopsin-digitonin after addition of digitonin (RD-1 + D-2). All solutions sedimenting in M/15 phosphate buffer, pH 6.3, at about 21°C. Photographs taken (*a*) 51 minutes, (*b*) 35 minutes, and (*c*) 55 minutes after beginning of centrifugation. The sharp sedimentation boundary of RD-1 becomes blurred by dialysis. The upward tilt of the boundary at the right of diagram (*b*) indicates that some of the heavier components are already beginning to accumulate at the bottom of the cell. Addition of digitonin to dialyzed rhodopsin-digitonin reestablishes the sharp RD-1 boundary. The excess digitonin sediments separately as D-2 (*viz.* Fig. 1 *d* and text).

RD-1 by paper ionophoresis and in the electrophoresis apparatus. The experiments on filter paper were made with phosphate buffer, at an ionic strength of 0.03 and pH 8.0; the others in a veronal-HCl buffer of 0.1 ionic strength and pH 8.5. On paper, rhodopsin migrated toward the anode as a single zone until the experiment was discontinued after 24 hours. In solution, RD-1 re-

mained homogeneous for 10 hours, travelling slowly toward the anode with a mobility of -1.79×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$. The schlieren diagrams of the ascending and descending boundaries after 9 hours are shown in Fig. 3.

The Micelle Weight of Rhodopsin-Digitonin (RD-1).—Since RD-1 is a well defined entity of constant composition, we can determine its micelle weight. Hecht and Pickels (1938) calculated that the micelle weight of frog rhodopsin-digitonin is about 270,000. For this, they chose 4×10^{-7} cm.^{2} sec.$^{-1}$ as the most

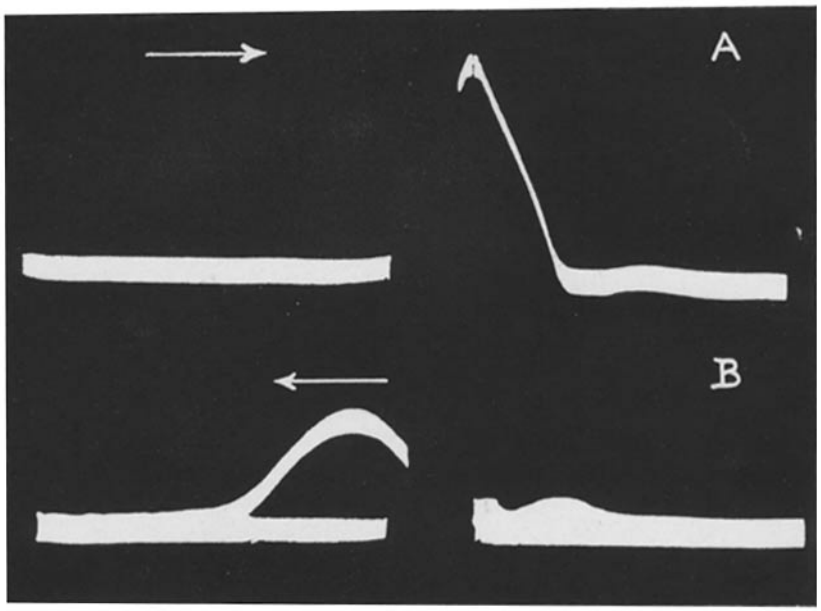

FIG. 3. Electrophoretic pattern of rhodopsin-digitonin in veronal-HCl buffer, pH 8.5, ionic strength of 0.1, 0°C. (*A*) Ascending and (*B*) descending boundaries; the direction of migration is indicated by arrows. Photographs taken 9 hours after the beginning of the experiment. RD-1 travels as a single component.

probable value for the diffusion constant (D_{20}), and assumed a particle density (ρ_{20}) of 1.33 gm. per ml. Their value of the diffusion constant was based on direct measurements yielding a D_{20} of 3.5×10^{-7} cm.^{2} sec.$^{-1}$ (Hecht, Chase, and Shlaer, 1937), and on estimates from the spreading of the sedimentation boundary. The latter method yields only very approximate results (Neurath, 1942), although it is somewhat more reliable with substances of high molecular weight. We have estimated the D_{20} from the spreading of the sedimentation boundaries in our own experiments, and find values ranging between 3.5 and 4.9×10^{-7} cm.^{2} sec.$^{-1}$, the low values being more reliable. It therefore seems

reasonable to assume that the D_{20} of RD-1 lies between the limits of 3.5 and 4.0×10^{-7} cm.^{2} sec.$^{-1}$.

We have also determined the partial specific volumes (V_{20}) of digitonin (D-1) and rhodopsin-digitonin (RD-1), and find that they occupy 0.738 and 0.766 ml. per gm., respectively.

From the three values, s_{20} of 9.77 to 9.86 Svedberg units, D_{20} of 3.5 to 4.0×10^{-7} cm.^{2} sec.$^{-1}$, and V_{20} of 0.766 ml. per gm., we find that the RD-1 micelle has a molecular weight of 260,000 to 290,000. Its frictional ratio (f/f_o) is about 1.2 to 1.4, indicating that the micelle has a somewhat ellipsoidal shape.[3]

We now need only know the proportions of rhodopsin and digitonin in RD-1, in order to arrive at an estimate of the molecular weight of rhodopsin.

V

The Molecular Weight of Rhodopsin, and the Number of Chromophores per Molecule

The Composition of RD-1.—The rhodopsin content of RD-1 defines the maximum molecular weight of rhodopsin. This is either the true molecular weight, or some multiple of it, depending upon the number of rhodopsin molecules in RD-1.

We have therefore determined the relative proportions of rhodopsin and digitonin in RD-1 by measuring its nitrogen and digitonin content (for methods, see section II). Since digitonin contains no nitrogen, all the nitrogen in RD-1 is contributed by rhodopsin. As shown in section III, the average nitrogen content of nine preparations of RD-1 was 0.22 mg. per ml. of solution with a K_{500} of 1.0. The lowest value for the best solution was 0.15 mg. per ml. Since contaminating impurities are likely to raise the nitrogen content of the solutions, we have used the lower value to calculate the molecular weight. Assuming that rhodopsin, like most proteins, contains about 15 per cent nitrogen by weight, 0.15 mg. nitrogen corresponds to 1 mg. of rhodopsin. A solution with a K_{500} of 1.0 therefore contains about 1 mg. of rhodopsin per ml.

We have also correlated the digitonin content of a number of solutions of RD-1 with their extinctions at 500 mμ. The results of these determinations are summarized in Table IV. Solutions with a K_{500} of 1.0 contain, on the average, 6.1 mg. digitonin per ml. The deviations from the average are considerable, but the ratio of rhodopsin (K_{500}) to digitonin is roughly constant in successive preparations.

The concentration of RD-1 is the sum of the individual concentrations of rhodopsin and digitonin. A solution with an extinction of 1.0 therefore contains 7.1 mg. RD-1 per ml. Only 1 mg. of this is due to rhodopsin, which is equivalent to 14 per cent. Since the molecular weight of RD-1 is 260,000 to 290,000, it contains 36,000 to 41,000 gm. rhodopsin and 220,000 to 250,000 gm. digitonin

per mole of micelles. The maximum molecular weight of rhodopsin is therefore about 40,000.

We are now in a position to test the assumption that rhodopsin contains about 15 per cent nitrogen. As RD-1 contains about 14 per cent rhodopsin, it should have a nitrogen content of about 2.1 per cent. This value has been checked by drying a solution of RD-1 and measuring its weight and nitrogen content. The dry weight was 10.037 mg., and it contained 0.221 mg. nitrogen, a nitrogen content of 2.2 per cent. Our assumption is therefore justified within experimental error.

The Molecular Weight of Rhodopsin.—We have shown above that RD-1 contains about 40,000 gm. rhodopsin per mole of micelles. If we can now discover how many moles this represents, we shall have determined the molecular

TABLE IV

Digitonin Content of Several Rhodopsin-Digitonin Solutions, and Its Relation to Their Rhodopsin Content

The rhodopsin concentration is expressed as the extinction at 500 mμ (K_{500}). The relative proportions of rhodopsin and digitonin are roughly constant in these solutions.

Preparation	Digitonin concentration	K_{500}	Digitonin concentration per K_{500} of 1.0
	mg. per ml.		*mg. per ml.*
30-10-52	13.6	2.00	6.80
21-11-52	13.7	2.35	5.83
28-11-52	15.2	2.74	5.55
11-2-53	15.4	2.46	6.25
Average			6.11

weight of rhodopsin. This is accomplished in the following manner: We have already shown that a rhodopsin solution with a K_{500} of 1.0 contains 7.1 mg. RD-1 per ml. This is equivalent to 2.4 to 2.7 $\times$ 10^{-8} mole RD-1 micelles. From the K_{500} we can also calculate the molar concentration of rhodopsin chromophores in this solution, if we know the molar extinction coefficient per chromophore. Actually, since it is not certain whether the chromophore contains one or two molecules of retinene (*cf.* Wald, 1953), it is more convenient to calculate the molar retinene equivalent of rhodopsin. Wald and Brown (1953) have recently determined the molar extinction coefficient of rhodopsin at 500 mμ and find that it has a value of 40,600 cm.^{2} per mole of retinene, so that a solution with a K_{500} of 1.0 contains 2.5 $\times$ 10^{-8} mole retinene per ml. The molar concentrations of RD-1 and retinene are therefore equal. This implies that RD-1 contains *one* molecule of rhodopsin, with a molecular weight of about 40,000, and carrying a *single* chromophore composed of *one* molecule of retinene.

The RD-1 micelle is therefore composed of 1 molecule of rhodopsin and 180

to 200 molecules of digitonin. Assuming that the digitonin micelle (D-1) consists of approximately 60 molecules of digitonin, RD-1 contains the equivalent of about three such micelles.

VI

DISCUSSION

The Molecular Weight of Rhodopsin.—Our value for the molecular weight of cattle rhodopsin may still be too high. We have, in fact, determined the amount of protein per RD-1 micelle. This is associated with one chromophore and can therefore contain no more than one molecule of rhodopsin. It may, however, include contaminating proteins which are consistently extracted with rhodopsin. The molecular weight will have to be determined in a number of ways before one can definitely settle this point. Until then, it seems reasonable to conclude that cattle rhodopsin has a molecular weight of about 40,000.

The rough agreement between the micelle weight determined by Hecht and Pickels for the RD-1 complex of frog rhodopsin and our value from cattle suggests that the molecular weight of frog rhodopsin is probably about the same. This is borne out also by the data of Broda, Goodeve, and Lythgoe (1940), who derived a minimum molecular weight for frog rhodopsin by comparing the extinction of rhodopsin solutions with their dry weight after removal of digitonin. For this computation, they chose too low a value for the molar extinction coefficient. When their data are recalculated with the correct extinction coefficient (40,600 $cm.^2$ per retinene equivalent), they yield a molecular weight of about 50,000 per chromophore. This is the true molecular weight, if frog rhodopsin also contains only one chromophore per molecule. It is almost sure to be a high estimate, being based on a dry weight, which is raised by any possible contaminant. It therefore seems probable also from these data that the molecular weight of frog rhodopsin is similar to that of cattle rhodopsin.

By an interpretation of absorption spectra on the basis of classic oscillator theory, Weale (1949 *a*, *b*) has estimated the molecular weight of rhodopsin from the shape and position of the main absorption band of the rhodopsin chromophore. His value for the molecular weight is 45,600. The theory and applicability of his method seem extremely doubtful to us and have been criticized in detail by Collins and Morton (1949). Thus in assigning a value to a constant p which Weale tells us varies between 0.1 and 2.7 for a number of compounds, he arbitrarily chooses $p = 1.0$ as the basis for his computation. Collins and Morton note furthermore that an application of this method to hemoglobin involves a tenfold discrepancy depending on whether the molecular weight is calculated from the absorption band at 555 $m\mu$, or from the Soret band. Under these circumstances, it is difficult to know what value to attach to Weale's estimate of the molecular weight of rhodopsin.

The Structure of the Rhodopsin Chromophore and the Molar Extinction Co-

efficient of Rhodopsin.—The incorporation of retinene into the rhodopsin chromophore involves a shift in spectrum of 116 mμ, and an increase of about 70 per cent in the extinction coefficient (Wald and Brown, 1953). This must somehow be explained from the structure of the chromophore and its mode of attachment to opsin. It has been suggested that the shift in spectrum is due to the incorporation of two retinene molecules into a single conjugated system (Wald, 1949), and a structure for such a chromophore has been proposed by Collins and Morton (1950). A number of considerations, however, have made it appear as though only one molecule of retinene were involved in the formation of the rhodopsin chromophore (Hubbard and Wald, 1952; Wald, 1953). The data presented above give the first direct answer to this question. They show clearly that rhodopsin contains only one molecule of retinene. Its absorption spectrum must therefore be derived from a single retinene unit.

It is apparent that the molar extinction coefficient of rhodopsin recently calculated per mole of retinene (Wald and Brown, 1953) is also the molar extinction coefficient of the chromophore and of rhodopsin itself. Since this has a value of 40,600 and the molecular weight of rhodopsin is about 40,000, rhodopsin has an E(1 per cent, 1 cm., 500 mμ) of about 10.

The Rhodopsin Content of the Rod Outer Limb.—We can now estimate the rhodopsin content of the rod outer limb. Collins, Love, and Morton (1952 *b*), in a recent analysis of cattle outer limbs, have shown that their rhodopsin content is equivalent to 22.6 mg. vitamin A per 100 gm. wet outer limbs, or 83 mg. vitamin A per 100 gm. on a dry weight basis. For this computation, they used the value of 48,000 for the extinction coefficient of rhodopsin per mole of vitamin A (or retinene). If their data are recalculated with the new figure of 40,600, the vitamin A equivalent becomes 26.7 mg. per 100 gm. wet weight, or 98 mg. per 100 gm. dry weight. Since there is a mole to mole correspondence between the vitamin A equivalent and the rhodopsin content, 286 gm. vitamin A corresponds to 40,000 gm. rhodopsin. The rhodopsin content of cattle outer limbs is therefore about 14 per cent of the dry weight, and 3.7 per cent of the wet weight.

Histological data (Steindorff, 1947) show that a cattle outer limb is about 10 μ long and 1 μ in diameter. The volume of the outer limb is therefore about 7.5 μ^3, and its wet weight 7.5×10^{-12} gm., assuming a density of 1.0. The rhodopsin content per outer limb is therefore about 2.8×10^{-13} gm., or 7×10^{-18} mole. Multiplying this value by Avogadro's number (6.06×10^{23} molecules per mole), we see that the cattle rod outer limb contains about 4.2×10^6 molecules of rhodopsin. This implies that the extinction (K_{500}) of an outer limb viewed end-on is about 0.037 cm.^{2}.

A similar calculation can be made for the rhodopsin content of the frog outer limb, if we assume that the molecular weight of frog rhodopsin is roughly the same as that of cattle rhodopsin. Here we know from the data of Broda

et al. (1940) that a frog retina contains about 570,000 outer limbs which, upon extraction, yield 0.1 ml. of a rhodopsin solution with a K_{500} of 0.814. This corresponds to 2×10^{-9} mole rhodopsin per retina, or 3.5×10^{-15} mole (1.4×10^{-10} gm.) per outer limb, which is equivalent to 2.1×10^{9} molecules.

The wet weight of a frog outer limb is about 1.4×10^{-9} gm., and its dry weight about 3.5×10^{-10} gm. (*cf.* Hubbard, 1954). Rhodopsin therefore accounts for about 10 per cent of the wet weight, and 35 per cent of the dry weight. Assuming that the frog outer limb is about 50 μ long, the extinction (K_{500}) along its axis is about 0.50 cm.^{2}.

It will be noted that the rhodopsin content per outer limb is about five hundred times higher for the frog than for cattle. The relative volumes of the two structures, however, are such that the ratio of the rhodopsin concentrations is only 2.5 to 1 on a weight basis.

Collins *et al.* (1952 *b*) have shown that no more than 70 per cent of the dry weight of the cattle outer limb is due to protein. Rhodopsin therefore constitutes at least one-fifth of the total protein. In the frog, this figure must be even higher.

VII

SUMMARY

The sedimentation behavior of aqueous solutions of digitonin and of cattle rhodopsin in digitonin has been examined in the ultracentrifuge. In confirmation of earlier work, digitonin was found to sediment as a micelle (D-1) with an s_{20} of about 6.35 Svedberg units, and containing at least 60 molecules. The rhodopsin solutions sediment as a stoichiometric complex of rhodopsin with digitonin (RD-1) with an s_{20} of about 9.77 Svedberg units. The s_{20} of the RD-1 micelle is constant between pH 6.3 and 9.6, and in the presence of excess digitonin.

RD-1 travels as a single boundary also in the electrophoresis apparatus at pH 8.5, and on filter paper at pH 8.0. The molecular weight of the RD-1 micelle lies between 260,000 and 290,000. Of this, only about 40,000 gm. are due to rhodopsin; the rest is digitonin (180 to 200 moles).

Comparison of the relative concentrations of RD-1 and retinene in solutions of rhodopsin-digitonin shows that RD-1 contains only one retinene equivalent. It can therefore contain only one molecule of rhodopsin with a molecular weight of about 40,000. Cattle rhodopsin therefore contains only *one* chromophore consisting of a *single* molecule of retinene. It is likely that frog rhodopsin has a similar molecular weight and also contains only one chromophore per molecule.

The molar extinction coefficient of rhodopsin is therefore identical with the extinction coefficient per mole of retinene (40,600 cm.^{2} per mole) and the E(1 per cent, 1 cm., 500 mμ) has a value of about 10.

Rhodopsin constitutes about 14 per cent of the dry weight, and 3.7 per cent of the wet weight of cattle outer limbs. This corresponds to about 4.2×10^6 molecules of rhodopsin per outer limb. The rhodopsin content of frog outer limbs is considerably higher: about 35 per cent of the dry weight, and 10 per cent of the wet weight, corresponding to about 2.1×10^9 molecules per outer limb. Thus the frog outer limb contains about five hundred times as much rhodopsin as the cattle outer limb. But the relative volumes of these structures are such that the ratio of concentrations is only about 2.5 to 1 on a weight basis. Rhodopsin accounts for at least one-fifth of the total protein of the cattle outer limb; for the frog, this value must be higher. The extinction (K_{500}) along its axis is about 0.037 cm.^{2} for the cattle outer limb, and about 0.50 cm.^{2} for the frog outer limb.

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