Spectrum and Purity of Bovine Rhodopsin*

Hitoshi Shichi

ABSTRACT: Cattle rhodopsin is generally believed to show three distinct absorption bands, α (498 mμ), β (345 mμ), and γ (278 mμ), in the visible and ultraviolet region. Since rhodopsin preparations purified by gel filtration do not show the β band, the validity of the β band has recently been questioned. The present investigation shows that further purification of such a preparation on calcium phosphate results in clear separation of the β band. The β band is also detected in absorption and circular dichroism spectra of sonically disrupted membrane fragments of the outer segments of the retina. On bleaching in the light, the β band in the circular dichroism spectrum of solubilized rhodopsin as well as of the membrane fragments disappeared completely. From these observations, the β band is concluded to be a part of the spectrum of unbleached rhodopsin. The molar extinction coefficient of the α band has been reported to be 23,000. From a spectral comparison of retinal oxime prepared from rhodopsin and from authentic *all-trans*-retinal, the molar extinction coefficient of the α band is found to be 39,000–42,000 in this investigation. Similar values are also obtained by colorimetric assays of retinal released from bleached rhodopsin. The spectral evidence obtained in this work suggests that the lower molar extinction coefficient and the absence of the β band previously reported on purified samples of rhodopsin may be ascribed to lesser purity of the preparations.

Rhodopsin, a visual pigment for night vision, is a glycoliprotein[1] present in the rod outer segment of the retina. Biochemical studies on the pigment have been greatly hampered by the difficulty of obtaining pure preparations. Wald (1953) has pointed out that purification of rhodopsin is complicated because of the need for detergents for solubilization and practical uselessness of conventional protein procedures for purification of extremely small amounts of the pigment. Sixteen years later, this is still a point in dispute. Heller (1968) has recently reported purification of bovine rhodopsin by gel filtration chromatography on Sephadex G-200. The purified preparation has been claimed to be homogeneous as judged by rechromatography, spectral data, and polyacrylamide gel disc electrophoresis, and it has been subjected to amino acid analysis. He has further applied the purification procedure to rhodopsin from rat and frog retinas and compared the amino acid composition of rhodopsin from different species (Heller, 1969). Hall *et al.* (1968) have shown that a part of the radioactive amino acids incorporated into rod outer segment disks is found in rhodopsin purified by Heller's method. The reliability of the amino acid analysis of rhodopsin from different species and of the data on the biosynthesis of rhodopsin rests primarily upon the purity of the rhodopsin preparation examined. Heller (1968) published a spectrum of rhodopsin showing a molar extinction coefficient at 500 mμ of 23,100 and a minimum at 410 mμ. The spectrum was devoid of the β band. These spectral features are in marked disagreement with those of preparations described by other workers (Wald, 1951; Collins *et al.*, 1952; Wald and Brown, 1953–1954; Shichi *et al.*, 1969) and seem to indicate possible heterogeneity of the preparation. Therefore, in this investigation, we will carefully compare spectral properties of Heller's rhodopsin preparation with those of other workers' samples and present evidence suggesting that his preparation is not of high purity.

Experimental Section

Preparation of Rhodopsin. Frozen bovine retinas, purchased from George Hormel Co., Austin, Minn., were ground to a homogeneous slurry and the outer segments of the retina were prepared either by the procedure of Heller (1968), or by flotation at the interphase between sucrose and buffer as described previously (Shichi *et al.*, 1969). Rhodopsin was extracted from the outer segments either with 0.04 M cetyltrimethylammonium bromide (CTAB, Eastman) in 6.6 mM potassium phosphate buffer (pH 6.5) or with 1% Emulphogene BC 720, a nonionic alkoxypoly(ethyleneoxy)ethanol (General Aniline and Film Corp.), and purified on Sephadex G-200 according to Heller (1968) and on calcium phosphate following the procedure previously described (Shichi *et al.*, 1969). All operations were carried out either under a dim red light or in total darkness.

Sonic Disruption of Bovine Retinal Outer Segment. Fresh dark-adapted bovine eyes (50–100) were obtained at a local abattoir and brought to the laboratory in ice in a dark container within 4 hr after slaughtering. Retinas were collected from the dissected eyes and gently homogenized with two volumes of 40% sucrose in 6.6 mM phosphate (pH 6.5) using a glass homogenizer and centrifuged at 2000*g* for 15 min and the supernatant was collected by pipetting. The sucrose concentration of the solution was lowered to 20% with

* From the Ophthalmology Branch, National Eye Institute, National Institutes of Health, U. S. Department of Health, Education, and Welfare, Bethesda, Maryland 20014. *Received December 2, 1969.*

[1] According to Heller (1968), little or no phospholipid is found in rhodopsin. Krinsky (1958) and Adams (1969), however, have reported that rhodopsin contains phospholipid and Poincelot *et al.* (1969) have suggested that phosphatidylethanolamine is involved in the binding of 11-*cis*-retinal in rhodopsin. Since the discrepancy has not been thoroughly investigated, rhodopsin is regarded here as a glycoliprotein.

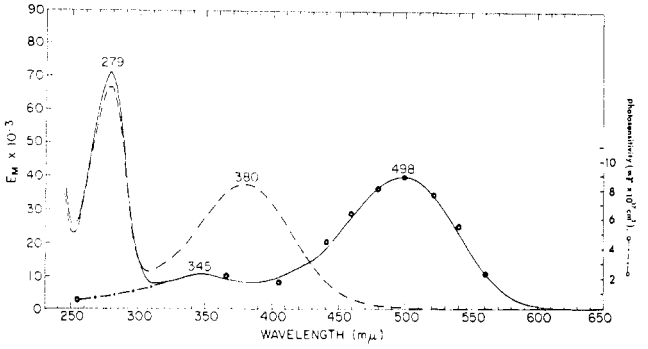

FIGURE 1: Absorption spectra of bovine rhodopsin in 0.04 M CTAB–0.066 M potassium phosphate (pH 6.5) at 20°. Rhodopsin collected in the first eluate from a calcium phosphate column was placed in the sample cuvet and the control cuvet contained CTAB-buffer which had been passed through the column prior to the application of crude rhodopsin on the column. (—) Unbleached; (---) bleached; (-●-) photosensitivity curve of Schneider *et al.* (1939) and Goodeve *et al.* (1942).

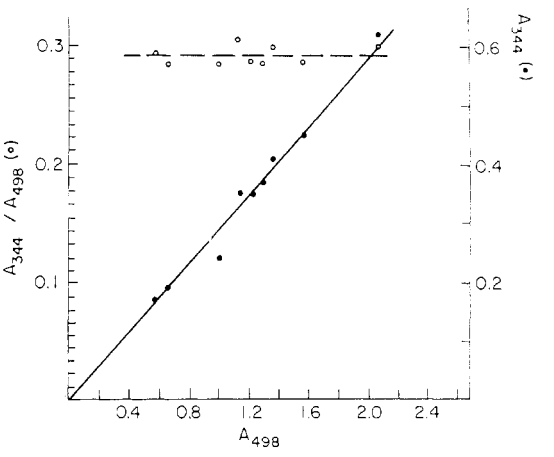

FIGURE 2: Linear relationship between absorption intensities at 498 and 344 mμ.

buffer and centrifuged at 25,000*g* for 20 min. The sedimented outer segments were purified by flotation in sucrose as described (Shichi *et al.*, 1969). The flotation procedure was repeated at least three times for purification. The outer segments were then suspended in about tenfold volume of distilled and deionized water and sonically disrupted for 3 min at 20 kc in an ice-water bath with a Sonifier (Heat System Co., Melville, N. Y.). The temperature of the suspension during the sonic treatment was maintained below 5°. The sonicated outer segment suspension was centrifuged at 100,000*g* for 60 min and the clear supernatant containing very fine outer segment particles was collected by pipetting and stored in the dark at 3°. If necessary, the suspension was concentrated by the freeze-thaw technique of Williams (1968).

Preparation of Retinal Oxime. One milliliter of 16.8 μM *all-trans*-retinal (Eastman) in ethanol was diluted to 2 ml with 0.5 M hydroxylamine (Fisher) and the absorption spectrum of retinal oxime was recorded against 50% aqueous ethanol. Purified rhodopsin in 1% Emulphogene was bleached in the presence of an equal volume of 0.5 M hydroxylamine and the spectrum of retinal oxime produced was recorded against 0.5% Emulphogene.

Spectral Measurements. Visible and ultraviolet absorption spectra were recorded at 20° using a Cary recording spectrophotometer. Circular dichroism spectra were recorded with a Cary 60 recording spectropolarimeter. In order to record spectra of a bleached sample, rhodopsin, kept at 20°, was irradiated with two Eltron cool white lamps (400 W) from a distance of 1 ft.

Determination of Protein and Retinal. Protein was assayed with the Folin–Ciocalteu reagent (Lowry *et al.*, 1951) and retinal by the method of Futterman and Saslaw (1961).

Results

Absorption Spectra. The absorption spectra of bovine rhodopsin extracted with CTAB and purified on a column of Sephadex G-200 were recorded against an aliquot of the buffer–detergent eluate collected during the washing of the column. The absorption minimum was found in the region of 400–410 mμ and the ratios of absorption at $A_{400}:A_{498}$ and $A_{278}:A_{498}$ (see below) were no less than 0.35 and 3.7, respectively. The β band was detected but it was considerably deformed probably because of the tailing absorption around 310 mμ of aromatic amino acid residues of contaminating protein. A similar preparation was obtained when Emulphogene was used as the solubilizer for the Sephadex chromatography. When such a preparation was further purified on calcium phosphate, the best eluate from the column showed absorption spectra like those shown in Figure 1. The absorption bands were found at 279, 345, and 498 mμ in the unbleached form and at 279 and 380 mμ in the bleached form. The replacement of the 498-mμ band by the 380-mμ band after bleaching is due to the liberation of *all-trans*-retinal from the chromophore of rhodopsin. The minimum absorption between the α and β bands was found around 390 mμ. The intensity of the 279-mμ absorption owing to the aromatic amino acid residues of opsin is decreased about 7% after irradiation. From the spectrum of rhodopsin and the determination of retinal concentration in the sample, the molar extinction coefficients of the bands at 279,345 and 498 mμ in CTAB were found to be 71.7×10^3, 10.6×10^3, and 39.7×10^3 cm² per mole, respectively. The spectral purity of rhodopsin is often indicated by the ratios of the extinction at 400 and 498 mμ ($A_{400}:A_{498}$) and at 278 and 498 mμ ($A_{278}:A_{498}$). The lower values of these ratios show the higher purity. The ratios of $A_{400}:A_{498}$ and $A_{278}:A_{498}$ for the purified rohdopsin were 0.21 and 1.75, respectively. From these results it is evident that the purity of the eluate from a Sephadex column can be significantly improved by the subsequent calcium phosphate chromatography, regardless of the types of solubilizing agent used.

Since the rhodopsin preparation obtained above was found to be pure enough for further spectral studies, a series of rhodopsin samples of different concentrations was prepared by independently extracting the outer segments with Emulphogene. Each sample was examined for the β band. The magnitude (Figure 2) of the β absorption was linearly proportional to the concentration of rhodopsin as indicated by the magnitude of A_{498} (closed circles). The ratio of $A_{344}:A_{498}$ was about 0.29 and constant with different preparations

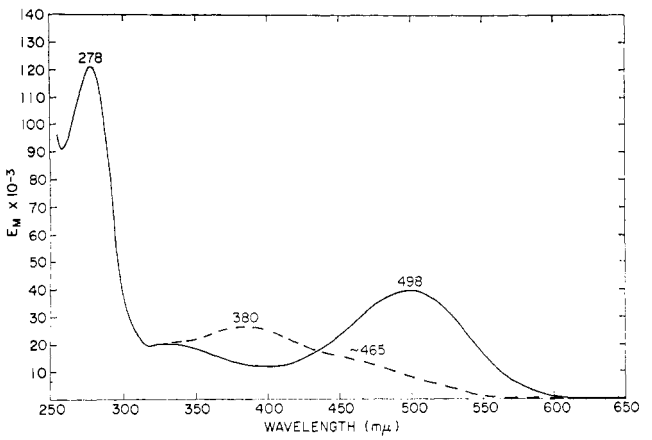

FIGURE 3: Absorption spectra of fine particles of the bovine retinal outer segment in distilled water at 20°. Solid line: unbleached; dotted line: bleached.

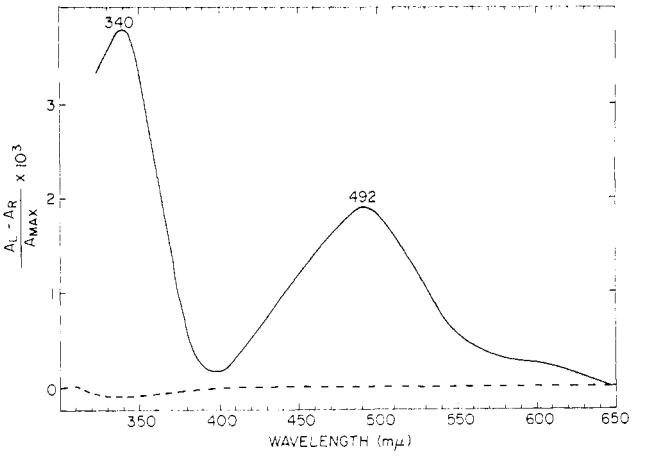

FIGURE 4: Circular dichroism spectra of bovine rhodopsin in 1% Emulphogene–6.6 mM potassium phosphate buffer (pH 6.5) at 20°. Rhodopsin (53 μM) was placed in an optical cell of 2-mm light path and circular dichroism spectra recorded for rhodopsin were corrected for the buffered Emulphogene solution. Solid line: unbleached; dotted line: bleached.

(open circles). The $A_{345}:A_{498}$ ratio of two independent preparations in CTAB was 0.27 and 0.29.

The absorption spectrum of a suspension containing fine particles of the outer segment showed bands at 278 and 498 mμ in addition to the distinct β band near 340 mμ (Figure 3). The molar extinction coefficient of the 498-mμ band was 40,000 cm²/mole of retinal.

Retinal oxime formed by mixing *all-trans*-retinal with hydroxylamine showed an absorption band at 362 mμ with the molar extinction coefficient of 59.3 × 10^3 cm²/mole. Retinal oxime produced by bleaching rhodopsin in the presence of hydroxylamine showed a band at 361 mμ. The concentration of rhodopsin was calculated from the intensity of the 361-mμ band and the molar extinction coefficient of rhodopsin at 498 mμ was estimated to be 41,000 cm²/mole. There was a single isosbestic point at 408 mμ during the bleaching process, as described by Adams *et al.* (1958), and reisomerization of *all-trans*-retinal was considered negligible.

Circular Dichroism. The circular dichroism spectrum of rhodopsin in buffered Emulphogene and that of a sonic extract of outer segments are shown in Figures 4 and 5. The pigment in detergent shows two positive bands at 492 and 340 mμ corresponding, respectively, to the α- and β-absorption bands. The magnitude of the 340-mμ band is approximately 1.8 times higher than that of the 492-mμ band. The aqueous suspension of sonicated outer segments also shows two positive bands at 340 and 495 mμ. However, the intensity of the 340-mμ band is approximately one-half of that of the 495-mμ band with the sonic extract. In each case, irradiation resulted in an irreversible disappearance of the two bands.

Discussion

Heller (1968) stated that bovine rhodopsin purified on Sephadex G-200 with CTAB as solubilizer did not have the β band in its absorption spectrum but partially bleached preparations showed a broad band at 350–360 mμ. These results led him to conclude that "the β band is not a part of the spectrum of the visual pigment but is due to free or bound retinal resulting from partial transformation to the light-exposed form of the pigment during isolation procedures."

The implication of this conclusion is of particular importance in considering the chromophore structure of rhodopsin because the α and β bands have been ascribed to the carotenoid prosthetic group of the pigment (Wald, 1953). The present spectral comparison showed that rhodopsin preparations purified on Sephadex appeared to be of lesser purity than those subjected to subsequent purification on calcium phosphate; the β band became clearly detectable only after the calcium phosphate chromatography. When rhodopsin samples prepared independently from the outer segments were spectrally examined, it was found that the intensity of the β band was directly proportional to the concentration of rhodopsin. The β band was little affected by the addition of hydroxylamine to rhodopsin; if the band was due to free or bound retinal as suggested by Heller (1968), it should immediately have shifted to 362 mμ with an increase in intensity.

It may be argued, however, that the β band is an artifact of solubilizing the pigment from the outer segment of the retina. The absorption and circular dichroism spectra of

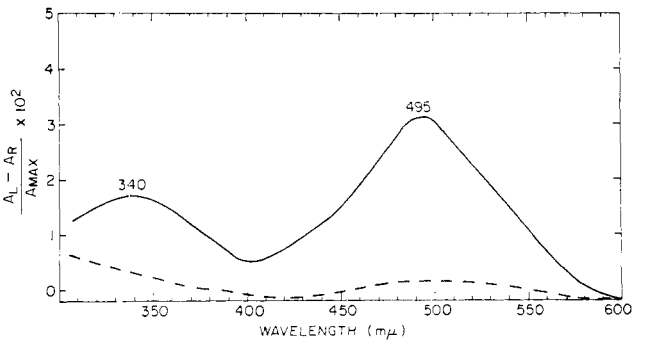

FIGURE 5: Circular dichroism spectra of sonically prepared fine particles of the bovine retinal outer segment in distilled water at 20°. The protein concentration of the solution was 0.86 mg/ml. The light path of the optical cell was 2 mm and recorded circular dichroism spectra were corrected for distilled water. Solid line: bleached; dotted line: bleached.

TABLE I: Molar Extinction Coefficients (ϵ) of *all-trans*-Retinal in Various Solvents.

Solvent	Band (mμ)	$\epsilon \times 10^{-3}$
Chloroform	390,[a] 389[b]	35.7,[a] 37.2[b]
Cyclohexane	373,[a] 373[b]	37.8,[a] 41.4[b]
Petroleum ether	370,[a] 370[b]	44.6,[a] 48.0[b]
Ethanol	382,[a] 386[b], 383[c]	40.0,[a] 39.9,[b] 42.9[c]
n-Heptane	370[d]	48.1[d]
1-Propanol	386[d]	42.9[d]
Digitonin[e]	389[c]	38.3[c]

[a] This work. [b] Calculated from data of Ball *et al.* (1948). [c] Wald and Brown (1953–1954). [d] Hubbard (1966). [e] A mixture of 2% aqueous digitonin and ethanol (1:1, v/v).

sonically prepared outer segment particles still showed the β band (Figures 3 and 5), thus excluding the possibility of artifact by solubilization. These fine particles, unlike solubilized rhodopsin, do not undergo a conformational change on bleaching[2] and resemble the intact outer segment (Shichi *et al.*, 1969). The validity of the β-absorption band of rhodopsin is also supported by the fact that there is a close correspondence between the absorption spectrum of rhodopsin and the relative photosensitivity curve determined by Schneider *et al.* (1939) and Goodeve *et al.* (1942) and the photosensitivity curve has a peak near 350 mμ (Figure 1 of this paper; Wald, 1949; Collins *et al.*, 1952).

Circular dichroism spectra of detergent-solubilized bovine rhodopsin showed a band around 340 mμ which disappeared after irradiation. The fact that *all-trans*-retinal demonstrated no appreciable band in the β-band region of the circular dichroism spectrum was inconsistent with the conclusion of Heller (1968) that the β absorption was due to retinal produced by light. Rhodopsin preparations from frog and squid also show similar circular dichroism bands which vanish in the light (Crescittelli *et al.*, 1966; Kito *et al.*, 1968).

Heller (1968) determined the molar extinction coefficient of purified bovine rhodopsin at 500 mμ to be 23,100 and considered the value of 40,600 previously obtained by Wald and Brown (1953–1954) too high. Heller (1969) also reported a value of around 23,000 for the ϵ_{500} of rhodopsin from rat and frog. His calculation of the coefficient (ϵ_{500}) involved the determination of a molecular weight of rhodopsin by gel filtration on a calibrated agarose column, and amino acid analysis of samples of known optical intensities at 500 mμ. The validity of the molecular sieving method for the determination of molecular weights depends on the similarity in shape of proteins and on the absence of adsorptive interaction between protein and gel. When the method is applied to proteins in the presence of detergents, the homogeneous behavior of detergent–protein complexes may not be expected because detergent molecules will be bound to proteins to varying degrees (Davison, 1968). Until the method is extensively tested with respect to the effect of detergent, the values of the molecular weight obtained by Heller (1968) and of ϵ_{500} derived from it remain open to question. It should be noted that the molar extinction coefficients estimated from protein concentrations and optical densities are seriously affected by purity of samples and the recovery of amino acid analysis. In contrast to Heller, Wald and Brown (1953–1954) obtained a value of 40,600 from spectral measurements only involving the recording of absorption spectra of *all-trans*-retinal and its oxime, and of retinal oxime prepared from the bleaching product of rhodopsin. In this investigation we have confirmed the result of Wald and Brown (1953–1954) and obtained a value of 42,000 for an ϵ_{498} in aqueous Emulphogene (Shichi *et al.*, 1969) and 39,700 in aqueous CTAB (in this investigation) from the measurement of the extinction at 498 mμ and the colorimetric assay of retinal with thiobarbiturate. From the molar extinction coefficient of 40,600 and the photosensitivity data of Schneider *et al.* (1939), Wald and Brown (1953–1954) estimated the quantum efficiency of bleaching rhodopsin to be 0.58. A similar treatment of the absorption coefficient found by Heller (1968) gives a quantum efficiency of 1.03, a value that is difficult to accept.

The molar extinction coefficients of *all-trans*-retinal near 380 mμ in various organic solvents are in the range of 35,000–40,000 (Table I). The value decreases slightly in aqueous digitonin–ethanol but it does not change appreciably in aqueous CTAB–ethanol or in aqueous Emulphogene–ethanol. Therefore, a value in aqueous detergent solution is probably no less than 90% of those in organic solvents, *i.e.*, 33,000. The relative ratio of the 498-mμ absorption of purified rhodopsin over the main absorption of *all-trans*-retinal produced as the product of bleaching is 1.18–1.2 (Wald and Brown, 1956; Shichi *et al.*, 1969). This indicates that an ϵ_{498} of rhodopsin will be close to 33,000 $\times$ 1.2 = 39.600, in good agreement with a value we have obtained above. For these reasons, we conclude that the value of 23,100 reported by Heller (1968) is a considerable underestimate.

When Heller (1968) assessed the purity of his preparation by the $A_{280}:A_{500}$ ratio, he made corrections on the absorption spectrum by "linear extrapolation of the absorption between 310 and 340 mμ into the 280-mμ range" for the nonspecific absorption of CTAB. Since this manipulation will reduce the magnitude of the 280-mμ absorption, it is possible that he overestimated the spectral purity. The spectral manipulation for correction and the overestimation of purity may account for the unusual spectral characteristics of Heller's rhodopsin preparation.

[2] H. Shichi, unpublished.

References

Adams, R. G. (1969), *J. Lipid Res. 10*, 473.

Adams, R. G., Kennedy, D., Wulff, V. J., and Zonana, H. V. (1958), *Arch. Biochem. Biophys. 75*, 534.

Ball, S., Goodwin, T. W., and Morton, R. A. (1948), *Biochem. J. 42*, 516.

Collins, F. D., Love, R. M., and Morton, R. A. (1952), *Biochem. J. 51*, 292.

Crescitelli, F., Mommaerts, W. F. H. M., and Shaw, T. I. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 1729.

Davison, P. F. (1968), *Science 161*, 906.

Futterman, S., and Saslaw, L. D. (1961), *J. Biol. Chem. 236*, 1652.

Goodeve, C. F., Lythgoe, R. J., and Schneider, E. E. (1942),

Proc. Roy. Soc. (London) B130, 380.
Hall, M. O., Bok, D., and Bacharach, A. D. E. (1968), *Science 161*, 787.
Heller, J. (1968), *Biochemistry 7*, 2906.
Heller, J. (1969), *Biochemistry 8*, 675.
Hubbard, R. (1966), *J. Biol. Chem. 241*, 1814.
Kito, Y., Azuma, M., and Maeda, Y. (1968), *Biochim. Biophys. Acta 154*, 352.
Krinsky, N. I. (1958), *Arch. Ophthal. 60*, 688.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Poincelot, R. P., Millar, P. G., Kimbel, R. L., and Abrahamson, E. W. (1969), *Nature 221*, 257.
Schneider, E. E., Goodeve, C. F., and Lythgoe, R. J. (1939), *Proc. Roy Soc. (London) A170*, 102.
Shichi, H., Lewis, M. S., Irreverre, F., and Stone, A. L. (1969), *J. Biol. Chem. 244*, 529.
Wald, G. (1949), *Doc. Ophthalmol. 3*, 94.
Wald, G. (1951), *Science 113*, 287.
Wald, G. (1953), *Ann. Rev. Biochem. 22*, 497.
Wald, G., and Brown, P. K. (1953–1954), *J. Gen. Physiol. 38*, 189.
Wald, G., and Brown, P. K. (1956), *Nature 177*, 174.
Williams, T. P. (1968), *Vision Res. 8*, 315.

Reversible Boundary Spreading as a Criterion of the Microheterogeneity of Plasma Albumins*

H. N. Bhargava† and Joseph F. Foster

ABSTRACT: Several samples of bovine plasma albumin of known microheterogeneity, as judged by the criterion of the solubility–pH profile method, have been examined by the reversible boundary spreading technique. For charcoal-defatted samples a linear relationship exists between h, the standard deviation of the mobility distribution, and ΔpH_{10}^{90}, the pH range required to alter the solubility of the protein in 3 M KCl from 10 to 90%. The straight line extrapolates to ΔpH_{10}^{90} approximately 0.1 when $h = 0$, *i.e.*, for a hypothetically homogeneous protein exhibiting no electrophoretic heterogeneity. This limiting value of ΔpH_{10}^{90} is shown to be consistent with published evidence that precipitation is associated with the N–F transition together with evidence on the difference in protonation behavior of N and F forms. Nondefatted and acid-defatted samples deviate from this simple linear relationship in such a way as to indicate that extrinsic contaminants make a greater contribution to ΔpH_{10}^{90} than to the electrophoretic heterogeneity, h.

The technique of electrophoresis is well known for detecting gross electrical heterogeneity or contamination in solutions of proteins (Alberty, 1948a; Hess, 1951; Taylor, 1953). A protein which is multimodally heterogeneous will exhibit multiple boundaries in a moving-boundary experiment (Longsworth, 1959). But the fact that a protein fails to develop more than one moving boundary in a given buffer solvent cannot be considered sufficient evidence that all protein molecules have the same electrophoretic mobility. As pointed out by Alberty (1948b) "if the molecules in a protein 'family' vary with respect to electrophoretic mobility because of differences in net charge or size or shape the protein gradient will spread faster in the electric field than expected for diffusion alone but will become sharper upon reversal of this field." This is the basic idea behind the criterion of reversible boundary spreading as a test of purity of proteins. Working with hemocyanins, Tiselius and Horsfall (1939) and Horsfall (1939) were the first to observe reversible boundary spreading. Later, Sharp *et al.* (1942) developed a theory which was modified by Alberty and associates (Alberty *et al.*, 1948; Anderson and Alberty, 1948) and Brown and Cann (1950). Alberty and coworkers applied this method to fourteen purified proteins and concluded (Anderson and Alberty, 1948) that none of the preparations satisfied this criterion of homogeneity. The method has received further elaboration and support by Longsworth (1959) who pointed out that fluctuations in charge and shape, to be expected in any isoelectric protein, should be rapidly reversible and should not contribute to the heterogeneity in mobility observed by this method.

Colvin *et al.* (1954) published a stimulating review on the microheterogeneity of proteins. They suggested that the data then available "provide a wide base for the conclusion that all protein preparations examined to date are microheterogeneous at best." They concluded much of this microheterogeneity to be inherent. Following Haurowitz (1950), they suggested that a native protein should be considered as a population of closely related but not identical molecules.

* From the Department of Chemistry, Purdue University, Lafayette, Indiana. *Received December 15, 1969.* This work was supported by Grant CA-02248 of the National Institutes of Health, U. S. Public Health Service. Presented in part at the 155th National Meeting of the American Chemical Society, San Francisco, Calif. 1968. This is paper VIII in the series on The Microheterogeneity of Plasma Albumins. See the preceding paper by Wong and Foster (1969).

† Present address: Department of Chemistry, Gorakhpur University, Gorakhpur, U. P., India.