Solubility of Rod Outer Segment Protein in Acidic Organic Solvents†

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ABSTRACT: Bovine retina rod outer segments were extracted with chloroform–methanol (2:1, 1:1, or 1:2, v/v) or methanol, each made 10^{-1}, 10^{-2}, 10^{-3}, or 10^{-4} M with anhydrous hydrogen chloride. All of the solvents solubilized a retinal Schiff base that had an absorption maximum at 465 nm, the maximum of *N*-retinylidenephosphatidylethanolamine (*N*-RPE). However, it was shown that the major component of the A_{465} material solubilized in the combinations of chloroform and methanol was a retinal–protein Schiff base and *not* *N*-RPE. Although *N*-RPE was found in all four of the extracting solvents, it was shown to be an artifact produced by transiminization during the extraction procedures. The degree of transiminization differed from solvent to solvent, the greates amount of *N*-RPE being formed when chloroform–methanol (1:2, v/v) or methanol was used. Solubility of the retinal–protein Schiff base was a function both of solvent and concentration of acid. The greatest solubility occurred in chloroform–methanol (2:1 and 1:1, v/v) at 10^{-1} and 10^{-2} M HCl. Plots of solubility *vs.* concentration of acid showed that a group with a p*K* of around 3 had to be protonated before solubilization could occur. These data are consistent with the protonation of the carboxyl groups of aspartic and glutamic acid residues of the protein.

Retina rod outer segments (ROS)[1] are composed of a series of flattened membraneous disks stacked one on top of the other. In cattle these disks contain upward to 20% (dry weight) rhodopsin, the photopigment of night vision (Anderson and Maude, 1970). The chromophoric group of rhodopsin is 11-*cis*-retinal covalently bound to the apoprotein opsin (Hubbard and Kropf, 1958).

A series of papers recently appeared claiming that a Schiff base of 11-*cis*-retinal and phosphatidylethanolamine is the chromophore of rhodopsin (Poincelo *et al.*, 1969, 1970; Poincelot and Abrahamson, 1970). According to these authors, *N*-RPE is part of a rhodopsin lipoprotein complex, and, after exposure of this complex to light, *all-trans*-retinal is transferred from the lipid to an ε-aminolysine residue of opsin.

Evidence that *N*-RPE is not the chromophore of rhodopsin has come from several laboratories. Hall and Bacharach (1970) injected [^{32}P]P_i into frogs, isolated the ROS, and purified rhodopsin and the ROS phospholipids. They found that pure rhodopsin contained no peak of radioactivity while the phospholipids were highly labeled. Our laboratory showed that the molar concentration of *N*-RPE in bovine ROS is less than that of rhodopsin (Anderson, 1970; Anderson and Maude, 1970). Recently, we injected labeled retinol into frogs, isolated pure rhodopsin, and extracted it with organic solvents (Anderson *et al.*, 1971). No *N*-RPE could be extracted from the pure visual pigment. From our experiments we concluded that retinal was covalently bound to opsin and that *N*-RPE, if it existed in ROS, served some function other than being the chromophore of rhodopsin.

Other evidence against *N*-RPE being the chromophore of rhodopsin has recently been given by Daemen *et al.* (1971). They showed that rhodopsin denatured with methanol in the presence of sodium borohydride had retinal covalently linked to lysine. No free retinol or *N*-RH_2PE were observed. In addition, they could not quantitatively extract the chromophore from native dark-adapted ROS with 0.1 N anhydrous HCl in methanol, the conditions claimed by Poincelot *et al.* (1970) to solubilize 90% of the chromophore.

In the present study, ROS were extracted with several combinations of chloroform and methanol containing varying concentrations of anhydrous HCl. The results agree with our earlier data and those of Daemen *et al.* (1971) showing that *N*-RPE is not the chromophore of rhodopsin. We also report the solubility of large amounts of ROS protein in the acidic organic solvents.

Materials and Methods

Preparation of ROS. ROS from dark-adapted bovine retinas (G. Hormel, Austin, Minn.) were prepared as previously described (Anderson and Maude, 1970) and lyophilized.

Extraction of Dark-Adapted ROS with Acidic Organic Solvents. Acidic methanol was prepared by bubbling anhydrous hydrogen chloride into anhydrous methanol until a 0.3 N solution was obtained. The pH of this solution was checked on a pH meter after diluting with water to a theoretical pH of 4.0; the actual pH was found to be 4.0 ± 0.1. This stock solution was diluted with various amounts of chloroform or methanol to give the desired acid concentrations and volumetric ratios of the extracting solvents. For convenience, acid concentrations are expressed as pHCl values (negative logarithm of the HCl concentrations).

Known weights (*ca.* 20 mg) of dry ROS were extracted in the dark with chloroform–methanol solutions in volumetric ratios of 2:1, 1:1, or 1:2, or with methanol. Each extracting solution was made up to pHCl 1, 2, 3, or 4. A total of 16 different combinations of solvents and acid concentrations were used. The ROS were extracted in an all glass homogenizer in exactly 4 ml of solvent. Each sample was given 15 brisk strokes with the pestle and immediately transferred to a glass tube and

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[1] Abbreviations used are: ROS, rod outer segments; *N*-RPE, *N*-retinylidenephosphatidylethanolamine; *N*-RH_2PE, *N*-retinylphosphatidylethanolamine; C, chloroform (table and figures); M, MeOH, methanol (table and figures).

centrifuged. The clear supernatant was removed and its absorption spectrum determined from 500 to 270 nm. Immediately after extraction, 0.1 ml of 0.3 N anhydrous HCl in methanol was added to the supernatant of the pHCl 3 and 4 extracts to assure protonation and stabilization of any Schiff base. In some cases, methanolic KOH was added to part of the extract and a spectrum of the basic solution determined.

Duplicate portions of two different concentrations of each extract were removed for protein determination (Lowry *et al.*, 1961). The remainder was washed with 0.1 N NaCl according to the procedure of Folch *et al.* (1957). The three solvents that did not contain the correct 2:1 ratios of chloroform to methanol necessary for the saline wash were adjusted to that ratio by the addition of chloroform. This resulted in a dilution in some instances and the absorbance values derived from these solutions were readjusted to the original 4 ml. The interface material between the chloroform and methanol–water layers was solubilized in 1.0 N NaOH and aliquots removed for protein determination. One-tenth milliliter of 0.3 N anhydrous HCl in methanol was added to each chloroform layer regardless of the acidity of the extracting solvent, and absorption spectra were determined on the acidified chloroform layer from each saline wash.

The absorbance values for all of the extracts and the chloroform layers resulting from the saline washes are reported both as total absorbance and as percent of the absorbance at 465 nm solubilized in 4 ml of solvent from 20 mg of ROS. One-hundred per cent represents the amount solubilized in chloroform–methanol (2:1, v/v, pHCl 1). The absolute amount of the chromophore that was solubilized was estimated by comparing the absorbances of the Schiff base at 465 nm and rhodopsin at 500 nm (described in the Results section). Values for protein concentration are reported as per cent of ROS dry weight.

Extraction of Light-Exposed ROS with Chloroform–Methanol (2:1, v/v). Known weights of ROS were suspended in deionized water and exposed to room light for 2 hr. They were then lyophilized, extracted with chloroform–methanol (2:1, v/v), and subjected to all of the manipulations outlined in the previous section.

Preparation of Synthetic N-RPE. Synthetic *N*-RPE was prepared in the dark from dipalmitoyl-PE and *all-trans*-retinal according to the procedure of Plack and Pritchard (1966).

Results

Solubility of Material Absorbing at 465 nm. Extraction of ROS with acidic organic solvents containing chloroform resulted in the solubilization of a substance that spectroscopically resembles the Schiff base *N*-RPE. Figure 1 contains the absorption spectra of protonated synthetic *N*-RPE and ROS extracted with the four different solvents at pHCl 2. All of the extracts have absorption maxima at around 465 nm, although decreasing the amounts of chloroform relative to methanol decreased the magnitude of the absorbance at 465 nm that could be solubilized. The chloroform–methanol (pHCl 3) extracts contained an additional maximum at 280 nm, which was so strong in the pHCl 1 and 2 extracts that it could be seen only after considerable dilution. No 280-nm peak was observed for synthetic *N*-RPE or the methanol extracts.

The synthetic *N*-RPE contains *all-trans*-retinal whereas the ROS extracts are assumed to contain 11-*cis*-retinal (this was not determined), since this isomer is found after a chemical bleach of rhodopsin. However, no differences were observed

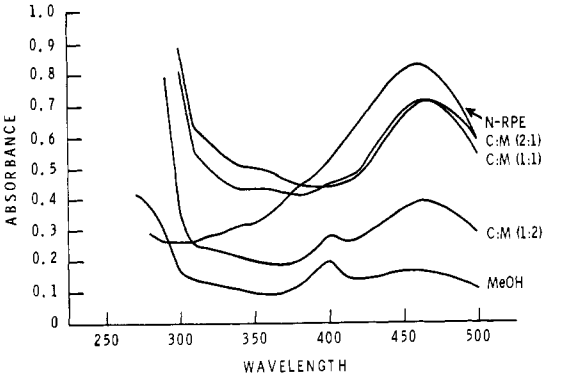

FIGURE 1: Absorption spectra of protonated *N*-RPE and pHCl 2 extracts of dark-adapted ROS.

in the absorption maxima of the ROS extracts and the synthetic *N*-RPE.

Solubility of Protein in the Extracting Solvents. The spectral data given in Figure 1 suggested that we were solubilizing protein. Therefore, we washed each extract with 0.1 N saline to remove any nonproteolipid protein from the organic solvents. Protein would precipitate at the interface of the chloroform and methanol–water layers, while *N*-RPE would remain in the chloroform. A bright orange material was observed at the interface which, after complete drying, could be partially dissolved in 5% sodium dodecyl sulfate (pH 2). This solubilized material exhibited typical retinal–Schiff base behavior. Determinations of protein revealed that as much as 35% of the dry weight of ROS could be solubilized in the organic solvents and that the degree of solubilization depended both upon the ratios of chloroform to methanol and the concentration of acid in the extracting solvent. Moreover, virtually all of the soluble protein was recovered at the interface following the saline wash. Insignificant amounts of protein remained in the chloroform layer and none was found in the methanol–water layer. These data are given in Figure 2A,B.

Effect of Solvent and Concentration of Acid on the Solubility of A_{465}. The solubility of material with a maximum of 465 nm (called A_{465}) paralleled that of protein solubility, being a function of both acid concentration and type of solvent. The data given in Figure 3A show the relative amounts of A_{465} material solubilized in the four solvents at the four pHCl values. Solvents containing chloroform solubilized more A_{465} at lower pHCl values whereas methanol solubilized more A_{465} at a higher pHCl. The data in Figure 3B are the A_{465} values recovered in the chloroform layer following the saline wash (adjusted for dilutions as described in the Methods section). Examination of these data shows that the bulk of the solubilized A_{465} is not *N*-RPE, since the values for A_{465} in Figure 3B are much less than those for the original extract (Figure 3A). However, some *N*-RPE could be solubilized in chloroform–methanol (1:2, v/v) and methanol at pHCl 2, 3, and 4, although it represents only about a third of the A_{465} solubilized by chloroform–methanol (2:1 and 1:2, v/v) at pHCl 1.

An estimate was made of the per cent of the total chromophore that was solubilized in the acidic organic solvents. If we assume that the molar extinction coefficient (ϵ_M) of rhodopsin is similar to that of a protonated Schiff base, we solubilized a maximum of 85% of the chromophore in the organic solvents. This assumption does not introduce a gross error since the ϵ_M of protonated Schiff bases of retinal has been estimated to be between 31,300 (Anderson and Maude, 1970) and 49,000

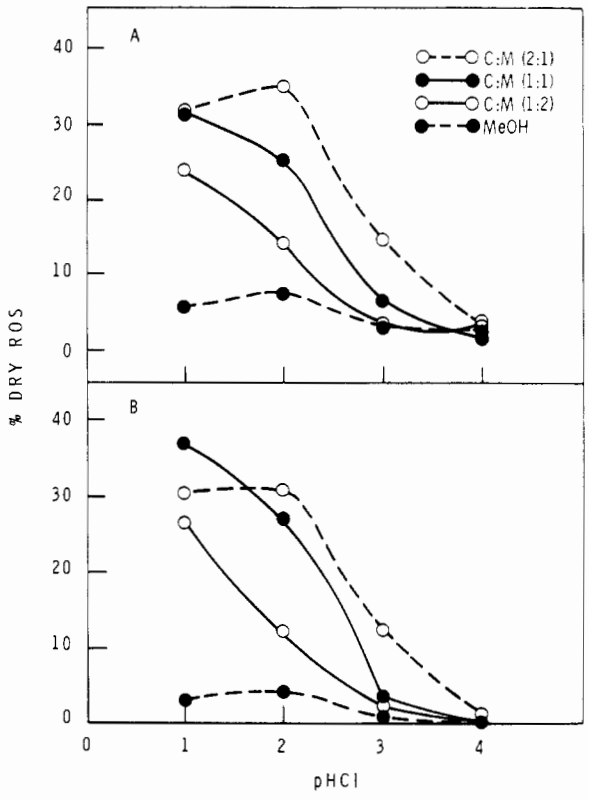

FIGURE 2: (A) Amounts of ROS protein solubilized in acidic organic solvents expressed as per cent of dry ROS weight. (B) Amounts of ROS protein recovered at the interface following a saline wash of the original ROS extract, expressed as per cent of dry ROS weight.

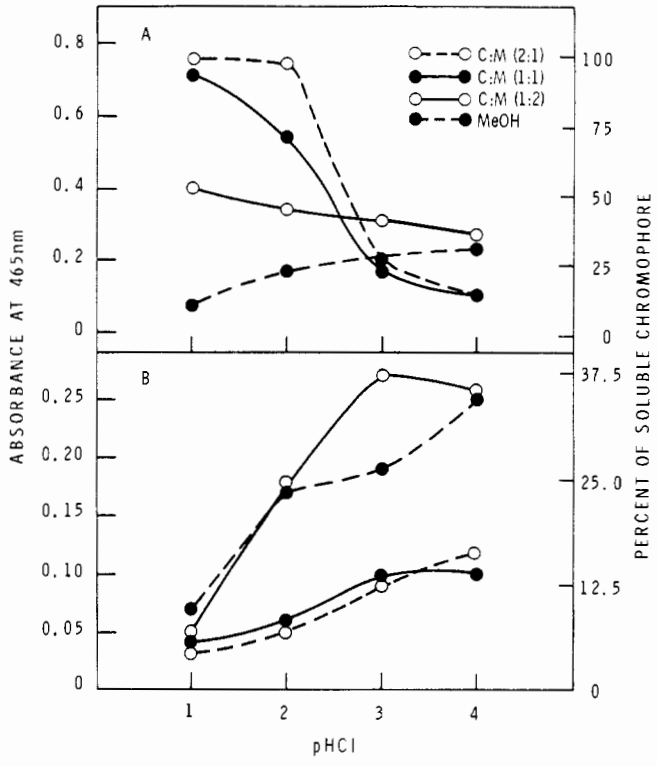

FIGURE 3: (A) Absorbance at 465 nm and per cent of soluble chromophore at 465 nm as a function of solvent and concentration of HCl. (B) Absorbance at 465 nm and per cent of soluble chromophore at 465 nm recovered in the chloroform layer following a saline wash of the original ROS extract.

(Daemen *et al.*, 1971) while the ϵ_M of rhodopsin is between 23,100 (Heller, 1968) and 42,000 (Shichi *et al.*, 1969). Dark-adapted ROS (20 mg) extracted with 4 ml of chloroform-methanol (2:1, v/v, pHCl 1) had an absorbance at 465 nm of 0.75; 20 mg of ROS solubilized in 4.0 ml of 1% Emulphogene (General Aniline) had an absorbance at 500 nm of 0.88.

The data given in Figure 3 are consistent with the solubilization of a retinal–protein Schiff base at low pHCl values in chloroform–methanol solvents, with high pHCl values and methanol favoring the formation of *N*-RPE. The latter is consistent with the observation of Daemen *et al.* (1971) that imine exchange can occur in low acid concentrations and is favored in methanol solutions.

Studies with Light-Exposed ROS. A series of aqueous ROS suspensions were exposed to room light for 2 hr, then lyophilized, and extracted with chloroform–methanol (2:1, v/v, pHCl 1, 2, 3, or 4). The purpose of this experiment was to determine whether or not the solubility of ROS protein depends upon bound retinal. The results of this experiment are given in Table I. It is apparent that the solubility of protein is not dependent upon bound retinal, since the values for protein in this table match those given in Figure 2A for the ROS protein extracted in the dark. In addition, the amount of protein recovered at the interface did not depend upon bound retinal. However, we did note that the protein at the interface was more difficult to solubilize in 1.0 N NaOH than that of dark-extracted ROS. This may explain the differences observed between soluble and interface protein concentrations.

The absorbance data given in Table I show that A_{465} material was recovered in the chloroform layer of the light-exposed ROS extract following the saline wash. Thin-layer chromatography was used to identify this material as *N*-RPE. Thus, *N*-RPE can be formed in ROS by two independent means; however, in each case it is produced as an artifact of either the solvent extraction or the ROS isolation procedures. Scans of the light-exposed extracts (chloroform layer after the saline wash) failed to reveal either a retinal (380–390 nm) or a retinal dimethyl acetal peak (325–340 nm). Therefore, most of the retinal released from protein must have immediately formed a Schiff base with phosphatidylethanolamine. A surprising observation was that even after 2 hr in water in the light less than half of the retinal had been released from protein. This is

TABLE I: Effect of Acid Concentration on the Extraction of Protein and Schiff Bases of Retinal from Light-Exposed ROS with Chloroform–Methanol (2:1, v/v).

	Protein (% Dry ROS)		Absorbance (OD/20 mg of ROS per 4 ml)	
pHCl	C–M[a]	Interface[b]	C–M[a]	$CHCl_3$[c]
1	35.4	28.5	0.653	0.256
2	35.6	33.6	0.735	0.243
3	10.5	16.9	0.393	0.287
4	3.8	3.7	0.242	0.225

[a] Protein and A_{465} solubilized in chloroform–methanol (2:1, v/v). [b] Protein recovered at the interface following a wash with 0.1 N saline. [c] A_{465} recovered in the chloroform layer following a wash with 0.1 N saline.

common for squid visual pigment (Hubbard and St. George, 1958) but vertebrate pigments are generally considered to decompose completely to opsin and retinal (Matthews *et al.*, 1963).

Discussion

Solubility of ROS Protein in Acidic Organic Solvents. Proteolipids are classical examples of proteins that are soluble in organic solvents. These compounds readily go into solution in chloroform–methanol (2:1, v/v), but usually remain in the chloroform layer following a saline wash. The lipid–protein bonding is considered to be hydrophilic with the acyl chains exposed to the solvent, which facilitates their being solubilized by organic solvents. The ROS protein dissolved by acidic organic solvents is not the classical proteolipid since the hydrophilic lipid–protein bonds of proteolipids would dissociate at high acid concentrations, making the protein less soluble at low pHCl values. Also, the retinas we used had been frozen and the ROS were lyophilized, all conditions that lead to the denaturation of proteolipids.

Fraenkel-Conrat and Olcott (1945) reported that mineral acid catalyzed the esterification of proteins with alcohols, a reaction that should favor solubilization of the protein in organic solvents. However, the time required for these reactions to proceed at room temperature is such that it renders this possibility unlikely in our case. Curtis (1969) has reported the solubility of mitochondrial membrane protein in acidic chloroform–methanol (2:1, v/v) and acidic methanol. However, the protein was also soluble in aqueous acidic or alkaline solutions. In contrast, ROS protein precipitates from alkaline solutions and is not soluble in acidic methanol or aqueous solutions.

The solubility of ROS protein is a function both of acid concentration and chloroform to methanol ratios. Examination of the solubility curve for chloroform–methanol (2:1, v/v) in Figure 2A shows an inflection point at around pHCl 3. If we equate pHCl with pH, this value is near the pK_1 of aspartic and glutamic acids. At pHCl 2 all of the carboxyl groups of the protein would be protonated. The drop in A_{465} at pHCl 3 and 4 shown in the curves in Figure 2A is not the result of a Schiff base not being protonated at these particular concentrations of acid since addition of 0.3 N HCl in methanol to these extracts did not significantly increase the magnitude of the absorption at 465 nm. Therefore, the most reasonable explanation of the solubility of ROS protein in acidic organic solvents may be that the protein behaves like a lipid once acidic amino acid residues have been protonated. However, prior to this the protein remains insoluble in both organic and aqueous solvents.

Although we did not identify the protein to which retinal was bound, it is most likely opsin. Supporting evidence comes from earlier work (Anderson *et al.*, 1971) in which we showed that tritiated retinol injected into frogs was bound only to rhodopsin in the outer segments (see Figure 1 of that paper), and that acidified aqueous detergent solutions of pure rhodopsin did not release free retinal. Therefore, extraction of ROS with acidic organic solvents should not release retinal from opsin. However, the linkage of retinal to opsin in rhodopsin, the exact nature of which is still unknown, is changed to a protonated Schiff base.

The number and relative amounts of the proteins solubilized in the acidic chloroform–methanol was not determined in this study. It remains to be seen whether or not this method can be used to prepare pure opsin free of lipid.

N-RPE as the Chromophore of Rhodopsin. The first paper claiming that *N*-RPE is the chromophore of rhodopsin (Poincelot *et al.*, 1969) was fortified by several others (Poincelot and Abrahamson, 1970; Poincelot *et al.*, 1970) as well as by data from two additional laboratories (Akhtar and Hirtenstein, 1969; Daemen and Bonting, 1969). However, Hirtenstein and Akhtar (1970) have recently expressed doubts about their initial position and Daemen *et al.* (1971) have presented a very strong case against *N*-RPE having a chromophoric role. Although the original group has found that retinal is bound to lysine in squid rhodopsin, they have not reversed their position about the binding site in the vertebrate retina (Fager *et al.*, 1971).

Our data and that of Daemen *et al.* (1971) show that *N*-RPE is an artifact formed during the isolation of ROS. When added to that previously presented by our laboratory (Anderson, 1970; Anderson and Maude, 1970; Anderson *et al.*, 1971) and others (Hall and Bacharach, 1970; Heller, 1968), these data give very strong, if not conclusive, evidence that *N*-RPE is not the chromophore of rhodopsin. The only possible way that *N*-RPE could be the chromophore would be if it were too tightly bound to the protein to be removed with the acidic organic solvents. However, Daemen *et al.* (1971) eliminated this possibility by denaturing rhodopsin with methanol in the presence of sodium borohydride. After alkaline hydrolysis, only retinyllysine was found. No free retinol or retinylethanolamine was observed.

Acknowledgments

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Added in Proof

We recently solubilized pure rhodopsin (a generous gift from Dr. Michael Hall) and ROS in chloroform–methanol (2:1, v/v, p(HCl)2) and washed with saline. The solid interface material was reduced with $NaBH_4$, thoroughly dried, and solubilized in 5% sodium dodecyl sulfate. Both solutions were subjected to polyacrylamide gel disc electrophoresis No differences were observed in the electrophoretic behavior of the two samples run on individual gels or run together. These data show that rhodopsin is the protein soluble in acidic organic solvents.

References

Akhtar, M., and Hirtenstein, M. D. (1969), *Biochem. J. 115*, 605.

Anderson, R. E. (1970), *Nature (London) 227*, 954.

Anderson, R. E., Hoffman, R., and Hall, M. O. (1971), *Nature (London) 229*, 249.

Anderson, R. E., and Maude, M. B. (1970), *Biochemistry 9*, 3624.

Curtis, P. J. (1969), *Biochim. Biophys. Acta 183*, 239.

Daemen, F. J. M., and Bonting, S. L. (1969), *Nature (London) 222*, 879.

Daemen, F. J. M., Jansen, P. A. A., and Bonting, S. L. (1971), *Arch. Biochem. Biophys. 145*, 300.

Fager, R., Kimbel, R. L., and Abrahamson, E. W. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 790.

Folch, J., Lees, M., and Sloan-Stanley, G. H. (1957), *J. Biol. Chem. 226*, 497.
Fraenkel-Conrat, H., and Olcott, H. S. (1945), *J. Biol. Chem. 161*, 259.
Hall, M. O., and Bacharach, A. D. E. (1970), *Nature (London) 225*, 637.
Heller, J. (1968), *Biochemistry 1*, 2906.
Hirtenstein, M. D., and Akhtar, M. (1970), *Biochem. J. 119*, 359.
Hubbard, R., and Kropf, A. (1958), *Proc. Nat. Acad. Sci. U. S. 44*, 130.
Hubbard, R., and St. George, R. C. C. (1958), *J. Gen. Physiol. 41*, 501.
Lowry, Q. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Matthews, R. G., Hubbard, R., Brown, P. K., and Wald, G. (1963), *J. Gen. Physiol. 47*, 215.
Plack, P. A., and Pritchard, D. J. (1969), *Biochem. J. 115*, 927.
Poincelot, R. P., and Abrahamson, E. W. (1970), *Biochemistry 9*, 1820.
Poincelot, R. P., Millar, P. G., Kimbel, R. L., and Abrahamson, E. W. (1969), *Nature (London) 221*, 256.
Poincelot, R. P., Millar, P. G., Kimbel, R. L., and Abrahamson, E. W. (1970), *Biochemistry 9*, 1809.
Shichi, H., Lewis, M. S., Irreverre, F., and Stone, A. L. (1969), *J. Biol. Chem. 244*, 529.

An Analysis of the Circular Dichroism of the Lysozyme–α-Lactalbumin Group of Proteins†

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ABSTRACT: Circular dichroism data are presented for eight lysozymes and α-lactalbumins. All have multiple Cotton effects in the near-ultraviolet region, associated with the absorption bands of the aromatic amino acids. All but the avian lysozomes have predominantly negative optical activity in this region. Hen and duck egg lysozymes are dominated by positive rotational strength, which is interpreted as arising from tryptophan residues. Calculations of on- and off-resonance interactions between the two adjacent active-site tryptophan residues show that these can give rise to sufficient rotational strength to explain the observed difference between these proteins and their homologs, which lack the two adjacent tryptophans. The apparently unrelated goose egg lysozyme, which has a low content of aromatic amino acids, shows only very small optical activity in the region of the aromatic absorption band, and marked differences in the peptide region. Differences between the egg lysozymes and the other proteins in the series in this region can be rationalized in terms of aromatic contributions. Similarities in the high-field proton magnetic resonance spectra of lysozymes, due to ring-current shifted aliphatic proton resonances, and similar effects in the same region of the inhibitor, *N*-acetylglucosamine, are described.

The interpretation of the optical activity of globular proteins in their native states is complicated by the presence of chromophores other than the peptide group, which frequently generate considerable, rotational strengths, and in some cases (Green and Melamed, 1966; Yang *et al.*, 1968) can even dominate the optical activity of the molecule as a whole. Little is known with any certainty about the nature of the perturbations that are responsible for such effects, and it is also difficult to relate the Cotton effects associated with the near-ultraviolet absorption bands of the aromatic residues to those at shorter wavelengths, where there is extensive overlap with peptide Cotton effects. We have approached this problem by considering the circular dichroism of a set of proteins, believed to possess essentially identical conformations, but differing considerably in amino acid composition and sequence. This is the lysozyme–α-lactalbumin group (Brew *et al.*, 1968; Browne *et al.*, 1969; Brew, 1970). In the light of comparisons between these molecules we consider, on the one hand, the effect of extrinsic chromophores on the interpretation of the circular dichroism of the peptide absorption bands, and also some possible explanations for the enhancement of optical activity observed in these chromophores.

Materials and Methods

Proteins. Hen egg lysozyme was the three-times-recrystallized product of Sigma Chemical Co. Duck egg and human lysozymes were gifts from Dr. C. C. F. Blake, and goose egg lysozyme from Drs. S. Kammerman and R. E. Canfield. Human and guinea pig α-lactalbumins were prepared as described elsewhere (Findlay and Brew, 1972; Brew and Campbell, 1967). Bovine α-lactalbumin was prepared following the procedure of Aschaffenburg and Drewry (1957), with the additional final step of gel filtration on Sephadex G-100 (4 × 140 cm) equilibriated with 50 mM ammonium bicarbonate. Camel α-lactalbumin was purified by a procedure essentially similar to that described by Kessler and Brew (1970) for pig α-lactalbumin. Acid-precipitated camel whey was dialyzed and lyophilized. The α-lactalbumin peak from

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