Structure of Visual Pigments. II. Binding of Retinal and Conformational Changes on Light Exposure in Bovine Visual Pigment$_{500}$*

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ABSTRACT: Previous observations (Bownds, D., and Wald, G. (1965), *Nature 205*, 254; Wald, G., and Brown, P. K. (1952), *J. Gen. Physiol. 35*, 797) showing that while native visual pigment is not susceptible to reduction with sodium borohydride, light-exposed visual pigment can be reduced with borohydride to produce a retinyl lysine derivative of visual pigment, and that only after exposure to light are free sulfhydryl groups detectable in visual pigment, have been quantitatively extended to show that purified bovine visual pigment$_{500}$ contains one retinal chromophore per molecule and that only one sulfhydryl group per molecule becomes titrable on exposure to light. The four other half-cystine residues in visual pigment are present as two disulfide bridges. Two conformational forms of visual pigment have been separated by gel filtration chromatography: a compact conformation shown by native visual pigment and an expanded conformation shown by the apoprotein. The light-exposed, borohydride-reduced visual pigment, in which retinal is covalently bound to the protein, also shows the expanded conformation. The Stokes radius of the native pigment is 23 Å, that of the apoprotein and the light-exposed, borohydride-reduced pigment is 25.5 Å, and their hydrodynamic volumes are in the ratio of 1:1.36. On the basis of these experiments it is proposed that the 11-*cis* isomer of retinal is bound to a unique lysine residue in native visual pigment through a *substituted* aldimine bond. The substituting moiety is a cysteine sulfur. The substituted aldimine bond functions as a cross-linking agent between a lysine and a cysteine residue, maintaining the native molecule in a compact conformation. Two photochemical reactions take place in the excited-state molecule: the C–S bond is broken and retinyl isomerizes to the all-*trans* form (Wald, G., and Hubbard, R. (1960), *Enzymes 3*, 369). This leads to a reversion of the substituted aldimine to a simple aldimine bond (making the linkage susceptible to borohydride reduction) and exposing a new titrable sulfhydryl group. This reversion of the aldimine bond breaks the cross-linkage between the lysine and cysteine residues thus transforming the molecule into its expanded conformation. It is further proposed that functioning as cross-linking agent is a general property of substituted aldimine bonds in proteins and that other cases of compact-expanded conformational transformations can be expected in this class of proteins.

Several attempts have been made to determine the number of chromophore prosthetic groups per molecule of visual pigment (Broda *et al.*, 1940; Hecht, 1942; Wald and Brown, 1953; Hubbard, 1954). These measurements were all indirect and involved certain assumptions about the molecular weight and molecular absorptivity of visual pigment. The present availability of purified visual pigment of known molecular weight and composition (Heller, 1968) has made it possible to reinvestigate this problem more directly.

It has been known for some time that when native visual pigment is exposed to light, titrable sulfhydryl groups appear. Wald and Brown (1952), who first described this phenomenon, estimated that two sulfhydryl per molecule are exposed by light. They postulated that either these sulfhydryl groups are involved in the binding of retinal to the apoprotein, or are made titrable by some "denaturation" process taking place on exposure to light, or both. Although the proposal of binding between retinal and a sulfhydryl group has been withdrawn, the assumption that a considerable conformational change takes place in the transformation of native to light-exposed visual pigment has been retained (Wald and Hubbard, 1960).

This paper reports the results of experiments on the number of retinal and sulfhydryl groups in bovine visual pigment$_{500}$ and describes the chromatographic separation of native an light-exposed visual pigments. On the basis of these experiments a proposal is presented on the mode of linkage between the chromophore and the protein in the native pigment.

Experimental Section

Native bovine visual pigment$_{500}$ (CTAB[1] complex)

* From the Jules Stein Eye Institute, University of California School of Medicine, Los Angeles, California 90024. *Received April 16, 1968.* This investigation has been supported by Research Grant NB-06592 from the National Institutes of Health, U. S. Public Health Service, and Research Grant G-358 from the National Council to Combat Blindness, Inc.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: CTAB, cetyltrimethylammonium bromide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PMB, *p*-mercuibenzoate.

was purified as previously described (Heller, 1968). Protein concentrations were estimated from the absorptions at 280 mμ (ε 37,000) and 500 mμ (ε 23,100), and the exact amount was always verified by amino acid analysis, assuming a molecular weight of 26,400 for the apoprotein (Heller, 1968).

Reduction with Sodium Borohydride. Native visual pigment (0.02–0.5 μmole) in 0.04 M CTAB–0.066 M sodium phosphate (pH 7.1) was added, in the dark, to a test tube containing a 100-fold molar excess of sodium borohydride (Sigma) dissolved in an amount of 2.5 N NaOH sufficient to bring the final pH to 8.5 (from 3 to 20 μl). The solution was then exposed at room temperature (20°) to ordinary laboratory illumination or to a 40-W white lamp at a distance of 40–50 cm. Within a few seconds the color of the solution changed to pale yellow, then became almost colorless. In some experiments the same reduction procedure was followed, with one exception: the pH was not adjusted with NaOH and the reduction was carried out at pH 7.1 instead. The reduction at both pH was immediate and complete as judged by appearance of the peak at 333 mμ and the disappearance of any peaks absorbing at longer wavelength.

Sulfhydryl groups were determined according to Ellman (1959) with the following modifications. Purified native visual pigment, 5.29×10^{-5} M in 0.04 M CTAB–0.066 M sodium phosphate (pH 7.1), was brought to pH 8.0 with 2.5 N NaOH (5 μl/ml) in the dark at 20°. The absorption at 412 mμ was read in the dark before, 15 and 30 min after, adding 20 μl of DTNB to both sample and reference cell. The visual pigment with DTNB was next exposed to white light at 20°, and after 15 to 30 min, the absorption at 412 mμ was read against the same reference cell.

As visual pigment has some absorption at 412 mμ and as this absorption changes somewhat on light exposure, another experiment was performed using an equivalent amount of visual pigment in detergent–buffer in the reference cell, adding 20 μl of DTNB reagent to the sample cell only, exposing both cells to white light, and after 15 and 30 min, reading the absorption at 412 mμ. In a third experiment the visual pigment in the sample cell was initially exposed to white light, and the absorption at 412 mμ was read against detergent–buffer in the reference cell. Next, 20 μl of DTNB was added to both sample and reference, and after 15 and 30 min, the absorption at 412 mμ was read.

To determine the total content of half-cystine residues, purified visual pigment in 0.04 M CTAB–0.066 M sodium phosphate (pH 8.0) was exposed to light, then treated with 0.3 M (final concentration) of 2-mercaptoethanol at 20° for 1 hr. At room temperature excess 2-mercaptoethanol was removed by gel filtration on a 1.5 × 11 cm column of G-25 (fine, Pharmacia) and the fraction corresponding to visual pigment was immediately examined with the DTNB reagent as above.

Chromatography of Native and Light-Exposed Visual Pigments. The gel filtration system on agarose was used as previously reported (Heller, 1968), with the following modifications. Column dimensions were 1.95 × 155 cm (bed volume 463 ml). Sample volume was generally 1 ml (0.22% of bed volume). In a few experiments the sample volumes were 2 and 3 ml. The same results were obtained, and within the sample volumes used (0.22–0.66% of bed volume), results were independent of sample volume. The effluent transmittance at 280 mμ was monitored with the Uvicord as reported. In the region of the peak(s) the absorption at 280, 500, or 333 mμ was recorded, as previously described, with the Hitachi EPS-3T spectrophotometer. The fractions collected were 2 ml in the region of interest. The effluent volume corresponding to maximun concentration of the test material (V_e, elution volume) was measured directly, with a measuring cylinder accurate to 0.5 ml. The recorded graph from the Uvicord was used merely as a guide to the fractions of interest.

The column was calibrated with known proteins as previously described. Molecular radii were calculated as described by Ackers (1967) using the relationship between the Stokes radius and the inverse error function complement of the column partition coefficient, $\sigma(\sigma = K_D)$. Radii of the calibrating proteins were either obtained from published value or calculated from the published diffusion coefficients with the relationship; $D = kT/6\pi\eta a$, where D is the diffusion coefficient, k is Boltzmann constant, T is absolute temperature, η is viscosity, and a is the Stokes radius. The void volume, V_0, was measured with Blue Dextran 2000 (Pharmacia), and the total volume, V_t, was measured with tritiated water. The difference between the total volume and the void volume thus determined is equal to the internal volume, V_i.

Results

Number of Retinal Prosthetic Groups in Visual Pigment. Collins (1953) first suggested that retinal is bound to visual pigment as a Schiff base. Bownds and Wald (1965) observed that sodium borohydride has no effect on native visual pigment. On the other hand, light-exposed visual pigment in which retinal is apparantly linked to the protein in an aldimine bond (Schiff base) is rapidly reduced by borohydride with a concomitant appearance of a new absorption peak at 333 mμ. The reduction binds retinal covalently to visual pigment and the chromophore cannot be extracted with organic solvents. Bownds (1967) has shown that retinal after reduction is bound to a lysine residue.

I have confirmed the above observations and used them to determine the number of prosthetic groups per mole of visual pigment. As shown in Table I, the reduction of visual pigment with sodium borohydride (at pH 8.5) leads to a loss of a single lysine residue. No other amino acids in visual pigments are affected by this reduction. No new ninhydrin-positive peaks were observed in either the short or long column runs after borohydride reduction; but this might be due to the instability of the resulting retinyl lysine to acid hydrolysis (Bownds, 1967). The nature of the products resulting from acid and alkaline hydrolysis of borohydride-reduced visual pigment is currently under investigation. The specific loss of a single lysine residue was found in several experiments performed with different preparations of visual pigment. The loss of a single lysine resi-

TABLE I: Amino Acid Composition of Native, and Light-Exposed, Sodium Borohydride Reduced Visual Pigment.[a,b]

	Not Reduced	Reduced
Lys	10.0	8.8
His	3.9	4.0
Arg	5.9	6.0
$CySO_3H$	5.0[c]	4.8[c]
Asp	15.1	15.1
$MetSO_2$	8.0[c]	8.3[c]
Thr	16.0	16.4
Ser	11.1	10.7
Glu	21.1	20.7
Pro	12.9	13.0
Gly	16.2	16.1
Ala	19.9	19.9
Val	17.0	18.0
Ile	11.6	12.0
Leu	19.1	19.3
Tyr	11.1	11.3
Phe	19.0	19.1

[a] Results are expressed as residues per mole, assuming mol wt 26,400. [b] Each figure is average of four analyses, 24-hr hydrolysis, 6 N HCl, 110°. [c] Results obtained on separate samples by performic acid oxidation.

due on reduction is taken as evidence that only one retinal chromophore is bound to each molecule of visual pigment.

Sulfhydryl Content. Native visual pigment shows 0.00–0.05 titrable sulfhydryl group/molecule when examined with the DTNB reagent. These results were obtained after incubations of more than 1 hr at 20° in the presence of 0.04 M CTAB. When native visual pigment, in the presence of DTNB, was exposed to light at 20°, there was an immediate appearance of 0.83–0.96 titrable sulfhydryl groups/molecule. The reaction was almost fully developed (better than 90%) in seconds, or as soon as it was practical to expose the visual pigment to light and reexamine it in the spectrophotometer. Incubations of light-exposed visual pigment for up to 3 hr at 20° in the presence of CTAB did not reveal more than 1 sulfhydryl group/molecule. Only after reduction with 2-mercaproethanol was it possible to titrate up to 4.5 sulfhydryl groups/molecule. Results were somewhat variable and probably represent rapid reoxidation of the sulfhydryl groups.

Conformational Changes on Light Exposure. The patterns obtained by gel filtration chromatography of native and light-exposed visual pigment are shown in Figure 1. The elution volumes, V_e, of native visual pigment (ten runs), light-exposed visual pigment (ten runs), light-exposed sodium borohydride reduced (three runs), and a mixture of native and light-exposed visual pigment (three runs) did not vary more than ±2 ml in each of these series and the general variation from run to run was ±1 ml (*ca.* 0.3% of V_e) which under the conditions employed is the limit of the experimental technique.

Light-exposed visual pigment had a consistently *smaller* V_e (average 341 ml) than native visual pigment (average V_e 352 ml). In other words, light-exposed visual pigment behaved as if it were a *larger molecule* than native visual pigment. The narrow and symmetrical shape of the peaks shows that only one species of molecules was present either in native or light-exposed visual pigment. Furthermore, the possibility that the apparent increase in size is due to association is ruled out by the fact that the smallest associating species, a dimer (mol wt 55,000), would have had a much smaller V_e (calculated 315 ml, see Heller, 1968) than the observed one. It was concluded that the observed chromatographic behavior is due to a change in *shape* and that light-exposed visual pigment has a larger hydrodynamic volume than native visual pigment. The light-exposed visual pigment in Figure 1B, actually underwent two processes. It was exposed to light which isomerizes the retinal from the 11-*cis* to all-*trans*. At neutral pH, this reaction leads ultimately, at a fairly rapid rate, to the liberation of the chromophore. Thus, the material shown in Figure 1B is actually the apoprotein. One possibility was that the removal of retinal induces the conformational change. In other words, perhaps the binding *per se* of retinal is responsible for the compact conformation of native visual pigment. However, the behavior of light-exposed, sodium borohydride reduced visual pigment shows that this is not so (Figure 1C). This molecule in which retinal is firmly bound to the protein showed the same increased size as did the apoprotein. This was interpreted to mean that the conformational change in going from native to apo visual pigment was not due to liberation of the prosthetic group but to some other process preceding the hydrolysis. Unfortunately in the present system the reverse experiment of re-forming native visual pigment is impossible because CTAB prevents the re-formation of native pigment (Snodderly, 1967).

It is possible to calculate the molecular radii and the relative hydrodynamic volumes of native and apo visual pigment using the V_e values obtained above. Ackers (1967) has shown a linear relationship between the Stokes radius and the inverse error function complement of the partition coefficient for columns of Sephadex and agarose. If a calibration curve is defined for a particular column by chromatographing known proteins, it is possible to obtain the molecular radius for an unknown from the experimental value of V_e. As seen in Figure 2, the Stokes radius of native bovine visual pigment is 23 Å while that of the apoprotein and the borohydride-reduced form is 25.5 Å. The relative hydrodynamic volumes, assuming spherical shape, of native and apo visual pigment are 1:1.36. Since the actual shapes of native and apo visual pigment molecule are unknown, these values should be regarded as representing approximations rather than absolute values.

Discussion

The experiments reported in this paper show that bovine visual pigment$_{500}$ has only one retinal prosthetic

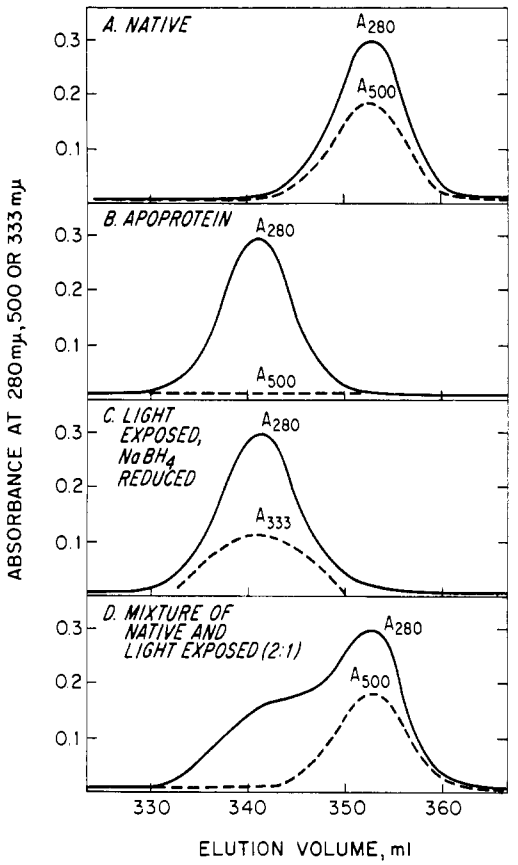

FIGURE 1: Chromatographic separation of native, apo, and light-exposed borohydride-reduced visual pigment on a column of agarose. For details, see Experimental Section. The results have been normalized so that each run will have the same A_{280} at the peak. The actual values were *ca.* 0.2–0.4.

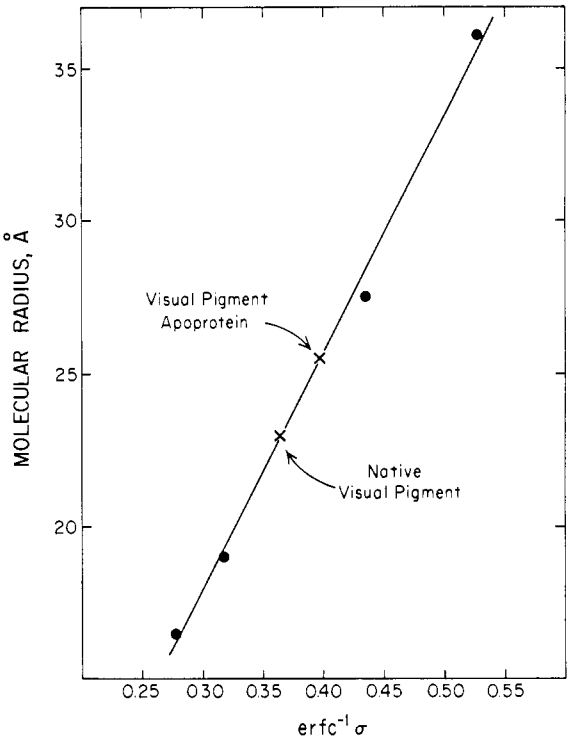

FIGURE 2: A plot of molecular radii as a function of the inverse error function complement of σ (Ackers, 1967). The value of σ was calculated from the elution volume of the test materials which were chromatographed on a column of agarose. For details, see Experimental Section. The experimental elution volumes were: V_0 (Dextran Blue 2000), 177 ml; V_t (tritiated water), 451 ml; V_e bovine serum albumin, 299 ml; V_e ovalbumin, 328 ml; V_e visual pigment apoprotein, 341 ml; V_e native visual pigment, 352 ml; V_e myoglobin, 370 ml; and V_e cytochrome *c*, 389 ml.

group per molecule. In the native pigment the covalently bound retinal *cannot* be reduced with sodium borohydride (Bownds and Wald, 1965; Akhtar *et al.*, 1965) and no free SH groups can be detected (Wald and Brown, 1952). On exposure to light (at 20°), two events take place simultaneously and almost instantaneously. The covalent bond between retinal and apoprotein reverts to the aldimine form (Schiff base) that can be reduced with sodium borohydride, and a single sulhydryl group becomes titrable. At the same time, through a major change in the protein conformation, the apoprotein and the borohydride-reduced forms show a considerably larger hydrodynamic volume than the native form shows.

These experiments can best be rationalized with the following proposal for the structure of the chromophore linkage region in bovine visual pigment.

In native visual pigment the 11-*cis* isomer of retinal is bound to a unique lysine residue through a substituted aldimine bond (Figure 3, I). The substituting group is the sulfur of a cysteine residue. In the dark native visual pigment is stable, it is not susceptible to borohydride reduction, and no free sulfhydryl group is detectable. The native form has a compact conformation. Although the lysine and cysteine residues are drawn close together (Figure 3, I) there is no reason to think they are actually neighbors in the polypeptide chain; on the contrary, several lines of evidence (see below) indicate that probably they are quite removed from each other in the primary structure and that a certain amount of folding and "strain" is involved in bringing the sulfhydryl to the proximity of the aldimine bond.

On absorption of light visual pigment is transformed into its excited-state counterpart. Two chemical reactions take place in the excited-state molecule. (1) The weakest bond in the molecule is broken. This is the bond between the terminal carbon of retinyl and the sulfur of cysteine. The exact mechanisim of this reaction is not known but it could very well be a free-radical mechanism. If this is so then the free-radical sulfur would next abstract a hydrogen from the nitrogen and return to its ground state. The free-radical nitrogen and free-radical carbon would then combine to form the aldimine bond. (2) The excited state (which can freely rotate around the carbon to carbon bond) isomerizes to the all *trans* form (Hubbard and Wald, 1952). These are then the two primary photochemical reactions, namely breaking of the C–S bond and isomerization. As most photochemical reactions these transformations are very fast. Concomitantly the substituted aldimine (I) reverts to an unsubstituted aldimine linkage (Schiff base) (II). The major consequence of these reactions is the transformation of the protein from the compact to the expanded

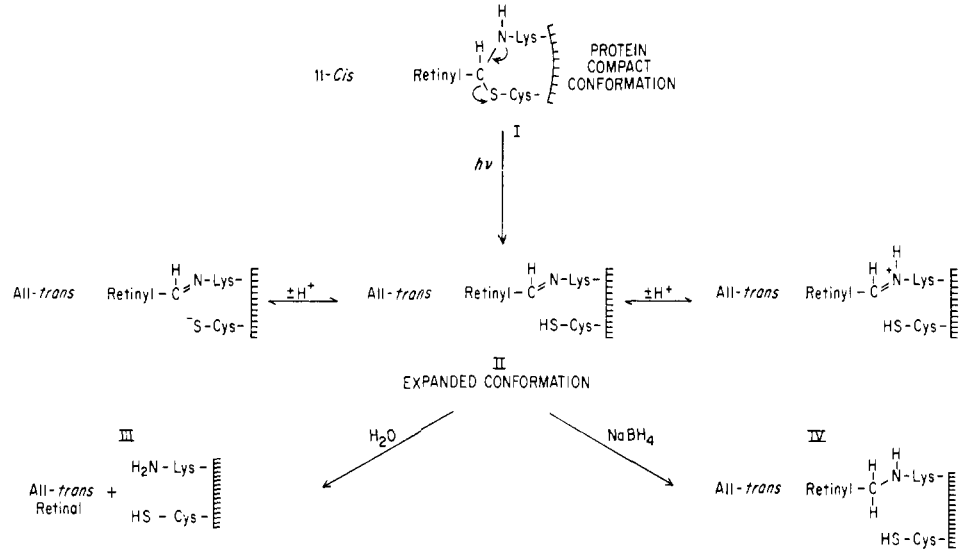

FIGURE 3: Schematic representation of the linkage between the retinal chromophore and protein in bovine visual pigment as discussed in the text.

conformation. The unsubstituted aldimine is rather unstable, due to the strong polarization of the bond, and rapdily hydrolyzes in neutral aqueous solutions to the free apoprotein (III), or it can be reduced with sodium borohydride to a stable derivative (IV).

Several other well-known properties of visual pigment bear additional evidence on the proposed mode of linkage between the chromophore and protein.

Whereas the visible spectrum of native visual pigment is not affected by pH changes between pH 5 and 10, the spectrum of light-exposed visual pigment (at 20°) shows a remarkable dependence upon pH, so much so that it was called indicator yellow (Lythgoe, 1937). This is in complete accord with the proposed structure, where the native form, I, a substituted aldimine, would not be expected to show any change in color with a change in pH, while the conjugated form, II, would be expected to show a strong dependence of color upon pH.

Wald and Brown (1952) showed not only that titrable sulfhydryl groups appear when native visual pigment is exposed to light, but that a free sulfhydryl(s) is necessary to the formation of pigment from the chromophore and the apoprotein. As reported by these investigators, PMB has no effect on native visual pigment and reacts only with the light-exposed form. The PMB-reacted apoprotein will not re-form the native pigment, but this inhibition is reversible, and removal of PMB with glutathione will enable the pigment to be re-formed. These experiments show that a free sulfhydryl group is not only made available when native visual pigment is exposed to light, but that, in some way, such a group is necessary for the actual formation of the pigment.

There is some evidence in the literature that at least one hydrogen ion is liberated from a functional group with a pK of 6.6 when native visual pigment is exposed to light (Radding and Wald, 1956). Again, this is compatible with the proposed structure where the change from I to II leads to formation of a new acid-dissociating group. At this point it is probably impossible to decide whether this apparent pK value represents the dissociation of the sulfhydryl or of the aldimine groups or both, as neither the true value of the sulfhydryl ionization constants in proteins (Cecil, 1963; Edsall, 1965) nor the value for the aldimine bond is known with any certainty. It is doubtful that model compounds can furnish these pK values; the environment of the linkage region is in all probability a highly specialized hydrophobic area where ionization constants could be expected to be quite different from those of model compounds.

Out of the several possible isomers of retinal only two (9-*cis* and 11-*cis*) bind to form native visual pigment (Wald and Hubbard, 1960). As shown in the present paper this binding of the prosthetic group to form native visual pigment results in a compact conformation of the protein, in contrast to the expanded conformation of the apoprotein. That this compact conformation is not due to the binding of the hydrophobic prosthetic group *per se* is shown by the fact that the borohydride reduced form, in which retinal is firmly bound to the protein, still has the expanded conformation. An attractive hypothesis is that the rather strict steric requirement for a particular isomer of retinal in forming native visual pigment is due to the necessity for approximating the aldimine bond to the sulfhydryl group so that a substituted aldimine can be formed. This "locks" the molecule in a strained, compact conformation.

Another aspect of the proposed structure of the substituted aldimine bond is that it offers an explanation for the bathochromic shift of the absorption spectrum from *ca.* 380 mμ of free retinal to 500 mμ of bovine visual pigment. The electronic configuration of the C–S–C bond is an sp^3-type hybridization with two lone-pair electrons in bonding positions (Cartmell and Fowles, 1961). These lone-pair electron oribitals overlap the π-orbital system of the polyene (retinal) and lead to further delocalization of the π system resulting in a shift of absorption to longer wavelength. An important contributing factor to the bathochromic shift is probably intra-

molecular charge transfer complex formation (Nagakura, 1955). The charge transfer complex is formed by interaction of the highest occupied orbital of the electron-donating center (sulfur) with the lowest vacant orbital of the electron-accepting center. The charges thus generated can again be delocalized over the whole polyene system.

A similar type of substituted aldimine linkage has been proposed for native muscle phosphorylase (Kent *et al.*, 1958; Fisher, 1964) and for some other pyridoxal enzymes (Fasella, 1967). It has been suggested that bound pyridoxal phosphate in muscle phosphorylase is functioning primarily as a structural element in maintaining the native conformation (Fischer *et al.*, 1958). It was recently shown by Hedrick *et al.* (1966) that apophosphorylase *b* has a less compact and less stable structure than the holoenzyme. The results of Hedrick *et al.* (1966) together with the experiments described in this paper suggest that substituted aldimines serve as cross-linking agents in proteins, very much like a disulfide bridge.

Considering alternative proposals for the linkage structure between retinal and native visual pigment, the experimental evidence seems to be quite conclusive that the aldimine bond is substituted. This is shown by the inability to reduce the native pigment with sodium borohydride, by the indefinite stability of the linkage (in the dark), and by the independence of the visible spectrum from pH effects. The problem is rather the identity of the substituting group. Assuming that it is not a sulfhydryl, there are three other possibilities. The aldimine bond is hydrated, the substituting function is an amino group of another lysine residue, or the substituting function is a hydroxyl group belonging to a serine or threonine residue. The hydrated form can be discounted on the grounds that it would be expected to be reduced by borohydride (as the hydration is probably reversible) and to show stability and color dependence upon pH; moreover, it is rather difficult to see how a hydration–dehydration sequence would have such a major influence on the protein conformation. The possibility of an amino or hydroxyl group being the substituting group is more difficult to disprove and it can only be said that there is no positive evidence of their involvement. In any case, if a substituting group other than SH is involved, it is still necessary to explain the appearance of a titrable sulfhydryl group on exposure to light and a requirement for a free SH for the formation of pigment from retinal and apoprotein. The assumption then would be that the conformational change resulting from light exposure, "unmasks" a "buried" sulfhydryl. A complimentary assumption will be that blocking the sulfhydryl with PMB prevents the proper refolding of the apoprotein necessary for the formation of native pigment. The simplest hypothesis and one which accounts for all the experimental facts with the minimal number of assumptions seems to be the one proposed initially, namely, that a sulfhydryl function substitutes the aldimine bond. This hypothesis has the added advantage of accounting in chemical terms for the variable reactivity of a sulfhydryl group in a protein, a well-known problem in enzymology and protein chemistry (Boyer, 1959; Cecil, 1963).

It is reasonable to assume that the light reaction, which affects only the relatively small chromophore, has to be transferred somehow to the protein in order to be propagated. The change from a substituted to an unsubstituted aldimine, with its concomitant effects on the protein shape might be such a mechanisim. A recent paper by Changeux *et al.* (1967) proposes a model for the amplifying cooperative effects seen in biological membranes in response to various agents. This model is based on the assumption that reversible conformational transitions are possible in the membrane subunits, the "protomers," and that the partition function of these conformational forms is influenced by binding with specific ligands. The response of the membrane as a whole to a conformational transition is one of the protomers depends upon the interaction between the subunits in the membrane. When certain assumptions are made on the interaction between neighboring subunits, the membrane is capable of an "all-or-none" response (phase transition) on binding of ligands. Substituting for ligand binding in the above model of Changeux *et al.*, the change in the substituted aldimine bond in response to light, and assuming that the interaction between visual pigment "protomers" is such that the conformational change will lead to an all-or-none response of the membrane, it is possible to formulate a working hypothesis, in biochemical terms, of the tremendous amplification taking place in the retina as a response to illumination.

Acknowledgments

It is a pleasure to thank Professor E. L. Smith and Dr. F. S. Markland for helpful discussions and critical reading of the manuscript. I gratefully acknowledge the expert technical assistance of Mrs. Biruta R. Vellutini.

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The Lipopolysaccharides of *Aerobacter aerogenes* Strains A3(S1) and NCTC 243*

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ABSTRACT: The lipopolysaccharides from *Aerobacter aerogenes* strains A3(S1) and NCTC 243 on mild hydrolysis each yield a polysaccharide fraction which is a complex mixture of fragments varying both in monosaccharide content and molecular weight but having a relatively constant ratio of glucose to heptose. This ratio is approximately 0.5 for the A3(S1) polysaccharide and 2 for that from NCTC 243. The polysaccharide fractions derived from both lipopolysaccharides contain a branched galactan which has 1→3-linked main chains and 1→4-linked branches; these polymers appear to differ structurally only in their degree of branching.

Isotopic methods are described (1) for quantitation of reducing saccharides present in hydrolysates at concentrations down to 0.1 μmole/ml and (2) for methylation analysis of milligram amounts of polysaccharides of unknown structure.

Cell wall lipopolysaccharides (LPS)[1] from *Enterobacteriaceae* are composed of a polysaccharide (PS) fraction and a lipid fraction (lipid A) which may be readily dissociated by mild acid hydrolysis. The polysaccharide portions consist of polymeric oligosaccharides (O-antigenic side chains) joined to a heptose phosphate backbone through a core oligosaccharide containing the sequence, GlcNAc→Glc→Gal→(Gal→)-Glc→. Gross structural features of these complex polysaccharides have been deduced from biosynthetic, immunochemical, and chemical analyses of *Salmonella* and *Escherichia coli* LPS's in a number of laboratories and are reviewed by Lüderitz *et al.* (1966b).

The LPS's from several strains of *Aerobacter* and *Klebsiella*, further members of the enteric group of organisms, have been analyzed by Sutherland and Wilkinson (1966), who found no uniform pattern of chemotypes similar to that in *Salmonella* species. In each of the organisms examined at least one of the core monosaccharides (glucose, glucosamine, or galactose) was present at a level much too low to account for a complete core structure. The analyses were, in fact, reminiscent of those found in the chemotypes, R_a, R_b, R_c, and R_d of the rough mutants of *Salmonella* (Lüderitz *et al.*, 1965, 1966a,b; Sutherland *et al.*, 1965; Osborn, 1966). In several of the strains, however, there was a fraction of the polysaccharide which might be considered analogous to the *Salmonella* O-antigenic side chain which is found covalently linked to the core and which is deleted from the LPS in strains not able to synthesize the complete core sequence. These authors suggested that the LPS's of *Klebsiella* and *Aerobacter* strains may contain polysaccharides directly linked to the heptose phosphate core.

This manuscript describes our initial examination of this suggestion. The data show that the polysaccharide fractions released from the lipopolysaccharides of *Aerobacter aerogenes* strains A3(S1) and NCTC 243 are mixtures of fragments of varying molecular size and monosaccharide content. The LPS's of both strains are shown to contain galactans having basically

* From the Division of Biochemistry, University of Illinois, Urbana, Illinois 61801. *Received March 11, 1968.* Supported by a grant from the U. S. Public Health Service, National Institute of Allergy and Infectious Diseases (AI 05696).

† Holder of a National Institutes of Health predoctoral traineeship on U. S. Public Health Service Training Grant GM-321.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: LPS, lipopolysaccharide; PS, polysaccharide; KDO, 2-keto-3-deoxyoctonate; TBA, thiobarbituric acid.