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Comparative Study of a Membrane Protein. Characterization of Bovine, Rat, and Frog Visual Pigments$_{500}$*

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ABSTRACT: Rat and frog visual pigments$_{500}$, which are membrane proteins found in the retinal rod outer segments, were purified and their properties compared with the previously isolated bovine visual pigment$_{500}$ (Heller, J. (1968), *Biochemistry 7*, 2906, 2914). The rat and frog visual pigments$_{500}$ are closely similar to the bovine pigment in all their physicochemical properties. These pigments have practically identical molecular weights and molar absorptivities at 280 and 500 mμ. They contain only one retinal prosthetic group per molecule, as determined by the loss of lysine residues after reduction with sodium borohydride, have two disulfide bridges, and are all glycoproteins. In the native pigments retinal is bound to the protein through a bond which is not susceptible to reduction with sodium borohydride, and is thus most probably a substituted aldimine. On exposure to light the substituted aldimine is converted into a simple aldimine which can be reduced with sodium borohydride and concomitantly one sulfhydryl group per molecule becomes titrable. Similarly to the bovine pigment, rat and frog visual pigments undergo a conformational change on light exposure such that the light-exposed form is more expanded than the native molecule. The magnitude of this conformational change is identical in all these pigments. The only differences that were found among these pigments were small variations in their amino acid composition. On the basis of these observations it is suggested that visual pigment$_{500}$, a membrane protein, from bovine, rat, and frog eyes forms a series of homologous proteins.

Native bovine visual pigment$_{500}$ was recently purified and has been shown to be a conjugated glycoprotein with a molecular weight of 27,700 (Heller, 1968a). Since native visual pigment$_{500}$ is one of the few noncatalytic membrane proteins that have been purified to a state of homogeneity and characterized as to several of its physicochemical properties it was interesting to compare the properties of this membrane protein in several other species. The present paper reports the purification of rat and frog visual pigments$_{500}$ and compares their properties with that of bovine visual pigment$_{500}$.

Experimental Section

Rat eyes were obtained from the Long Evans strain of *Rattus norwegicus*. Frog eyes were obtained from *Rana pipiens*. The retinas were dissected under dim red light. The isolation and purification of visual pigment and other experimental techniques have been previously reported (Heller, 1968a,b). Several hundred retinas were used for each preparation and the reported results thus represent a pooled sample.

Results

Isolation and Purification. Rat and frog visual pigment$_{500}$ could be purified by essentially the same procedure used to purify bovine visual pigment$_{500}$. All the pigments were stable in the presence of the dissociating agent cetyltrimethylammonium bromide and showed identical elution patterns after gel filtration. The visual pigments emerged as a single, symmetrical peak with a constant A_{280}/A_{500} ratio across the peak and were completely separated from a high molecular weight, colorless, protein contaminant. In the case of the frog visual pigment, an additional peak was observed in the "small molecules" fraction. This chromatographic peak was yellow and had absorption maxima at 328, 450, and 480 mμ. The material was thus probably a mixture of carotenoids and is derived from the yellow oil globules present in the amphibian retina (Wolken, 1966). When the visual pigments were concentrated in the dark by pressure dialysis and rechromatographed, a single peak with the same elution volume was obtained.

Some of the properties of the purified visual pigments are summarized in Table I.

* From the Jules Stein Eye Institute, University of California School of Medicine, Los Angeles, California, 90024. *Received August 20, 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.

TABLE I: Some Physicochemical Properties of Purified Visual Pigment.

	Bovine	Rat	Frog
Molecular weight			
Estimated by gel filtration	27,000–28,000	27,000–28,000	27,000–28,000
Calculated[a]	27,707	27,769	28,023
Stokes molecular radius (Å[b])			
Native	23	23	23
Light exposed	25.5	25.5	25.5
ϵ_{500}	23.100 ± 800	22,300 ± 1,000	23,100 ± 1,000
ϵ_{280}	37,000 ± 1,000	36,000 ± 1,000	39,600 ± 1,000
Ratio A_{280}/A_{500}	1.6 ± 0.08	1.6 ± 0.1	1.7 ± 0.08
Number of retinal prosthetic groups per molecule[c]	1	1	1
Number of titrable sulfhydryl groups per molecule in native pigment[d]	None	1	1
Number of additional sulfhydryl groups per molecule after light exposure[d]	1	1	1

[a] Calculated using amino acid and carbohydrate composition data (Table II), including retinal. [b] Calculated from gel filtration data according to Ackers (1967). [c] Calculated as loss of lysine residues after reduction with $NaBH_4$ of light-exposed visual pigment. [d] Determined by titration with 5,5-dithiobis(2-nitrobenzoic acid) at pH 8.0 according to Ellman (1959).

Molecular Weight. The rat and frog visual pigments were eluted from a calibrated column of agarose at exactly the same volume as bovine visual pigment. Since the molecular weight of bovine visual pigment is 27,700 (Heller, 1968a) the molecular weight of the other pigments, as judged by gel filtration, was in the range of 27,000–28,000.

Spectra. The purified pigments had closely similar spectra. The ϵ_{500} of all the pigments was around 23,000, and the ϵ_{280} was in the range of 36,000–40,000 (Table I).

The Number of Chromophores per Molecule. While the native visual pigments had 10 lysine residues/molecule, there were only 8.9–9.1 lysine residues/molecule after reduction with sodium borohydride of the light-exposed visual pigments. No other amino acids were affected by this treatment. Since reduction with sodium borohydride binds the retinyl group to an ϵ-amino group of lysine through secondary amine linkage that is stable to acid hydrolysis, these experiments showed that the visual pigments had only one retinal prosthetic group per molecule.

Nature of Linkage between Retinal and Apoprotein-Conformational Changes. Similarly to the bovine pigment, the rat and frog visual pigments were not susceptible to reduction with sodium borohydride in their native form, thus indicating that no aldimine bond (Schiff base) is present in the molecule. The visual pigments could be reduced with borohydride only after first being exposed to light. The light-exposed, borohydride-reduced pigments displayed a new spectral peak absorbing maximally at 333 mμ. Again, similarly to the bovine pigment, in the rat and frog visual pigments one previously masked sulfhydryl group per molecule became titrable with 5,5-dithiobis(2-nitrobenzoic acid) after light exposure. It has been previously shown (Heller, 1968b) that accompanying the reversion of the substituted aldimine bond in native bovine pigment to a simple aldimine bond, there was a major change in the conformation of the proteins. The change was such that the light-exposed pigment was more expanded than the native form as judged by gel filtration chromatography. The change in Stokes molecular radii in going from the native to light-exposed pigment was from 23 to 25.5 Å. Assuming a spherical shape for both molecules this represents a 36% increase in volume. The rat and frog visual pigments showed exactly the same conformational change in being transformed from the native to the light-exposed pigment. The calculated Stokes molecular radii for the compact and expanded forms of bovine, rat, and frog visual pigment were identical (Table I).

Composition. As can be seen from Table II, despite the great similarity in amino acid composition between the three pigments, there seem to be present some significant variations. Except for serine, these variations were small and represent one or two residues out of 10–20 present. Only three amino acid residues (Lys, His, and Arg) had the same frequency in all three pigments. Both native rat and frog visual pigment had one titrable sulfhydryl group and were thus different from the native bovine pigment which had none. Titration of native rat and frog pigment with 5,5-dithiobis(2-nitrobenzoic acid) in the dark did not alter their visible spectrum. On the other hand, all three visual pigments showed one additional titrable sulfhydryl group per molecule after light exposure.

Since the bovine pigment had five half-cystine residues per molecule, four of which were present in two disulfide bridges and one that was possibly the substituting group of the aldimine bond (Heller, 1968b), and since both rat and frog pigments had six half-cystine residues per molecule, one of which could be titrated in the dark and one which became titrable after light exposure, it was concluded that rat and frog visual pigments have one free sulfhydryl group (titrable in the native molecule), one sulfhydryl group presumably substituting the aldimine bond, and four half-cystine residues present in two disulfide bridges, similar to the bovine pigment.

Interestingly, the very high mole per cent content of nonpolar residues (Pro, Ala, Cys, Val, Met, Ile, Leu, Phe) was practically identical for the three pigments (50.2, 49.6, and 49.4).

TABLE II: Composition of Visual Pigments.[a]

	Bovine	Rat	Frog
Lys	10	10	10
His	4	4	4
Arg	6	6	6
$CySO_3H$[b]	5	6	6
Asp	15	16	15
$MetSO_2$[b]	8	9	9
Thr[c]	17	18	16
Ser[c]	12	13	17
Glu	21	21	20
Pro	13	14	14
Gly	16	16	15
Ala	20	19	18
Val[d]	20	18	18
Ile[d]	13	13	15
Leu[d]	20	20	19
Tyr	11	11	13
Phe	19	18	18
Trp[e]	5	4	4
Glucosamine[f]	3	3	3
Neutral sugar[g]	3	3	3
Total number of amino acid residues	235	236	237
Number of nonpolar residues	118	117	117

[a] Values are reported as residues per molecule. Duplicate samples were hydrolyzed in 6 N HCl at 110° for 24, 48, and 72 hr. [b] Determined on separate samples after performic acid oxidation (Moore, 1963). [c] Values extrapolated to zero time. [d] Values extrapolated to infinite time. [e] Determined on separate samples by titration with *N*-bromosuccinimide at pH 4.0 (Patchornik *et al.*, 1958). [f] Determined on the long column of the analyzer after hydrolysis in 4 N HCl, at 100° for 6 and 10 hr. [g] Determined by the phenol–sulfuric acid method (Dubois *et al.*, 1956) with a mannose–galactose (2:1) mixture as standard.

Discussion

The visual pigments examined in the present investigation are of vertebrate origin (two mammalian and one amphibian) and have their visible absorption maximum at 500 mμ. That visual pigments are membrane proteins is shown by several lines of evidence. Studies with polarized light show that visual pigment is highly oriented (Schmidt, 1938) and the orientation is such that the visual pigment chromophore is perpendicular to the long axis of the rod. Electron microscopy and low-angle X-ray diffraction studies reveal an ordered membrane structure of stacked disks in the rod outer segments (Sjøstrand, 1953), and this membrane is apparently composed of an ordered array of particles about 40 Å in diameter (Blasie *et al.*, 1965). Visual pigments are completely insoluble in ordinary aqueous media and can be brought into solution only with the aid of strong dissociating agents. Finally, the properties of purified visual pigments bear some striking resemblances to other membrane proteins from erythrocyte and mitochondria, as will be more fully discussed below.

For this particular group of visual pigments the experiments reported in this paper lead to some general conclusions. *The over-all physicochemical structure of these pigments was identical.* They all had practically the same molecular weight (Table I), possessed only one retinal prosthetic group per molecule, and had closely similar spectra and molar absorptivities at 500 mμ (Table I). They were all glycoproteins and had two disulfide bridges per molecule (Table II). The visual pigments that were examined were all stable in the presence of cetyltrimethylammonium bromide.

Moreover, this structural identity goes even further and was expressed as a functional similarity. All the visual pigments examined probably had a substituted aldimine linkage between retinal and a lysine residue in the native pigment and consequently were not susceptible to reduction with sodium borohydride. The pigments underwent the same compact to expanded conformational change on exposure to light with concomitant reversion of the substituted aldimine bond to a simple aldimine bond (Schiff base) which could be reduced with sodium borohydride. The calculated Stokes molecular radii of the native (compact) and light exposed (expanded) conformers of these visual pigments were identical. This reversion of the aldimine bond was accompanied by the appearance of a single titrable sulfhydryl group per molecule.

Erhardt *et al.* (1966) and Ostroy *et al.* (1966), using the amperometric silver titration method, found that one to four sulfhydryl groups are titrable in the native pigment and that three to four more sulfhydryl groups become titrable after illumination. The reason for detection of variable amounts of sulfhydryl groups in the native bovine pigment as determined by these investigators is not clear. It might be due to the existence of some of the pigment as the light-exposed form, the presence of a sulfhydryl-containing contamination, or both. The higher values of sulfhydryl groups found by these investigators in the illuminated bovine pigment

are probably due to binding of an additional silver ion to the initial silver mercaptide (Benesch and Benesch, 1962).

In other words, using the above criteria, namely, molecular weight, number of retinal prosthetic groups per molecule, nature of the linkage between retinal and the apoprotein, conformational changes on exposure to light, molar absorptivity at 500 mμ, and glycoprotein nature of molecule, it was impossible to distinguish any one of these visual pigments from any other. The only differences found between these visual pigments were in their amino acid composition (Table II). The number of differences in the amino acid composition between any particular pair of pigments indicates the *minimal* number of amino acid substitutions possible in comparing the sequences. Over and above these differences there was a fundamental similarity in the amino acid composition. They all had a high content of nonpolar residues, lysine, histidine, and arginine had the same frequency, while aspartic and glutamic acids, methionine, half-cystine, proline, glycine, leucine, phenylalanine, and tryptophan showed only a variation of one residue. Unfortunately it was impossible to obtain comparative peptide maps from these visual pigments due to their extreme resistance to proteolytic digestion (J. Heller, unpublished results).

Shields *et al.* (1967) have recently reported the isolation and purification of bovine visual pigment (sodium borohydride reduced derivative) as the digitonin complex. The amino acid composition of visual pigment reported by these investigators is significantly different from the one reported here, and this variation is probably due to the use of a different dissociating agent in solubilizing and purifying the pigment and by a different method of estimating the molecular weight of the protein.

Although the designation of a series of proteins as homologous can only be made definitvely on the basis of comparing their complete amino acid sequences, it is felt that the cumulative evidence of the results presented in this paper strongly suggest that visual pigments$_{500}$ from bovine, rat, and frog retinas form a series of homologous proteins (Neurath *et al.*, 1967). This is defined as a series of proteins closely similar in their function and over-all structure yet varying in details of their amino acid sequence. Such a series are, for example, the cytochromes *c* (Smith and Margoliash, 1964), hemoglobins (Hill and Buettner-Janusch, 1964), and lactic dehydrogenases (Wilson *et al.*, 1964). The homology in all these cases is between proteins derived from different species. It should not be assumed that *all* visual pigments are homologous. There is evidence that some visual pigments, *e.g.*, squid visual pigment$_{480}$, that are superficially similar to vertebrate visual pigments, have quite a different structure (J. Heller, unpublished results). This is probably a reflection of the very diverse phylogenetic origin of vertebrate and squid visual pigments.

Similarities between Visual Pigments and Other Membrane Proteins. Many of the properties of visual pigments$_{500}$ described in this paper bear a striking resemblance to the so-called "structural proteins" isolated from various membrane systems (Green *et al.*, 1968; Rosenberg and Guidotti, 1968; Schneiderman and Junga, 1968). Similarly to mitochondrial "structural proteins" and the proteins of erythrocyte membrane, visual pigments$_{500}$ are insoluble in aqueous buffers, and as we have observed are insoluble in 8 M urea or 6 M guanidine hydrochloride, but are soluble in 70% formic acid. Further, we have found that denatured visual pigments are highly resistant to proteolysis by tryspin, chymotrypsin, subtilisin, pepsin, and Pronase. Similar to "structural proteins" and the proteins of erythrocyte membrane, visual pigments$_{500}$ have a high content of nonpolar amino acid residues and they are glycoproteins.

Whether these similar properties of mitochondrial "structural proteins," the erythrocyte membrane proteins, and visual pigments$_{500}$ are an expression of a common origin, or a necessary attribute of a membrane-forming protein *per se*, or both, cannot be answered at this time.

Acknowledgment

I am deeply indebted to Dr. M. O. Hall and D. Bok for their expert help in obtaining the retinal material, to Mr. A. Bacharach for his superb preparation of the visual pigments, and to Mrs. Lawrence for her help in some of the experiments. It is a pleasure to thank Professor E. L. Smith and Dr. F. S. Markland for helpful discussions and critical reading of the manuscript.

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The Composition of the Glycolipids in Dog Intestine*

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ABSTRACT: Whole lipid extracts of dog small intestine were chromatographed on silicic acid, yielding all the glycolipids in three major fractions. Five classes of glycolipid were isolated in high yield from the fractions by column chromatography with Florisil, DEAE-cellulose, and silicic acid and by thin-layer chromatography. Cerebrosides, ceramide di-, tri-, and pentahexosides, and gangliosides were isolated in amounts of 0.162, 0.098, 0.041, 0.070, and 0.171 μmoles per g of fresh tissue, respectively, and 0.026 μmole-/g of sulfatide was found by direct analysis. A minor glycolipid fraction, estimated at 0.014 μmole/g, contained longer oligosaccharide chains. This fraction was separated from all other lipids and appeared to be a mixture of ceramide hexahexosides containing glucose, galactose, hexosamine, and fucose. These seven classes of lipids accounted for 82% of the original whole lipid hexose, and the remaining 18% was distributed among unresolved side fractions. All the isolated lipids were glycosides of glucosylceramide with long-chain fatty acids. The intestine glycolipids are distinguished from those of several other tissues by the absence of ceramide tetrahexosides and presence of penta- and hexahexosides.

The resolution of the glycolipids of dog small intestine into three silicic acid fractions was described in a previous publication (Vance *et al.*, 1966). The glycolipid mixture from one of these was isolated from all other lipids and partially resolved into at least four classes of glycolipid. Two of these were characterized as a novel ceramide pentaglycoside and a monosialoganglioside[1] This paper describes the other glycolipids of this fraction together with those of the other two silicic acid fractions. An estimate is included of the distribution of the types of glycolipid in intestine based on these isolations.

Experimental Section

Analytical Methods. Methods for the determination of phosphorus, primary amine, long-chain nitrogen, chromic acid uptake, ester groups, sialic acid, hexosamine, glucose, total hexose, and the distribution of fatty acids were described previously (Vance *et al.*, 1966). The fatty acid methyl esters were further purified from sphingosine contaminant by silicic acid chromatography modified from the method of Hirsch and Ahrens (1958). Hydroxy fatty acids were then determined by acetylation of the free acids and hydroxamate formation using a modification of the method of Gutnikov and Schenk (1962). Fucose was determined on glycolipid fractions low in phospholipid by the method of Dische and Shettles (1948) applied to a water suspension of the lipid. Sulfatides were determined by the method of Kean (1968) and reducing sugar by the method of Somogyi (1945).

Preparation and Composition of the Glycolipids. The extraction, purification, and initial resolution of the lipids by preparative silicic acid chromatography were all carried out as described previously. The fractions containing glycolipid were eluted from the column by mixtures of 10, 17, and 50% methanol in chloroform, respectively, and were designated S-10, S-17, and S-50.

Preparation and Resolution of the S-10 Glycolipids This fraction contains two glycolipid classes, ceramide mono- and dihexosides. These were isolated by two-stage column chromatography using Florisil.

Florisil Chromatography. Florisil (450 g; Floridin Co., 60–100 mesh) were placed in a 41-mm i.d. column and washed with 2 l. of chloroform. Fraction S-10 lipids containing 9.8 matoms of lipid K and 0.61 mmole of lipid galactose were placed on the column in 500 ml of chloroform solution. The column was then eluted with 1750 ml of 5%, 3150 ml of 38%, and finally 2100 ml of 60% methanol in chloroform, respectively. The

* From the Department of Biochemistry, University of Alabama Medical Center, Birmingham, Alabama. *Received August 22, 1968.* This work was supported by a grant from the Life Insurance Medical Research Fund and by Public Health Service Grant HD-02184 from the National Institute of Child Health and Human Development, National Institutes of Health. A preliminary report of this work has been presented (McKibbin, 1967).

[1] In this paper the following terminology is used: "ganglioside" is a glycolipid which contains sialic acid, "ceramide oligohexoside" is a ceramide oligoglycoside which does not contain sialic acid. The composition of all solvent mixtures is expressed on a volume per volume basis. The author is indebted to Miss Martha D. Fendley for valuable technical assistance.