11-cis-Retinaldehyde bound to cellular retinaldehyde-binding protein (CRALBP) is unaffected in bovine eyecup preparations by illumination that bleaches 70% of the rhodopsin. Illumination of retinal homogenates to which CRALBP-[3H]11-cis-retinaldehyde had been added did not result in a reduction of the specific activity of recovered 11-cis-retinaldehyde, ruling out a bleaching regeneration cycle. The quantum efficiency of photoisomerization for CRALBP-11-cis-retinaldehyde was determined by comparing the rate of photoisomerization of 11-cis-retinaldehyde bound to purified CRALBP and opsins. The low value obtained (0.07), coupled with a low molar extinction coefficient (15,400 M⁻¹ cm⁻¹), results in a photosensitivity only about 4% that of rhodopsin.

CRALBP binds 9-cis- and 11-cis-retinaldehyde, producing complexes with absorption maxima at 405 and 425 nm, respectively. No complexes were detected with 13-cis- and all-trans-retinaldehyde. Following incubation of CRALBP-11-cis-retinol with an equimolar mixture of 9-, 11-, 13-cis-, and all-trans-retinaldehydes, only 11-cis-retinaldehyde and residual 11-cis-retinol are present on the protein following separation from excess retinoids. A similar result is obtained following incubation of CRALBP-11-cis-retinol with mixtures of 9- and 11-cis-retinaldehyde ranging in composition from 9:1 to 1:9 (9-cis:11-cis, mol/mol). The results indicate that CRALBP-11-cis-retinol is sufficiently stereoselective in its binding properties to warrant consideration as a component of the mechanism for the generation of 11-cis-retinaldehyde in the dark.

Regeneration of bleached visual pigment in the dark depends on the production of 11-cis-retinaldehyde from an all-trans-precursor. The overall process must be stereospecific since only 11-cis-retinaldehyde is found in visual pigments even though opsin is able to bind other geometrical isomers of retinaldehyde (Derguini et al., 1984; Hubbard and Wald, 1952). This strict stereospecificity led most investigators to assume that isomerization must be catalyzed by a stereospecific enzyme. However, the failure of several laboratories to produce this isomerization in vitro at reasonable rates leaves open the question of the existence of this isomerase. (Akhtar et al., 1973; Bernstein et al., 1986; Futterman 1974; Ostapenko and Furayev 1973; Rotmans et al., 1972). In principle, stereospecificity could be introduced after isomerization takes place. Since an equilibrium mixture of geometrical isomers of retinaldehyde contains only 0.1% 11-cis-retinaldehyde (Rando and Chang, 1983), a mechanism would be needed for the selection of this isomer. Hubbard, one of the early investigators in this field, recognized that stereospecificity could be introduced by a protein that bound 11-cis-retinaldehyde specifically (Hubbard and Colman, 1959). Several years ago a protein that carries endogenous 11-cis-retinaldehyde or 11-cis-retinol was discovered in extracts of retina cellular retinaldehyde-binding protein, CRALBP) (Futterman et al., 1977; Stubbs et al., 1979). This paper describes studies that examine the photochemical properties of 11-cis-retinaldehyde bound to CRALBP and the stereoselectivity of the binding site. The results demonstrate that bound 11-cis-retinaldehyde is protected from photoisomerization and indicate that the binding site is sufficiently discriminating to select 11-cis-retinaldehyde from a mixture of geometrical isomers of retinaldehyde. An abbreviated account of this work has been published previously (Saari and Bredberg, 1986).

EXPERIMENTAL PROCEDURES

Materials—The sources and purification of retinoids and CRALBP have been given in previous publications (Saari, 1982; Saari et al., 1982; Hubbard et al., 1972). Bovine eyes were obtained from PD & J Meats (Kent, WA) and HPLC solvents from Burdick and Jackson. The HPLC column used for retinoid separations was purchased from The Separations Group (C-18, 5-μm, Vydac 201TP 54). Phamacia was the source of the Mono Q column. Emulphogene BC-70 (polyoxyethylene-10-tridecyl ether) was purchased from Sigma.

Methods—All procedures involving light-sensitive materials were performed under red illumination (40 watt ruby red bulbs, Westinghouse) at 5°C unless specified. Illumination of Intact Bovine Retina (RPE)—Bovine eyes were placed in a light-tight, chilled container within 15 min of the death of the animal and used for experimentation within 2 h. The eyes were hemisedected and the posterior half including the vitreous humor exposed to white light from a slide projector at room temperature (ca. 21°C). Eyecups that received no illumination served as controls. Separate homogenates were prepared from retina and RPE and centrifuged (Saari, 1982). CRALBP was partially purified from these supernatants by gel filtration with Sephadex G-100 (Saari, 1982), and bound retinoids were extracted, identified, and quantified by reversed-phase HPLC as previously described (Saari et al., 1984). A portion of the retinal homogenate was solubilized in 2% Emulphogene BC-70, and rhodopsin concentration was determined from the decrease in absorption at 500 nm in the presence of 0.1 M NH₄OH following exposure of the detergent solution to illumination. The amount of bleached rhodopsin in control retinas was 10–15%. Illumination
increased this amount to 50-73% (see Table 1).

Illumination of Bovine Retinal Homogenates—[\(^{3}H\)]11-cis-Retinaldehyde complexed with CRALBP was added to a homogenate of bovine retina to give a final concentration of 3 \(\mu\)M. A portion of the homogenate was exposed to illumination 60 cm from a slide projector (Sylvania CLX 300 watt bulb, 3150K) directly or after passage through a combination of a Schott GG-4 and a Corning CS 5-57 filter (maximum transmittance at 465 nm). Illuminated and unilluminated portions of the homogenate were centrifuged and CRALBP partially purified as described (Saari and Bredberg, 1982). Solutions of CRALBP and rhodopsin were prepared in 20 mM Tris, pH 7.5, 150 mM NaCl. CRALBP solutions contained an equimolar concentration of bovine serum albumin to trap liberated all-trans-retinaldehyde, whereas rhodopsin solutions contained 2% Enulophogene BC-70. CRALBP was purified and charged with 11-cis-retinaldehyde as previously described (Saari and Bredberg, 1982). Solutions of CRALBP and rhodopsin were illuminated with 2.5 or 10 \(\mu\)mol of light filtered through a combination of filters with a transmittance that was maximal at the wavelength (Schott GG-4 plus Corning CS 6-57). The intensity of irradiance on the sample as measured with a digital radiometer was 640 microwatts/cm\(^2\) with this filter combination. The concentration of CRALBP and rhodopsin ranged from 2–8 \(\mu\)M. In other experiments, solutions of CRALBP and rhodopsin were illuminated with 425 and 500 nm light, respectively, at intervals of 1 min. At intervals of 1 min, samples were removed for analysis. The fraction of bleached rhodopsin was determined from the decrease in absorption at 500 nm. Photoisomerization of 11-cis-retinaldehyde bound to CRALBP was determined by HPLC after extraction of retinoids.

First order rate constants (\(k\)) were calculated from the slopes of plots of \(\log a_{t} / a_{0}\) versus time (see Fig. 1). Data were corrected for the overlap of the filter transmittance, complex absorption, and spectral radiance emittance of a black body radiator at 3150°K (Wyszecki and Stiles, 1967).

\(\phi_{\text{CRALBP}}\) was calculated assuming that the ratio of the first order rate constants for photoisomerization of the two complexes is proportional to the ratio of their photosensitivities. Since photosensitivity = \(\psi\) (Dartnall, 1972)

\[
\phi_{\text{CRALBP}} = \frac{k_{\text{CRALBP}}}{k_{\text{rhodopsin}}} = \frac{\psi_{\text{CRALBP}}}{\psi_{\text{rhodopsin}}}
\]

In addition to the determined rate constants, the following values were used for \(\phi_{\text{CRALBP}}\) (Hubbard et al., 1971; \(\phi_{\text{CRALBP}} = 15,400\) (Stubbels et al., 1979); \(\phi_{\text{rhodopsin}} = 0.67\) (Dartnall, 1972; Waddell et al., 1976).

Preparation of CRALBP—11-cis-Retinol or CRALBP-9-cis-Retinaldehyde—The published purification procedure (Saari, 1982) was followed except that the supernatant obtained after homogenization of the retinas was illuminated with 465 nm light for 20 min, followed by incubation of either 2 \(\mu\)mol of 11-cis-retinol or 5 \(\mu\)mol of 9-cis-retinaldehyde per retina. No complex could be isolated after incubation with 13-cis- or all-trans-retinaldehyde.

Stereo selectivity Experiments—CRALBP-11-cis-retinol (10 nmol, 8 \(\mu\)M) in 20 mM Tris, pH 7.5, was incubated with either 2.5 or 10 \(\mu\)mol each of 9-, 11-, 13-cis, and all-trans-retinaldehyde for 1 h at room temperature. CRALBP was then separated from excess retinoids by chromatography on a Pharmacia Mono Q Sephadex column (see below). Retinoids were extracted and analyzed by reversed-phase HPLC as previously described (Saari et al., 1984). In other experiments 10 nmol of CRALBP-11-cis-retinol in the same buffer were illuminated with an intensity of either 2 \(\mu\)mol of 11-cis-retinol or 5 \(\mu\)mol of 9-cis-retinaldehyde per retina. No complex could be isolated after incubation with 13-cis- or all-trans-retinaldehyde.

### RESULTS

Sensitivity of CRALBP to Bleaching Illumination—Bovine eyecups were exposed to white illumination (see "Experimental Procedures"), and the extent of photoisomerization of 11-cis-retinaldehyde bound to opsin and to CRALBP was determined and expressed as the ratio of 11-cis-retinaldehyde recovered from illuminated and unilluminated eyecups. The results, presented in Table 1, experiments 1 and 2, demonstrate that 11-cis-retinaldehyde bound to CRALBP is not photoisomerized by illumination that bleaches ~70% of the rhodopsin since the ratio of 11-cis-retinaldehyde recovered from the unbleached control eyes was close to unity for CRALBP from both sensory retina and RPE.

The results of experiment 1 did not rule out the possibility that 11-cis-retinaldehyde had been photoisomerized to all-trans-retinaldehyde and then converted back to 11-cis-retinaldehyde in either a light or dark process. To test for this possibility, a retinal homogenate to which [\(^{3}H\)]11-cis-retinaldehyde complexed with CRALBP had been added was illuminated. Light with maximal absorbance at 465 nm was used to minimize photoisomerization of liberated all-trans-retinaldehyde (maximum absorbance at 381 nm). A bleaching-regeneration cycle would be reflected in decreased specific radioactivity of 11-cis-retinaldehyde even though the amount of the retinoid bound to the protein might not change. The results of this experiment (Table 1, experiment 2) demonstrate that there is a decrease in the specific activity after bleaching. However, the error associated with these experiments is appreciable (10–15%), and the decrease in specific activity (17%) is not comparable to the amount of rhodopsin bleached during the experiment (73%). Thus, there is little or no photoisomerization of 11-cis-retinaldehyde bound to CRALBP.

Quantum Efficiency of Photoisomerization of 11-cis-Retinaldehyde Bound to CRALBP—The first order rate constants (\(k\)) for the photoisomerization of 11-cis-retinaldehyde bound to CRALBP and opsin, and the known values of \(\phi_{\text{rhodopsin}}, \phi_{\text{CRALBP}}\) (Saari et al., 1984) were used to evaluate \(\phi_{\text{CRALBP}}\) (see "Experimental Procedures" for details). The time course of photoisomerization by 465 nm of light of 11-cis-retinaldehyde bound to purified CRALBP or opsin is illustrated in Fig. 1.

The results from four experiments employing three experi-

### TABLE 1

| Experiment | Tissue | 11-cis-Retinaldehyde illuminated/unilluminated | Analytical method used* |
|------------|--------|-----------------------------------------------|-------------------------|
| 1          | White retina | 1.10 | 0.50 | Absorbance | Absorbance |
| 2          | 465 nm retina | 0.92 | 0.27 | Absorbance | Specific radioactivity |

*The ratio of 11-cis-retinaldehyde in illuminated and unilluminated samples was obtained by determining its absorbance or specific radioactivity after extraction and isolation by HPLC.
Binding Specificity of CRALBP—In order to ascertain which of the isomers of retinaldehyde were able to complex with CRALBP, the complex was photoisomerized at an early stage in its purification and 9- 11-cis-, and all-cis-retinaldehyde were added, each to separate portions. Subsequent purification, spectral analysis, and HPLC demonstrated complex formation with 9- and 11-cis-retinaldehyde. No complexes were detected with all-cis-retinaldehyde.

The results indicate that CRALBP is not totally specific for 11-cis-retinaldehyde but gives no indication of the relative affinities of the two retinaldehydes, termed here the stereoselectivity of the binding site of CRALBP for retinaldehydes, were investigated by incubating mixtures of retinaldehyde with the CRALBP.

Table II
Stereoselectivity of CRALBP-11-cis-retinol

| Retinaldehydes | 9-cis-retinol | 11-cis-retinol | 13-cis-retinol | All-trans-retinaldehyde |
|----------------|---------------|----------------|----------------|-------------------------|
| 11-cis-retinol | 100           | 95             | 90             | 100                     |
| 9-cis-retinol  | 95            | 90             | 80             | 95                      |
| 13-cis-retinol | 90            | 80             | 7              | 90                      |
| All-trans-retinaldehyde | 100 | 95 | 90 | 95 |

*9-, 11-, 13-cis-, and trans-retinaldehyde.
TABLE III  
Stereoactivity of CRALBP-11-cis-retinol  
Mixtures of 9- and 11-cis-retinaldehyde. CRALBP-11-cis-retinol (10 nmol) was incubated with the retinoids shown for 2 h at 21°C. After removal of excess retinoids, those associated with CRALBP were extracted and analyzed by HPLC. A dash (—) indicates that the retinoid could not be detected.

| Retinoids associated with CRALBP (%) | Nmol of each retinaldehyde |  |
|-------------------------------------|---------------------------|---|
|                                     | 9-cis | 11-cis | 9-cis | 11-cis |
| 0                                   | 0    |       | 95    |       |
| 1                                   | 9    |       | 20    |       |
| 5                                   | 5    |       | 59    |       |
| 9                                   | 1    |       | 88    |       |

DISCUSSION

CRALBP-11-cis-retinaldehyde displays many properties usually associated with visual pigments including a red-shifted chromophoric maximum and an occurrence restricted to light-sensitive tissues (Futterman and Saari, 1977). In addition, illumination of the complex produces a relatively stereospecific isomerization of 11-cis-retinaldehyde to all-trans-retinaldehyde (Saari et al., 1984). These observations led us to question whether 11-cis-retinaldehyde bound to CRALBP is photoisomerized by light that bleaches rhodopsin. The results presented in Table I demonstrate that in bovine eyecups and retinal homogenates, up to 70% of the rhodopsin could be bleached without significant photosomization of CRALBP-11-cis-retinaldehyde. The ratio of 11-cis-retinaldehyde recovered in the bleached eyes relative to that recovered from the unbleached control eyes was close to unity for CRALBP from both sensory retina and RPE. Previous studies had shown the binding protein to be present in extracts of both tissues (Saari et al., 1982). The lack of photosomization of CRALBP-11-cis-retinaldehyde from RPE cannot be attributed to screening by pigment since approximately two-thirds of the area of bovine RPE is nonpigmented.

The possibility that photosomization and regeneration had occurred is ruled out in the retinal homogenate since the specific activity of added [3H]11-cis-retinaldehyde was not significantly reduced during the experiment. We conclude CRALBP-11-cis-retinaldehyde is only weakly photosensitive since there is little or no photosomization of the complex in these experiments.

Photosensitivity is defined as the product of the molar extinction coefficient (ε) and the quantum efficiency for photosomization (ψ) (Dartnall, 1972). The ε of CRALBP at 425 nm is 15,400 M⁻¹ cm⁻¹ (Stubb et al., 1979), whereas that for rhodopsin at 500 nm is 40,600 M⁻¹ cm⁻¹ (Dartnall, 1972; Hubbard et al., 1971). This difference would account for approximately a 3-fold reduction in photosensitivity if ψ for CRALBP and rhodopsin were comparable. The more than 3-fold difference in photosensitivities observed in the results of Table I suggested that ψ for the two complexes had to differ as well.

The ratio of the first order rate constants for photosomization of CRALBP and rhodopsin, the known values for the extinction coefficients of both protein complexes and ψrhodopsin were used to evaluate ψCRALBP. The value obtained (0.07) is comparable to that reported for 11-cis-retinaldehyde in methanol (0.04) and less than that reported in a hydrocarbon (0.24) (Table IV) (Waddell et al., 1976). The difference in the photosensitivities of the two 11-cis-retinaldehyde complexes (approximately 17-fold) would lead to an expected 4% photosomization of CRALBP-11-cis-retinaldehyde with a rhodopsin bleach of 70%, less than the estimated experimental error of the experiments.

The requirements for solubilization and photosomization of 11-cis-retinaldehyde complexed with rhodopsin and CRALBP are different. Rhodopsin must be solubilized with a detergent whereas the CRALBP-retinoid complex is disrupted at high detergent concentrations. In addition, all-trans-retinaldehyde, generated during the photosomization, precipitates CRALBP after completion of about one-third of reaction. Addition of an equimolar amount of bovine serum albumin prevents precipitation, probably by binding the liberated aldehyde. However, it is unlikely that these factors are responsible for major errors in the evaluation. The quantum efficiency value employed for rhodopsin was determined in the presence of detergent (Waddell et al., 1976), and measurements of the initial rate of photosomization of CRALBP-11-cis-retinaldehyde (i.e., before precipitation) indicate that serum albumin does not affect the rate of this process. In addition, three experimental arrangements were used, employing different concentrations of proteins and illumination at three wavelengths.

A comparison of the photosensitivities of uncomplexed 11-cis-retinaldehyde in solution or bound to either opsin or CRALBP is presented in Table IV. The high extinction coefficient and quantum efficiency for rhodopsin results in a pigment with high photosensitivity, in keeping with the physiological function of rhodopsin in the visual process. In contrast, ψ and ε for 11-cis-retinaldehyde complexed to CRALBP are lower than the corresponding values for rhodopsin and for 11-cis-retinaldehyde in solution, and the resulting photosensitivity is only 4% that of the visual pigment. Thus, even though CRALBP superficially resembles a visual pigment, it appears unlikely that its function depends on photosomization of bound retinoid. The low photosensitivity of the complex suggests that the physiological role of CRALBP may be related to its ability to solubilize 11-cis-retinaldehyde and protect it from photosomization.

11-cis-Retinaldehyde in solution exists as an equilibrium mixture of distorted 12-s-cis and 12-s-trans-forms (Birge, 1981; Birge et al., 1976; Callender et al., 1976; Rowan et al., 1974). Cooling solutions of the retinoids to 75°C results in a large increase in the oscillator strength of the 360-nm absorption band and a 3-fold increase in φ (Birge, 1981; Birge et al., 1976). Since reduced temperature preferentially stabilizes the 12-s-trans-form (Rowan et al., 1974), a higher φ and ε are likely to be intrinsic features of this conformation. Resonance Raman spectroscopy suggests that this is the conformer that is bound by opsin (Callender et al., 1976; Cookingham and Lewis, 1978), perhaps contributing to the formation of a complex with high photosensitivity. An extension of this line of reasoning suggests that preferential binding of the 12-s-cis-form by CRALBP could contribute to the formation of a complex with low φ and ε. However, the polarity of the binding site could also contribute to the described properties and
additional spectral studies will be required to assess the validity of this argument.

Earlier studies of the binding specificity of CRALBP employing displacement of bound ligand by zolot excesses of the various geometrical isomers (Futterman et al., 1977), indicated that the binding site was relatively specific for 11-cis-retinaldehyde; however, other cis-isomers were active as well. In this study we were unable to isolate complexes of CRALBP and 13-cis- or all-trans-retinaldehyde. However, both 9-cis- and 11-cis-retinaldehyde formed isolatable complexes with the protein (Fig. 2). In both complexes the maximum of ligand absorption is shifted relative to that of the free ligand dissolved in an organic solvent.

Although this result would seem to indicate that CRALBP cannot differentiate between 9- and 11-cis-retinaldehyde, the possibility existed that the affinities of the protein for the two ligands might be sufficiently different to permit selection of one over the other. The most rigorous way to investigate this question would be to determine the association constants for the protein-retinal complexes. However, since we were unable to prepare apo-CRALBP that could be regenerated, a different experimental approach was necessary. Accordingly, CRALBP was first complexed with 11-cis-retinol, a ligand that binds with lower affinity, and then incubated with a mixture of geometrical isomers of retinaldehyde. It is clear from the results of these experiments that CRALBP-11-cis-retinol is very stereoselective. Only 11-cis-retinaldehyde was selected from a mixture of four geometrical isomers including all-trans-retinaldehyde (Table III) and from mixtures of 9- and 11-cis-retinaldehyde that included a 9-fold molar excess of 9-cis isomer (Table IV). The results of the experiments provide a plausible explanation for the stereoselectivity as well. With mixtures consisting of an excess of 9-cis- over 11-cis-retinaldehyde, only 11-cis-retinaldehyde was found associated with the protein. However, some 11-cis-retinol was also bound to the protein, suggesting that 9-cis-retinaldehyde cannot displace 11-cis-retinol (Table IV). The results thus suggest the affinity ranking order: 11-cis-retinaldehyde > 11-cis-retinol > 9-cis-retinaldehyde and provide an explanation for the observed stereoselectivity of CRALBP-11-cis-retinol.

Production of 11-cis-retinoid in the dark remains enigmatic despite decades of research (Akhtar et al., 1973; Bernstein et al., 1985; Futterman, 1974; Ostapenko and Furayev, 1973; Rotmans et al., 1972). Although the stereosepecificity of the overall process suggests that a stereospecific enzyme process is involved, the possibility remains that stereospecificity is introduced after isomerization has taken place (Bernstein et al., 1985). Continued removal of 11-cis-retinaldehyde from an equilibrium mixture of aldehydes would also produce the requisite stereospecificity of the process. Results of this study suggest that CRALBP is sufficiently stereoselective to be considered in this functional role.

The unique occurrence of CRALBP in retina and its complement of endogenous 11-cis-retinoid strongly suggests that the protein is associated with the visual cycle. The low photosensitivity of bound 11-cis-retinaldehyde suggests a protective role for the protein rather than one of promotion of photoisomerization. The ability of the binding protein to select 11-cis-retinaldehyde from a mixture of isomers indicates that CRALBP could function in conjunction with an isomerization catalyst to generate the stereospecificity found in the visual system. Future experimentation will be directed toward determining whether the presence of the binding protein influences the composition of retinaldehydes isomers generated by isomerization catalysts and toward determining a quantitative estimation of the affinities of CRALBP for retinoids of interest. Recent experiments suggest the 11-cis-configuration may be generated by isomerization of all-trans-retinol (Bernstein and Rando, 1986). It will also be of interest to determine if the stereoselective properties of CRALBP extend to retinols as well as retinaldehydes.

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