Further Studies on the Acquisition of Novel Optical Activity on Interaction of Lutein and Other Carotenoids with Proteins

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Lutein had novel spectroscopic properties in the visible region on the formation of complexes with several proteins [S. Takagi, M. Shiroishi and T. Takagi, Agric. Biol. Chem., 44, 2111 (1980)]. The effects of pH, molar ratio of lutein to protein, and the variety of protein on the phenomenon was studied. The phenomenon was insensitive to these parameters. Solubilization into micelles of deoxycholate was found to induce no optical activity in contrast to bilirubin by Perrin et al. [J. H. Perrin and M. Wilsey, Chem. Commun., 769 (1971)].

It is strongly suggested in this paper that the observed changes in spectroscopic properties including the novel one in circular dichroism come chiefly from mutual interactions between lutein molecules in the complexes. Changes in spectroscopic properties comparable to those for lutein were observed with β-cryptoxanthin but not with canthaxanthin or ethyl β-apo-8'-carotenoate, although the latter two formed complexes with ovalbumin. The presence of at least one asymmetric carbon atom in the ionone rings seems to be essential for the novel spectroscopic changes to be observed. The possible correlation of the trans-cis conformational change in the conjugated double bond system was discussed. The optical activity was presumed to come from the intermolecular dipole-dipole coupling with the chiral spatial orientation.

Lutein is a major carotenoid in higher plants with the structure shown in Fig. 1(I). The xanthophyll family including lutein is exclusively located in the chloroplast1,2) and presumed to play various important roles in photosynthesis and protection of tissues against light.3) One of the present authors (S.T.) has found that lutein isolated from spinach leaves4) inhibits the lipoxygenase of spinach chloroplast.5) Inhibition of oxidation of lipids in chloroplasts might be another function of lutein. In previous papers,6,7) we reported that strongly lipophilic lutein can be made water-soluble in the presence of several proteins, and ascribed the “solubilization” to the formation of lutein-protein complexes in which both are oligomerized. It was tentatively presumed that the lutein molecules are mutually associated therefore having a chiral nature, and the hydrophobic surface of the aggregate is shielded from water by the association of the protein molecules.

Biochemical Studies on Carotenoids. Part X. For Part IX, see ref. 6. A part of this work was carried out while S. Takagi was a visiting research associate at the Institute for Protein Research.
The most significant observation was the acquisition of a novel circular dichroic (CD) spectrum in the visible region by lutein on complex formation. Spectral change was also observed with the absorption in the visible region. The spectrum was devoid of the fine structure characteristic of the spectra of carotenoids in organic solvents. We have since carried out further studies on this peculiar phenomenon. The present paper presents our interpretation of the phenomenon based on the newly obtained results as well as the previous ones.

MATERIALS AND METHODS

Crystalline lutein was prepared from spinach leaves as described previously. Canthaxanthin and ethyl β-apo-8'-carotenoate were obtained from Japan Roche Co., and β-cryptoxanthin was isolated from peel of Unshu citrus. All of the carotenoid preparations were confirmed to be chromatographically pure. Their concentrations were determined spectrophotometrically assuming values of $E_{1%\text{cm}}^{1\text{cm}}$ for the free forms in ethanol and those for the complexes in 0.1 M sodium phosphate buffer, pH 7.0, to be 2340 at 446 nm and 1470 at 384 nm for lutein; 2110 at 470 nm and 1370 at 455 nm for canthaxanthin; 2390 at 450 nm and 1020 at 450 nm for ethyl β-apo-8'-carotenoate; and 2390 at 455 nm and 910 at 418 nm for β-cryptoxanthin. Ovalbumin was isolated from hen egg white by the method of Kekwick et al. Lysozyme was purchased from Seikagaku Industry Co. Protein concentration was determined by the method of Lowry using ovalbumin as a standard protein. Lutein and other carotenoids were complexed with ovalbumin and lysozyme by the method described previously.

Absorption and CD spectra were measured with a spectrophotometer, Shimadzu Multipurpose model 3000, and a spectropolarimeter, JASCO model J-500, respectively, at room temperature, 25 ± 3°C.

Gel filtration was carried out with a Sephadex G-200 column, 1.2 x 50 cm. One ml of the complex solution was applied to the column followed by elution with 0.1M phosphate buffer, pH 7.0. Aliquots of 2 ml each were collected, and the absorbance at $\lambda_{\text{max}}$, 384 nm for lutein and 455 nm for canthaxanthin, and that at 750 nm (protein, with Lowry’s method) were measured.

RESULTS AND DISCUSSION

Stability of the solubilized lutein and protein concentration

As described previously, a new optical activity was acquired as the result of association of lutein molecules solubilized by the co-association of protein molecules. The CD spectra in the visible region were insensitive to the kind of proteins used. It was strongly suggested, therefore, that the protein molecules involved in the complex protected the associated lutein molecules from further aggregation which might lead to the phase separation of lutein from the aqueous phase as a red precipitate. The protective action of ovalbumin, for example, reached a limit at the lutein–ovalbumin molar ratios of 1.0 and 1.2 in the presence of 0.1 and 0.2% ovalbumin, respectively.

Further experiments were carried out. The concentration of lutein was now fixed at $5.1 \times 10^{-6}$ M and that of ovalbumin was widely changed from 0.01 to 2.0% (0.2 x 10^{-5} to 40.3 x 10^{-5} M). Above the lutein–ovalbumin ratio of 0.5 (when the protein concentration was below 0.05%), red precipitates were formed making spectroscopic measurements impossible. Ellipticity and absorbance at the maximum wavelengths are shown in Fig. 2.

The molar ellipticity at the maximum wavelength was independent of the concentration of ovalbumin. This is apparently in accord with the view that protein molecules only play a role as “solubilizer” and the mode of association of lutein molecules is invariable throughout. A somewhat complicating fact is that the absorbance at the maximum wavelength increased with the protein concentration as is
also shown in Fig. 2. Since the optical rotatory strength, a direct measure of optical activity, is the scalar product of the magnetic transition moment and the electric transition moment, the constancy of the ellipticity mentioned above might be an accidental product of the compensating changes in both of the two moments. Further discussion must await more detailed studies. It can be, however, safely concluded that the lutein molecules form an aggregate with a chiral nature and its conformation approaches a limiting one as the protein concentration is decreased.

**Effect of pH**

Among the proteins tested in the previous study, lysozyme and RNase formed complexes with lutein at pH 7.0, but the products were red precipitates.

Both of the proteins have isoelectric points higher than 7. To get some insight into the effect of the charge on a protein, lutein and ovalbumin were mixed using the same procedure as that in the previous study except that the pH was changed between 5 and 11 (Fig. 3). Below pH 5.5, the isoelectric point of the protein, ovalbumin formed precipitates with lutein. Although lutein is apparently without a charged group, it is clear that lutein forms insoluble complexes with positively charged proteins. This phenomenon suggests the probability that the two hydroxyl groups on the lutein molecule dissociate to give the molecule negative charges. Above pH 5.5, lutein formed a complex with ovalbumin soluble in the aqueous media. In the pH region tested, no appreciable change was observed for the light absorption spectrum. The molar ellipticity at the maximum wavelength, however, progressively decreased as shown in Fig. 3. From these results, the charge of proteins seems to have a slight influence on the acquisition of the new optical activity by lutein at pHs above the isoelectric points of the proteins.

Lysozyme has been shown to form a complex with lutein at pH 7.0 to form a red precipitate. In 0.1 M NaOH-phosphate buffer, pH 12.0, higher than 11 the isoelectric point of the enzyme, a soluble complex was formed between lutein and lysozyme. As shown in Fig. 4A, the absorption spectrum of the complex had a maximum at the same wavelength as that of the cross-over point in the corresponding CD spectrum (Fig. 4B). The above results are in good agreement with those previously obtained with several other pro-

![Fig. 3. Effect of pH on Molar Ellipticities of Lutein-Ovalbumin Complex in Aqueous Solution.](image)

Lutein concentration was 0.51 x 10^{-5} M, ovalbumin concentration, 4.03 x 10^{-5} M in 0.1 M phosphate buffer, pH 5.5 to 8.0, and 0.1 M borate buffer, above pH 9.0. Molar ellipticities were calculated from ellipticities at 374 nm.

![Fig. 4. Absorption Spectrum (A) and CD Spectra (B) of Lutein in Ethanol and Lutein-Lysozyme Complex in an Aqueous Solution.](image)

Lutein concentration was 0.70 x 10^{-5} M, lysozyme concentration, 1.2 x 10^{-4} M (curves 1 and 3). (1) absorption spectrum of lutein-lysozyme complex in 0.1 M NaOH-phosphate buffer, pH 12.0, (2) CD spectrum of lutein in ethanol, and (3) CD spectrum of the complex in the same buffer.
teins. Thus the water-soluble lutein–protein complexes may be stabilized only in the condition in which the protein surface is negatively charged.

**Solubilization of lutein by deoxycholate**

The optical activity of lutein acquired on the complex formation with several proteins was insensitive to the kind of protein. It is strongly suggested that the optical activity chiefly comes from the mutual interaction of lutein molecules rather than that between protein and lutein. This assumption was supported by the observation that lutein acquired no optical activity on solubilization with sodium deoxycholate (1.4 × 10⁻² M in 0.1 M phosphate buffer, pH 7.0). As shown in Fig. 5, the absorption spectrum of lutein solubilized by deoxycholate slightly shifted to a longer wavelength region compared to that in ethanol, and retained the characteristic fine structure.

**Other carotenoids**

Three carotenoids other than lutein were tested to see whether they formed complexes with ovalbumin acquiring an optical activity similar to that observed with lutein.

Canthaxanthin, 4,4'-diketo-β-carotene (Fig. 1(II)), a widely distributed red carotenoid in invertebrates, has eleven conjugated double bonds, but has no asymmetric carbon on its ionone rings. Ethyl β-apo-8'-carotenoate (Fig. 1(III)) is a synthetic carotenoid having 10 conjugated double bonds. β-Cryptoxanthin, 3-hydroxy-β-carotene, (Fig. 1(IV)) has a hydroxyl group on the one-sided ionone ring to introduce an asymmetric carbon.

The complex formed between canthaxanthin and ovalbumin at pH 7.0 was soluble in water and the protein was highly associated on complex formation as judged from the elution pattern on gel chromatography (Fig. 6). The absorption spectrum of canthaxanthin was devoid of the fine structure and had a maximum at 446 nm in ethanol (Fig. 7). The complex between canthaxanthin and ovalbumin showed a peak at 458 nm with less absorptivity than that in ethanol. No CD absorption, however, appeared on complex formation. The spectroscopic and gel filtration behaviors of ethyl β-apo-8'-carotenoate complexed with ovalbumin were the same as those of canthaxanthin except for the difference of the spectrum pattern and its slight shift to a higher wavelength. β-Cryptoxanthin formed a complex with ovalbumin as judged from the elution pattern on gel chromatography suggesting the association of the protein (not shown). As shown in Fig. 8A, the absorption spectrum of β-cryptoxanthin complexed with ovalbumin was definitely different from that in...
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Fig. 7. Absorption Spectra of Canthaxanthin in Ethanol and Canthaxanthin–Ovalbumin Complex in an Aqueous Solution.
Canthaxanthin concentration was $1.47 \times 10^{-5}$ M, ovalbumin concentration, $4.03 \times 10^{-5}$ M. (1), absorption spectrum of canthaxanthin–ovalbumin complex in 0.1 M phosphate buffer, pH 7.0.

ethanol. As shown in Fig. 8B, the complex showed a CD spectrum in the visible region. The spectrum differs from those observed on the formation of the complexes between lutein and several proteins. It should be noted that the CD spectrum (Fig. 8B-2) as well as the absorption spectrum (Fig. 8A-2) is more complex than those observed with lutein, and has a shape which seems to be composed of several subcomponents. It should also be noted that the CD band in the longer wavelength region is positive in the present case while the corresponding ones in the cases of lutein were always negative, and vice versa for the case of the CD band in the shorter wavelength region.

The above results indicate that the presence of asymmetric carbon atoms at the positions of 3 or 3' or both is a prerequisite for the acquisition of the optical activity.

Origin of the change of absorption spectrum
One of the characteristic changes observed on the formation of complexes between lutein and proteins was the attenuation in intensity and the shift of the absorption spectrum to a shorter wavelength region by as much as 60 nm and the concomitant disappearance of the fine structure compared to the spectrum in ethanol. By the way, a subsidiary peak in the near UV region observed in ethanol could not be detected with the complexes. These phenomena might possibly be correlated with the trans-cis transformation of the conjugated double bonds. It has been shown that several carotenoids with the all-ds conformation exhibit an absorption spectrum with an intensity lower than and a maximum shorter than those of corresponding all-trans isomers. The absorption maximum shifts 2 to 5 nm for every increase of one cis-double bond. A carotenoid with a single unhindered cis-double bond shows an absorption spectrum with fine structures in the visible region and a subsidiary peak in the near ultraviolet region. On the other hand, a carotenoid with a "sterically hindered" double bond gives a spectrum devoid of fine structure in the visible region and no sub-peak in the near-ultraviolet region. The main absorption peak appears at a wavelength much shorter than that of a
carotenoid with the all-trans conformation.

Taking the above phenomena into consideration, the lutein molecules are presumed to be converted from an all trans-conformation to a hindered cis-conformation as a result of their mutual association.

**Origin of the optical activity**

Lutein could be solubilized into micelles of deoxycholate, an optically active surfactant (Fig. 5). In the case of solubilization of bilirubin into the micelles, a strong optical activity apparently similar to those of the lutein-protein complexes was observed and ascribed to the electric transition dipole–dipole interaction within a single molecule.\(^{15}\) This fact shows that bilirubin in the bilirubin-deoxycholate complex acquires an optical activity by being solubilized into deoxycholate micelles with a chiral environment. On the contrary, lutein acquired no optical activity on solubilization into the micelle. This observation strongly indicates that the optical activity of the lutein–protein complexes originates from the mutual interaction between lutein molecules. The intramolecular interaction of the single lutein molecules and the chiral environment of a deoxycholate micelle are totally unable to induce any optical activity.

In conclusion, the origin of the optical activity of the lutein–protein complexes must be ascribed predominantly to the mutual interaction between the lutein molecules. The conjugated double bond system of lutein may be assumed to be a unitary chromophore having absorption in the visible region. A new optical activity showing a CD spectrum with a roughly point-symmetrical shape like that in Fig. 4 is expected to appear when a pair of such chromophores is mutually located in a position near a chiral arrangement.\(^{16,17}\) The lutein molecule has asymmetric carbons with 3S, 6R and 3'R configurations on each of the two ionone rings, and, therefore, is intrinsically asymmetric. It is likely that the lutein molecules prefer either a pair with possible mutual orientation of left or right handedness for a specific mutual orientation. The protein molecules involved in the complex formation are certainly playing an important role in the solubilization of the lutein molecule, otherwise insoluble in water. The protein molecules seem, however, not to be important in the selection of the preferred orientation of the pair of lutein molecules. The insensitiveness of the acquired optical activity to the kind of protein and the pH value of the medium supports the above presumption. In the case of the bilirubin-bovine serum albumin complex in which an intramolecular interaction is predominantly responsible for the acquired optical activity, it is highly sensitive to pH and even the sign of the optical activity is changed with pH.\(^{18,19}\) These results also support the above presumption.

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**REFERENCES**