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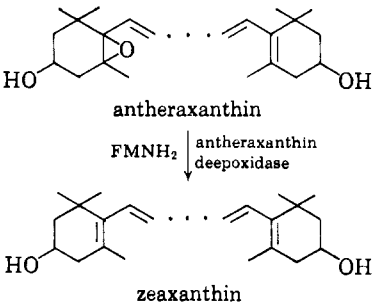
Reduction of Carotenoid Epoxides with Lithium Aluminum Hydride*

B. P. Schimmer† and N. I. Krinsky

ABSTRACT: We have undertaken a study of the chemical reduction of antheraxanthin and neoxanthin, the epoxide carotenoids of *Euglena gracilis*, in an attempt to elucidate the mechanism of the enzymatic reductive deepoxidation of carotenoids (Bamji, M. S., and Krinsky, N. I. (1965), *J. Biol. Chem. 240*, 467). These epoxide carotenoids were treated with a large excess of $LiAlH_4$, and the resultant reaction mixtures were separated into several fractions by gradient elution from silica gel G–Celite (1:1). The individual fractions were characterized by absorption spectra, relative polarity values, and dehydration reactions with acidichloroform. The 5,6-epoxide groups of antheraxanthin and neoxanthin, upon reduction with $LiAlH_4$, yield, along with the expected 5-hydroxyl derivatives, equal amounts of the unexpected 5,6-olefins *via* a mechanism which does not involve dehydration of an hydroxylated intermediate. The mechanism of enzymatic deepoxidation may be like the $LiAlH_4$ reaction reported here. Based on our results, we suggest that the 3-hydroxyl and 5,6-epoxide groups of antheraxanthin and neoxanthin are in a *cis* configuration on the ionone ring.

The deepoxidation of epoxide carotenoids in photosynthetic tissue has been described as an anaerobic light-induced reaction (Sapozhnikov *et al.*, 1957; Yamamoto *et al.*, 1962b; Krinsky, 1964). Recently, Bamji and Krinsky (1965) have demonstrated that in *Euglena gracilis* the anaerobic deepoxidation of antheraxanthin to zeaxanthin can occur in the dark upon the addition of $FMNH_2$.[1]

These authors concluded that light functions in this type of reaction by generating reducing potential.



They have suggested that the deepoxidation of antheraxanthin might proceed *via* reduction of the 5,6-epoxide to an hydroxyl intermediate followed by dehydration to the 5,6-olefin. The proposed hydroxyl intermediate has not as yet been demonstrated as a participant in this reaction, nor has it been demonstrated as a naturally occurring component of *E.*

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[1] Abbreviation used: $FMNH_2$, reduced flavin mononucleotide.

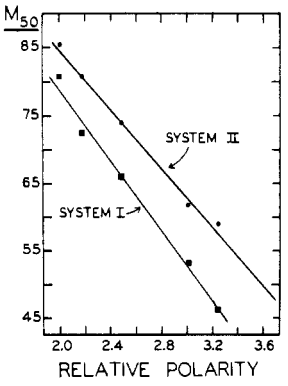


FIGURE 1: Relationship of M_{50} values to relative polarity values for systems I and II.

gracilis (Schimmer and Krinsky, 1966). In an attempt to prepare and characterize this hypothetical hydroxyl intermediate, we have investigated the reduction of antheraxanthin by $LiAlH_4$. Along with the expected hydroxyl intermediate, this chemical reduction also forms the 5,6-olefin, zeaxanthin. Neoxanthin, the other epoxide carotenoid of *E. gracilis*, also forms both the 5-hydroxyl and 5,6-olefin derivatives upon treatment with $LiAlH_4$. The formation of the 5,6-olefin from the 5,6-epoxide by $LiAlH_4$ does not involve the hydroxyl derivative as an intermediate.

During the course of this work, Grob and Siekmann (1965) have treated 5,6-epoxy-β-carotene with $LiAlH_4$ and have produced 5-hydroxy-β-carotene as the major reaction product. In contrast to our results, these authors do not detect the formation of the 5,6-olefin derivative.

Materials and Methods

Reaction of Epoxide Carotenoids with $LiAlH_4$. The procedure described by Brown (1951) was employed to treat antheraxanthin and neoxanthin with $LiAlH_4$. A slurry (3 ml) prepared with 1 g of $LiAlH_4$ and 10 ml of tetrahydrofuran was added to the dried pigment. This mixture was reacted in the dark at 0° for 1–2 hr, and then neutralized by the cautious dropwise addition of absolute ethanol. The pigment was transferred to diethyl ether, washed with water, and evaporated to dryness at 40° under reduced pressure. The dried pigment was dissolved in 5% acetone in petroleum ether (bp 30–60°), placed on a column of silica gel G–Celite (1:1), and chromatographed by gradient elution with acetone in petroleum ether under 7 psi of nitrogen pressure. When necessary, the isolated fractions were rechromatographed on columns of silica gel G–Celite (1:1), magnesium oxide–Celite (1:1), and Micro-Cel C, and on thin layer plates of silica gel G–magnesium oxide (1:1) developed with either 100% acetone or 20% ethanol in petroleum ether.

Reaction of Carbonyl Carotenoids with $NaBH_4$. The procedure described by Krinsky and Goldsmith (1960) was used to reduce carbonyl carotenoids with $NaBH_4$.

Reaction of Carotenoids with Acidic Chloroform. A modification of the procedure of Karrer and Leumann (1951) as previously described (Schimmer and Krinsky, 1966) was employed to dehydrate carotenoids containing tertiary hydroxyl groups. The products of reaction with acidic chloroform were separated by chromatography on thin layer plates of silica gel G. The separated pigments with adsorbant were scraped from the plate and the pigment was eluted from the adsorbant with ethanol.

Determination of Relative Polarity Values. The partition system described by Krinsky (1963) (system I) or a modification thereof (system II) was employed to determine relative polarity values of carotenoid pigments. In system I, petroleum ether and aqueous methanol served as the partitioning solvents; in system II, 3:1 (v/v) petroleum ether–diethyl ether and aqueous methanol served as the partitioning solvents. The pigment to be partitioned was dissolved in the methanolic phase, and its partition distribution and M_{50} values were determined as previously described (Krinsky, 1963). The partition distribution and M_{50} values determined for several carotenoid standards in system II are listed in Table I along with the relative polarity values determined in system I (Krinsky, 1963). Figure 1 shows the relationship of M_{50} values (that percentage methanol in which the partition distribution for a carotenoid is 50:50) to relative polarity values in both systems I and II. This relationship was used in the determination of relative polarity values for unknown carotenoids. With this modified partition system one can more readily determine the relative polarity values of very polar carotenoids, *i.e.*, with relative polarity

TABLE I: Partition Distribution and M_{50} Values in a Petroleum Ether–Diethyl Ether–Aqueous Methanol Partition System (System II).

Carotenoid	% Methanol	Partition Distribution	M_{50}[a]	Rel Polarity Value[b]
Zeaxanthin	85	50.6:49.4	85.5	2.0
	80	64.6:35.4		
Antheraxanthin	80	42.4:57.6	78.3	2.24
	75	65.1:34.9		
Violaxanthin	75	45.3:54.7	74.0	2.48
	70	67.1:32.9		
Trollein-like carotenoid ex. *E. gracilis*	65	32.6:67.4	61.7	3.00
	60	61.0:39.0		
Neoxanthin	60	42.9:57.1	59.0	3.24
	55	94.7:25.3		

[a] M_{50} is that percentage methanol in which a carotenoid has a partition distribution of 50:50 and is determined graphically from the partition values. [b] The values are those determined in system I by Krinsky (1963).

TABLE II: Products of Reaction of Antheraxanthin with $LiAlH_4$.

Fraction	% Recovd	Rel Polarity Value[a]	Wavelength Max (mμ)[b]	5,6-Epoxide[c]
Antheraxanthin		2.24	475,445,(423)	+
A-1	5.9			
A-1a		2.09	(470), 445	−
A-1b		1.83	465	−
A-2	22.0	2.00	474,447.5,(425),340	−
A-3	44.0	2.24	472,445,(422)	+
A-4	21.0	2.85	471,442,(421),337	−
A-4a[d]		2.00	468,440	
A-5	5.6	2.96	472,444	−
A-5a[d]		2.00	(468),445	
A-5b[d]		1.40	468,440	

[a] Relative polarity values were determined in system I (see Methods). [b] Wavelength maxima were determined in ethanol; items in parentheses indicate shoulders. [c] The 5,6-epoxide group was identified by a characteristic 15–20-mμ hypsochromic spectral shift in the presence of traces of hydrochloric acid in ethanol (Karrer and Jucker, 1950). [d] Product of reaction with acidic chloroform.

values greater than 3.0. The relative polarity value represents the total contribution of individual functional groups to the over-all polarity of a carotenoid molecule. The individual functional groups are assigned polarity values based on a scale of 1.00 unit for the nonallylic hydroxyl group (Krinsky, 1963). Spectral characteristics of all purified fractions were determined in absolute ethanol with a Cary Model 14 spectrophotometer.

Materials. Adsorbants included silica gel G (Brinkmann Instruments, according to Stahl), magnesium oxide (Sea Sorb 43, Fisher Scientific Co.), Celite (Johns-Manville Sales Corp.), and Micro-Cel C (Johns-Manville Sales Corp.). Antheraxanthin, neoxanthin, zeaxanthin, and a trolleinlike carotenoid were isolated from *Euglena gracilis* as previously described (Krinsky, 1963; Krinsky and Goldsmith, 1960; Krinsky *et al.*, 1964). Antheraxanthin and neoxanthin were crystallized from diethyl ether–petroleum ether. Violaxanthin was isolated from spinach purchased through local markets (Yamamoto *et al.*, 1962a). Neochrome was prepared from a solution of neoxanthin in ethanol by the addition of traces of hydrochloric acid in ethanol (Goldsmith and Krinsky, 1960). The resulting 5,8-furanoid was purified on a column of silica gel G–Celite (1:1).

Results

Reaction of Antheraxanthin with $LiAlH_4$. Antheraxanthin (1.88 mg), when treated with $LiAlH_4$ for 2 hr and chromatographed on silica gel G–Celite, gives the elution pattern shown in Figure 2. The individual separated fractions are indicated in this figure. Recovery, based on total absorbancy at 440 mμ, is 35% of the starting material. The per cent yield, relative polarity value, and wavelength maxima for each fraction are listed in Table II.

Fraction A-1 separates into two major subfractions, A-1a and A-1b, when chromatographed on magnesium oxide–Celite (1:1). Neither subfraction has a 5,6-epoxide group (Table II). Fraction A-1a has very little spectral fine structure in ethanol and a single λ_{max} (Table II). In petroleum ether, the λ_{max} for fraction A-1a does not change, but increased spectral fine structure appears [λ_{max} 470, 445, and (420) mμ]. In 95% ethanol, this fraction undergoes reduction with $NaBH_4$ to yield a compound with spectral fine structure (λ_{max} 448, 422, and 400 mμ). This hypsochromic spectral shift of 23 mμ is characteristic of a carotenoid with an in-chain keto group reduced to an hydroxyl group (Jensen and Schmidt, 1963). The relative polarity value for the reduced compound is 0.10 unit greater than that for fraction A-1a and the R_F value on silica

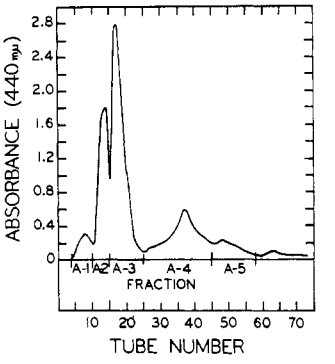
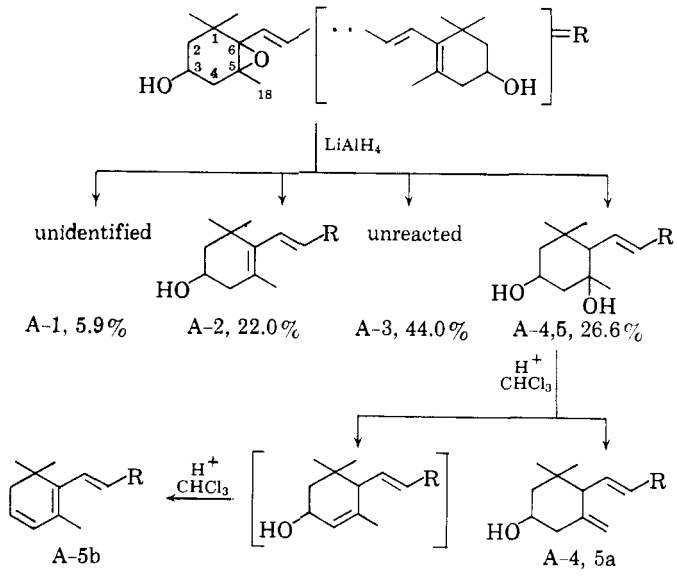


FIGURE 2: Gradient elution pattern of $LiAlH_4$-treated antheraxanthin. The pigments were eluted from a silica gel G–Celite (1:1) column using a linear gradient of 15–50% acetone in petroleum ether. Tube contents under each peak were pooled and treated as individual fractions.

SCHEME I: Reaction of Antheraxanthin with Lithium Aluminum Hydride.



gel G thin layer plates developed with 25% acetone in petroleum ether changes from 0.34 to 0.16, further substantiating the reduction of a keto group to an hydroxyl group. Fraction A-1b has a very symmetrical shape with a single wavelength maximum (Table II). In petroleum ether, the λ_{max} remains unchanged and fine structure is absent; however, spectral asymmetry appears. This fraction appears to be resistant to reduction with $NaBH_4$ as determined by the absence of changes in R_F values on silica gel G thin layer plates, relative polarity values, and spectrum. *Fraction A-2*, as determined by its spectral and partition characteristics and its lack of a 5,6-epoxide group (Table II), is the product of replacement of the 5,6-epoxide of antheraxanthin with a 5,6-double bond, *i.e.*, zeaxanthin. When compared with naturally occurring zeaxanthin, fraction A-2 behaves identically on columns of silica gel G–Celite (1:1), Micro-Cel C, and magnesium oxide–Celite (1:1). On thin layer plates of silica gel G and on Keiselguhr-impregnated paper (Jensen and Jensen, 1959), fraction A-2 chromatographs with zeaxanthin. On Keiselguhr-impregnated paper, zeaxanthin and fraction A-2 split into two similar isomeric forms as determined by R_F values in 10% acetone in petroleum ether and by spectral characteristics in ethanol. The minor spectral differences between fraction A-2 and zeaxanthin are due to a relatively high concentration of *cis* isomers in fraction A-2. *Fraction A-3*, based on its spectral properties, 5,6-epoxide group, and relative polarity value (Table II), is unreacted antheraxanthin. *Fraction A-4*, based on its spectral properties, its lack of a 5,6-epoxide group, and its relative polarity value (Table II), as well as its position behind antheraxanthin on silica gel G–Celite (1:1) (Figure 2), appears to be the product of reduction of the 5,6-epoxide group of antheraxanthin to the corresponding 5- (or 6-) hydroxy-6- (or 5-) hydro derivative. Upon treatment of fraction A-4 with acidic chloroform, a product, fraction A-4a, is formed with a similar λ_{max} (Table II), but with two nonallylic hydroxyl groups as determined by its relative polarity value (Table II) and its mobility ahead of fraction A-4 on thin layer plates of silica gel G. The behavior of fraction A-4 with acidic chloroform establishes the hydroxyl group on the 5 position, yielding 3,3′,5-trihydroxy-6-hydro-β-carotene (5-hydroxyzeaxanthin), and indicates that fraction A-4a is a result of dehydration of the 5-hydroxyl group to the $\Delta^{5(18)}$ derivative as is observed when neoxanthin is treated with acidic chloroform (Schimmer and Krinsky, 1966).

Fraction A-5, based on spectral properties, absence of the 5,6-epoxide group, and relative polarity value (Table II), is also a product of reduction of the 5,6-epoxide to the 5- (or 6-) hydroxy-6- (or 5-) hydro derivative. Treatment of fraction A-5 with acidic chloroform results in the separation of two fractions, A-5a and A-5b, along with unreacted material. The λ_{max} for fraction A-5a is similar to that of fraction A-5, but its relative polarity value is indicative of a compound with two nonallylic hydroxyl groups (Table II). This behavior of fraction A-5 with acidic chloroform establishes the hydroxyl group on the 5 position and indicates that fraction A-5 is 5-hydroxyzeaxanthin and is isomeric with fraction A-4. Fraction A-5a appears to be the product of dehydration of the 5-hydroxyl group of fraction A-5 to the $\Delta^{5(18)}$ derivative. Fraction A-5b, based on its spectral and partition properties (Table II), appears to be the result of elimination of both the 5-hydroxyl and 3-hydroxyl groups *via* a Δ^4 intermediate as seen in the dehydration of neoxanthin with acidic chloroform (Schimmer and Krinsky, 1966). Although the relative polarity value of fraction A-5b (Table II) would be expected to be closer to 1.00, such a low value is difficult to determine.

Acetylation of pooled fractions A-4 and A-5 to completion as described by Kuhn and Sørensen (1938)

TABLE III: Products of Reaction of Neoxanthin with $LiAlH_4$.

Fraction	% Recovd	Rel Polarity Value[a]	Wavelength Max (mμ)[b]	5,6-Epoxide[c]
Neoxanthin		2.96	466,438,414,328	+
N-1	2.4			
N-1a		2.86	468,440,(418)	−
N-1b		2.75	468	−
N-2	54.6	2.99	465,437,413,330	+
N-3,4	22.1	2.72	472,444,(422)	−
N-3,4a[d]		1.96	473,445,(424)	
N-3,4b[d]		1.37	472,444,(425)	
N-5	20.9	3.70	469,440,416	
N-5a[d]		3.04	468,440,416	
N-5b[d]		2.12	467,439,415	

[a] The relative polarity values were determined in system II (see Methods). [b] Wavelength maxima were determined in ethanol. [c] The 5,6-epoxide group was identified by a characteristic 15–20-mμ hypsochromic spectral shift in the presence of traces of hydrochloric acid in ethanol (Karrer and Jucker, 1950). [d] Product of reaction with acidic chloroform.

gives a product with a relative polarity value of 1.46 units in system I. Tertiary hydroxyl groups acetylate to a very small extent (<10%) under these conditions (Jensen, 1962), so that the major product formed in 3,3′-diacetyl-5-hydroxyzeaxanthin. The theoretical relative polarity value for this product is 1.94 units (Krinsky, 1963), significantly higher than the observed value. The calculated value for the 3-acetyl-5-hydroxyl moiety is thus 0.99 unit (1.46 units − 0.47 unit), in good agreement with the value of 0.98 unit observed earlier for the 3′-acetyl-5′-hydroxyl moiety of acetylated neoxanthin (Schimmer and Krinsky, 1966). This low value has been ascribed to hydrogen bond interaction between the 3-acetyl and the 5-hydroxyl groups, and suggests that they exist in a *cis* configuration on the β-ionone ring. Thus the 3-hydroxyl and 5,6-epoxide groups of antheraxanthin would also be expected to be in a *cis* configuration. The reactions of antheraxanthin with $LiAlH_4$ are outlined in Scheme I.

Reaction of Neoxanthin with $LiAlH_4$. Neoxanthin (0.593 mg) when treated with $LiAlH_4$ for 1 hr and chromatographed on silica gel G–Celite gives the elution pattern shown in Figure 3. The individual separated fractions are indicated in this figure. Recovery, based on total absorbancy at 440 mμ, is 38% of starting material. The per cent yield, relative polarity value, and wavelength maxima of each fraction are listed in Table III.

Fraction N-1 can be further separated into two major subfractions, N-1a and N-1b. Fraction N-1a, a compound with spectral fine structure (Table III), is converted to a compound without spectral fine structure and with a λ_{max} at 460 mμ in the presence of hydrochloric acid in ethanol. The relative polarity value for this product is 2.63 units in system II. Fraction N-1b has an asymmetrical spectral shape with a single wavelength maximum in ethanol (Table III). In petroleum ether, this fraction exhibits a spectrum with more pronounced fine structure [λ_{max} (492), 468, and (435) mμ]. In the presence of hydrochloric acid in ethanol, there is no spectral change observed, but a product is recovered with similar spectral properties and a relative polarity value of 2.15 units in system II. Fraction N-1b, upon treatment with $NaBH_4$, is converted to a compound with a neoxanthin-like spectrum and a λ_{max} at 439 mμ. The relative polarity value for this fraction is 3.04 units in system II. The reduction product of fraction N-1b, upon subsequent treatment with acidic ethanol, shows no spectral change but does result in the isolation of a product with a similar spectrum and a relative polarity value of 2.66 units in system II. *Fraction N-2*, on magnesium oxide–silica gel G (1:1) thin layer plates developed with 100% acetone, chromatographs with neoxanthin. Based on spectral properties, relative polarity value, presence of a 5,6-epoxide group (Table III), and chromatographic behavior, fraction N-2 is unreacted neoxanthin. *Fractions N-3 and N-4* are apparently isomers. Their spectral properties, relative polarity values, and lack of a 5,6-epoxide group indicate that the 5,6-epoxide group of neoxanthin has been replaced by a 5,6-double bond, yielding 3,3′,5′-trihydroxy-6′-hydro-β-carotene (5-hydroxyzeaxanthin). Treatment of pooled fractions N-3 and N-4 with acidic chloroform results in the isolation of two products, N-3,4a and N-3,4b, both of which have the same spectral properties as fraction N-3,4 (Table III). Subsequent treatment of fraction N-3,4a with acidic ethanol gives no change in relative polarity value, indicating the absence of allylic hydroxyl groups (Petracek and Zechmeister, 1956). The unchanged spectral characteristics and relative polarity value, indicative of two nonallylic hydroxyl groups (Table III), confirm

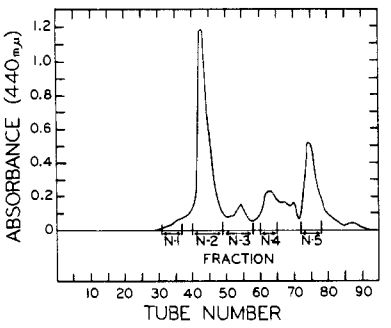


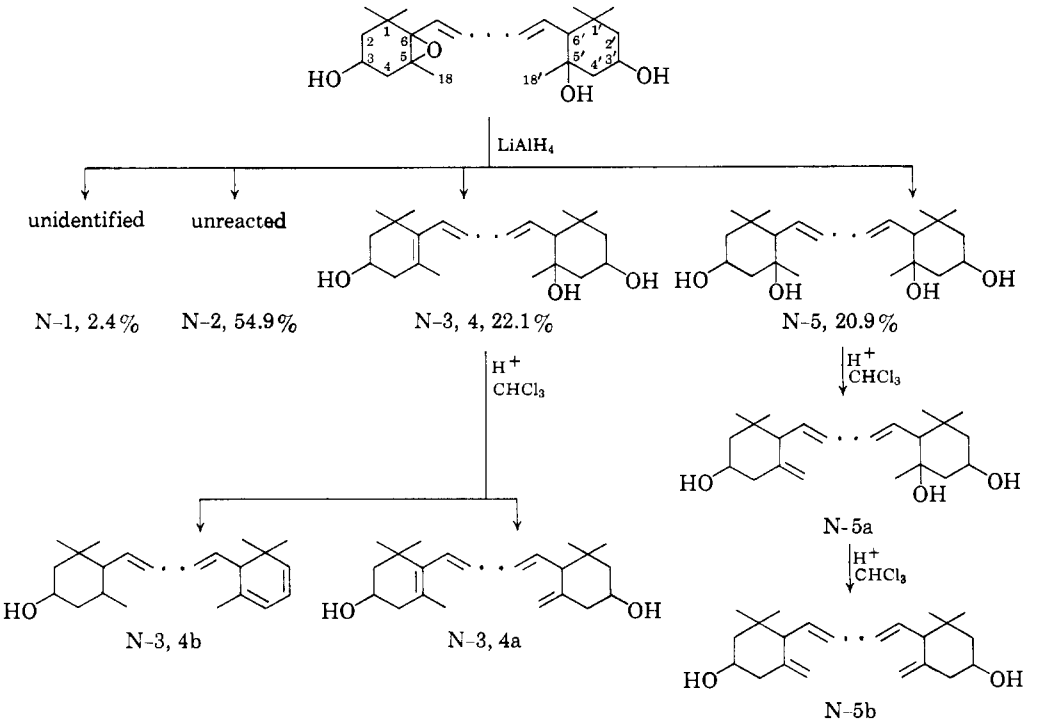
FIGURE 3: Gradient elution pattern of $LiAlH_4$-treated neoxanthin. The pigments were eluted from a silica gel G–Celite (1:1) column using a linear gradient of 20–50 % acetone in petroleum ether. Tube contents under each peak were pooled and treated as individual fractions.

the presence of the 5′-hydroxyl group on fraction N-3,4 and indicate that fraction N-3,4a is the product of dehydration of the 5′-hydroxyl group to the $\Delta^{5'(18')}$ derivative. Fraction N-3,4b, based on spectral and partition properties (Table III), appears to be the product of elimination of both the 5′-hydroxyl and 3′-hydroxyl groups *via* a $\Delta^{4'}$ intermediate. The pattern of dehydration of fraction N-3,4 with acidic chloroform is identical with that previously observed for neoxanthin (Schimmer and Krinsky, 1966). *Fraction N-5*, based on its lack of a 5,6-epoxide, its spectral properties, and its relative polarity value, is the product of reduction of the 5,6-epoxide of neoxanthin to an hydroxyl group. Treatment of fraction N-5 with acidic chloroform results in the isolation of two reaction products, N-5a and N-5b. As determined by its relative polarity value and spectral properties, fraction N-5a contains only three nonallylic hydroxyl groups and nine conjugated double bonds (Table III). By the same criteria, fraction N-5b contains only two nonallylic hydroxyl groups and nine conjugated double bonds (Table III). These results indicate that fraction N-5 is 3,3′,5,5′-tetrahydroxy-6,6′-dihydro-β-carotene, and that dehydration by acidic chloroform occurs from the 5 position to give the $\Delta^{5(18)}$ derivative, N-5a, and then from the 5′ position to give the $\Delta^{5(18),5'(18')}$ derivative, N-5b.

Acetylation of fraction N-5 (Kuhn and Sørensen, 1938) forms 3,3′-diacetyl-5,5′-dihydroxy-6,6′-dihydro-β-carotene. The relative polarity value predicted for this compound is 2.94 units (Krinsky, 1963), significantly higher than the observed relative polarity value of 1.95 units in system II. As observed previously (Schimmer and Krinsky, 1966), the 3′-acetyl-5′-hydroxyl moiety of 3,3′-diacetylneoxanthin has a relative polarity value of 0.98 unit due to hydrogen bond interaction between these two groups which presumably are in a *cis* configuration on the ionone ring. Essentially, this same value is calculated for the 3-acetyl-5-hydroxyl moiety of acetylated fraction N-5 (1.95 units − 0.98 unit = 0.97 unit), suggesting that the 5-hydroxyl group of fraction N-5 formed by reduction of the 5,6-epoxide of neoxanthin is *cis* to the 3-hydroxyl group. Therefore, the 3-hydroxyl and 5,6-epoxide groups of neoxanthin would also be in a *cis* configuration. The reaction of neoxanthin with $LiAlH_4$ is outlined in Scheme II.

Reaction of Neochrome with $LiAlH_4$. Reaction of 0.965 mg of neochrome with $LiAlH_4$ for 1 hr results

SCHEME II: Reaction of Neoxanthin with Lithium Aluminum Hydride.



in the recovery of 67% of the pigment based on absorption at 420 mμ. Chromatography on silica gel G–Celite (1:1) yields one major fraction followed by a more polar fraction on the column. The major fraction (70%), based on its neochrome-like spectrum and a relative polarity value of 3.02 units in system II is unreacted neochrome. No pigment is observed ahead of neochrome on the column, indicating that the 5′-hydroxyl group is not removed under these conditions. The more polar pigment reflects the possible reaction of $LiAlH_4$ on the 5,8-furanoid oxide portion of the neochrome molecule.

Reaction of 5-Hydroxyzeaxanthin with $LiAlH_4$. Reaction of 0.383 mg of 5-hydroxyzeaxanthin, prepared by $LiAlH_4$ reduction of antheraxanthin as described above, with $LiAlH_4$ for 1 hr results in the recovery of 75% of total pigment based on absorption at 440 mμ. Chromatography on silica gel G–Celite (1:1) yields only the starting material, indicating that the 5-hydroxyl group is not removed under these conditions.

Reaction of Violaxanthin with $LiAlH_4$. Violaxanthin, a diepoxide found in algae and higher plants, when treated with $LiAlH_4$ and chromatographed on silica gel G–Celite also yields gradient elution patterns suggestive of conversion of the 5,6-epoxides to hydroxyl and olefin derivatives.

Discussion

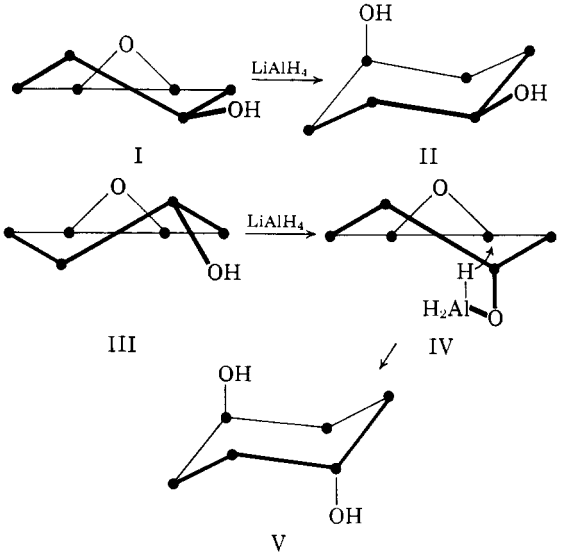
The reduction of a substituted epoxide with $LiAlH_4$ usually results in the formation of the most highly substituted carbinol *via* an S_N2 mechanism (Brown, 1951). In the case of carotenoid 5,6-epoxides, where both C-5 and C-6 are fully substituted, hydride attack would be expected to take place preferentially at the most electrophilic carbon atom, *i.e.*, C-6, leading to the formation of the 5-hydroxyl derivative (Pullman and Pullman, 1963). This derivative is indeed one of the major products of $LiAlH_4$ reduction of antheraxanthin and neoxanthin, as reported here, and is also the major product of $LiAlH_4$ reduction of 5,6-epoxy-β-carotene (Grob and Siekmann, 1965).

The formation of the 5,6-olefin at the same concentration as the 5-hydroxyl derivative from the reduction of both antheraxanthin and neoxanthin was not predictable from our knowledge of hydride reduction of epoxides. Inasmuch as the 5-hydroxyl group formed is tertiary and therefore susceptible to base-catalyzed elimination, we explored the possibility that the 5,6-olefin was formed *via* dehydration of the 5-hydroxyl intermediate. Neochrome, the 5,8-furanoid oxide of neoxanthin which contains a tertiary 5′-hydroxyl group (Curl, 1965; Schimmer and Krinsky, 1966), and 5-hydroxyzeaxanthin, prepared by $LiAlH_4$ reduction of antheraxanthin, were treated with $LiAlH_4$ under experimental conditions similar to those which produced a 5,6-olefin from either antheraxanthin or neoxanthin. An olefin derivative was not formed from either 5-hydroxyl group under these conditions, indicating that the formation of the 5,6-olefin from the 5,6-epoxide does not involve the 5-hydroxyl group as an intermediate. Inasmuch as Grob and Siekmann (1965) did not observe olefin formation during $LiAlH_4$ reduction of 5,6-epoxy-β-carotene, our results indicate that the 3-hydroxyl group on both antheraxanthin and neoxanthin influences the course of reduction and directs the reaction toward olefin formation. In lithium metal reductions of epoxides, an analogous influence of vicinyl *cis*-hydroxyl groups leading to olefin formation has been observed (Hallsworth and Henbest, 1960).

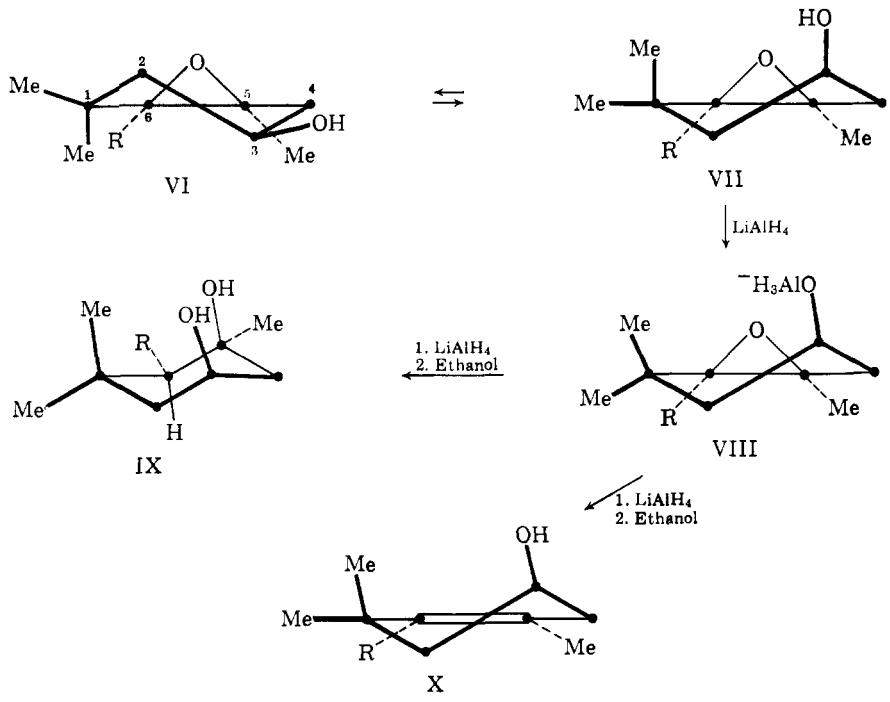
Henbest and Nicholls (1957) have shown that $LiAlH_4$ reduction of *cis*-3,4-epoxycyclohexanol (I) forms the *cis*-1,4-diol (II) whereas reduction of the *trans*-3,4-epoxycyclohexanol (III) forms the *trans*-1,4-diol (V) instead of the expected *trans*-1,3-diol. They interpreted the formation of the *trans*-1,4-diol as being due to an intramolecular reduction of the epoxide by the $OAlH_3^-$ group on the C-1 position (IV) (Scheme III). If the 3-hydroxyl group on antheraxanthin or neoxanthin were *trans* to the 5,6-epoxide, we might expect the formation of the 6-hydroxyl derivative by an analogous intramolecular reduction of the epoxide with $OAlH_3^-$. We do not observe this intramolecular direction by the 3-hydroxyl group, further supporting the suggestion that the 3-hydroxyl and 5,6-epoxide groups on both antheraxanthin and neoxanthin are in a *cis* configuration as described in Results.

There are two possible chair conformations that the 3-hydroxy-5,6-epoxyionone ring of antheraxanthin and neoxanthin could assume: one with the hydroxyl group equatorial (VI) and one with the hydroxyl group axial (VII) (Scheme IV). With the 3-hydroxyl group equatorial, the epoxide would be expected to open at the 5 position to give the axial C-6 alcohol, whereas with the 3-hydroxyl group axial, the epoxide would be expected to open at the 6 position to yield the axial C-5 alcohol IX (Eliel, 1956). Since the C-5 alcohol is the derivative formed, we conclude that the 5,6-epoxide of both antheraxanthin and neoxanthin open with the ionone ring in the conformation depicted by structure

SCHEME III



SCHEME IV



VII. In this conformation, the axial anionic aluminum hydride intermediate (VIII) must exert a strong inhibitory effect on the formation of the 5-hydroxyl derivative IX due to charge repulsion. Inhibition of the formation of the 5-hydroxyl derivative might thus permit the second type of reaction to occur, *i.e.*, formation of the 5,6-olefin X (Scheme IV).

The first fractions isolated from the reactions of antheraxanthin and neoxanthin with $LiAlH_4$ have not been completely characterized. These unexpected carbonyl-like compounds may arise during the decomposition of the aluminum hydride complex with ethanol. These fractions no longer contain the 5,6-epoxide group, but the first fraction from the reaction of neoxanthin appears to have the 5′-hydroxyl group still intact, suggesting that these fractions are products of the reaction of $LiAlH_4$ with the 5,6-epoxide portions of the carotenoids.

That both antheraxanthin and neoxanthin appear to contain a 3-hydroxy-5,6-epoxyionone ring in *cis* configuration is indicative of their close biosynthetic relationship. The biosynthesis of neoxanthin might proceed *via* hydration of the 5′,6′ double bond of antheraxanthin. This type of reaction has been proposed by Jensen *et al.* (1961) for the formation of tertiary hydroxyl groups on the carotenoids of purple bacteria.

The conversion of antheraxanthin to zeaxanthin by $LiAlH_4$ duplicates the reaction of antheraxanthin deepoxidase described by Bamji and Krinsky (1965). If the mechanisms of the chemical and biochemical reactions are analogous, the biochemical deepoxidation need not proceed through an hydroxylated intermediate as proposed by these authors. That the possible hydroxylated intermediate has not been isolated as a naturally occurring compound from *E. gracilis* lends support to this analogy.

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Purification and Characterization of Phytochrome from Oat Seedlings*

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ABSTRACT: Phytochrome was extracted from etiolated oat seedlings and purified 750-fold by a four-step procedure involving chromatography on calcium phosphate, chromatography on Sephadex G-200, continuous electrophoresis on a free-flowing film, and finally gel filtration on Bio-Gel P-150. The product obtained was homogeneous to electrophoresis on cellulose polyacetate or acrylamide gel. Exclusion chromatography indicated that phytochrome had a molecular weight of about 60,000. The molecular extinction coefficients for the red-absorbing form maxima were ϵ_{280} 82,000, ϵ_{382} 26,000, and ϵ_{664} 76,000.

The action spectra of several light-induced morphological changes in plant development, including germination and the inception of flowering and of dormancy, suggest that they are all controlled by a single light-sensitive pigment which exists in two readily interconvertible forms (Borthwick *et al.*, 1952a,b; Hendricks *et al.*, 1956; Hendricks and Borthwick, 1959; Borthwick and Hendricks, 1960). The one form, P_R, has an absorption maximum at about 665 mμ; the other form, P_{FR}, has a maximum at about 725 mμ. These forms are interconverted by irradiation with light of the appropriate wavelength.

$$P_R \underset{\text{far-red light}}{\overset{\text{red light}}{\rightleftharpoons}} P_{FR}$$

This pigment, called phytochrome, was first detected in plant extracts by Butler *et al.* (1959) and was subsequently partially purified by Siegelman and Firer (1964). This paper describes the preparation and characterization of high-purity phytochrome from oat seedlings.

Experimental Section

Assays. The unit of phytochrome was previously defined as that quantity which dissolved in 1 ml of solution gave a ΔA_{665} (absorbancy P_R − absorbancy P_{FR}) of 1 in a 1-cm path (Mumford, 1966). For routine assay of column eluates, ΔA was estimated by use of a "Ratiospect" spectrophotometer with a built-in sample irradiator (Model R-2 Agricultural Specialty Co., Inc., Hyattsville, Md.). To obtain red light for sample irradiation the output of a Sylvania DFA T12 "Tru-Flector" projection lamp was passed through a 660-mμ Bausch and Lomb interference filter; far-red light was obtained by use of a 730-mμ interference filter. In column monitoring samples were irradiated for 0.5-min periods. However, in assays for final specific activity, 2.5-min irradiation periods were used. When the phytochrome concentration was greater than 40 units/l., the sample cup was only partially filled and the ΔA calculated by using the appropriate factor.

For reasons of convenience and sample preservation, protein was usually estimated by ultraviolet absorption at 280 mμ using the nomograph prepared by E. Adams (California Corp. for Biochemical Research, Los Angeles, Calif.) which is based on the extinction coefficients for enolase and nucleic acid given by Warburg and Christian (1942). Determination of the protein

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