# Optical Properties of Astaxanthin Solutions and Aggregates*

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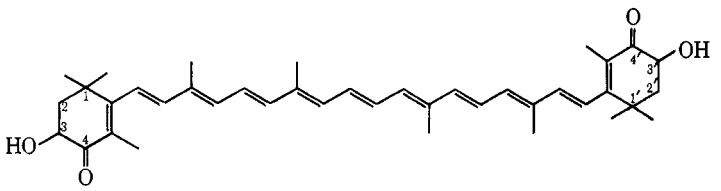
ABSTRACT: The absorption maximum of astaxanthin is dependent upon the refractive index, but shows no relationship to the dielectric constant or the charge solvating ability of the solvent. Astaxanthin is reversibly protonated by trichloroacetic and trifluoroacetic acids in benzene to give a product with maximal absorption at 840 mμ and reacts irreversibly with hydrochloric acid in aqueous ethanol to give a product with maximal absorption at 648 mμ. Dispersions of astaxanthin in predominantly aqueous media have an absorption maximum near 455 mμ, which is shifted to near 555 mμ in the presence of sodium chloride. In the presence of sodium perchlorate and related salts the dispersions are converted into a new yellow species with an absorption maximum near 400 mμ. Astaxanthin exhibits a normal optical rotatory dispersion spectrum, but the yellow aggregate has a markedly increased maximum molar rotation of 90,000 near the absorption maximum and Cotton effects above and below the maximum. It is suggested that the optical properties of the yellow aggregate result from π-electron interactions between the carotenoid molecules, some of which may be described by the exciton model, and that the same type of interactions are responsible for the similar properties of the yellow lobster shell pigment.

The prosthetic group of the blue lobster shell pigment, crustacyanin, and the yellow lobster shell pigment is the orange-red carotenoid, astaxanthin (3,3′-hydroxy-4,4′-oxo-β-carotene, I) (Kuhn and Sorensen, 1938a,b; Wald *et al.*, 1948; Jencks and Buten, 1964; Cheesman *et al.*, 1966).

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In this paper we report the results of an investigation of the optical properties of this carotenoid under a variety of experimental conditions, which was carried out in the hope of obtaining information regarding the factors which are responsible for the remarkable spectral perturbations undergone by this molecule and other carotenoids upon combination with their apoproteins. It has previously been shown that astaxanthin is converted into a blue pigment upon treatment with strong base in the absence of oxygen (Kuhn and Sorensen, 1938a,b). Some of the results described in this and the accompanying paper have been reported in a preliminary communication (Buchwald and Jencks, 1967).

## Experimental Section

Materials were of reagent grade when available and organic reagents were generally recrystallized or redistilled before use. Pyridine was redistilled from barium oxide and benzene was stored over soduim metal. Hexane was spectral grade. Generous samples of cantaxanthin and lutein were gifts of Dr. Norman I. Krinsky. Crustaxanthin was prepared by the reduction of a methanolic solution of astaxanthin with sodium borohydride (Krinsky and Goldsmith, 1960). Astacene was prepared by allowing a solution of astaxanthin in 12% methanolic potassium hydroxide to undergo air oxidation at 37° for 1 hr. Silica gel was Grade 950 (60–200 mesh) from Will Corp.

Astaxanthin was extracted from carefully cleaned and ground lobster shells with acetone at room temperature. The astaxanthin was extracted from acetone into methylene chloride, which was then washed with concentrated aqueous sodium chloride, dried over sodium sulfate, and evaporated to an oil with a rotatory evaporator. Astaxanthin was crystallized from methylene chloride–petroleum ether (bp 30–60°) at −20°. Crystals were also obtained by a similar procedure in which petroleum ether was substituted for methylene chloride, but the product was impure, as judged by its extinction coefficient. The crystals were stored in the dark at −20°. Exposure to excessive light, base, or temperatures higher than 35° was avoided during the purification procedure.

Absorption spectra were taken with a Perkin-Elmer Model 350 recording spectrophotometer, which was calibrated for wavelength and absorbance with filters

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TABLE I: Effect of Solvents on the Absorption Spectrum of Astaxanthin.

| Solvent | $\lambda_{max}$ (mμ) | $\nu'_{max}$ ($cm^{-1}$) | $\nu^1/_2$ ($cm^{-1}$)[a] | $n^b$ |
|---|---|---|---|---|
| Methanol | 473 | 21,140 | 4,250 | 1.329 |
| Acetonitrile | 474 | 21,100 | 4,150 | 1.345 |
| Acetone | 475 | 21,050 | 4,100 | 1.359 |
| Ethanol | 476 | 21,010 | 4,200 | 1.362 |
| Hexane | 472 | 21,190 | 4,200 | 1.365 |
| 1-Propanol | 478 | 20,920 | 4,200 | 1.384 |
| Dioxane | 482 | 20,750 | 4,100 | 1.420 |
| Dichloromethane | 486 | 20,580 | 4,100 | 1.424 |
| Cyclohexane | 478 | 20,920 | 4,150 | 1.429 |
| Chloroform | 489 | 20,450 | 4,100 | 1.446 |
| Carbon tetrachloride | 485 | 20,620 | 4,150 | 1.460 |
| Dimethyl sulfoxide | 492 | 20,330 | 4,200 | 1.479 |
| Benzene | 488 | 20,490 | 4,100 | 1.501 |
| Pyridine | 492 | 20,330 | 4,200 | 1.509 |
| Chlorobenzene | 493 | 20,280 | 4,100 | 1.525 |
| Iodomethane | 496 | 20,160 | 4,150 | 1.530 |
| Ethyl cinnamate | 496 | 20,160 | 4,150 | 1.560 |
| Carbon disulfide | 506 | 19,760 | 4,100 | 1.624 |
| Petroleum ether (36–52°) | 473 | 21,140 | 4,250 | |

[a] Half-band width: frequency difference between positions of half-maximal absorbance; ±100 $cm^{-1}$. [b] Refractive index.

supplied by the manufacturer. Kinetic experiments were usually carried out with a Zeiss PMQ-II spectrophotometer fitted with a brass cell holder in the cell compartment which was thermostated at 25°. Spectra were taken in 1-cm path-length cells against a solvent blank. Spectra of astaxanthin in different solvents were obtained by dissolving astaxanthin crystals in methylene chloride, evaporating aliquots of this solution to dryness to give the more soluble amorphous form of astaxanthin in the bottom of each tube, and finally adding the desired solvent. Spectra in hexane were taken with a solution saturated with astaxanthin crystals. The spectrum in ethanol solution of crystalline astaxanthin was shown to be identical with that of astaxanthin obtained from acetone-denatured α-crustacyanin.

The extinction coefficient of astaxanthin in pyridine was determined directly with crystalline material which was dried under vacuum in the dark for 2 days over paraffin and phosphorous pentoxide and was then weighed on a microbalance. The extinction coefficient in other solvents was obtained by adding 10 μl of a concentrated solution of astaxanthin in pyridine to 1.0 ml of the desired solvent and comparing the absorbance at the $\lambda_{max}$ with that of a solution diluted into pyridine. These experiments were carried out in quadruplicate.

Optical rotatory dispersion spectra were obtained with a Cary Model 60 recording spectropolarimeter at 22° with 1-cm path-length cells. The optical rotatory dispersion spectra are given in terms of the molar rotation [M] per molecule of astaxanthin, obtained from the relationship [M] = [α](molecular weight)/100, in which [α] is the specific rotation and [M] is in units of degrees $cm^2$/dmole (Fasman, 1963).

Solutions of trichloroacetic and trifluoroacetic acids in benzene were prepared each day for experiments on the reaction of astaxanthin with these acids. The spectra were obtained immediately after the addition of astaxanthin. Absorbance measurements for determination of the equilibrium constants were extrapolated to zero time and each experiment was carried out in triplicate. The absorbance at complete reaction was obtained from the reciprocal of the ordinate intercept of a reciprocal plot of the change in absorbance against acid concentration. The equilibrium constant was calculated for each experimental point from in

$$K = \frac{A - A^0}{A_\infty - A} \frac{1}{[\text{acid}]} \qquad (1)$$

which $A$ is the measured absorbance, $A^0$ is the absorbance in the absence of acid, and $A_\infty$ is the absorbance at the extrapolated end point of the reaction. The reported value of $K$ is the average of the individual determinations.

## Results

*Spectra of Astaxanthin in Solution.* The spectral properties of astaxanthin in a series of 19 solvents

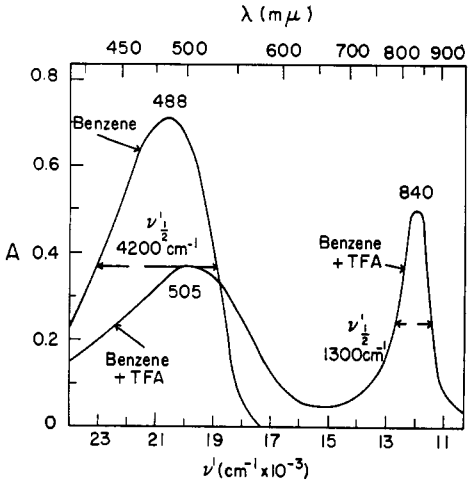



FIGURE 1: Spectra of astaxanthin in benzene and in 0.95 M trifluoroacetic acid in benzene.

are summarized in Table I. The absorption maximum ranges from 472 mμ in hexane to 506 mμ in carbon disulfide. Where comparisons are possible, the maxima agree with values reported by Cheesman (1958), but are 0–2 mμ higher than those reported by Goodwin and Srisukh (1949). The half-band width of 4200 $cm^{-1}$ and the shape of the absorption band do not vary significantly in the different solvents. The single broad absorption maximum is characteristic of polyenes containing carbonyl groups which are conjugated with the double-bond chain (Jaffee and Orchin, 1962). The ratio of absorbance in the visible to that in the ultraviolet at 280 mμ is 6.6; this is larger than the value from a previously reported spectrum (Karrer and Wurgler, 1943).

The absorption maximum of astaxanthin adsorbed on silica gel (Leermakers *et al.*, 1966) is at 500 mμ, an increase of 22 mμ over that in the cyclohexane used to suspend the silica gel. There is an increase of about 20% in the band width, which may represent heterogeneity of the bound dye. A very similar color was obtained when astaxanthin was adsorbed onto activated alumina, but it was not possible to obtain a spectrum of this turbid suspension.

Several experiments were carried out to determine whether molecules which could serve as π-electron donors or acceptors would influence the spectrum of astaxanthin. Durene (1 M) and chloranil (0.01 M) in methylene chloride and 0.04 M tryptophan in methanol (Ishigami *et al.*, 1966) were found to have no effect on the spectrum of astaxanthin. Indole (5 M) in ethanol causes an increase from 477 to 496 mμ and 2 M phenanthrene in methylene chloride causes an increase from 487 to 494 mμ in the absorption maximum, but these compounds cause no change in the extinction coefficient or half-band width of astaxanthin. In the presence of tetracyanoethylene, astaxanthin undergoes rapid decomposition to a product absorbing at 380 mμ, which then undergoes further decomposition to products with no visible absorption. No new absorption bands appeared in the near-infrared region (700–2000 mμ) in the presence of these reagents. At a concentration of $5 \times 10^{-6}$ M astaxanthin was found to exhibit no detectable fluorescence at wavelengths up to 700 mμ when the solution was excited at 475, 320, and 270 mμ.

TABLE II: Molar Extinction Coefficient ($\epsilon_{max}$) of Astaxanthin.

| Solvent | λ (mμ) | $\epsilon_{max}$ Solvent/ $\epsilon_{max}$ Pyridine | $\epsilon_{max}$ |
|---|---|---|---|
| Pyridine | 492 | 1.00 | $1.12 \times 10^5$ |
| Hexane | 472 | 1.11 ± 0.02 | $1.24 \times 10^5$ |
| Methanol | 473 | 1.10 ± 0.02 | $1.23 \times 10^5$ |
| Benzene | 489 | 1.01 ± 0.02 | $1.13 \times 10^5$ |

The molar extinction coefficient of astaxanthin in pyridine was found to be $1.12 \times 10^5$ (Table II), which is in agreement with the value of $1.13 \times 10^5$ reported by Kuhn *et al.* (1939), but appears to be smaller than a value obtained from a figure published by Karrer and Wurgler (1943) and is larger than a value of $0.98 \times 10^5$ obtained by Wald.[1] The extinction coefficient is 10% higher in hexane and methanol than in the aromatic solvents, pyridine and benzene (Table II). Wald has obtained values of $1.08 \times 10^5$ and $1.27 \times 10^5$ for the extinction coefficients of astaxanthin in ethanol and hexane, respectively. The value of the extinction coefficient was also estimated by determining the ratio of the extinction coefficients of astaxanthin and crustaxanthin from measurements of the absorption of an astaxanthin solution in methanol before and after reduction to crustaxanthin by sodium borohydride. From this ratio (0.89) and the extinction coefficient of $1.40 \times 10^5$ for crustaxanthin in hexane (Bodea *et al.*, 1966) a value of $1.25 \times 10^5$ for the extinction coefficient of astaxanthin in hexane is obtained. The value in hexane is identical with the extinction coefficient of canthaxanthin (4,4′-oxo-β-carotene) in hexane (Davies, 1965), as might be expected because nonconjugated hydroxyl groups should not affect the extinction coefficient. The addition of 5% water to pyridine solutions was found to have no effect on the extinction coefficient of astaxanthin.

It was noted that there is a tendency for preparations of astaxanthin in apparently clear solutions of hexane or 50–70% dimethyl sulfoxide in water to exist as aggregates or crystals which are dispersed in the solvent in such a way that the apparent extinction coefficient is reduced manyfold. The normal absorption returns if additional dimethyl sulfoxide or another solvent is added so as to permit true solution of the pigment. This phenomenon is similar to the almost

[1] Personal communication from Professor G. Wald.

TABLE III: Equilibrium Constants for the Reaction of Astaxanthin and Acids in Benzene.

| Acid | Concn of Acid (M) | Astaxanthin (M × $10^6$) | Wavelength | $K$ ($M^{-1}$) |
|---|---|---|---|---|
| TCA[a] | 0–5.5 | 4.4 | Visible | 0.4 ± 0.1 |
| TFA[b] | 0–0.95 | 6.3 | 840 mμ | 1.2 ± 0.2 |
| TFA | 0–0.95 | 4.9 | Visible | 1.5 ± 0.2 |
| TFA | 0–0.95 | 9.6 | 840 mμ | 1.1 ± 0.2 |
| TFA | 0–0.95 | 9.6 | Visible | 1.6 ± 0.2 |

[a] Trichloroacetic acid. [b] Trifluoroacetic acid.

complete disappearance of color observed with crustacyanin in the presence of certain solvents (Jencks and Buten, 1964).

*Reactions of Astaxanthin with Acids.* The addition of trifluoroacetic acid to a solution of astaxanthin in benzene results in the appearance of a new absorption peak with a maximum at 840 mμ and a decrease in the absorption in the visible region (Figure 1). The new peak is much narrower ($\nu'_{1/2}$ = 1300 $cm^{-1}$) than the peak in the visible region and has an extinction coefficient about 1.5 times larger. At high concentrations of acid the peak in the visible region is shifted to 505 mμ and is slightly broadened. Similar results were obtained with higher concentrations of trichloroacetic acid. The addition of an equal volume of dioxane causes an immediate disappearance of the 840-mμ peak and the reappearance of the absorption in the visible region. The addition of dioxane within a period of 5 min after the addition of acid gives a recovery of more than 90% of the original absorbance, but acid causes a decomposition of the pigment on longer standing and 9 M trifluoroacetic acid causes an irreversible destruction of astaxanthin in 1 min.

Approximate values of the equilibrium constants for the reactions of astaxanthin with trichloroacetic and trifluoroacetic acids in benzene were obtained from measurements of the absorption in the visible and near-infrared regions at zero time after mixing, as described in the Experimental Section. An example of a reciprocal plot which was used to estimate these equilibrium constants, from measurements at 840 mμ in the presence of trifluoroacetic acid, is shown in Figure 2. The equilibrium constant of 0.4 ± 0.1 $M^{-1}$ for the reaction of trichloroacetic acid with astaxanthin (Table III) is similar to the value determined by Wassermann (1954) for the reaction of trichloroacetic acid with β-carotene. The larger value of 1.4 ± 0.2 $M^{-1}$ for the reaction of trifluoroacetic acid with astaxanthin shows the expected increase for this stronger acid.

In contrast to these reactions, the reaction of astaxanthin with 4 M hydrochloric acid in 50% ethanol–water occurs with a slow appearance of a sharp new absorption maximum at 648 mμ ($\nu'_{1/2} \sim$ 2600 $cm^{-1}$) and a concomitant decrease of absorbance at 476 mμ (Figure 3). This reaction is irreversible on neutraliza-

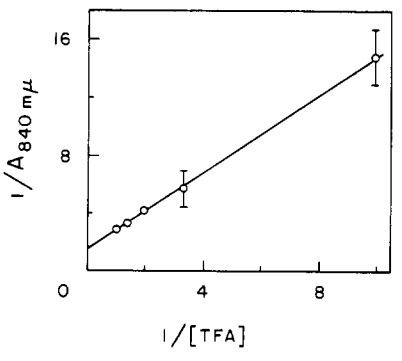



FIGURE 2: Reciprocal plot of the absorbance at 840 mμ against trifluoroacetic acid concentration used to determine the equilibrium constant for the reaction of trifluoroacetic acid with astaxanthin in benzene.

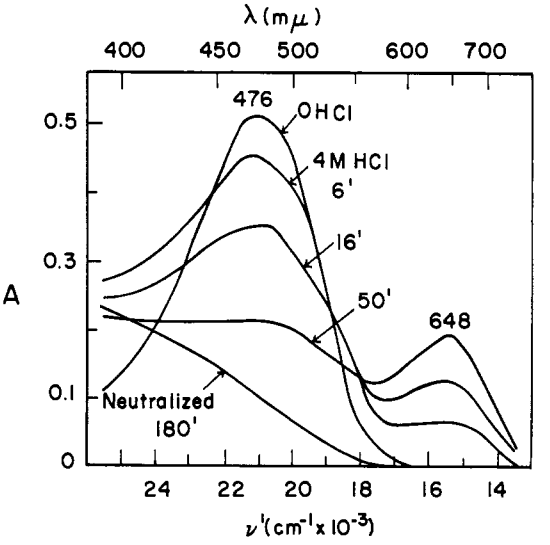



FIGURE 3: The change in the spectrum of astaxanthin with time in 50% ethanol (v/v) containing 4 M hydrochloric acid. The absorbance of the sample containing no acid has been multiplied by 0.55.

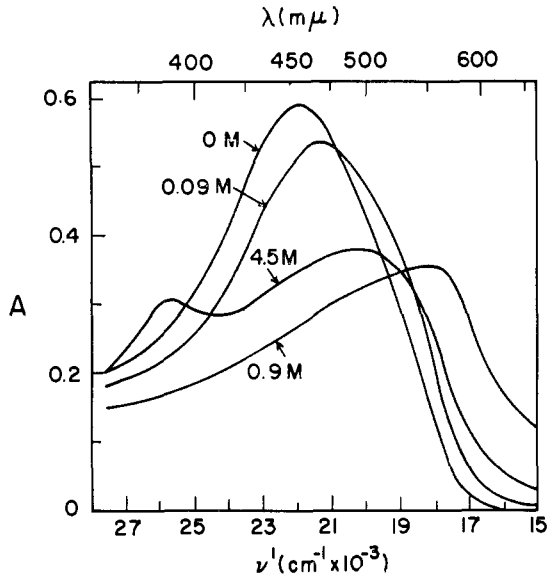



FIGURE 4: The effect of different concentrations of sodium chloride on the spectrum of a suspension of astaxanthin in water containing 10% ethanol.

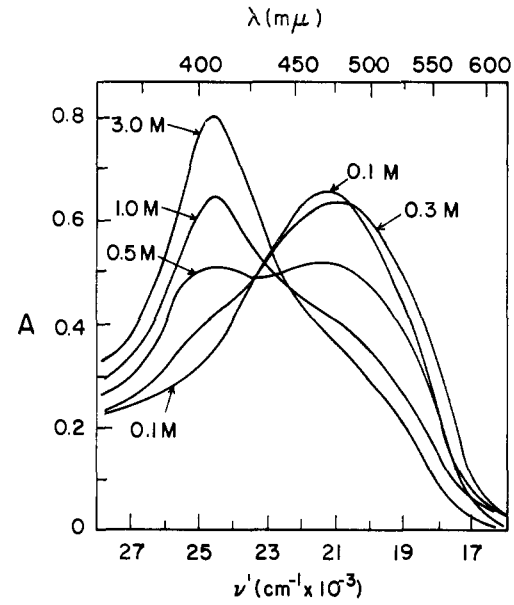



FIGURE 5: The effect of different concentrations of sodium perchlorate on the spectrum of a suspension of astaxanthin in water containing 10% ethanol.

tion and is followed by a further decomposition reaction which leads to the disappearance of visible absorption.

*Spectral Properties of Astaxanthin in Predominantly Aqueous Medium.* The addition of small quantities of concentrated solutions of astaxanthin in water-miscible solvents, such as ethanol and pyridine, to a large volume of water gives a stable suspension of pigment aggregates or crystals, which is usually almost or completely clear to the eye and which can be examined spectroscopically without special equipment. The absorption maximum of such a preparation in 10% ethanol is near 455 mμ, compared to 475 mμ in ethanol, and shows a broadening of the peak and a decrease in the extinction coefficient of about 35%.

The presence of sodium chloride causes a shift in the absorption maximum of such preparations to longer wave'engths, as shown in Figure 4. In the presence of 0.9 M sodium chloride the absorption maximum is near 555 mμ. The normal spectrum of astaxanthin is observed if ethanol is added to these preparations to a concentration of 50% within a few minutes, but on prolonged standing there is an irreversible decomposition of the astaxanthin. Similar preparations may be obtained from astaxanthin and very small quantities of the nonionic detergent Brij 98 in the absence of salt. Astaxanthin in pyridine and detergent in water were evaporated to dryness and resuspended in water with vigorous shaking. The product with a molar ratio of astaxanthin to detergent of between 5:1 and 8:1 has an absorption maximum at 500 mμ.

At very high concentrations of sodium chloride a small peak near 390 mμ appears (Figure 4). A yellow product with maximum absorption in this region is formed upon the addition of astaxanthin to more dilute solutions of a number of salts, including sodium iodide, sodium perchlorate, and sodium hydroxide. Spectra at a series of different concentrations of sodium perchlorate are shown in Figure 5. The extinction coefficient at the absorption maximum of 410 mμ of this product in 7.2 M sodium iodide is 80% of that in ethanol. The formation of the yellow product is fully reversible upon further addition of an equal volume of ethanol. The order of addition of the components does not affect the results: similar results are obtained if astaxanthin is dispersed into water followed by the addition of salt, if it is dispersed into concentrated salt and then diluted with water, or if it is dispersed directly into the dilute salt solution. The presence of detergents such as desoxycholate (an anionic detergent) or Brij 98 (a neutral detergent) at concentrations of 0.5–1% completely prevents the appearance of the yellow product. The preparations in the presence of 4.3 M sodium perchlorate were shown to follow Beer's law at the absorption maximum of 407 mμ up to an absorbance of 1.3.

The effectiveness of different salt solutions in bringing about the formation of the yellow product, as measured by the ratio of the absorbance of astaxanthin suspensions at 407 and 475 mμ, generally follows the Hofmeister series, with the notable exception of sodium hydroxide, which is the most active salt examined (Table IV). The differences in effectiveness are primarily a function of the nature of the monovalent anion; variation of the cation or the addition of salts of polyvalent anions has little effect. Variation of the pH from 1.5 to 12 has no significant effect on the spectral properties of the suspensions in sodium chloride or sodium perchlorate, except that at the

TABLE IV: Effect of Salts on $A_{407}/A_{475}$ of Suspensions of Astaxanthin in Water Containing 5% Ethanol.

| Salt Concn | $A_{407}/A_{475}$ 0.48 M | 1.9 M | 3.8 M |
|---|---|---|---|
| | Varying Anion | | |
| NaOH | 1.2 | 2.1 | |
| $NaClO_4$ | 1.0 | 1.9 | 2.25 |
| NaSCN | 0.7 | 1.55 | 1.85 |
| NaI | 0.85 | 1.45 | 1.75 |
| $NaClO_3$ | 0.5 | 0.8 | 1.35 |
| NaBr | 0.5 | 0.5 | 1.1 |
| $NaNO_3$ | 0.5 | 0.55 | 1.0 |
| NaCl | 0.45 | 0.45 | 0.65 |
| NaHCOO | 0.5 | 0.5 | 0.5 |
| $NaH_2PO_4$ | 0.5 | 0.5 | 0.45 |
| $NaCH_3COO$ | 0.5 | 0.5 | 0.4 |
| $Na_2SO_4$ | 0.5 | 0.5 | 0.5 |
| $Na_2S_2O_3$ | 0.5 | 0.45 | |
| $Na_3$ Citrate | 0.5 | | |
| | Varying Cation | | |
| NaCl | 0.5 | 0.5 | |
| $NH_4Cl$ | 0.5 | 0.5 | |
| LiCl | 0.55 | 0.55 | |
| CsCl | 0.5 | 0.5 | |
| $MgCl_2$ | 0.55 | 0.55 | |
| $CdCl_2$ | 0.55 | 0.5 | |
| $BaCl_2$ | 0.55 | 0.5 | |
| Water | 0.5 | | |
| Ethanol | 0.35 | | |

highest pH values the preparations in sodium chloride show the appearance of a yellow peak caused by the increase of sodium hydroxide concentration.

The color could be completely removed from preparations of astaxanthin in 2 M sodium nitrate, 2 M sodium chloride, or 3 M sodium perchlorate by centrifugation for 40 min at 35,000*g*. A preliminary experiment revealed no difference in the sedimentation rate of suspensions prepared in 2 M sodium chloride and 4 M sodium perchlorate, suggesting that there is not a large difference in the size of the aggregates in these two solvents, but a detailed study of the size of the aggregates has not been carried out.

Dispersion of astaxanthin in ethanol into 8 M urea, 3 M guanidine hydrochloride, or 6 M propionamide or into desoxyribonucleic acid (2 mg/ml), protamine sulfate (6 mg/ml), bovine serum albumin (7 mg/ml), or several synthetic polypeptides (7 mg/ml) gave spectra very similar to that of a dispersion in water.

A series of similar experiments was carried out with β-carotene, cantaxanthin, astacene, lutein, and crustaxanthin in the presence of 1 M sodium chloride and 3 M sodium perchlorate. These suspensions showed a broadening of the absorption and, in some cases, the appearance of a new peak at higher a wavelength

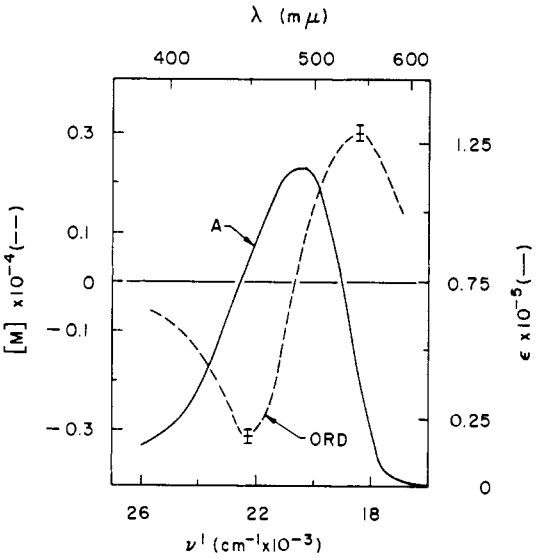



FIGURE 6: Optical rotatory dispersion (- - -) and absorption (—) spectra of axtaxanthin (2.7 and 4.9 × $10^{-5}$ M) in methylene chloride.

similar to that observed previously in aqueous suspensions of carotenoids (Karrer and Straus, 1938; Shibata, 1956), but in no case was the appearance of a strong absorption band near 400 mμ similar to that of astaxanthin observed.

*Optical Rotation.* Astaxanthin does not exhibit detectable optical rotation at the concentrations ordinarily used for spectroscopy, but in more concentrated solutions it exhibits a normal optical rotation curve with a Cotton effect at the same wavelength as the absorption maximum and a maximum molar rotation of 3000 (Figure 6).

In contrast, dilute dispersions of astaxanthin in sodium hydroxide show a much stronger optical rotation with a maximum molar rotation of 90,000 in the region of the absorption maximum (Figure 7; note the difference in the ordinate scales of Figures 6 and 7). This optical rotation is remarkable in that the maximum positive rotation occurs at 400 mμ, very close to the absorption maximum of the astaxanthin dispersion at 402 mμ, and crosses the base line at wavelengths above and below the absorption maximum. Red dispersions of the type formed in the presence of sodium chloride do not exhibit detectable optical rotation at the concentrations ordinarily used for spectroscopy.

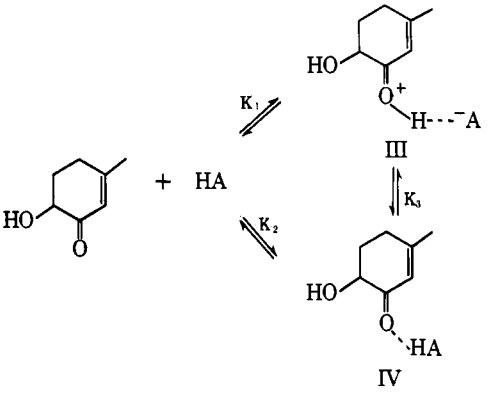
## Discussion

*Solvent Dependence of the Spectrum of Astaxanthin.* In order to evaluate the possibility that the differences between the spectra of astaxanthin in solution and in the lobster shell pigments might arise from a solvent effect of the protein environment, the spectrum of astaxanthin was examined in a number of solvents.

The sensitivity of the absorption band to the nature of the solvent is small and, as shown in Figure 8, the position of the absorption maximum shows only the dependence on the refractive index of the medium which is expected of any electronic transition and which is exhibited by cyanine dyes (Bayliss, 1950; West and Geddes, 1964). Plots of the excitation energy against $z$ values, based on the excitation energies of the charge-transfer band of pyridinium halides (Kosower, 1958), $x_R$ and $x_B$ values, based on merocyanine dyes (Brooker *et al.*, 1965), and the dielectric constant of the solvent resemble scatter diagrams and show no correlation with these parameters. Adsorption onto silica gel, which causes large shifts in the absorption maxima of compounds with differing polarities of their ground and excited states (Leermakers *et al.*, 1966), also does not cause a large spectral shift. It is concluded that there is little or no difference in the polarity of the ground and excited states of astaxanthin and that there is no evidence for the existence of specific interactions between solvent molecules and astaxanthin which differentiate between the ground and excited states.

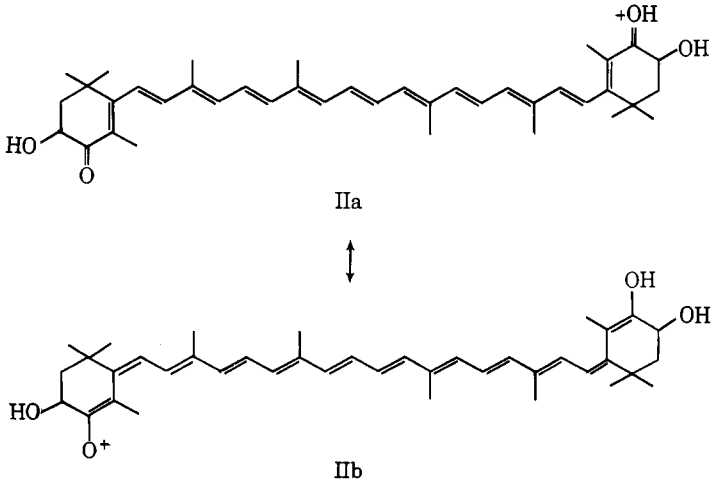
No new absorption maxima or large changes in spectrum which might be indicative of charge transfer or other types of interaction with aromatic molecules were observed in the presence of durene, chloranil, indole, phenanthrene, or tryptophan. No spectral change was observed in the presence of acid and tryptophan under the conditions in which the spectrum of retinal undergoes a change which was ascribed by Ishigami *et al.* (1966) to the formation of a charge-transfer complex. The 10% decrease in the extinction coefficient of astaxanthin in the aromatic solvents benzene and pyridine is probably caused by dispersion interaction between the carotenoid and the $\pi$-electron systems of the solvents, similar to that which accounts for the hypochromicity of the stacked bases of polynucleotides (Tinoco, 1960, 1961; Rhodes, 1961).

*Reactions of Astaxanthin with Acids.* The reversible reaction of astaxanthin with trichloroacetic and trifluoroacetic acids in benzene gives a new absorption maximum at 840 m$\mu$ accompanied by a decrease in the visible absorption band. The product is assigned the structure II, similar to the products of the reactions of acids with other carotenoids examined by Wassermann (1954, 1959a–d). The position of the new absorption band at 840 m$\mu$ is intermediate between that of protonated methylbixin, a carbonyl containing carotenoid, at 750 m$\mu$ and that of protonated $\beta$-carotene at 960 m$\mu$. Calculations based on Kuhn's free-electron model are consistent with protonation at a terminal position for the carotenoids and terminal protonation is supported by measurements of electronic and nuclear magnetic resonance spectra of protonated shorter chain polyenes (Wassermann, 1959a,c; Sorenson, 1965). Conductivity measurements indicate that the protonated carotenoids exist as ion pairs with dissociation constants on the order of $10^{-8}$ M (Wassermann, 1959d). Double-reciprocal plots of the decrease in visible absorption against acid concentration show that in the case of astaxanthin there is still a significant amount of visible absorption remaining at infinite acid concentration, at which complex formation must be complete. This fact and the observation that there is a shift in the visible absorption maximum to longer wavelengths in the presence of acid suggest that in the reaction with astaxanthin the product exists as a hydrogen-bonded complex with acid (IV) as well as a hydrogen-bonded ion pair (III). The observed equilibrium constant includes the equilibrium constants




for the formation of both III and IV. Alternatively, the remaining visible absorption may represent a species which is protonated on a hydroxyl group.

The increase in wavelength and the decrease in band width of the carotenoid absorption band upon protonation are attributed to the near equivalence of the resonance structures IIa and b, which results in a similar bond order for all of the bonds in the conjugated system of the protonated carotenoid. The differences in bond order and potential of the alternating single and double bonds of a carotenoid are believed to be responsible for the fact that the increase in the wavelength of the absorption maximum with increasing chain length levels off at relatively short chain lengths, whereas the absorption maximum of cyanine dyes, which have almost equivalent bond orders along the chain, increases steadily with increasing chain length



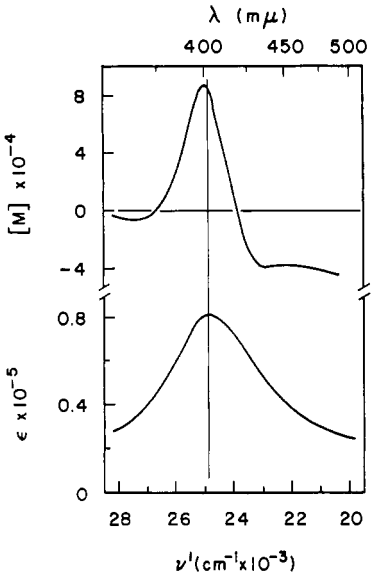



FIGURE 7: Optical rotatory dispersion (upper curve) and absorption (lower curve) spectra of an astaxanthin suspension in 1 M sodium hydroxide in water containing 5% pyridine.

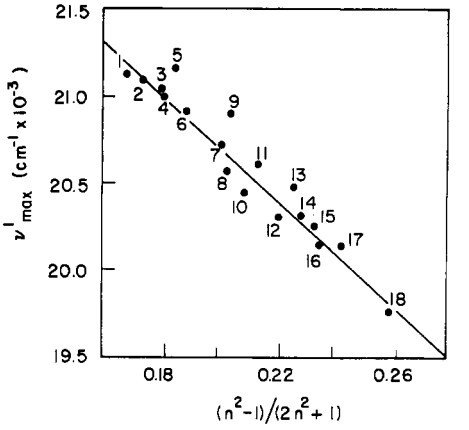



FIGURE 8: The effect of the refractive index of the solvent on the absorption maximum of astaxanthin. The numbers refer to the solvents listed in Table I.

(Kuhn, 1949; Platt, 1961). Consequently, cyanine dyes absorb at higher wavelengths than polyenes of equal chain length. The fact that the absorption maxima of protonated polyenes are at shorter wavelengths than predicted by the free-electron model suggests that the bonds are still not entirely equivalent (Wassermann, 1959a,c; Sorensen, 1965). The narrowing of the absorption band upon protonation is caused, in part, by loss of the vibrational coupling to the electronic transition arising from the alternation of single and double bonds, which is responsible for the splitting or broadening of the absorption band of free carotenoids (Kuhn, 1949). The protonated polyenes have a single electronic transition similar to that of the cyanine dyes and might be regarded, according to Platt's (1959) terminology, as strongly polarized polyenes.

The product which is formed in the time-dependent reaction of astaxanthin with hydrochloric acid in aqueous alcohol has an absorption maximum at 648 mμ, which is similar to that of the crustacyanins, but the shape of the absorption band is entirely different from that of astaxanthin or the protein pigments. This compound appears to be a degradation product of astaxanthin in which the alternation of bond order has been reduced compared to astaxanthin and which is similar to the products of reactions of carotenoids with Lewis acids, such as antimony trichloride and boron trifluoride (Carr and Price, 1962; Caldwell and Hughes, 1946; Wallcave *et al.*, 1953).

*Properties of Astaxanthin in Aqueous Media.* The spectra of dispersions of astaxanthin in water and in the presence of "hard" salts at one end of the Hofmeister series, such as sodium chloride, exhibit a broadening and change in shape which is suggestive of a change in the relative importance of the several vibrational transitions which are responsible for the broad absorption bands of carotenoids. Carotenoids with the usual triple-peaked absorption band show similar behavior in aggregates or crystalline suspensions and may exhibit a fourth peak under these conditions (Karrer and Straus, 1938; Shibata, 1956). The susceptibility of the carotenoids to oxidative degradation is greatly increased in such suspensions.

The presence of sodium chloride induces the formation of aggregates in which mutual interaction of the carotenoid molecules causes a shift in the absorption maximum to wavelengths as high as 555 mμ. The shape of the absorption band is different from that of monomeric astaxanthin, but heterogeneity in the particles may contribute to the broadening. The interaction between the π-electron systems of the astaxanthin molecules which is responsible for the spectral shift is presumably the same as that which is responsible for the spectral shifts of other unsaturated molecules, such as the cyanine dyes, in dimers or higher aggregates (Franck and Teller, 1938; Simpson and Peterson, 1957; DeVoe, 1964; West and Carroll, 1966). According to the exciton theory an increase in absorption maximum to higher wavelengths is expected if the molecules are arranged in the aggregate with a predominantly head-to-tail relationship of the transition dipoles (Levinson *et al.*, 1957; Kasha, 1963). However, the structure and π-electron interactions of these aggregates are not such as to cause an enhanced optical rotation.

The most interesting spectral change of astaxanthin, because of the similarity of the product to the native yellow lobster pigment and the yellow denaturation products of the crustacyanins, is the conversion into a yellow aggregate with an absorption maximum near 400 mμ, which is induced by high concentrations of sodium chloride and by lower concentrations of "soft" salts at the opposite end of the Hofmeister series from sodium chloride, such as sodium perchlorate. The

formation of this product is dependent on the nature of the anion of the salt. The order of effectiveness of the anions in the series $ClO_4^- > SCN^- \sim I^- > ClO_3^- > Br^- \sim NO_3^- > Cl^- > HCOO^-$, $H_2PO_4^-$, $CH_3COO^-$, $SO_4^{2-}$, $S_2O_3^{2-}$, citrate$^{3-}$ is essentially the same as has been observed for the interaction of salts with other compounds which contain carbonyl groups, such as acetyltetraglycine ethyl ester, acetic anhydride, and acetylimidazolium cation (Robinson and Jencks, 1965; Bunton *et al.*, 1962; Marburg and Jencks, 1962). This suggests that the action of these salts may involve an interaction with the carbonyl groups of astaxanthin to induce the formation of the particular structure of the aggregate which is responsible for the yellow color. The great effectiveness of hydroxide ion is anomalous according to the Hofmeister series, but hydroxide ion may have a specific tendency to interact with carbonyl groups. Salts are known to influence the tendency of cyanine dyes to undergo aggregation and changes in spectrum, presumably by salting out the uncharged portion and interacting directly with the charged groups of the dye molecule (Leermakers *et al.*, 1938). The yellow aggregate was obtained only with astaxanthin, but the yellow color is not dependent on the presence of carbonyl groups once the aggregate is formed, because it is maintained when the carbonyl groups of astaxanthin are reduced with sodium borohydride.

The absorption band of the yellow product is different in shape and narrower compared to that of astaxanthin, which means that there is a change in the nature as well as the energy of the electronic transitions in the aggregate. The decreased extinction coefficient of the yellow aggregate and the yellow lobster shell pigment (Buchwald and Jencks, 1968) compared to astaxanthin in hexane or methanol is similar to the hypochromism of interacting nucleotide bases and to the decrease in the extinction coefficient of astaxanthin in the aromatic solvents pyridine and benzene. These spectral changes are indicative of an interaction of the $\pi$-electron systems in the yellow aggregate which is different from that in the aggregates formed in water or moderately concentrated sodium chloride solutions. According to the exciton model, this type of spectral change may be caused by interaction of strongly coupled transition dipoles which are aligned in a parallel manner in the aggregate (Levinson *et al.*, 1957; Kasha, 1963).

The optical rotatory dispersion spectrum of astaxanthin is normal, with a Cotton effect at the absorption maximum and maximum positive and negative rotations above and below the absorption maximum, respectively. In contrast, the maximum molar rotation of astaxanthin in the yellow aggregate is increased in magnitude by 30-fold and occurs at almost the same wavelength as the absorption maximum, with Cotton effects above and below the absorption maximum. Such enhanced optical rotatory strength is well known in other aggregates or polymers with a helical or other definite structure, including polynucleotides, polypeptides, and dyes which undergo aggregation in the presence of asymmetric inducing agents (Tinoco *et al.*, 1963; Stryer and Blout, 1961; Mason, 1964). Although the situation is often complicated by the presence of multiple absorption bands, the optical rotatory dispersion and circular dichroism spectra of dyes which are aggregated in contact with optically active polymers or small molecules usually show a normal relationship to the absorption maximum; this is true for the narrow, intense *J* bands as well as other absorption bands of these aggregates which are commonly attributed to exciton interaction (Styrer and Blout, 1961; Mason, 1964). The dependence on wavelength of the optical rotation of the yellow aggregate and of the optical rotation and circular dichroism of the yellow pigment (Buchwald and Jencks, 1968) is indicative of a splitting of the main absorption band into two neighboring absorption bands of opposite rotational direction. This type of behavior is seen also in di- or polynucleotides and may be explained by an exciton interaction which causes a splitting of the absorption band if there is an angle between the transition dipoles, as in a helical structure (Tinoco *et al.*, 1963; Warshaw *et al.*, 1965; Van Holde *et al.*, 1965). However, the same exciton splitting cannot be invoked to explain both the large shift in the wavelength of the absorption maximum and the small splitting which causes the optical rotatory behavior of the yellow pigments.

The close similarities of the absorption and optical rotatory properties of the yellow aggregate to those of the yellow lobster pigment (Buchwald and Jencks, 1968) suggest that the mechanism of alteration of the optical properties of astaxanthin is the same in these two systems. In the lobster pigment the protein serves to replace the salt in the model system and to keep the pigment aggregate in solution, but does not appear to be directly responsible for the altered absorption and rotational spectra of the pigment.

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