Absorption Spectra of Copper and Zinc Complexes of Pheophytins and Pheophorbides

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This study has been made to establish the molar absorbance coefficients of the zinc and copper(II) complexes of the pheophytins and pheophorbides. Spectral curves and metal analyses have been made for each of the four mentioned chelates of zinc and copper(II) following purification by chromatography. Chelation was accomplished with ease in aqueous acetone solutions of the respective pheophytins and pheophorbides. Thin-layer chromatography procedures, developed during this study, provided reliable indication of the purity of the complexes. Zinc and copper(II) salts and their solutions promoted rapid allomerization of the pigment during chelation. Molar absorbance coefficients are presented.

Zinc and copper pheophytins and pheophorbides may be contaminants in processed foods. Sweeney and Martin (1958) observed changes in the shape of spectral curves of pigments (in acetone) of cooked broccoli on addition of zinc chloride. Schanderl *et al.* (1965a) reported the formation of zinc and copper complexes in glass-packed pea purees. Fischbach and Newburger (1943) made a spectrophotometric study of the green pigment in canned okra and found zinc complexes present. Fischbach (1943) determined the zinc content of pigment extracted from okra to which zinc salts had been added during processing for preservation. Lamort (1956) made a spectrophotometric study of many metal complexes of pheophytin, including those of zinc and copper. Tonomura (1955) compared the spectra of metal complexes of chlorins and porphyrins. Schanderl *et al.* (1965b) tabulated absorptivities of copper complexes of pheophytins *a* and *b* and pheophorbide *a*, calculated from the absorptivities of pheophytins *a* and *b* reported by Zscheile and Comar (1941).

This study, to determine the molar absorbance coefficients of the zinc and copper complexes of chlorophyll derivatives, has been conducted to enhance our knowledge of the formation of zinc and copper complexes during food processing. The concentration of purified preparations of metal complexes has been estimated from their absorption spectra and from their metal content. Molar absorbance coefficients and spectral curves for the zinc and copper(II) pheophytins are presented.

MATERIALS

Chlorophyll was obtained from fresh English ivy leaves. This rich pigment source was available year around and was relatively free of chlorophyllase. Peroxide-free diethyl ether (Mallinckrodt code 0848, delivered and stored in metal cans) was used. Coarse granular, anhydrous sodium sulfate (Mallinckrodt code 8024) was used for drying pigment extracts. The coarse-grained product did not retain chlorophylls and certain derivatives to the extent that the fine-grained salt did. Basic impurities in sodium sulfate, even in trace amounts, cause absorption of the pheophorbides, free or complexed. Sodium sulfate solution, 5%, adjusted to pH 6.4 with HCl, was used for washing acetone from extracts. Skellysolve B was purified with silica gel following the procedure of Graf *et al.* (1944). Reagent grade petroleum ether, b.p. 65° to 110° C., was preferred in these studies. Polyethylene powder, linear polyethylene of low melt index designated as *MI*, 0.025, M_v, 2.3×10^5 was obtained from the Dow Chemical Co., Midland, Mich. Powdered sugar was 4X confectioners' sugar containing 3% cornstarch. All other chemicals used were reagent grade quality. Deionized water was used for all aqueous solutions.

EXPERIMENTAL

Chlorophylls *a* and *b* were separated by the method of Zscheile and Comar (1941) with modifications by the authors based, in part, on the method of Anderson and Calvin (1962). Fifty grams of fresh English ivy leaves were blended with 250 ml. of acetone and 5 grams of calcium carbonate. A 200-ml. volume of diethyl ether was added to the filtrate from the blended mixture, and the acetone was washed from the mixture with sufficient 5% sodium sulfate solution. The ether solution was dried by passing through anhydrous sodium sulfate and concentrated to a low volume under reduced pressure in an atmosphere of nitrogen, and 50 ml. of acetone were added. The diethyl ether was completely removed by further volume reduction, and water was added to make an acetone content of 70%. This procedure for reducing the volume of the aqueous acetone total pigment extract for application to the polyethylene column avoided the annoying formation of a two-phase system and the accompanying danger of pigment alteration.

The pigment in 70% aqueous acetone was placed on a polyethylene resin column which had been previously wetted with 70% acetone. The column was developed first with 70% acetone until the xanthophylls had moved away from the green pigments, then with 80 to 90% acetone until the chlorophylls moved to the bottom. The receiving vessel was changed, and the chlorophylls were eluted. The chlorophylls were again transferred to diethyl ether, which was then dried by passing through anhydrous sodium sulfate, concentrated under reduced pressure in an atmosphere of nitrogen, and diluted with Skellysolve B to yield a mixture of Skellysolve B and diethyl ether (9 to 1).

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The pigment mixture was placed on a powdered sugar column, previously wetted with a 10% diethyl ether solution in Skellysolve B, and was developed with a diethyl ether solution in Skellysolve B. The diethyl ether concentration, initially 15%, was increased at 5% increments to 30%. Chromatography was continued until the chlorophyll *a* had separated from the chlorophyll *b*, the bands were mechanically removed, and the pigments were extracted with diethyl ether. The *a* and *b* fractions were rechromatographed until pure pigments were obtained. The purity of the chlorophyll *a* and chlorophyll *b* fractions was checked by the thin-layer chromatography technique of Jones *et al.* (1966) and by the Molisch (1896) phase test. When necessary to store solutions of chlorophylls or other prepared pigments, they were held in diethyl ether at −20° C.

Pheophytins *a* and *b* were prepared from the purified chlorophylls in 100 ml. of 80% acetone containing 1 ml. of concentrated hydrochloric acid. Conversion of chlorophyll to pheophytin was complete in approximately 1 hour as verified by thin-layer chromatography. The pheophytins were transferred to diethyl ether, which was repeatedly washed with water to remove the acid as verified by testing the wash water with litmus paper. The pheophytins were transferred to acetone.

Copper(II) pheophytin *a* or *b* was prepared by adding 20 ml. of 1*M* copper(II) chloride to about 80 ml. of the acetone solution of the desired pheophytin. To minimize oxidative changes, a few crystals of ascorbic acid were added to the reacting solutions, and chelation was conducted in an atmosphere of nitrogen. Zinc pheophytin *a* or *b* was prepared in a similar manner, except that 5 grams of crystals of zinc chloride rather than a solution of this salt were added to the pheophytin solution to be chelated. Gentle mixing action during chelation was provided by a magnetic stirrer.

The progress of chelation with either copper or zinc was followed by thin-layer chromatography. Chelation was complete in 1.0 to 1.5 hours. The metal chelates were transferred from acetone to diethyl ether, which was then dried with anhydrous sodium sulfate.

Each copper or zinc pheophytin was purified by chromatography on a sugar column. The pigment was transferred to petroleum ether, and 20 ml. of diethyl ether were added to 80 ml. of the petroleum ether solution. The column was developed with 20% diethyl ether in petroleum ether for the zinc pheophytins and the copper(II) pheophytin *a*. For copper(II) pheophytin *b*, the column was developed with 30% diethyl ether in petroleum ether. More than one band was obtained for each preparation. These bands were removed separately, and the pigments were eluted for characterization as discussed later.

The free pheophorbides were prepared from purified chlorophylls by the method of Willstätter and Stoll (1908). The pigments were transferred to acetone. The pheophorbides *a* and *b* of zinc and copper(II) were prepared by the chelation procedure outlined above for the pheophytins. Purification of zinc pheophorbide *a* or *b* or copper(II) pheophorbide *a* or *b* was carried out by chromatography on a sugar column from suitable diethyl ether–petroleum ether (b.p. 65° to 110° C.) solutions. A few crystals of ascorbic acid in the developing mixtures facilitated banding and reduced streaking on the column. For the zinc complexes, chromatography was started with diethyl ether–petroleum ether (60 to 40), and the mixture was changed to 65 to 35 as necessary; for copper *a*, the beginning ratio was 60 to 40 and was changed to 70 to 30 for completion; and for copper *b*, the initial ratio of 70 to 30 was increased to 80 to 20 as necessary. If present, traces of pheophytin complexes were eluted during the development of the pheophorbide complexes.

The absorbances of suitable dilutions of the pigments in diethyl ether were determined with a Beckman DK2-A recording spectrophotometer with the following settings: scale expansion 1×, scanning time dial 10, time constant 0.1, sensitivity dial 50. The position of absorption maximum was determined with hydrogen or mercury lines. The 656.3-mμ hydrogen line was especially useful for verification of peak points in the red region.

The zinc complexes were analyzed for zinc content by the method of Rush and Yoe (1954) with some modification. The samples were prepared for analysis by wet ashing with a mixture of concentrated nitric acid and 70% perchloric acid (10 to 1), evaporation to dryness, and dissolving the residue in 1*M* hydrochloric acid. Absorbances were determined at 620 mμ on a Beckman B spectrophotometer.

The copper content of the copper(II) complexes was determined by the method of Christie *et al.* (1957) with slight modification. The samples were prepared for analysis by digestion with a mixture of concentrated nitric and sulfuric acids and neutralization with ammonium hydroxide. Absorbances were determined by a Beckman B spectrophotometer at 650 mμ.

RESULTS

Molar absorbance coefficients (ϵ_M) were calculated from concentrations, as determined by metal analysis, and absorbances of solutions according to the equation,

$$\epsilon_M = \frac{A}{c \times l}$$

where A is the absorbance of the solution used for metal analysis, c is the concentration of the metal complex in moles per liter, and l is the path length in centimeters. The values of A were calculated from the absorbances of suitably diluted solutions. The concentrations of the zinc and copper complexes were based on a 1-to-1 ratio of metal to complex. Schanderl *et al.* (1965b) reported that 1 mole of copper complex contained 1 mole of copper.

Spectral curves for copper(II) pheophytins *a* and *b* and for zinc pheophytins *a* and *b* are given in Figures 1 and 2, respectively. Copper(II) and zinc pheophorbide curves were identical to those of the corresponding pheophytin. Millimolar absorbance coefficients of the copper and zinc pheophytins and pheophorbides are given at their red maxima in Table I. Included also in Table I are the wavelengths of the absorption maxima for the copper and zinc chelates in the blue and red regions of the spectrum. The observed maxima were the same for corresponding pheophytins and pheophorbides with respect to either the *a* or *b* derivative. The ratio of absorbances for corresponding pheophytins and pheophorbides was similar.

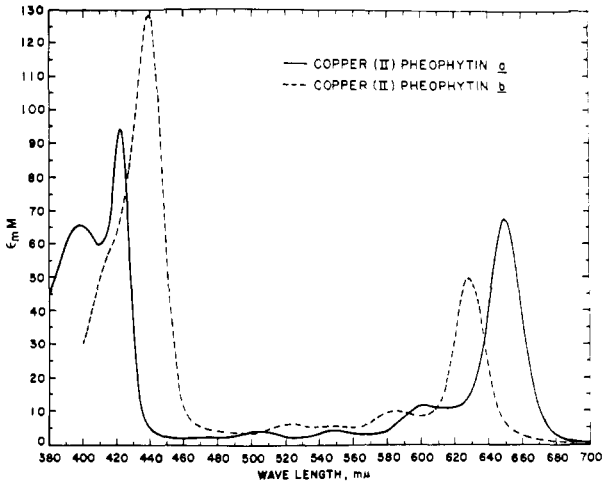

Figure 1. Spectral curves of copper pheophytins *a* and *b* in diethyl ether

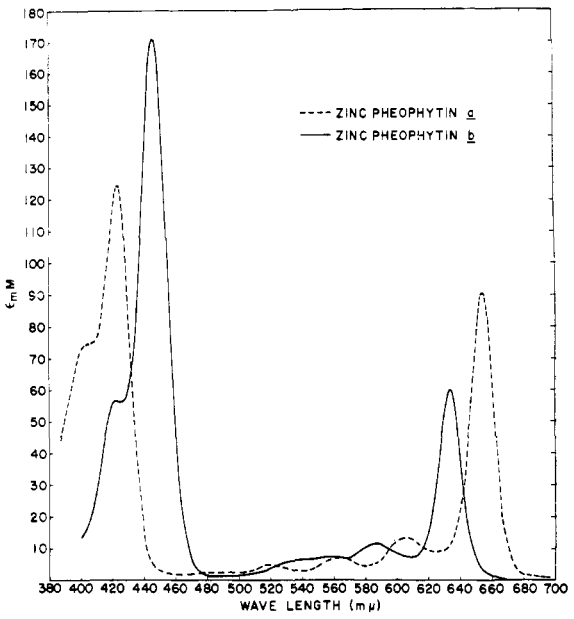

Figure 2. Spectral curves of zinc pheophytins *a* and *b* in diethyl ether

Table I. Millimolar Absorbance Coefficients (ϵ_{mM}) of Copper(II) and Zinc Complexes of Pheophytins and Pheophorbides

Metal Complex	ϵ_{mM}, Red Max.	Blue Max., λ, Mμ	Red Max., λ, Mμ	ϵ_{mM}, Blue Max. / ϵ_{mM}, Red Max.
Cu pheophytin *a*	67.9[a]	421	648	1.36
Cu pheophorbide *a*	69.3[b]	421	648	1.35
Cu pheophytin *b*	49.8[c]	438	627	2.53
Cu pheophorbide *b*	50.7[d]	438	627	2.48
Zn pheophytin *a*	90.3[e]	423	653	1.38
Zn pheophorbide *a*	90.9[f]	423	653	1.38
Zn pheophytin *b*	60.2[g]	446	634	2.94
Zn pheophorbide *b*	59.0[h]	446	634	2.94

[a] 67.0, 70.5, 66.2, 67.0, 68.6, and 67.8.
[b] 68.1 and 70.5.
[c] 48.8, 50.6, and 50.4.
[d] 51.9 and 49.5.
[e] 90.1, 90.3, and 90.6.
[f] 91.0 and 90.8.
[g] 61.6, 57.7, 59.4, 61.8, and 60.4.
[h] 58.9 and 59.0.

DISCUSSION

The molar absorbance coefficients of the zinc and copper(II) complexes of pheophytins and pheophorbides *a* and *b* have been estimated from rigorously purified preparations. Pigment changes attributed to allomerization were induced readily and to an appreciable extent during formation of the zinc and copper complexes. Purification was effected by sugar column chromatography.

For copper(II) pheophytins *a* and *b* and copper(II) pheophorbide *a*, the previously reported molar absorbance coefficients of Schanderl *et al.* (1965b) are about 18% lower than those presented here. A large proportion of the difference between values may be attributed to preparation procedures and to the method of calculation of molar absorbance coefficients. The copper chelate preparations of Schanderl *et al.* (1965b) were not purified and may have been comprised wholly or in a large part of the allomerized form of the respective pigment investigated. The present studies showed that the coefficients of the allomerized pigments were approximately 6% lower than those for the corresponding nonallomerized compounds.

The molar absorbance coefficients of Schanderl *et al.* (1965b) were based on molar absorbance coefficients for pheophytins *a* and *b* as determined by Zscheile and Comar (1941), which were low when compared with those reported by Smith and Benitez (1955), by Davidson (1954), and as determined in this laboratory. Using the pheophytin coefficients of Davidson (1954), the calculated coefficients of the copper complexes reported by Schanderl *et al.* (1965b) are approximately 9% lower than those reported in Table I.

Allomerized chelates were distinguished from nonallomerized pigments by the phase test, location on a sugar column or thin-layer chromatogram, and by their spectral curves. The allomerized (Molisch phase test negative) pigments were more strongly adsorbed on a sugar column and banded above the nonallomerized compounds. Spectral curves for nonallomerized pheophytins and pheophorbides of zinc *a*, zinc *b*, and copper *a* complexes had either a secondary peak or a plateau at a wavelength shorter than that of the major peak in the blue region. This regional characteristic was not present in curves of the corresponding allomerized complexes and served as an indication of purity of a given preparation with respect to the relative amount of allomerized product present. Spectral curves of the copper *b* complexes have neither a secondary peak nor a plateau in the blue region. The difference in the shape of curves for nonallomerized and allomerized chelates is shown in Figure 3.

The absorption maxima of the allomerized pigments

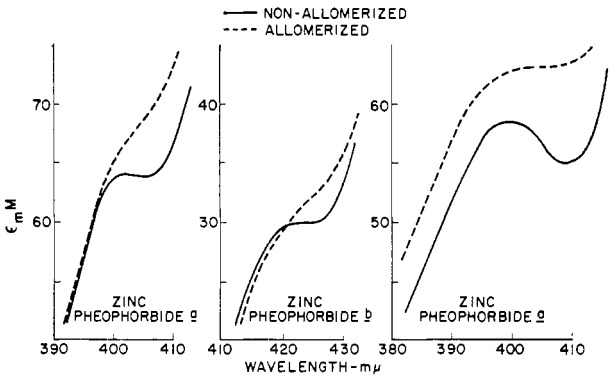

Figure 3. Comparison of absorption spectra of nonallomerized and allomerized zinc and copper (II) pheophorbides in the blue region

Solvent, diethyl ether

were at wavelengths about 2 mμ longer than those of the corresponding nonallomerized compounds in the red region of the spectrum. The ratios of absorbances at the blue maxima and the red maxima were a good indication of allomerization. Allomerized chelates in diethyl ether yielded ratios which were about 10% larger than the ratios listed in Table I. Since the purity of these preparations has been demonstrated, the larger ratios cannot be attributed to carotenoid impurities.

Chelation of the pheophytins and pheophorbides in acetone solution took place with copper or zinc chloride without difficulty. The reaction proceeded essentially to completion in 30 to 90 minutes. The zinc chelates were formed more readily if solid zinc chloride was added to an acetone solution of the pigment which had not been diluted with water.

Copper and zinc pheophorbides may be crystallized readily from petroleum ether–diethyl ether mixtures. Thin-layer chromatography indicated that crystallization was effective for the removal of impurities other than allomerized fractions, but that it was necessary to chromatograph the pigments to separate nonallomerized and allomerized metal complexes.

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