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Interaction of Sunset Yellow with Copper(II) Ion

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Reactions between Sunset Yellow (FD&C No. 6) and cupric ion were examined. When the dye combined with cupric ion, a shift in the visible absorption spectrum occurred and hydrogen ion was liberated. The hydrogen ion liberated per dye molecule was determined by potentiometric and spectrophotometric titration. The dye–Cu(II) complex ratio was determined from spectrophotometric data by means of the Scatchard plot, Job's method, and the slope–ratio method. Sunset Yellow reacted with cupric ion in a ratio of 2:1 with the liberation of 0.5 equiv of hydrogen ion and a ratio of 1:1 with the liberation of one hydrogen ion. Earlier reports of a 1:2 complex could not be confirmed. The dissociation constants for the complexes are 8.87×10^{-4} and 5.37×10^{-4}, respectively.

Banerjee et al. (1977) reported that the food dyes, FD&C Red No. 2, FD&C Red No. 4, and FD&C Yellow No. 6 formed complexes with cupric ion. The authors presented arguments for their conclusions that the dyes reacted with cupric ion in the ratios of 1:1 and 1:2 dye to cupric ion. The 1:1 dye–Cu(II) complex is consistent with the findings of other authors, while the 1:2 dye–Cu(II) complex had not been previously reported.

It is our view that the evidence for the formation of a 1:2 dye–Cu(II) complex is subject to other interpretation. Indeed, Banerjee et al. (1977) expressed the hope that their findings would stimulate further investigation. It is in keeping with this hope that we reexamined this complexation reaction.

In solution the azo dye is involved in three equilibriums: acid–base equilibrium, tautomeric equilibrium, and polymeric equilibrium.

The acid–base equilibriums (Figure 1) involve the sulfonic acid groups and the *o*-hydroxyazo group. The sulfonic acid groups are strong acids and dissociate around pH 2.00 (Jablonski, 1951). The *o*-hydroxyazo hydrogen is usually more basic than a phenolic group and dissociates around pH 12.00 (Zollinger, 1961). Its more basic character is attributed to proton tautomerism.

Historically, the theory of proton tautomerism was first demonstrated by using a hydroxyazo–hydrazone equilibrium (Zollinger, 1961). The equilibrium is a transfer of a proton (from the *o*-hydroxyl group) between the oxygen and the β-nitrogen (Figure 1). The change in the electronic configuration of the azo chromophore changes the absorbance spectrum. Likewise, the removal of the hydrogen in the acid–base equilibrium will also change the spectrum (Mason, 1970). The position of the tautomeric equilibrium is solvent dependent. The hydroxyazo form dominates in organic, nonpolar solvents. The hydrazone form is favored in polar solvents which undergo hydrogen bonding. In aqueous solution essentially 100% of the dye is present as the hydrazone (Krueger, 1975). Both forms aggregate (Zollinger, 1961).

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Banerjee et al. (1977) indicated the formation of 1:1 and 1:2 dye–Cu(II) complexes:

$$Cu^{2+} + D \rightleftarrows (CuD)^{+} + H^{+} \text{ at pH } 4.50$$

$$Cu^{2+} + (CuD)^{+} \rightleftarrows [Cu(CuD)]^{+} + 2H^{+} \text{ at pH } 6.00$$

They propose that at pH 4.50 the *o*-hydroxyazo hydrogen is replaced. The reaction, because it involves the chromophore of the dye molecule, would produce a spectral shift and such a shift was observed. They proposed that at pH 6.00 the cupric ion neutralizes the two sulfonic acid groups via a "salt formation equilibrium" with the exact location of the second cupric ion being unknown. The reaction would produce little or no spectral change. Because of their location, the sulfonic acid groups exert little or no influence on the chromophore of the dye (Zollinger, 1961; Blumberger, 1940). At pH 6.00 only a slight spectral change was observed with the production of two protons. The sulfonic acid groups being strong acids should be dissociated at pH 4.50 and certainly at pH 6.00. A "salt-forming equilibrium" between cupric ion and the acid groups would be expected at a lower pH and the reaction with the basic hydroxyazo hydrogen at a higher pH, rather than the reverse as suggested by the authors. The reverse order could be explained if the salt-forming equilibrium could only occur after the 1:1 complex had formed. Such a mechanism is not indicated in this case, because the sulfonic acid groups and the *o*-hydroxyazo group have little or no influence on each other (Zollinger, 1961; Blumberger, 1940). This is also confirmed by the slight spectral shift (which we calculated to be statistically insignificant) that Banerjee et al. observed. Therefore, the ordered mechanism suggested by Banerjee et al. appears questionable.

Figure 2 illustrates previously reported dye–Cu(II) complexes (Jarvis, 1961; Krueger, 1975; Zollinger, 1961). The 1:1 complex presented by Banerjee et al. (1977) associates the cupric ion with the α-nitrogen. The interaction between the azo group and cupric ion should be with the β-nitrogen (Jarvis, 1961; Krueger, 1975; Zollinger, 1961). The β-nitrogen is slightly more electronegative than the α-nitrogen because the oxygen ortho to the α-nitrogen tends to draw electrons from the α-nitrogen. Also, the six-membered ring formed by the interaction of the β-nitrogen with cupric ion is more stable than a five-membered ring would be (Krueger, 1975).

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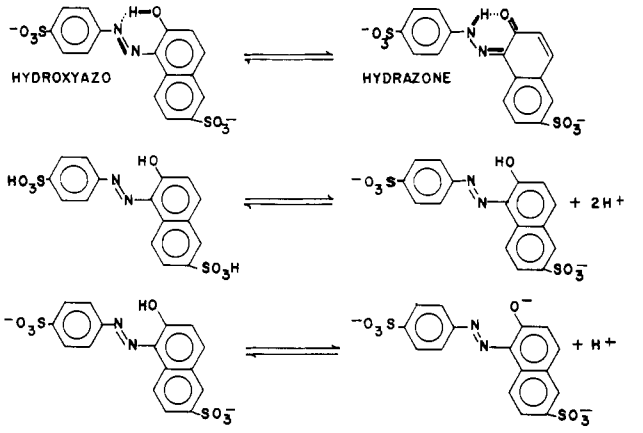

Figure 1. Tautomeric and acid–base equilibriums for Sunset Yellow.

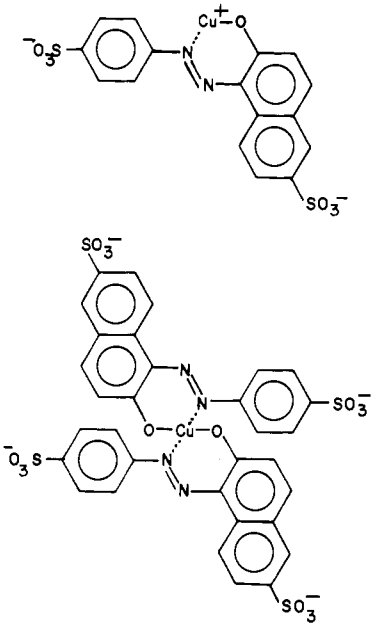

Figure 2. Dye–Cu(II) complexes.

The 1:2 complex presented by Banerjee et al. (1977) is most questionable. If the sulfonic acid groups are dissociated, then two protons cannot be produced by reaction with cupric ion. Sterically, how one would explain a 1:2 complex is not clear, for the sulfonic acid groups are too distant in the 1:1 complex (Figure 2). In the 2:1 complex (Figure 2), one could imagine one cupric ion sandwiched between the sulfonic acid groups at one end of the complex and another cupric ion sandwiched between the sulfonic acid groups at the other end as well. Since the two sites are electronically identical, there seems no reason why cupric ion would interact with only one site.

It is important to study the reaction of these dyes with cupric ion, for they belong to a class of coloring agents important to the food industry and numerous food products do contain cupric ion. Since some of this class of coloring agents are thought to produce adverse reactions in man (Collins and McLaughlin, 1973), further investigation of the reaction chemistry could lead to a better understanding of their possible biological activity.

EXPERIMENTAL SECTION

The standard certified food color used was FD&C Yellow No. 6, Sunset Yellow (Warner-Jenkinson Co., lot G-2516). All other chemicals used were reagent grade and all water was twice deionized. The food color was further purified by an extraction procedure (AOAC, 1960), followed by a precipitation procedure (Jablonski, 1951). Purity of the resulting dye was determined by paper and TLC chromatography, and visible and infrared spectra of the purified dye were compared with those of authentic samples of the dye.

The ionic strength of the solutions was held constant for all experiments by using appropriate amounts of 0.010 N $NaNO_3$. All pH values were determined by using a Beckman Model 4500 digital pH meter. Absorbance readings were measured by using a Perkin-Elmer Model 545 UV–visible recording spectrophotometer.

RESULTS AND DISCUSSION

Titration Studies. The nature of the absorption spectra of metal–azo dye solutions is strongly influenced by such factors as reactant concentration, pH, temperature, etc. Thus, two of our initial priorities were to examine systematically these effects and also to find specific conditions such that we could distinguish between the spectrum of the dye and those of the metal–dye complexes.

Dye Only. The acid–base equilibriums of the dye involve the sulfonic acid groups and the *o*-hydroxyazo group. A conventional titration of the dye was made. The results confirmed that the sulfonic acid groups are dissociated above pH 2.50. The *o*-hydroxyazo group should have an end point at 3 equiv of KOH. The end point of the dissociation of the *o*-hydroxyazo group was not observed due to limits of the glass electrode.

Dye–Cu(NO_3)$_2$ Mixtures. The overall reaction between dye and cupric nitrate liberates hydrogen ion. When titrating the 1:1 dye–cupric nitrate mixture, a color change occurred between pH 5.00 and pH 5.44. The solution went from orange-red to bright orange. This could indicate an end point at 1.5 equiv of KOH, though the end point was not clearly defined by the titration curve.

In the dye–Cu(II) mixture at pH 6.10, a dark brown precipitate formed, obscuring the color of the solution. When the solution was filtered, the filtrate was orange-red. The filtrate was diluted and the absorbance at 485 nm showed that all the dye was present in the filtrate. The IR spectrum (KBr pellet) confirmed an inorganic precipitate, rather than an insoluble dye–Cu(II) complex.

The end point for all reactions in the dye–Cu(II) mixture occurred at 3.5 equiv; the end point for cupric nitrate was at 3.0 equiv. Half an equivalent of hydrogen ion was produced by the reaction of dye with cupric nitrate.

As pointed out above, the color of dye solution changed at 1.5 equiv. Since the mixture had been acidified with 1 equiv of HCl, the color change could therefore indicate the production of 0.5 equiv of hydrogen ion from the reaction of cupric nitrate with dye.

Similar titrations done in excess cupric nitrate or without HCl yielded identical data.

The dye–Cu(II) mixture contained 1 equiv of dye. The dye could liberate 1 equiv of hydrogen ion. The liberation of only 0.5 equiv of hydrogen ion may indicate that only half the dye reacted. An alternate conclusion would be that the dye–Cu(II) complex is basic enough to form a hydrogen complex.

Titrations were performed in 95% ethanol to test whether the extent of reaction was only 50%. Large amounts of low molecular weight alcohols inhibit polymerization of the dye (Krueger, 1975). Known dye–Cu(II) complexes are either the monomeric or dimeric form of the dye. Polymerization of the dye would inhibit complex formation.

Ethanol Titration. Titration data in ethanol were idential with those obtained in aqueous solution. Since the extent of reaction did not change in ethanol, the for-

mation of a hydrogen complex seems a more adequate explanation of the data.

Equilibrated Dye–Cu(II) Mixture. In the pH range 3.75–6.10 the titration curve for the dye–Cu(II) mixture was not definitive. There is no buffer region for determining the pK and no sharply defined end point. These results could indicate that the mixture did not have time to equilibrate between each addition of base.

The dye–Cu(II) mixture was allowed to equilibrate at room temperature (25 °C) in vials containing varying amounts of 0.10 N KOH. After 24 h there was no further change in pH. There was still no sharp end point evident or clearly defined buffer region. These results could be attributed to the complex having a small dissociation constant or to the weakly acidic properties of the *o*-hydroxyazo group. The *o*-hydroxyazo group is implicated in the reaction because a change in color occurred.

Spectrophotometric Titrations. *o-Hydroxyazo Group.* Though the pK was too high to determine by conventional titration, it can be determined spectrally. The anion (D^{3-}) absorbs differently from the DH^{2-} form, since the hydrogen is part of the azo chromophore.

A determination of the pK was made and found to have a value of 10.00 which is low for an *o*-hydroxyazo group. This pK is questionable due to the presence of CO_2 from air.

Dye–Cu(II) Mixture. Since the reaction between dye and cupric nitrate involves a color change, spectrophotometric titration is an alternative method for determining the end point. Assuming the validity of Beer's law (shown to be true later) for the dye (D) and the complex (x), the absorbance (A) will be the sum of the concentrations of the absorbing species (C_D and C_x) times their respective absorptivities (a_D and a_x):

$$A = a_D C_D + a_x C_x$$

The total concentration of dye (C_T) is held constant in the titration; thus

$$C_T = C_D + C_x \text{ or } C_D = C_T - C_x$$

By substituting into the Beer's law relationship and rearranging to fit the form $y = Ax + B$, we find

$$A = (a_x - a_D)C_x + a_D C_T$$

From the above equation the linear response of absorbance to concentration (C_x) can yield an appreciable break in a spectrophotometric titration even when small changes in concentration do not give well-defined end points with the log scale of a potentiometric titration (Vogel, 1961). In addition, spectrophotometric titration has the advantage of being a direct measure of the reaction between dye and cupric ion.

In spectrophotometric titration, the total absorbance (A) is not of interest because it contains two unknowns (C_x and a_x); only the changes are interesting. As an added benefit, such titration is not hindered by the presence of other absorbing species as they would become part of the constant B.

Like equations can be derived for other complexes and the straight lines (Figure 3) will intersect at the end points (Vogel, 1961). The data in Figure 3 showed two breaks, at 0.5 and at 1.1 equiv per dye molecule. After 1.1 equiv no further change in absorbance was observed, indicating the reactions were complete. Because there are two breaks, two complexes are indicated. The system can be described as follows.

From 0–0.5 equiv the absorbance is defined by the function

$$A = (a_{x_1} - a_D)C_{x_1} + a_D C_T$$

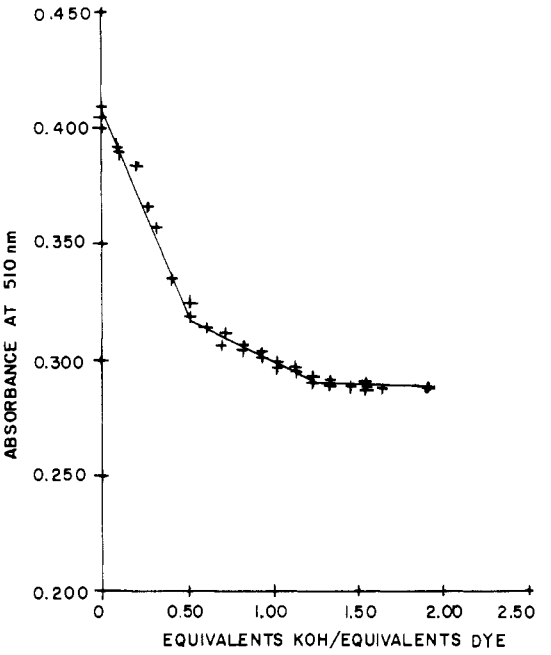

Figure 3. Spectrophotometric titration of 5×10^{-5} M dye plus 1×10^{-3} M $Cu(NO_3)_2$ in 0.01 N $NaNO_3$.

The slope is steep due to the greater difference between a_{x_1} and a_D.

From 0.5–1.0 equiv absorbance is defined as

$$A = (a_{x_2} - a_{x_1})C_{x_2} + a_{x_1} C_T$$

where

$$C_T = C_{x_1} + C_{x_2}$$

The slope is less than that for 0–0.5 equiv, indicating a smaller difference between a_{x_1} and a_{x_2}.

After 1.1 equiv the reaction is complete and

$$A = a_{x_2} C_{x_2}$$

where

$$C_T = C_{x_2}$$

The constant absorbance is 0.288. This absorbance divided by the total dye concentration gives a value of 6.17×10^3, the absorptivity of one of the complexes.

Absorption Spectra. *Cupric Nitrate.* Figure 4 shows the change in the spectrum of the dye with the addition of cupric nitrate. Addition of cupric nitrate produces a hypsochromic shift of 10 nm. The shift also results in a hypochromic effect. Two isosbestic points are present. Banerjee et al. (1977) only reported one by beginning their spectrum at 380 nm, instead of 330 nm. Two isosbestic points might indicate two dye–Cu(II) complexes. Generally, isosbestic points are not a reliable method for determining the number of unresolved species (Kolthoff et al., 1969; Zollinger, 1961). The nature of the peaks will determine how many isosbestic points occur.

pH. As indicated above, Figure 5 shows the spectra of the acid–base forms of the dye. Because of their location in the dye molecule, the sulfonic acid groups have little influence on the spectrum of the dye. So little, in fact, that a salt-forming equilibrium between them and cupric nitrate could probably not be detected spectrally.

The spectrum of the dye was also studied at different temperatures and in different solvents. The purpose was to see if, by a manipulation of conditions, the spectrum of the dye could be shifted such that the peak of the dye

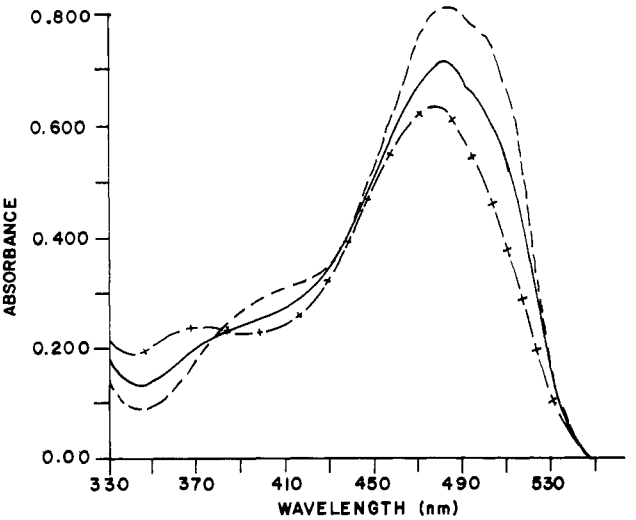

Figure 4. Effect of cupric ion on the dye spectrum. 4.60×10^{-5} M dye in 0.01 N $NaNO_3$ for all samples; (---) dye only; (—) dye:Cu $(NO_3)_2$, 1:2; (+-+) dye:Cu $(NO_3)_2$, 1:10.

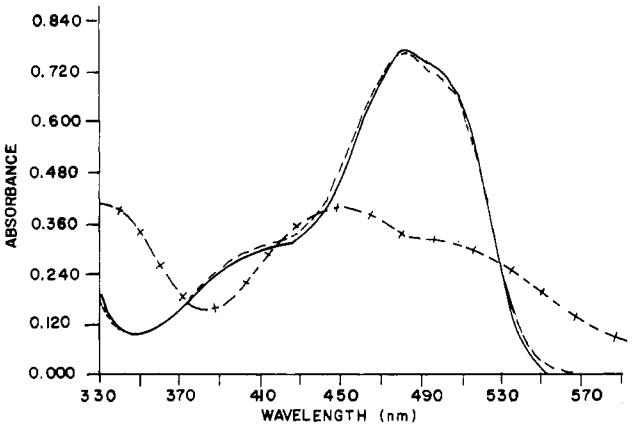

Figure 5. Effect of pH on the dye spectrum. 4.60×10^{-5} M dye in 0.01 N $NaNO_3$; (---) pH 1.36 (DH_3); (—) pH 5.52 (DH^{2-}); (+-+) pH 13.50 (D^{3-}).

could be resolved from the peak of the complex.

Temperature. An increase in temperature decreases the absorbance of the dye and of the dye–Cu(II) mixture. The decrease in the absorbance of the dye is proportional to the decrease in the absorbance of the dye–Cu(II) mixture. Since temperature affects the spectra in the same way, one temperature has no advantage over any other temperature. For convenience, room temperature (24 °C) was chosen for subsequent studies.

While temperature was of no aid in distinguishing between the peaks of the dye and complex, it is an important consideration for reproducibility of results. The temperature of the instrument room was constant, but the tungsten lamp light source heated the sample chamber. For optimum reproducibility, solutions should be allowed to equilibrate to the temperature of the instrument room. Samples should then be read immediately upon being placed in the sample chamber. In cases where the change in absorbance is desired, the reference dye solutions must be maintained at a fixed temperature (in this instance 24 °C) by a thermal control chamber. Allowing the reference dye solution to heat in the sample chamber results in errors as large as 0.035 in absorbance readings.

Solvent. The polymeric equilibrium of the dye involves an intermolecular hydrogen bond and the hydrogen bonding involves the azo chromophore of the dye molecule. The polarity of the solvent and its ability to hydrogen bond will thus affect the spectrum of the dye.

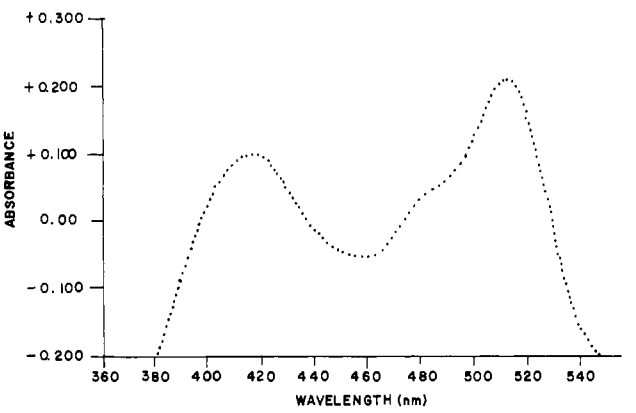

Figure 6. Wavelength of maximum difference. 5×10^{-5} M dye vs. 5×10^{-5} M dye plus 1×10^{-4} M $Cu(NO_3)_2$, both in 0.01 N $NaNO_3$.

Addition of salts promotes aggregation (Krueger, 1975). In $NaNO_3$, the location of the maximum of the dye does not change, but the intensity increases. In 0.01 N $NaNO_3$, the spectrum of the dye is the same as that in water.

High molecular weight alcohols and large amounts of low molecular weight alcohols inhibit aggregation. The maximum at 485 nm for the dye does not change, but the intensity at the maximum decreases. In polyglycol the spectrum was the same as that in *n*-amyl alcohol. In methanol the spectrum was the same as the spectrum in 95% ethanol.

The dye–Cu(II) complex was soluble in both methanol and ethanol. The wavelength of the maximum of the complex was exactly the same in water. The intensity of the maximum decreased. As with temperature the decrease was the same for the dye and complex. Resolution of the peaks did not improve.

Since it was not found possible to resolve the spectral peaks by the physical or chemical methods outlined above, the spectrum of the dye against that of the dye–Cu(II) complex was determined (Figure 6). The peaks in the difference spectrum indicate wavelengths where the differences between the spectra are maximum. At the wavelengths of maximum difference, changes in absorbance are an indication of complex formation.

Validity of Beer's Law and Determination of Absorptivities. Methods for determining the stoichiometric ratio assume the validity of Beer's law. In the Scatchard plot (Scatchard et al., 1957) the absorptivities are also required. Studies were done at two wavelengths where large differences in the spectra of the complex and dye occurred, 410 and 510 nm. Attempts were also made to use 350 nm where the difference is also large (refer to Figure 4). Neither dye nor complex follow Beer's law in this region.

Dye Only. Absorption data at 410 and 510 nm for dye in the DH^{2-} form and at 510 nm for dye in the D^{3-} form were treated by regression methods ($r^2 = 0.998$). The interval for the intercept, A, in all cases included zero; thus the dye solutions followed Beer's law.

Dye–Cu(II) Complex. The absorptivities of the complexes were determined by studying the effect of changing the dye concentration with fixed concentration of excess cupric ion.

Assume absorbance is equal to the sum of the concentration of the dye and the complex times their respective absorptivities (a):

$$A = a_D C_D + a_x C_x$$

If the cupric concentration is constant and in excess, then as dye is added, the reaction is

$$DH^{2-} + Cu^{2+} \xrightarrow{K} \text{complex} + H^+$$

The concentrations of free dye (C_D) and complex (C_x) will be related to each other by a constant, K_{eq}. A plot of absorbance vs. the total dye concentration (C_T) will be nonlinear.

When cupric ion is large enough to drive the reaction to completion, all the dye will be complexed:

$$C_x = C_T \qquad C_D = 0$$

The absorbance will be linear with C_T, and the slope will be the absorptivity of the complex.

With a large excess of cupric nitrate (1×10^{-2} M), our experiments indicated only one complex with $a_{510} = 5.97 \times 10^3$ M^{-1} cm^{-1}. The intercept interval for the linear regression included zero, confirming that the complex follows Beer's law. The absorptivity at 510 nm for the complex formed in 1×10^{-2} M cupric ion is the same ($\alpha = 0.1$) as the absorptivity (6.17×10^3 M^{-1} cm^{-1}) for the complex formed in excess KOH. Furthermore, the complex formed under the above conditions does not absorb at 410 nm.

With less cupric nitrate (5×10^{-4} M), at 410 nm Beer's law is followed with the absorptivity being $a_{410} = 4.90 \times 10^3$ M^{-1} cm^{-1}. This complex is not the same complex that is observed at 510 nm in 1×10^{-2} M cupric ion.

Analogous to driving the reaction to completion by adding excess reactant, cupric nitrate, would be to drive the reaction by removing the product, hydrogen ion. This was achieved by adding base which reacts with the hydrogen ion. Since the titration study at spectral concentrations showed the liberation of 0.5 and 1.0 equiv of hydrogen ion/dye molecule, two solutions were prepared, one dye–Cu(II) solution containing 0.5 equiv and a second dye–Cu(II) solution containing 1 equiv of base per dye molecule. The resulting solutions were diluted.

Examination of the spectral data revealed that neither slope gave the expected absorptivity, 5.97×10^3. In this case the slope is not necessarily the absorptivity of the complex, for the interval for the intercept $A = 0.265$ did not include zero.

For verification that these results were not due to an artifact of the methods, the effect of total dye concentration on fixed ratios of dye to cupric nitrate without base was examined. Here the data showed a linear relationship between absorbance and total dye concentration. This implies that the composition of the complex or complexes is not affected by concentration.

Only one complex absorbs at 410 nm and a different complex absorbs at 510 nm in an excess of cupric nitrate (1×10^{-2} M). The complex ratios can be determined by using different levels of cupric nitrate and different wavelengths.

Scatchard Plots for Determining Dissociation Constants and Complex Ratios—Derivation Form Used. The Scatchard plot for equilibrium ligand binding (Scatchard et al., 1957) takes the form

$$\frac{[ES]}{[S]_F[E]_0} = \frac{-1}{K_s}\frac{[ES]}{[E]_0} + \frac{n}{K_s}$$

where [ES] = bound substrate concentration, $[S]_F$ = free substrate concentration, $[E]_0$ = total enzyme concentration, K_s = substrate dissociation constant, and n = number of ligand binding sites per molecule of enzyme. This was applied to the reaction between cupric ion and dye where $[ES] = C_x$ = complex concentration, $[S]_F = [Cu]_F$ = free cupric ion concentration, $[E]_0 = C_T$ = total dye concentration, K_s = dissociation constant of the complex, and n = number of binding sites for cupric ion per molecule of dye. The complex concentration C_x can be determined by using Beer's law and the difference between the absorbance of a dye solution with and without cupric ion.

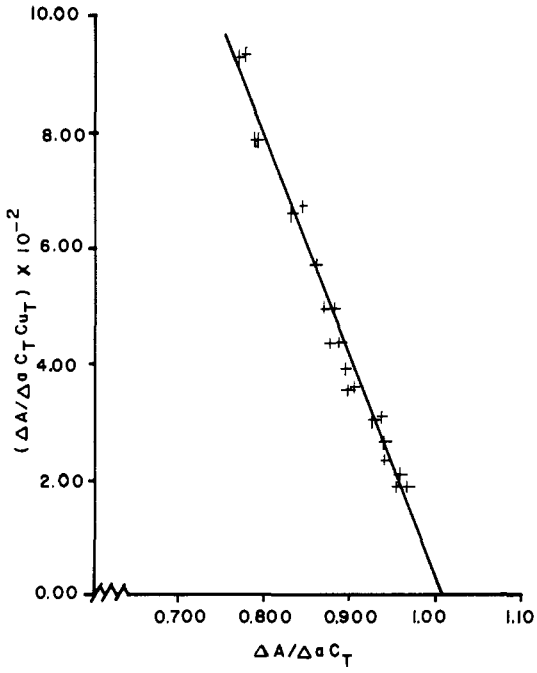

Figure 7. Scatchard plot at 510 nm.

As previously derived, the absorbance of a dye–cupric ion mixture is

$$A = (a_x - a_D)C_x + a_D C_T$$

The absorbance of dye solution is

$$A' = a_D C_T$$

Subtracting the two equations and solving for C_x

$$\frac{\Delta A}{\Delta a} = \frac{A - A'}{a_x - a_D} = C_x$$

The term $[Cu]_F$ is unknown but can be approximated as the total cupric ion concentration ($[Cu]_T$) by making the cupric ion concentration much larger than the dye concentration.

The Scatchard plot for equilibrium dye binding in excess cupric nitrate is transformed to

$$\frac{\Delta A}{\Delta a[Cu]_T C_T} = \frac{-1}{K_s}\frac{\Delta A}{\Delta a C_T} + \frac{n}{K_s}$$

Scatchard Plot at 510 nm. Since the Scatchard plot (Scatchard et al., 1957) is a reciprocal plot, cupric concentrations were chosen to yield more equal reciprocal increments. More samples were taken at lower values of cupric nitrate, since small errors in the cupric nitrate concentration are magnified when the reciprocals are taken, especially at lower concentrations.

From the determination of the absorptivities for the dye complexes at 510 nm, it was determined that the reactions are essentially complete at ratios of 1:200, or greater, dye to cupric nitrate ratios; at ratios of 1:20, two complexes are present.

For the Scatchard plot at 510 nm, ratios varied from 1:15 to 1:200. Low ratios were deliberately taken. When two complexes are present, the Scatchard plot is a curve, the sum of the linear regressions of each individual complex. The Scatchard plot over the entire range was a curve, verifying the formation of two complexes.

At very high concentrations of cupric nitrate, the Scatchard plot assumes linearity (Figure 7), thus indicating that only one complex is present in a large excess of cupric ion. The dissociation constant (the negative reciprocal of the slope) was determined to be $K_s = (5.37 \pm 0.382) \times 10^{-4}$ at $\alpha = 0.05$. The ratio of cupric ion to dye (n) is the intercept (B) divided by the slope (A):

$$(n/K_s)(-K_s) = B/A = n = 1.010$$

To determine the confidence limits ($\alpha = 0.05$) for n, it would be best to determine the limits at $y = 0$, since the data are closer to the y intercept than to the x intercept and the confidence limits for n will be smaller.

Since x is the independent variable and y the dependent variable, to determine the range of x at $y = 0$, the prediction interval for y at fixed values of x was calculated. For $0.990 < x < 1.030$, the prediction interval includes $y = 0$. The line very closely defines $n = 1.0$. The complex ratio is 1:1 for the complex in excess of cupric nitrate with an absorptivity $a_x = 5.97 \times 10^3$ M^{-1} cm^{-1}.

Scatchard Plot at 410 nm. The concentration range used for the observation made at 510 nm cannot be used at 410 nm, since only one complex absorbs at 410 nm. That complex forms at lower ratios of dye to cupric nitrate. At high concentrations of cupric nitrate absorbance at 410 nm decreases as cupric nitrate concentration increases.

For observations made at 410 nm, the ratios of dye to cupric nitrate employed were quite low, 5:1 to 1:8. An assumption of the Scatchard plot is that $C_T \ll [Cu]_T$, so that $[Cu]_F \sim [Cu]_T$. In this case the assumption can still be used, but the changes in absorbance are small, for most of the cupric ion is unbound. For example, given $[Cu]_T = 2.00 \times 10^{-4}$ M, $\Delta A = 0.088$. The approximate amount of complex formed is

$$C_x = \frac{\Delta A}{\Delta a} = \frac{0.088}{9.34 \times 10^3} = 9.4 \times 10^{-6} \text{ M}$$

$$[Cu]_T - C_x = 2.00 \times 10^{-4} - 9.4 \times 10^{-6} \text{ M}$$

$$[Cu]_F = 1.91 \times 10^{-4} \text{ M}$$

This is an acceptable error of 5%. The change in absorbance being small, there are only two significant digits. The concentrations of cupric nitrate used for the plot are those greater than 6.00×10^{-5} M, an error of 8% or less.

The points of the Scatchard plot were more scattered than those at 510 nm. The scattering was due to (a) loss of a significant digit in the change of absorbance, (b) the amplification by the reciprocal plot of errors in the small concentrations of cupric nitrate, and (c) the lesser error introduced by assuming essentially all the cupric ion is free. Because of the scatter of the data points on the Scatchard plot, the slope was tested and the regression found to be significant.

The interval for n was larger than that found for the experiments at 510 nm. The interval did not include $n = 1.00$, confirming that the absorbing complex at 410 nm was different from the complex found in excessive cupric nitrate and observed at 510 nm. The only meaningful value of n included in the interval was $n = 0.500$. The data indicated that a 2:1 dye–Cu(II) complex absorbs at 410 nm.

Job's Method for Determining Complex Ratio. *Total Concentration.* In the Job's method (Job, 1928), the total concentration of dye plus cupric nitrate is held constant, and the ratios of the two are varied. Three total concentrations were used, 6×10^{-5}, 5×10^{-5}, and 4×10^{-5} M.

Smaller concentrations were found to be unsuitable, because the change in absorbance was quite small. Larger concentrations were deemed unsuitable, because the total absorbance readings were too large to be reliable.

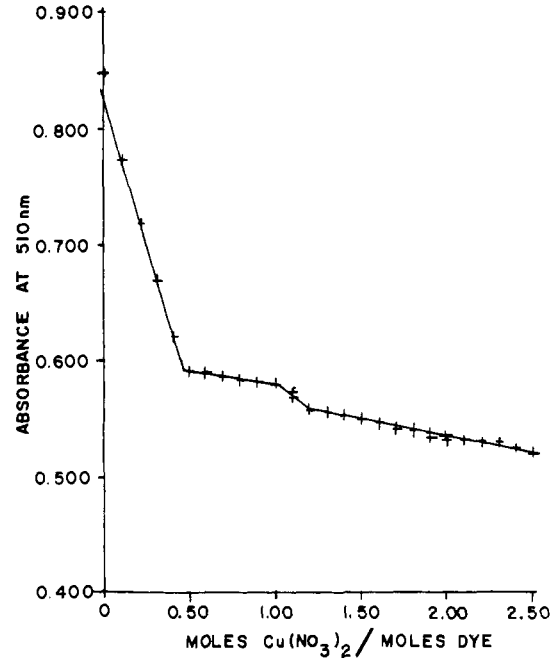

Figure 8. Slope–ratio data at 510 nm. Dye was constant at 5×10^{-5} M in 0.01 N $NaNO_3$.

In theory, the total concentration should not influence where the maximum of the peak occurs, only the amplitude. This was true at 410 nm but not at 510 nm. At both 410 and 510 nm, plots of total absorbance vs. total dye concentration were linear, indicating Job's method can be used for determining the complex ratios.

Data were treated by polynomial regression analysis. The vertexes at 410 nm were the same ($\alpha = 0.05$) and indicated a complex ratio of 2:1 dye to cupric ion. The vertexes at 510 nm were not the same ($\alpha = 0.05$). This indicated that there was more than one complex and that the form of the complex depends on the total concentration. At high total concentrations, the vertex approached those obtained at 410 nm. This indicates that more 2:1 dye–Cu(II) complex is formed at high total concentrations.

At the lowest total concentration, the vertex approached 0.500. This would indicate that a 1:1 complex predominates at this concentration.

Slope–Ratio Method for Determining Complex Ratio. With the slope–ratio method (Bjerrum, 1944), the total dye concentration was held constant at 5×10^{-5} M and different amounts of cupric nitrate were added. In effect the slope–ratio method is a spectrophotometric titration of dye with cupric nitrate.

A plot revealed that at 410 nm only one break occurred at 0.500, which is a complex ratio of 2:1 dye to cupric ion. At 510 nm two breaks occurred (Figure 8), one at 0.5 and one near 1.00. These indicated the formation of two complexes: the 2:1 complex, as found at 410 nm, and a 1:1 complex. These results appear to confirm the complex ratios found by Job's method and the Scatchard plots.

In the slope–ratio plots, plateau regions do not occur. This indicates that with the dilute concentrations used, the reactions do not go to completion; however, the breaks in the plots were still pronounced.

CONCLUSIONS

pH and Complex Formation. *Titrations.* Conventional titrations showed an end point at 0.5 equiv/dye molecule. Spectrophotometric titrations showed two end points, at 0.5 and 1 equiv/dye molecule. The difference

in results can be rationalized as a concentration effect.

Microtitrations were done to avoid use of a concentrated dye solution and to minimize the dilution effect of adding titrant. Dye solutions of 1×10^{-3} M were used, more dilute solutions giving insufficient pH changes. Spectrophotometric titrations were done with concentrations of 5×10^{-5} M dye. Microtitration dye solutions (1×10^{-3} M) were orange in color, even in ethanol, while spectrophotometric dye solutions (5×10^{-5} M) were yellow in color. Solution color indicates aggregation at higher concentrations.

The known dye–Cu(II) complexes are either monomeric or dimeric forms of the dye. At 1×10^{-3} M dye, the tendency of the dye to aggregate would inhibit the monomeric complex. At 5×10^{-5} M dye, the dye is still aggregated as shown by the effect of low molecular weight alcohols on the spectrum of the dye. The spectral concentrations being less, the tendency to aggregate is diminished. Thus, both the monomeric and dimeric complex would form.

pH of Spectral Solutions. For determination of the absorptivities and the stoichiometric ratios for the complexes, pH was not controlled. The pH was recorded for all solutions.

The pH of dye solutions containing high concentrations of cupric nitrate was low. For the Scatchard plot of data taken at 510 nm, the pH ranged from 4.30 to 4.06. The pH was directly related to the addition of cupric nitrate, indicating that hydrolysis of cupric ion ($K_h = 1 \times 10^{-8}$) dominated the pH of the solutions.

Lower concentrations of cupric ion were used for Job's method. The pH of dye–Cu(II) mixtures had no relationship to the change in absorbance or the concentration of cupric ion. Thus, at spectral concentrations, the pH of the solutions at both low and high concentrations of cupric nitrate had no direct relationship to complex formation. Complex formation was only directly related to the amount of cupric nitrate present in the reaction mixture.

Amount of Cupric Nitrate and Complex Formation. At dye to cupric nitrate ratios of 1:30 or greater, the Scatchard plot of data obtained at 510 nm showed a 1:1 complex. At lower ratios of dye to cupric nitrate, 1:10 or less, the Scatchard plot of data obtained at 410 nm implied a complex ratio of 2:1.

At ratios of 1:19 or less, Job's method using data obtained at 510 nm suggested a mixture of 1:1 and 2:1 complex. The 2:1 dominated at higher concentrations and the 1:1 at lower concentrations. The Job's plots of data taken at 410 nm showed only a 2:1 complex.

At intermediate dye to cupric nitrate ratios of 5:1 to 1:30, the slope–ratio method showed a 2:1 complex with a maximum at 410 nm and both a 1:1 ratio and a 2:1 ratio of complex with a maximum at 510 nm.

The results indicate the formation of two complexes: a 1:1 at high concentrations of cupric nitrate and a 2:1 dye–Cu(II) complex at low concentrations of cupric nitrate.

The results do not discount the possibility of a 2:2 dinuclear Cu(II) complex. [Crystallography has shown a mononuclear 2:1 complex for an analogous dye (Jarvis, 1961).] A large number of crystalline structures have shown complexes which contain two cupric ions connected with one or two oxygen atoms from an organic ligand. Such behavior is usually found in dihydroxy dimers of the type $L_2Cu(OH)_2CuL_2$ or $L_2CuCl_2CuL_2$. Dimers with direct Cu–Cu bonds have not been found in ligands with only one ligand atom (Jørgenson, 1966). The 1:1 stoichiometric ratio likely indicates the molecular ratio as well.

Proposed Reactions between Dye and Cupric Nitrate. Combining the results from the titrations and the determinations of the complex ratios, the following overall reactions for the dye with cupric nitrate are proposed:

$$(DH)_2^{4-} + Cu^{2+} \rightleftarrows D_2HCu^{3-} + H^+ \quad (1)$$

$$(DH)_2^{4-} + 2Cu^{2+} \rightleftarrows 2DCu^{1-} + 2H^+ \quad (2)$$

The dye is assumed to be in the dimeric form, since it is the monomer–dimer equilibrium which exists at spectral concentrations (Monahan and Blossey, 1970). Low molecular weight alcohols which favor the monomer do affect the spectrum of the dye, showing aggregation does occur.

As the reactions show, at high concentrations of cupric ion, the 1:1 complex forms. Reaction 2 describes the results but is probably not indicative of the mechanism. It would seem more likely that as cupric ion is added to the dye solution, the 2:1 complex forms (reaction 1). When sufficient complex is formed, the dye concentration decreases, shifting the monomer–dimer equilibrium:

$$(DH)_2^{4-} \rightarrow 2DH^{2-}$$

Cupric ion would then react as follows:

$$DH^{2-} + Cu^{2+} \rightarrow DCu^{1-} + H^+$$

This mechanism is indicated by the microtitration studies which suggest that the 1:1 complex does not form in aggregated dye solutions.

The mechanism is also supported by the fact that the 1:1 complex forms when the pH of the dye solutions is low due to the hydrolysis of cupric ion. A reaction liberating one hydrogen at low pH is more plausible than a reaction liberating two hydrogens.

Both titration studies indicate the presence of the hydroxy complex, D_2HCu^{3-}, in reaction 1. Such hydroxy complexes usually occur when the ligand has three or more ligand atoms or when chelate rings are many membered or strained (Bjerrum et al., 1957). The former is not the case; a strained ring is likely.

The reactions proposed are as follows:

$$(DH)_2^{4-} + Cu^{2+} \rightleftarrows D_2HCu^{3-} + H^+ \qquad K_d = 8.87 \times 10^{-4}$$

$$D_2HCu^{3-} + Cu^{2+} \rightleftarrows 2DCu^{1-} + H^+ \qquad K_d = 5.37 \times 10^{-4}$$

No results obtained indicate the formation of the 1:2 dye–Cu(II) complex found by Banerjee et al. (1977) or the liberation of more than one hydrogen per dye molecule.

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Methanol, Ethanol, and Acetaldehyde Contents of Citrus Products

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The three major citrus volatiles methanol, ethanol, and acetaldehyde were quantitatively determined for various citrus products by gas chromatography. Methanol concentrations varied from 10 to 80 ppm, ethanol from 90 to 900 ppm, and acetaldehyde from 50 to 190 ppm (w/v). Correlations were examined between composition of volatiles and storage history or other quality factors. A positive correlation was observed between methanol content and storage time of canned grapefruit sections and between ethanol content and storage time of non-heat-treated, glass-packed grapefruit sections. Composite data for all single-strength juices (fresh and processed) showed that acetaldehyde concentration was higher and ethanol and methanol concentrations were lower in grapefruit than in orange juice. Similarly, reconstituted commericial concentrates contained less methanol and ethanol and more acetaldehyde than single-strength juices. Similarity between the profiles of volatiles for some concentrates and the profile for single-strength juice suggested that these concentrates contained essence. Volatiles in single-strength juice did not correlate with Brix, acid pulp, or storage history, but a possible relationship between ethanol and the processing date for orange juice was found. Some of these correlations might be useful in quality evaluation.

Volatiles are routinely determined when quality and storage abuse of citrus products are evaluated (Lund and Dinsmore, 1978). Diacetyl content is related to the condition of the fruit and the presence of microorganisms in processing equipment. Peel oil content is evaluated on the basis of its limonene content. Furfural in stored juice is related to heat-induced off-flavors. For determination, all three of these compounds are recovered by distillation and analyzed by titration or colorimetry.

In the early work on citrus products (Kirchner et al., 1953; Kirchner and Miller, 1957), volatiles were analyzed by distillation and derivative formation. These studies established that methanol, acetaldehyde, and ethanol predominate in fresh and canned grapefruit and orange juices. Since concentrations varied widely in fresh, freshly canned, and stored canned juices, the authors implied that volatile concentration might be related to processing variables and storage treatment.

More recently, a gas chromatographic (GC) headspace procedure was employed for analysis of ethanol and acetaldehyde in citrus fruit (Davis and Chace, 1969; Davis, 1970, 1971; Davis et al., 1974; Roe and Bruemmer, 1974). Ethanol content was found to increase considerably during the growing season and was proposed as a quality indicator in addition to the presently used Brix/acid ratio (Davis, 1970, 1971). Acetaldehyde also increased, but not as sharply. In a related study, Norman and Craft (1971) determined ethanol, acetaldehyde, and methanol in intact oranges and correlated production of these volatiles with storage of fresh fruit in air and nitrogen.

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Other GC techniques have been used to quantitate limonene and other abundant volatiles (Lund and Shaw, 1979). These various studies of citrus volatiles demonstrated that they may be analyzed by a single GC determination.

We therefore undertook to develop an improved procedure for determination of methanol, acetaldehyde, and ethanol in a variety of citrus products. These included raw, fresh juices and freshly processed single-strength (canned and glass-packed) juices, concentrated juices, and freshly processed (canned and glass-packed) grapefruit sections. Storage tests were also carried out on samples of processed single-strength juice and sections.

MATERIALS AND METHODS

General. A Hewlett-Packard Model 7620A gas chromatograph equipped with a flame ionization detector was employed. The carrier gas (helium) flow rate was 37 mL/min, and residual oxygen in the gas was removed with an Oxytrap (Altech Associates, Arlington Heights, IL). Injection port and detector block temperatures were 220 °C. The column temperature was 100 °C. The column consisted of a 1.5 m (5 ft) × 3.1 mm ($^1/_8$ in.) Teflon-lined stainless steel tube packed with 50/80 Porapak Q (Waters Associates, Milford, MA). The ends were plugged with silanized glass wool.

The injection port was modified: a removable glass liner was incorporated for easier cleaning of nonvolatile residues (Figure 1). A Teflon seat was installed at the column end, and two Teflon washers were inserted as supports for the liner, as shown in the figure (Lund and Shaw, 1979). The liner was loosely plugged at the column end with a 1-cm silanized glass wool plug and fastened at the other end with a wire loop. The stainless steel adapter between the injection port and column (6.2-3.1 mm, $^1/_2$-$^1/_8$ in.) was treated with ThetaKote (The Theta Corp., Media, PA),