STUDIES OF COLLOIDAL CHLOROPHYLL IN AQUEOUS DIOXANE

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ABSTRACT The preparation and properties of a colloidal state of pure chlorophyll *a* in aqueous dioxane are described. The red absorption maximum is at 685± 1 mμ, depending on buffer concentration. The typical 672 mμ colloid (obtained by diluting an acetone solution with water) can be converted directly to the 685 mμ colloid by the addition of 1 M dioxane. The 672 → 685 mμ conversion is irreversible and is second order with respect to both 672 colloid and dioxane. It is shown that the formation of the 685 mμ colloid of chlorophyll *a* requires the Mg atom; no dioxane species is obtained with pheophytin or ethyl pheophorbide. Furthermore, of the transition metal salts of chlorophyll, Cu, Co, Ni, and Zn, only the Zn salt interacts with dioxane.

INTRODUCTION

An association between dioxane and chlorophyll was first suggested by the work of Takashima (1), who found that crystals of chlorophyll asociated with protein precipitated from leaf extracts containing dioxane, α-picoline, and water. Later, H. Tamiya (2) reported in conversations at the International Botanical Congress in 1959 that acicular crystals of chlorophyll and protein precipitated from aqueous carbitol leaf extracts after adding dioxane and standing in the cold. Krasnovsky and Kosobutskaya (3) and Krasnovsky and Brin (4) provided evidence that the association of dioxane and chlorophyll was a specific one and did not depend on the presence of protein. When they diluted a small volume of chlorophyll dissolved in dioxane with a large volume of water, a colloid formed having a 690 mμ absorption maximum. In contrast, as is widely known, dilution of chlorophyll solutions in other water-miscible solvents (*e.g.*, alcohols, acetone, and tetrahydrofuran) with water leads to colloids with absorption maxima at about 673 mμ. Krasnovsky and Brin, and later Belavtseva and Krasnovsky (5) reported that crystalline residues remained after evaporating dioxane solutions and aqueous dioxane colloids of chlorophyll. They suggested that the crystals contained both chlorophyll and dioxane molecules.

The nature of the interaction of dioxane with chlorophyll is of interest for two reasons. First, there appears to be no basis in chlorophyll chemistry for a specific effect of dioxane. While traces of polar solvents *e.g.*, diethyl ether, tetrahydrofuran,

acetone, alcohols, and presumably dioxane activate the fluorescence of chlorophyll, presumably by forming a complex (6), the interaction leading to the 690 mμ colloid is specific for dioxane. Second, the mechanism underlying shifts of the absorption peak of chlorophyll from 660 to 670 mμ—the region characteristic of the dissolved and ordinary colloidal chlorophyll—to longer wavelengths requires further elucidation. While the 730 to 740 mμ peak of crystalline chlorophyll has been attributed to a delocalization of excitation among regularly oriented pigment molecules (7), the positions of the peaks (at about 682 and 694 to 705 mμ) of two of the *in vivo* fractions of chlorophyll have not been explained. As the existence of the 690 mμ colloid shows, the *in vivo* peaks need not be attributed, as has often been done, to specific chlorophyll-protein associations.

Neither the nature of the chlorophyll-dioxane interaction nor the basis for the absorption peak at 690 mμ has been completely clarified in the present study. Our results do, however, indicate the nature of the complex—namely, a colloidal particle of unknown size in which chlorophyll interacts *via* its magnesium ion with dioxane. In addition, the preparation, spectrum, kinetics of formation, and other properties of the dioxane colloid have been determined.

MATERIALS AND METHODS

Crystalline, chromatographically pure chlorophyll *a* and ethyl chlorophyllide *a* were prepared from spinach and tree of heaven (*Ailanthus altissima*) respectively, by the methods of Jacobs, Vatter, and Holt (8). Commercial dioxane was employed in most cases. Provided commercial dioxane was not contaminated by acids or peroxides in high concentrations, it gave results identical to those obtained with dioxane purified by the following procedure. Commercial dioxane was refluxed with 10 per cent of its volume of 1 N HCl for 8 hours, then distilled. The distillate was refluxed over solid NaOH, distilled, shaken with $SnCl_2 \cdot 2H_2O$, and distilled again. The distillate was then refluxed over metallic sodium for several hours and distilled for use.

Pheophorbide *a* and pheophytin *a* were prepared by adding 0.01 N HCl to acetone solutions of chromatographically pure ethyl chlorophyllide *a* and chlorophyll *a*.

Divalent Cu, Zn, Co, and Ni salts of pheophorbide were prepared by adding methanol solutions of the metal chloride salt (A.R. grade) to a chloroform solution of ethyl pheophorbide *a*, containing 2 per cent acetone, and refluxing the mixture. The concentration of metal ions was much in excess of the pheophorbide concentration. Refluxing was continued until green color was restored. This required 1 or 2 hours, except in the case of $NiCl_2$, which required 8 hours' refluxing and was then allowed to stand overnight. After refluxing, the solutions were dried under vacuum, using a water aspirator. The solid material was collected in a Büchner filter and washed extensively with distilled water to remove all excess inorganic salt. The metal pheophorbide was then air-dried, dissolved in acetone, and its spectrum recorded. The red absorption maxima of acetone solutions were observed at 651 mμ (Cu), 647 mμ (Ni), 656 mμ (Zn), 652 to 655 mμ (Co.).

Samples of the dioxane-chlorophyll colloid were prepared either by mixing a small volume of a dioxane solution of pigment with a large volume of buffer (0.05 tris or KH_2PO_4—K_2HPO_4, pH 7.0), or by mixing a small volume of an acetone or tetrahydrofuran solution of pigment with a large volume of buffer containing dioxane.

The earlier spectrophotometric data were obtained with a Beckman DU instrument. Later a Cary 14 instrument became available and was employed thereafter. The wavelength calibration of the Beckman was checked regularly using the 656 mμ emission line of hydrogen, and wavelength settings were always approached from longer wavelengths in order to avoid backlash, which in our instrument corresponded to about 1 mμ. The calibrations of the Beckman and the Cary were also checked using the emission of a neon Geissler tube as light source. With the Cary the positions of the emission lines on the recorded spectrum could be determined with a precision of ± 1 A at the lowest scanning speed. For 18 emission lines between 6029.9 and 7032.4 A. the positions estimated from the Cary record were 2.1 to 3.6 A longer than the true values (9). Thus, the Cary calibration involved a systematic error of 2 to 3 A. The low sensitivity of the phototube of the Beckman permitted only 7 of the neon lines to be detected. The positions of lines indicated by the Beckman were identical to the true values to within ±2.5 mμ. This range of deviation we estimate exceeds the experimental error in setting the wavelength and may be due to shortcomings in the mechanical or optical components of the instrument, or, more probably, to errors introduced by the relatively large slit widths (0.1 to 0.3 mm) needed to detect the emission lines. The slit widths employed during studies of the colloid were 0.045 mm (Beckman) and 0.1 mm (Cary). The position of the absorption maximum of the dioxane colloid of chlorophyll *a* in 0.10 M phosphate buffer was usually found at 682 to 683 mμ ± 1 mμ with the Beckman, but at 685 mμ ± 1 mμ with the Cary. This discrepancy, which is larger than expected from the above discussed errors in calibration and reading, is unexplained; possibly it is due to an effect of light scattering on the position of the absorption maxima, as has been discussed by Latimer (10).

RESULTS

Spectrum of the Dioxane-Chlorophyll Colloid. In Fig. 1 are shown spectra of chlorophyll (1) dissolved in acetone, (2) dispersed in 0.01 M phosphate buffer, and (3) dispersed in 1.0 M dioxane buffered with 0.01 M phosphate. The spectrum of the 672 mμ colloid is characterized by a low, broad, assymetric absorption band; the relatively high absorbance at 700 to 750 mμ indicates moderately strong scattering by the suspension. The spectrum of the dioxane colloid is striking because of its high, narrow, red absorption band which is, in contrast to other states of chlorophyll *a*, higher than the blue band; the suspension is only slightly scattering.

In disagreement with other reports (11), we found that the molar decadic extinction coefficient of chlorophyll *a* in acetone is 8.4×10^4 liters moles^{-1} cm^{-1} at 662 mμ, assuming that the coefficient for chlorophyll *a* in ether is 9.1×10^4 at 660 mμ (11). Using this value for acetone solutions the extinction coefficients (uncorrected for scattering) of the 672 and 685 mμ colloids are, respectively, 4.2×10^4 and 9.0×10^4 at the absorption peaks. The values are precise to $\pm 0.3 \times 10^4$.

There are small shifts in the absorption maximum of the dioxane colloid which depend on the electrolyte concentration. With buffer concentrations less than 0.01 M the absorption maximum is at 684 mμ, at 685 mμ with 0.05 M buffer, and at 686 mμ with 0.10 M buffer. Another effect of electrolyte concentration can be shown by preparing the dioxane colloid in the absence of buffer, then adding NaCl to give

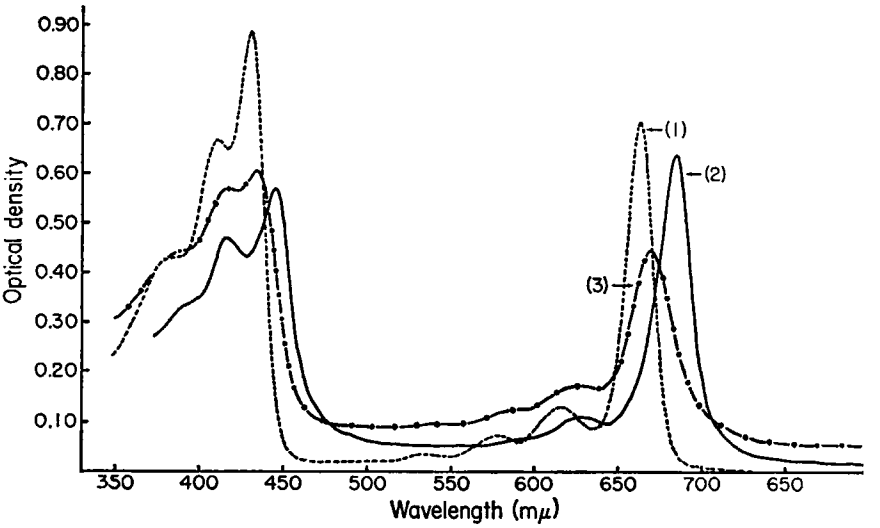


FIGURE 1 Absorption spectra of chlorophyll *a* in (1) acetone, (2) 0.2 M dioxane buffered with 0.05 M tris pH 7.0, (3) 0.05 M tris buffer pH 7.0. The concentrations are not equimolar.

a final concentration of 0.2 M. After adding the salt, the red absorption maximum shifts from 684 mμ to 690 mμ, broadens considerably, and falls in height. Concurrently the optical density at 700 mμ increases and greater scattering is seen. We assume these effects are due to aggregation of the colloid particles caused by partial neutralization of their charge by the electrolyte.

The absorption spectrum (Fig. 1) of the chlorophyll-dioxane colloid was obtained whenever acetone or tetradydrofuran solutions of chlorophyll were rapidly mixed in buffer containing dioxane at a concentration of 0.6 M. With buffers containing smaller dioxane concentrations, spectra were found which analysis showed were mixtures of the 672 and 685 mμ colloids. The analysis consisted of the following: the absorption spectra of identical concentrations of the 672 and 685 mμ colloids were plotted on semilog paper along with spectra of mixtures calculated from the spectra of the pure colloids. To within a small experimental error, it was found that measured spectra of suspensions in buffer containing less than 0.6 M dioxane always coincided with one of the calculated spectra. Thus with dioxane concentrations less than 0.6 M suspensions were proved to be mixtures of the 672 mμ and 685 mμ colloidal species. In Fig. 2 are shown several of the calculated spectra of mixtures and also spectra of suspensions measured in 0.13 M dioxane. (These spectra, obtained with the Beckman DU gave peaks at 670 mμ and 683 mμ as previously noted.) Of interest are the two isobestic points at 670 and 697 mμ.

By matching the spectrum of a suspension with one of the calculated spectra, the relative amounts of the two colloidal species could be estimated. In this way, the

fraction of the chlorophyll present as dioxane-colloid could be assayed. The points in Fig. 3 show how this fraction varies with dioxane concentration. In the same figure, are functions derived from kinetic studies to be discussed later.

Nature of the Dioxane-Chlorophyll Interaction. Elucidation of the nature of the dioxane-chlorophyll association was attempted through studies of interactions of (1) chlorophyll with dioxane analogs and (2) dioxane with chlorophyll derivatives in which the central metal ion and the phytol alcohol side chain were removed or replaced.

Attempts to observe a colloidal state, different from the 672 mμ one and similar to the 685 mμ dioxane colloid, were unsuccessful when various molecules were substituted for dioxane. The following substances were examined: 5-amino-5-ethyl,

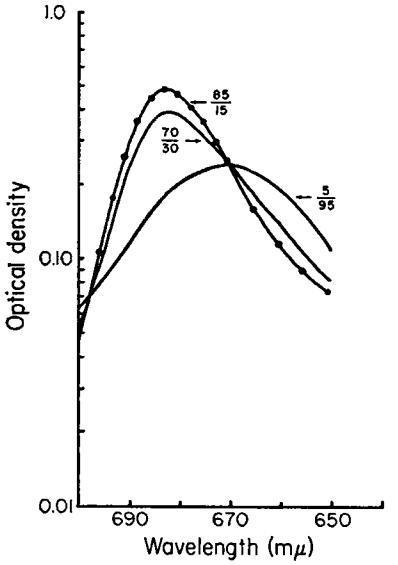


FIGURE 2 A semilog plot of the calculated absorption spectra (solid lines) for different mixtures of the 670 mμ colloid and the 685 mμ dioxane colloid. The ratios shown are

$$\frac{\text{Per cent dioxane colloid}}{\text{Per cent 670 m}\mu\text{ colloid}}$$

The points are experimental (Beckman DU, 0.13 M dioxane).

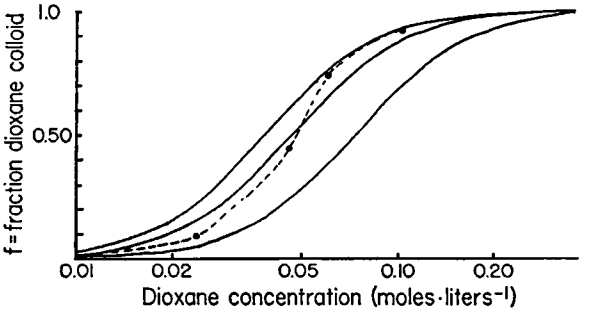


FIGURE 3 Fraction (*f*) of chlorophyll present as dioxane colloid as a function of the log (dioxane) concentration. Solid curves are theoretical; dashed curve is experimental.

1,3-dioxane, *s*-trioxane, 1,3-dioxolane, 4-methyl-1,3-dioxolane, and tetrahydrofuran (these are heterocyclic compounds containing one, two, or three oxygen molecules in five- or six-membered rings); morpholine, *n*-methyl morpholine, 3,5-dimethyl pyrazole, imidazole, and piperidine (these are heterocycles containing nitrogen); 2-methoxy ethanol, ethylene glycol, triethylene glycol, triethylene glycol diethyl ether, and 1,3-butylene glycol (linear molecules containing ether linkages). With all but two of these substances chlorophyll assumed the 672 mμ form. With the basic substances piperidine and morpholine, 655 mμ maxima were observed, which probably indicates the occurrence of the reported reaction (12) between chlorophyll and amines. Unfortunately, neither unsubstituted 1,3-dioxane nor substituted 1,4-dioxanes were available. In summary these studies failed to indicate the specific properties of dioxane required in the association with chlorophyll.

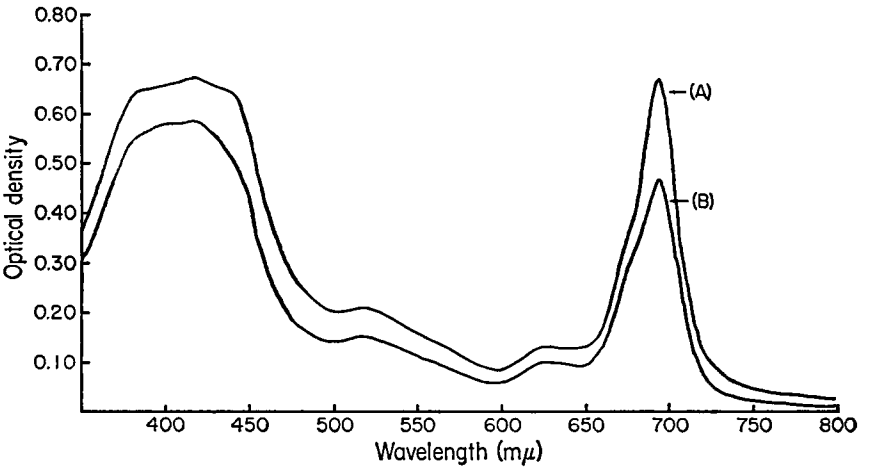


FIGURE 4 Absorption spectra of pheophytin *a* in (A) 1 M dioxane buffered with 1 M phosphate pH 7.0, (B) 1 M phosphate buffer pH 7.0. The concentrations are not equimolar.

In experiments with pheophytin, essentially identical spectra (Fig. 4) were found for suspensions in both aqueous buffer and in 1 M aqueous dioxane. The spectra, for somewhat different pheophytin concentrations both have red absorption maxima at 692 mμ. This shows that pheophytin does not form a dioxane colloid, and suggests that, in the case of chlorophyll, dioxane interacts with the magnesium atom. Ethyl pheophorbide also did not form a dioxane colloid. In contrast, ethyl chlorophyllide in aqueous dioxane possessed a spectrum similar to that of the chlorophyll-dioxane colloid, but the entire spectrum was shifted 4 to 5 mμ to the red giving a peak at 689 to 690μ. In the absence of dioxane, acetone solutions of ethyl chlorophyllide added to buffer immediately formed microcrystalline suspensions. This difference in behavior indicates that ethyl chlorophyllide also forms a dioxane colloid,

as is expected if the magnesium ion is the basis of the reaction. The dioxane colloid of ethyl chlorophyllide is, however, unstable and if followed for 1 or 2 hours shows a slow decay into microcrystals with the characteristic absorption at 735 to 740 mμ.

Not all metal pheophorbides will form a dioxane complex. Thus Cu, Ni, and Co pheophorbides did not form a distinct colloidal state (*i.e.* the spectra were identical in the presence or absence of dioxane). However, Zn pheophorbide does appear to form a dioxane colloid. In 10 per cent (v/v) dioxane in buffer the red absorption band (Fig. 5) of Zn pheophorbide was asymmetric, with a definite broadening on the long wavelength side of the band. In buffer without dioxane, the red absorption band of the Zn salt was symmetric and centered at 661 mμ When the concentration of dioxane was increased to 20 per cent, a shoulder developed at 680 mμ, which was quite pronounced after 20 minutes standing (Fig. 5). These observations sug-

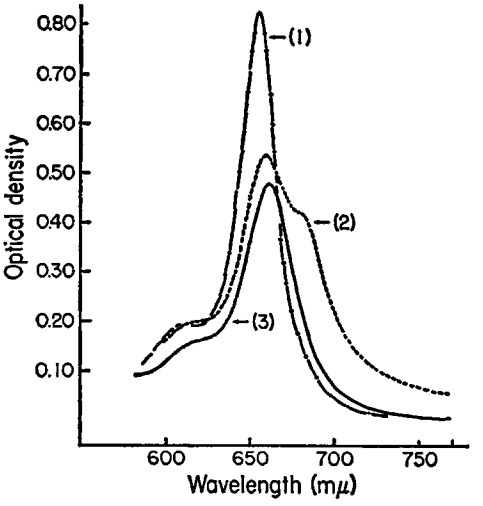
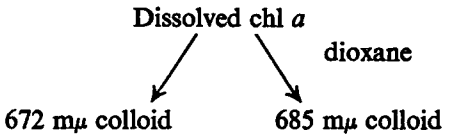


FIGURE 5 Absorption spectra of Zn pheophorbide in (1) acetone, (2) 20 per cent dioxane, (3) water.

gest that a dioxane–Zn pheophorbide complex occurs, but at somewhat higher concentrations than those required for forming the chlorophyll-dioxane colloid.

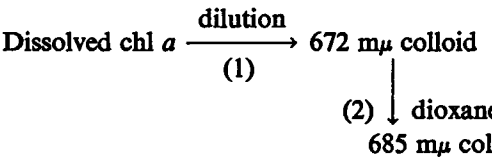
Formation of the Chlorophyll-Dioxane Colloid. As noted earlier, mixtures of the 672 mμ and 685 mμ colloidal states were obtained with dioxane concentrations below 0.6 M, and the relative amount of the 685 mμ colloid increased with increasing dioxane concentration. One mechanism which could account for this distribution is an equilibrium, *i.e.* 672 mμ colloid + dioxane $\rightleftharpoons$ 685 mμ colloid. Such an equilibrium was ruled out, however, by finding that when a suspension of the dioxane colloid was diluted with enough buffer to reduce the dioxane concentration 100-fold, none of the dioxane colloid was converted back into the 672 mμ form. Thus, the formation of the dioxane colloid is an irreversible process, and the previously observed distribution between the two forms must result from a dependence of the rate of formation of the 685 mμ species upon dioxane concentration. Plausible mecha-

nisms of formation of the dioxane colloid are (1) direct formation from dissolved chlorophyll (in which case the 672 mμ and 685 mμ colloids compete for dissolved chlorophyll)



(i)

and (2) rapid formation of the 672 mμ colloid at the time of dilution of the dissolved pigment and subsequent, slower conversion of the 672 mμ to the 685 mμ colloid . . .



(ii)

Observations which support the second, but not the first, mechanism are that the formation of the 672 mμ colloid is very fast while the formation of the 685 mμ colloid is slower, and that when 1.0 M dioxane in buffer (which does not dissolve chlorophyll) is added to the 672 mμ colloid, the latter is converted to the 685 mμ species.

Kinetics of Conversion of Colloidal to Dioxane-Complexed Colloidal Chlorophyll. The kinetics of the reaction were investigated in order to obtain additional support for the mechanism established above, in order to prove that the observed distribution between colloid and complex is a consequence of the dependence of the rate of reaction 2 on dioxane concentration, and in order to determine the order of reaction with respect to colloid and dioxane concentrations.

A preliminary experiment showed that the conversion of the colloid to complex could be conveniently followed by measuring the optical density at 683 mμ (Beckman) as a function of time, over periods ranging from 5 to 50 minutes depending on dioxane concentration. Samples were prepared by pipetting 0.20 ml aliquots of a solution of chlorophyll in tetrahydrofuran into 3.5 ml volumes of 0.05 M tris buffer (pH 7.0) containing varying quantities of dioxane. The optical density was then measured at intervals until the reaction was nearly completed. The time curves for several dioxane concentrations are shown in Fig. 6.

The adherence of the kinetic data to the following rate law was tested.

$$-\frac{dC}{dt} = kC^n d^m \qquad (1)$$

Here, C is the concentration in moles liter^{-1} of chlorophyll present as the 672 mμ colloid, d is the concentration of dioxane in moles liter^{-1}, and k, n, and m are constants. Since, in the samples studied, the concentration of dioxane was at least 10^4

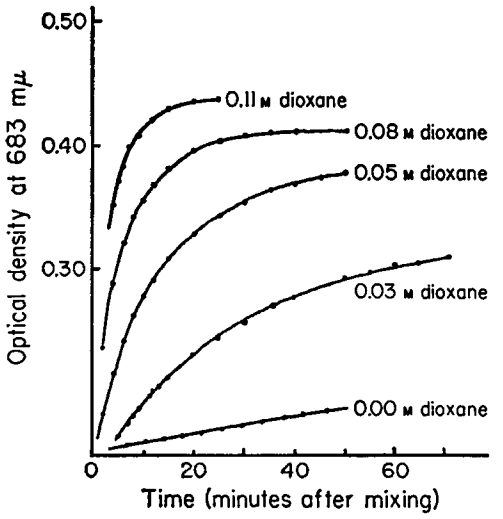


FIGURE 6 Formation of the dioxane colloid. Change in optical density at 683 mμ as a function of time and dioxane concentration.

larger than the chlorophyll concentration, equation (1) reduces to

$$-\frac{dC}{dt} = KC^n \tag{2}$$

where

$$K = k\,d^m \tag{3}$$

and K is a constant for a given dioxane concentration. Upon integration equation (2) becomes

$$\frac{1}{C^{n-1}} = (n-1)Kt - \frac{1}{C_0^{n-1}} \tag{4}$$

where C_0 is the concentration of chlorophyll in 672 mμ colloidal form at zero time. As is evident from equation (3), the assumed dependence of rate on colloid concentration will be verified if a value of the constant n exists such that $1/C^{n-1}$ is a linear function of time.

From the spectrophotometric data of Fig. 6, the concentration (C) of chlorophyll present as colloid was calculated from

$$C = \frac{A_t - E^1\frac{A_0}{E}}{E - E^1} \tag{5}$$

Here, $E(= 3.0 \times 10^4)$ and $E^1(= 9.0 \times 10^4)$ are the extinction coefficients at 683 mμ of colloidal and dioxane-complexed chlorophyll, respectively; A_0 is the absorbancy at time zero when, presumably, all chlorophyll is colloidal. Since the formation of the dioxane compex was rapid, values of A_0 were obtained from preparations lacking dioxane.

Values of $1/C^{n-1}$ were calculated for $n = 1, 2, 3$, at dioxane concentrations of 0.03, 0.05, and 0.08 M and plotted against time. As seen in Figs. 7 and 8 the second order plots ($n = 2$), but not the plots for other orders, were convincingly linear. At dioxane concentrations of 0.17 M and 0.22 M, the reaction is so rapid that it was not possible to obtain accurate kinetic data with the Beckman spectrophotometer. It should also be noted that there is a very slow increase in absorbancy of colloidal chlorophyll in the absence of dioxane (Fig. 6) which has not been corrected for in calculating the concentrations of colloidal chlorophyll. From a comparison of the first, second, and third order test plots, we conclude that the order with respect to colloidal chlorophyll is 2, to the nearest integral order.

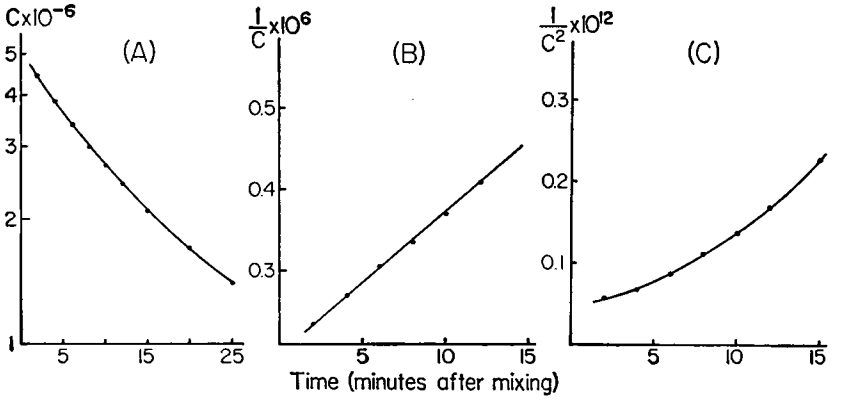


FIGURE 7 Comparison of test plots for (A) first order, (B) second order, (C) third order reaction with respect to the 672 mμ colloid. C is the concentration of 672 mμ colloid in moles-liter^{-1} at time t.

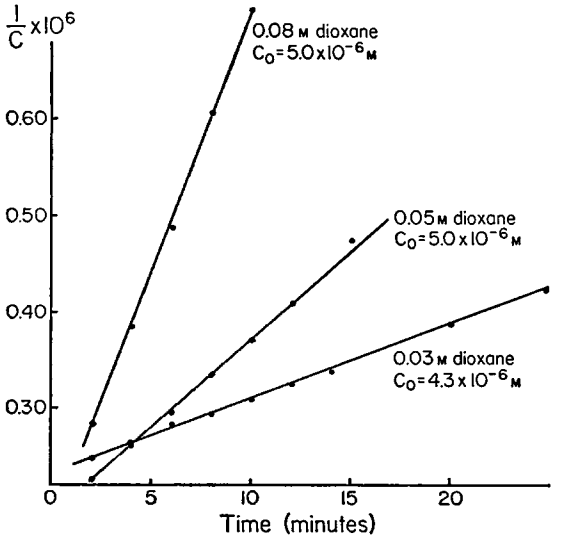


FIGURE 8 Second order test plots for the conversion of the 672 mμ colloid to the 685 mμ colloid. C is the concentration of the 672 mμ colloid at time t and C_0 is the initial concentration of chlorophyll.

From the slopes = $(n-1)\ K = K$ of the second order test plots (Fig. 8), the values of K at different dioxane concentrations were calculated. The values obtained are as follows:—

Dioxane	$K \times 10^6$
moles/liter	*moles*$^{-1}$ *liter*$^{+1}$ *min.*$^{-1}$
0.03	0.0078
0.05	0.019
0.08	0.054

According to equation (3), log K should be a linear function of log (dioxane concentration). This dependence, to within experimental error, was found when K was graphed against dioxane concentration (Fig. 9). The slope of the line, equal to

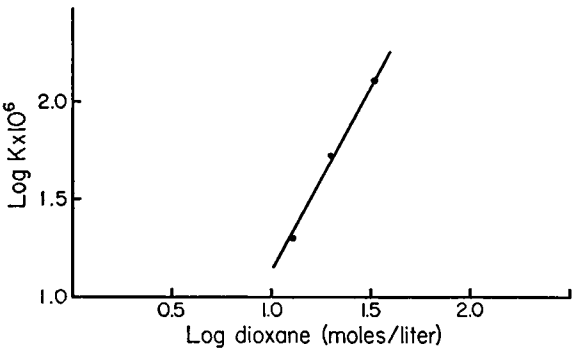


FIGURE 9 Graph showing the relation of the log of the dioxane concentration to the log of K.

the order (m) of the reaction with respect to dioxane, was found to be 1.9 ± 5 per cent, or 2 to the nearest integral order. From equation (3) the average value of k was calculated and found to be 8.3×10^6 ± 5 per cent (moles^{-3} liter^{+3} min.$^{-1}$). Both rate constants, K and k, are for colloid concentrations expressed as concentration (in moles liter^{-1}) of chlorophyll present as colloid.

In order to show that the kinetics of the reaction are consistent with the previously observed distribution of pigment between colloid and dioxane complex (Fig. 3), an equation for the fraction (f) of chlorophyll present as dioxane complex at a time t after adding chlorophyll to a dioxane solution was derived from equation (4);

$$f = 1 - \frac{C}{C_0} = 1 - \frac{1}{k(\text{dioxane})^2\, C_0 t + 1} \qquad (6)$$

Here k is the rate constant ($= 8.3 \times 10^6$) and C_0 is the concentration of chlorophyll present as colloid at time zero. For the data of Fig. 3, C_0 was equal to 7.8×10^{-6} M. The fraction, f, as a function of dioxane concentration was plotted for times of 1,

3, and 5 minutes. As seen in Fig. 3, the points for the fraction of complex determined earlier by analysis of absorption spectra fall on or between the curves for about 1 and 5 minutes. Considering that the earlier data were obtained without strict control of the time elapsing between preparation of the sample and completion of its spectrum, the derived curves fit the points very well. Thus, the distribution between colloid and complex appears to be wholly accounted for by the dependence of the rate of formation of the dioxane complex on dioxane concentration.

DISCUSSION

The dioxane-chlorophyll association studied here occurs only when chlorophyll is in the colloidal state. This is indicated by two observations; first, a failure to find any effect of dioxane on the solution absorption spectrum when dilute or concentrated solutions of chlorophyll were studied, and second, the flocculation of the dioxane colloid by NaCl.

It has been shown that the formation of the dioxane-chlorophyll association requires the presence of the Mg atom, since neither pheophytin nor ethyl pheophorbide form a spectroscopically recognizable complex. It is perhaps not unexpected that chlorophyll should complex with dioxane, since complexes between chlorophyll and ketones, ethers, and alcohols are known to activate fluorescence (6). However, in the present case dioxane behaves differently in aqueous solution from any of the fluorescence activators studied. When chlorophyll is dissolved in acetone, ethanol, or any other fluorecence activator studied (except dioxane), and then added to a large excess of water, the typical 672 mμ colloidal state is formed. Only in the case of dioxane was a distinct colloidal species absorbing in the 685 mμ region obtained upon dilution with water.

From the standpoint of direct complex formation between chlorophyll and dioxane—with the electrons of the two heterocyclic oxygens of dioxane acting as donor and the magnesium atom acting as acceptor—it would be attractive to postulate that each molecule of dioxane is bound to two chlorophyll molecules through their magnesium atoms in a type of dimer formation. With the present data, it would not be possible to decide whether this type of interaction *i.e.*, . . . chl–dioxane–chl . . . , could be extended to give large particles with a pseudocrystalline character. There is evidence from recent work that dioxane does complex directly with transition metal ions. Chandy and Moosath (13) have shown that metallic halide dioxanates of divalent Ni and Co are formed from $Co(OH)_2$ and Ni $(OH)_2$ in dioxane solutions swept with HCl. Also Cardinaud (14) has presented spectroscopic evidence of complex formation between *p*-nitrophenol and dioxane.

It is difficult, however, to invoke direct complex formation as an explanation of the colloidal phenomena studied here, even though it may be a predominant factor when chlorophyll is dissolved in dioxane solution. First, no splitting or broadening of the red absorption band is observed in the colloid, as might be expected (15, 16)

if a true dimer had been formed. Second, it is difficult to understand why structurally similar molecules *e.g.,* 1,3-dioxolane or 5-amino-1,3-dioxane, or 6-membered ring compounds with nitrogens in the para positions, do not participate in a similar type of association with chlorophyll. It is certainly well known that nitrogen, as well as oxygen, is a good donor in complex formation. In the case of the 1,3-oxygen heterocycles, even though there may be some steric differences, it is not clear why some distinct type of complexes should not have formed. Finally no spectroscopically recognizable complex was formed between dioxane and the Cu, Ni, or Co salts of chlorophyll. From the work of Chandy *et al.,* cited above, it might be expected that some complex formation would take place, while in fact only in the case of the Zn salt was there spectroscopic evidence of an interaction with dioxane.

An alternative mechanism which is believed to be more compatable with these results, is based on the ability of dioxane to lower the dielectric constant of aqueous solutions (17). In the present case the lowering of the solvent dielectric constant, leading to increased electrostatic repulsion between charged particles, is believed to be responsible for the formation of a new colloidal state with a distinct absorption spectrum. Thus, no direct complex need be formed, and as is typical of most phase transitions, it would be expected to be an irreversible process. Doty *et al.* (18) have used this lowering of solvent dielectric constant by dioxane to study the properties of polyglutamic acid. Foster and Yang (19), and Van Holde and Sun (20) have attributed the observed increase in the volume of bovine serum albumin in aqueous dioxane to a lowering of the solvent dielectric constant, and the results of Van Holde and Sun are in semiquantitative agreement with this mechanism, rather than with a direct interaction of dioxane with the protein. In the present case this mechanism is in agreement with the failure to find complexes between chlorophyll and 1,3-dioxolane and substituted 1,3-dioxane, since it is well known that both of these molecules possess considerable dipole moments (21). Furthermore, it has been shown that chelates of transition metal ions with *bis*-salicylaldehyde ethylenediimine have stability that decreases, and ionic character that increases in the series Cu, Ni, Zn, Mg (22). The results of Barnes and Dorough (23) on the stability of metalloporphin chelates indicate that in general the order of stability is, small divalent > large divalent > alkali metal. It is also known that the correlation of the stability constants and second ionization potential for chelates formed by 5-salicylaldehyde sulfonate and the metals Co, Ni, Cu, and Zn shows a discontinuity at Zn, where the stability falls (24). Hence, in the present case, the non-ionic chelates *e.g.,* Cu chlorophyll as well as the metal-free derivatives pheophytin and pheophorbide, may lack sufficient ionic character for the lowering of the solvent dielectric constant (by dioxane) to have an effect. Under these circumstances, of course, there would be no appearance of an interaction with dioxane. The appearance of a dioxane-dependent colloidal state of the Zn salt may be attributed to its anomalous stability characteristics, and presumably greater ionic character.

Although the order of magnitude of the charge existing on a non-isoelecric protein molecule might not be comparable to that existing on a colloidal chlorophyll particle, and although the dioxane concentrations employed here (*ca.* 6 per cent) are the minimum concentrations used in the protein studies cited above, nevertheless this mechanism is believed to be more compatible with the experimental results than a mechanism involving specific complex formation.

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