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Ring V Reactions of Chlorophylls and Pheophytins with Amines

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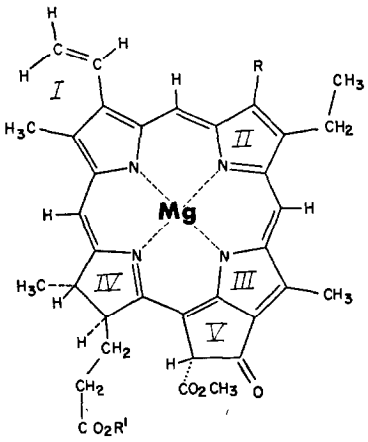
A study of the kinetics of the reaction of chlorophyll *a* with propylamine and isobutylamine indicates a low activation energy (~5 kcal) and high negative entropy (~60 eu). Propylamine and isobutylamine react with Ring V cleavage more readily with chlorophyll *b* and pheophytin *b* compounds than with the *a* compounds, and more readily with the pheophytins than with chlorophylls.

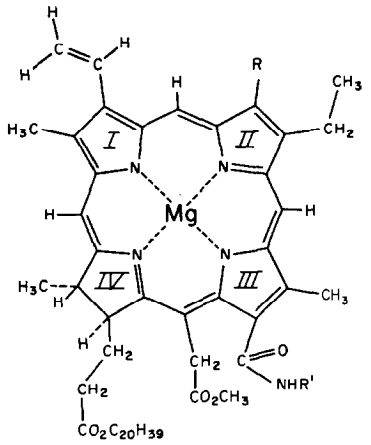
Two features of special interest in chlorophyll **1** are the isocyclic Ring V and the central magnesium atom. Coordination of basic (nucleophilic) compounds such as water or amines at those sites may play a significant role in photosynthesis. The import-



1a Chlorophyll *a*, R = $-CH_3$
1b Chlorophyll *b*, R = $-CHO$

ance of water in micelle formation (*1*) in aliphatic hydrocarbon solvents and photoreduction experiments *in vitro* utilizing the carbonyl reagent phenylhydrazine (*2*) point to the need for a better understanding of both the coordination of bases to the central magnesium atom of chlorophyll and of reactions at the carbonyl group of Ring V.

While ordinary β-keto esters, such as methyl-2-cyclopentanonecarboxylate, react with amines to give carbinolamines and enamines (*3*), Ring V in chlorophyll is readily cleaved and chlorinamides **2** are formed (*4*, *5*). Ring V cleavage is favored, probably because of the steric relief offered in breaking the ring. The bond in Ring V undergoing



2a R = $-CH_3$
2b R = $-CHO$

rupture is slightly longer than an ordinary C—C bond and it is generally agreed that such bonds are more subject to cleavage than are shorter bonds. Weller and Livingston (*5*) were the first investigators to obtain kinetic data for the amine reaction with chlorophyll, although ring opening of other porphyrins possessing isocyclic rings had been reported earlier (*6*).

In our efforts to establish unequivocally the structures of the products (*4*) we were not able to duplicate the kinetic data of Weller and Livingston. We also noted that trace amounts of water or alcohol markedly affect the reaction rates. As it was possible that the differences in rates were due to (a) interaction of hydrogen bonding agents (H_2O) with Ring V, or (b) coordination of water with the magnesium, we have carried out a more thorough investigation of the kinetics. Our interest has been intensified by the recent reports of Ballschmiter and Katz (*1*) on the unique and complex interactions of chlorophyll with water and by the suggestion that chlorophyll–water adducts may be involved in the photoactivity of chlorophyll (P700) *in vivo*. (See Ref. 7 for pertinent references.)

There are primarily two aspects that we have considered in studying the kinetics of the chlorophyll–amine reaction. First, we have determined the rate of the reaction at several temperatures with varying concentrations of added water or alcohol in order to understand the role of these agents on the kinetics and the entropy of activation. Second, we have established the relative rates of reaction of amines for chlorophylls *a* and *b* and pheophytins *a* and *b*. These data give a general indication of the extent to which magnesium and the formyl group affect the π systems and thereby the reactivity of the carbonyl group in Ring V.

RESULTS AND DISCUSSION

Tables 1 and 2 summarize our results for the reaction of chlorophyll *a* with *n*-propylamine and isobutylamine. Visible absorption spectra of chlorophyll *a* and its *n*-propylamine derivative are shown in Fig. 1, and an example illustrative of the decrease in the

TABLE 1

LOGARITHM OF PSEUDO-VELOCITY CONSTANTS[a] FOR THE REACTION OF CHLOROPHYLL *a* WITH SOLUTIONS OF ISOBUTYL- AND *n*-PROPYLAMINE WITH ALCOHOL

Amine	%ETOH (v/v)	Log k' sec^{-1} at 26°C[b]
Isobutylamine	0.0	-3.48 ± 0.02
	0.0010	-3.10 ± 0.08
	0.010	-2.97 ± 0.08
	0.10	-3.03 ± 0.02
	1.0	-3.10 ± 0.01
	2.0	-3.27 ± 0.05
	5.0	-3.10 ± 0.08
	10.0	-2.90 ± 0.08
n-Propylamine	0.0	-3.74 ± 0.04
	0.0010	-2.91 ± 0.00
	0.010	-2.79 ± 0.02
	0.10	-2.80 ± 0.03
	1.0	-3.09 ± 0.03
	4.0	-2.75 ± 0.02
	10.0	-2.68 ± 0.01

[a] $k't = \log(E - E_\infty)/(E_0 - E_\infty)$, where k' is the pseudo-first-order constant, E is the optical density at time t, E_0 is the initial OD, and E_∞ is the OD at reaction completion.

[b] Most of the data are based on at least three kinetic runs.

red absorption maximum at 660 nm of chlorophyll *a* and the concurrent growing in of the 641-nm peak of the *n*-propylamine derivative as a function of time is shown in Fig. 2. The reaction rate in the form of a pseudo-first-order rate constant k' was calculated from the decrease in the red absorption maximum at 660 nm; k' is defined as in Ref. *4*. The concentration of chlorophyll *a* in these experiments was 10^{-6} *M*. The addition of water or ethanol had a marked effect on the reaction rates, generally increasing them. Very significant increases in rates were observed when reactions in anhydrous amines were compared with reactions in amines containing 0.0010% water or ethanol. The molar ratio of water to chlorophyll is about 100:1 (*8*) in a 0.0010% solution. Additional 10-fold increases in water concentrations did not increase the rates as markedly.

We also examined the reaction of chlorophyll *a* with propylamine and isobutylamine at several different temperatures, both under anhydrous conditions and with two different concentrations of water (0.010% and 1.0%). These data are included in Table 2. A plot of log k' vs $1/T$ did not give good straight lines, but energies of activation were clearly low (5 ± 1 kcal). Weller and Livingston observed curvature in their

TABLE 2

LOGARITHM OF PSEUDO-VELOCITY CONSTANTS FOR THE REACTION OF CHLOROPHYLL *a* WITH SOLUTIONS OF ISOBUTYL- AND *n*-PROPYLAMINE WITH WATER

Amine	% H_2O (v/v)	Log k' sec^{-1} at 26°C[a]	Log k' sec^{-1} at 1°C	Log k' sec^{-1} at 10°C	Log k' sec^{-1} at 40°C
Isobutylamine	0.00	−3.48 ± 0.02	−3.74 ± 0.02	−3.64 ± 0.07	−3.10 ± 0.05
	0.0010	−3.02 ± 0.04			
	0.010	−3.00 ± 0.03	−3.60 ± 0.01	−3.47 ± 0.04	−2.91 ± 0.01
	0.10	−3.02 ± 0.02			
	1.0	−3.07 ± 0.04	−3.43 ± 0.03	−3.37 ± 0.03	−2.82 ± 0.03
	2.0	−2.91 ± 0.02			
	2.5	−2.88 ± 0.01			
	5.0	−2.53 ± 0.00			
	10.0	−2.19 ± 0.01			
n-Propylamine	0.00	−3.74 ± 0.04	−3.79 ± 0.1		−3.36 ± 0.01
	0.0010	−3.02 ± 0.05			
	0.010	−3.04 ± 0.06	−3.50 ± 0.07	−3.36 ± 0.00	−3.04 ± 0.03
	0.10	−3.00 ± 0.06			
	1.0	−2.89 ± 0.06	−3.30 ± 0.04	−3.30 ± 0.04	−2.85 ± 0.02
	4.0	−2.44 ± 0.03			
	10.0	−1.94 ± 0.01			

[a] Most of the data are based on at least three kinetic runs.

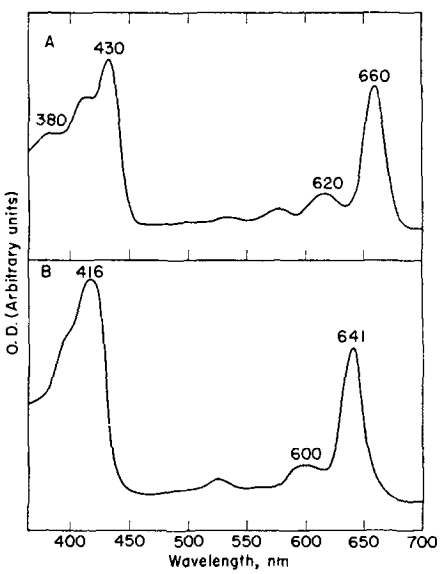


FIG. 1. Visible absorption spectra in diethyl ether of (A) chlorophyll *a* (10^{-6} *M*), and (B) the reaction product of chlorophyll *a* and *n*-propylamine.

variable temperature studies (*5*) and deduced an approximate value of 3 kcal for the energy of activation.

In interpreting these data it should first be noted that in pure amine solution it is expected that the chlorophyll molecule will have at least one amine molecule coordinated in the Mg axial position. A rather weak coordination interaction of amine with Ring V should also occur. Water added in small amounts competes with the amine for coordination sites on the magnesium. Snellgrove and Plane (*9*) explained water

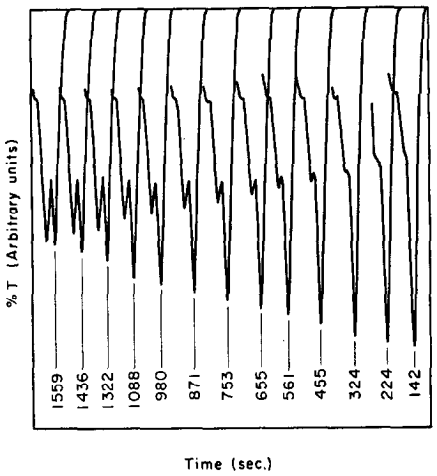


FIG. 2. Kinetics of chlorophyll *a*–*n*-propylamine reaction at 10°C, followed by decrease in the optical density at 660 nm as a function of time.

catalysis of magnesium displacement from various porphyrins in terms of competitive coordination of pyridine, water, and alcohols. With aliphatic amines, however, displacement of amines by water would be considerably more difficult, if only from mass action considerations. At Ring V, however, the possibility of competitive hydrogen-bonding with water is a distinct possibility.

We consider, therefore, that the effects of small amounts of water or alcohol should first be sought in coordination effects at Ring V resulting from hydrogen-bonding at the carbonyl functions of Ring V: keto C═O---HOR. Although the keto C═O and the carbomethoxy function of Ring V constitute a β-keto ester, we need not be concerned with the role of the enol form in the reaction under study. Proton magnetic resonance studies, not otherwise described here, show that chlorophylls and pheophytins occur predominantly in the keto form; the proton at C-10 is clearly visible throughout the reaction. The infrared data also establish the presence of the keto C═O group. There is evidence from models that the C—C bond in Ring V is unusually long. A shortened double bond that would result from enolization would thus not be favored sterically. It is, therefore, difficult to avoid the conclusion that the keto form is by far predominant, and that the role of enol in these reactions must be of lesser importance. Moreover, rate enhancement by water is also observed with pheophytin *a*, which lacks the central magnesium atom. At 1°C the log k' for pheophytin *a* and isobutylamine is -2.2 under anhydrous conditions, compared with -1.4 with 1% water present for the enhancement.

Hence, some kind of a Ring V interaction by water is the most likely interpretation for the results, but association of the macrocycle with water cannot be excluded.

At the beginning of the reaction of chlorophyll with amines, the environment near the reaction site is not highly polar. Weller and Livingston noted that the reaction of isobutylamine with chlorophyll in methylcyclohexane had a $\Delta S\dagger$ of about −50 eu, not greatly different from our value of about −60 eu. A possible explanation of the large negative entropy of activation is that solvation by water or alcohol of a dipolar reaction intermediate is responsible for the rate enhancement. However, varying the amount of water from as anhydrous as possible to 1.0% does not lead to a major change in the entropy of activation. It seems to us more likely, therefore, that coordination of chlorophyll with water or alcohol occurs both initially and in the transition state. Ring V may be especially adapted, both sterically and electronically, to association with water, or perhaps water is coordinated to both the magnesium in chlorophyll and to the imine groups in pheophytin. Thus, the high negative entropy is not due to marked differences in coordination but to the inherently greater order provided by a dipolar transition state.

Weller and Livingston (*5*) reported that the presence of magnesium makes Ring V more reactive, and observed a faster reaction of chlorophyll *b* than pheophytin *b* with isobutylamine. We have observed the opposite. By examing the reaction of chlorophyll *a* and pheophytin *a* in the infrared at about 0.1 *M* using a solution of 3.5% *n*-propylamine in diisobutylamine, it was possible to follow the reaction quantitatively by following the rate of disappearance of the free keto C=O absorption near 1700 cm^{-1} under conditions that were comparable to those that could be employed in the visible, except for the differences in concentrations. Reaction of chlorophyll *a* and pheophytin *a* with diisobutylamine proved to be extremely slow. Consequently, the observed reaction was essentially that of *n*-propylamine with pheophytin *a*. Under these conditions, the reaction of pheophytin *a* with propylamine ($\log k = -3.6$) was about 10 times as fast as the reaction of chlorophyll *a* with propylamine ($\log k = -2.6$). Thus, contrary to Weller and Livingston's observation with chlorophyll *b* and pheophytin *b*, we observed that the presence of magnesium led to slower reaction rates. We did not see any major shifts in the red absorption maximum of pheophytin *b* in visible absorption spectral studies. Weller and Livingston do not give the details of their rate studies with pheophytin *b*, and it is not clear what data they use as a basis for their conclusion that chlorophyll *b* is more reactive than pheophytin *b*.

Changes in the satellite peaks of the red maximum of pheophytin *a* were observed as the ring opening occurred. The intensities of peaks at 525, 550, and 600 nm decreased, and by following these intensity changes the reaction of pheophytin *a* with isobutylamine at 10°C was found to have a $\log k'$ of about −1.9 compared to a $\log k'$ of −3.6 for chlorophyll *a* under the same conditions. Therefore, our studies both in the visible at 10^{-6} *M* and in the infrared at 10^{-1} *M* revealed that pheophytin *a* is more reactive than chlorophyll *a*. The slower rate of reaction of chlorophyll may be a consequence of an increase in the negative charge in the π-cloud of the macrocycle, with a resultant decrease in the partial positive charge on the carbonyl carbon, which makes it less susceptible to nucleophilic attack.

We previously noted that reaction of primary amines and chlorophyll *b* occurs at both the formyl group and at the ketone carbonyl function, and that the kinetic data

were not clearly first order (*4*). Thus, the large visible spectral changes observed with chlorophyll *b* dissolved in amines do indeed reflect cleavage of Ring V, but the spectral changes could involve not only chlorophyll *b* itself but also its aldimine derivative resulting from reaction of a primary amine with the formyl group. We considered it desirable to clarify this situation.

Phenylhydrazine appeared to be a useful tool for investigating the reactions involving chlorophyll *b* because its reaction with the formyl group was much faster than its reaction with the keto C=O function of Ring V. A 20% solution of phenylhydrazine dissolved in tetrahydrofuran reacted rapidly with the formyl group as judged from the infrared spectra, the reaction being essentially complete in 5 min. For analysis, samples were removed periodically from the infrared cell and transferred into diethyl ether, and the visible spectra of the ether solutions were determined immediately. As the formyl group absorption diminished in the infrared, a new absorption peak appeared at 650 nm.

After the formyl carbonyl absorption in the infrared had nearly disappeared, a sample of the solution from the infrared cell was removed and placed in pure isobutylamine. Reaction was immediate and rapid as indicated by the reduction of the absorption maximum at 650 nm. A reaction rate of $\log k' = -3.4$ was obtained, which is not the reaction rate for chlorophyll *b*, but rather that for the phenylhydrazone derivative of chlorophyll *b*.

The reaction of phenylhydrazine with chlorophyll *b* is thus complicated by phenylhydrazone formation. Phenylhydrazine has been widely used as a sensitizer in photochemical reactions involving chlorophyll. Livingston, Sickle, and Uchiyama (*2*) observed that a 0.05 *M* methanol solution of phenylhydrazine altered the shape of the red peak of chlorophyll *b*. It seems to us reasonable to suggest that in many photochemical studies sensitization may not have been mediated by chlorophyll *b*, but by its phenylhydrazone. A multitude of other photochemical studies (*10*) involving phenylhydrazine and chlorophyll are likewise complicated by the possibility of Ring V phenylhydrazone formation.

In order to establish the relative rates of Ring V cleavage for the chlorophylls and pheophytins, changes in infrared spectra were observed using a 5% piperidine solution in tetrahydrofuran. Piperidine reacts rapidly with both chlorophyll *a* and *b*, but reaction occurs only at Ring V (*4*) because piperidine is a secondary amine. Our studies indicated that both pheophytins are more reactive than the corresponding chlorophylls and the "*b*" compounds are more reactive than the "*a*" compounds. Therefore, replacing magnesium with hydrogen and replacing a methyl group by a formyl group both increase Ring V reactivity. The formyl group has the expected effect of withdrawing electrons from the π-system of the macrocycle and thus effectively increasing the positive charge on the Ring V carbonyl carbon.

EXPERIMENTAL

Isolation of chlorophylls. Chlorophylls *a* and *b* were isolated from spinach essentially by the procedure reported by Strain, Thomas, and Katz (*11*). The presence of water in chlorophyll *a* preparations was detected by dissolving the sample in cyclohexane and very quickly scanning the spectrum from 750 to 650 nm. As noted by Ballschmiter and

Katz (*1*), chlorophyll *a* in aliphatic hydrocarbons exhibits absorption at 740–745 nm if it contains water. We observed that the 740 to 745-nm absorption in cyclohexane decreased with time and that the decrease in intensity followed first-order kinetics. As this rate was variable with a log k' of −1.56–1.77, the observed rate may be a measure of the size of the chlorophyll–water adducts of Ballschmiter and Katz (*1*).

Thorough drying of the pigments was accomplished by dissolving them in carbon tetrachloride, concentrating the solution in vacuum (10^{-2} mm), heating the dry pigments for 30 min at 60–70°C in vacuum, followed by pumping in vacuum overnight. Samples dried in this manner showed no absorption at 740–745 nm in aliphatic hydrocarbon solvents. The amines were purified and dried in the namner previously described (*4*), and reactions with anhydrous amines were carried out by a final distillation of the amines over molecular sieves on a vacuum line. It should be apparent that thorough drying of both the solvents and reagents and of the chlorophyll and pheophytins is essential.

Isolation of pheophytins. Ethereal solutions of chlorophyll were shaken with 6 *N* hydrochloric acid until the green color turned to brownish green (usually 5–10 min). The ether was washed with water, dried (Na_2SO_4) and concentrated in vacuum. The pheophytin was purified by chromatography on powdered sugar and eluted from the sugar with ether. The ether was washed well with water, dried (Na_2SO_4), and concentrated in vacuum. The residual pigment was dried in the same manner as the chlorophylls.

Procedures for following the reaction of chlorophylls with amines. Pseudo-first-order rate constants were determined by means of the equation $k't = \log (E - E_\infty)/(E_0 - E_\infty)$ used previously (*4*) (see footnote *b*, Table 1). The concentration of amine was determined by density measurements in order to obtain rate constants, since $k = k'/[\text{Amine concn}]$, where k is the true rate constant.

1. Visible spectrum investigations. The chlorophyll was dissolved in a small amount of the amine solution being investigated, and the concentrated solution quickly diluted to the desired concentration by transferring it to amine solution in 1-cm quartz cells.

A Beckman DK-2A spectrophotometer with a thermostated cell compartment was used for all measurements. To obtain reproducible results it was essential that all solutions of water or alcohol dissolved in amine be freshly prepared. Different preparations of chlorophyll gave comparable data, and for many of the experiments not involving anhydrous amines thoroughly dry chlorophyll samples gave the same results as chlorophyll that shows the 740 to 745-nm absorption in cyclohexane. For experiments with anhydrous amine the chlorophyll was thoroughly dried in vacuum, the amine was distilled onto the chlorophyll on the vacuum line, and the reaction tube sealed off in vacuum. Dry chlorophyll and dry amine handled without delay gave results comparable to those obtained in sealed tubes. Many observations led us to conclude that dry air or oxygen had no significant effect on the chlorophyll–amine reactions, and that reaction rates observed in sealed, degassed systems were comparable to those carried out in dry air.

2. Infrared spectrum investigations. A Beckman IR-8 Spectrophotometer equipped with a very slow scan motor was used for the infrared studies. Most readings were in sodium chloride 0.1-mm cavity cells; with solutions containing water, calcium fluoride cells were employed. Absorbance readings were roughly comparable to those obtained

by measuring the areas of the absorption bands as displayed on an external recorder. In the infrared studies relative rates were of particular interest, so no attempt was made to thermostat the cell compartment, which was about 40°C.

To compare results in the infrared with those in the visible, small samples were removed from the infrared cell, diluted with ether and the visible spectra determined at 40°C. An approximate half-life was obtained by this procedure which was in good agreement to that obtained from the infrared observations.

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