[CONTRIBUTION FROM THE PHOTOSYNTHESIS LABORATORY, UNIVERSITY OF ILLINOIS]

Fluorescence Properties of the Intermediates in the Photoreduction of Chlorophyll *a* and Evidence for Complex Formation in Solution

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It is shown that there are (at least) two intermediate forms of chlorophyll formed during its photoreduction by phenylhydrazine. One form has a fluorescence maximum at 625 mμ; the action spectrum for exciting this fluorescence has maxima at 420, 570 and 615 mμ. The other form has a fluorescence maximum at 545 mμ; the action spectrum for exciting this fluorescence has maxima at 410, 500 and 520 mμ. Fluorescence and action spectra at −193° are also given. Evidence is presented in support of the contention that the first stable intermediate in the photoreduction of chlorophyll is the form which has a fluorescence maximum at 625 mμ and absorption maxima at 420, 570 and 615 mμ. Fluorescence data are presented which substantiate the theory that a complex between chlorophyll and phenylhydrazine is required for this photoreduction.

I. Introduction

It has been observed[2] that chlorophyll can be reversibly reduced by ascorbic acid in pyridine solutions in the absence of oxygen. The reduced "pink" form of chlorophyll is relatively stable and has an absorption band at 520 mμ. It was found in the present study that if in place of pyridine, the reduction is carried out in diethyl ether or in a mixture of 5 parts ether, 5 parts isopentane and 2 parts ethanol (henceforth to be referred to as EPA), there is an additional weak absorption maximum at 585 mμ.

Evstigneev and Gavrilova[3] have discussed the fluorescence of reduced chlorophyll in toluene. At room temperature they observed a single band between 600–635 mμ; at the temperature of liquid nitrogen two additional bands appeared, a strong one between 525–545 mμ and a weak one between 550–570 mμ.

In the present study, it was found that when chlorophyll *a* in EPA is reduced with excess phenylhydrazine at 20°, two fluorescence maxima appear, one at 625 and another at 545 mμ. At the temperature of liquid nitrogen, these fluorescence maxima are shifted to 625 and 530 mμ. The action spectrum for exciting the fluorescence of these two species will be described below. (The fluorescence band at 550–570 mμ reported by Evstigneev and Gavrilova[3] for toluene was not observed.)

Evstigneev and Gavrilova[3] have proposed that the absorption bands at 520 and 585 mμ correspond to two different forms of reduced chlorophyll—a dissociated and non-dissociated form of a semiquinone. The evidence they present is that the absorption bands at 520 and 585 mμ can be independently modified by chemical treatment. The present findings support their hypothesis.

Fluorescence data are given which indicate that the primary photoproduct in the reduction of chlorophyll is the form whose absorption maximum is at 585 mμ. These data support the contention that a complex between chlorophyll and phenylhydrazine is involved in the primary photochemical reaction.

II. Methods

A description of the equipment used to obtain fluorescence and action spectra has been given elsewhere.[4] (Essentially, two monochromators are used, one to provide a monochromatic source of illumination for exciting fluorescence, the other for observing fluorescence. To excite fluorescence the 365, 405 and 436 mμ mercury lines are used.)

All fluorescence spectra have been corrected for the spectral response of the photomultiplier tube used as the fluorescence detector. In the figures showing action spectra, the ordinates are equal to $F(\lambda)/I_0(\lambda)$, which corresponds to the energy (in relative units) required to excite fluorescence. $I_0(\lambda)$ is the number of quanta of incident light as a function of wave length. $F(\lambda)$ is the fluorescence intensity from a particular molecular species as a function of the wave length of the incident light and is given in relative units.

Both the absorption and fluorescence spectra of the solution of reduced chlorophyll possess several bands. To determine which absorption bands correspond to a particular fluorescence band, the action spectrum for exciting its fluorescence was measured. This action spectrum is a close approximation to the absorption spectrum of the particular species giving rise to the fluorescence. The maxima of action spectra readily can be correlated with similar maxima occurring in the absorption spectrum of a solution of reduced chlorophyll. The quantitative relationship between action and absorption spectra is discussed below.

In general, the absorption spectrum of a molecule and the action spectrum for exciting its fluorescence are identical, provided all quanta absorbed are equally efficient in exciting fluorescence. A difference can exist between the two spectra, as in the case of a solution containing several species of fluorescent (and non-fluorescent) pigments. This difference results from some of the incident light being absorbed by species which do not give rise to the particular fluorescence band under investigation.

It can be shown that if reabsorption of fluorescence is negligible (as is the case with the low concentrations used in the experiments described below), eq. 1 describes the relationship between action and absorption spectra.

$$F(\lambda) = \frac{C\epsilon(\lambda)}{A(\lambda)} \phi\, KI(\lambda) \qquad (1)$$

The action spectrum of the fluorescent molecule is given by $F(\lambda)$, its absorption spectrum by $C\epsilon(\lambda)$ (where C is concentration), and the total absorption spectrum of the several pigments in solution by $A(\lambda)$ (in optical density units). $I(\lambda)$ is the total number of quanta absorbed, ϕ is the fluorescence yield of the molecule in question and K is a geometry factor. (A correction for reabsorption of fluorescence, which is necessary at high concentrations, enters primarily as a constant term added to $A(\lambda)$.)

III. Experimental Results

A. Fluorescence and Action Spectra of Reduced Chlorophyll. Experiments at 20°.—A solution of chlorophyll *a* (10^{-6} *M*) and phenylhydrazine (10^{-3} *M*) in EPA was deoxygenated by evacuation and then irradiated with white light, isolated from infrared by means of glass filters. Upon irradiation, the green chlorophyll was converted into a pink solution of reduced chlorophyll having absorption maxima at 665, 615, 585, 520 and 410 mμ (Fig. 1). The absorption maximum at 665 mμ is that of unreduced chloro-

(1) S. S. Brody, Air Force Cambridge Research Center, Bedford, Mass.

(2) A. A. Krasnovsky, *Compt. rend. (Doklady), Acad. Sci. U. S. S. R.*, **60**, 421 (1948).

(3) E. V. Evstigneev and V. A. Gavrilova, *ibid.*, **91**, 899 (1953).

(4) S. S. Brody and M. Brody, *Arch. Biochem. and Biophys.*, **82**, 161 (1959).

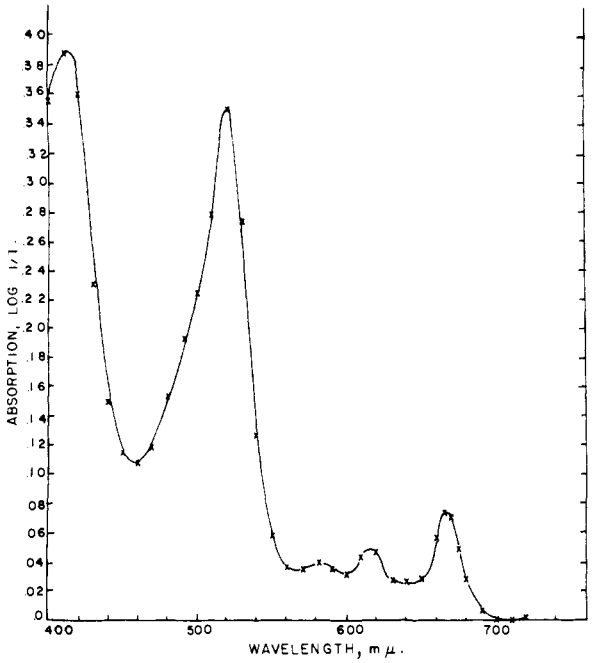

Fig. 1.—Absorption spectrum of chlorophyll *a* in EPA after photoreduction by phenylhydrazine (10^{-3} *M*) at 20°. Absorption is given as the logarithm of the reciprocal of the transmission *T*. The absorption maximum at 665 mμ is from unreduced chlorophyll *a*.

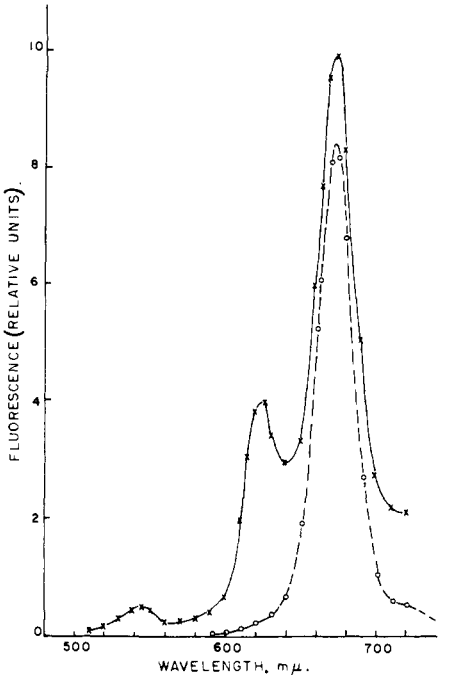

Fig. 2.—Fluorescence spectrum of chlorophyll *a* (10^{-6} *M*) in EPA at 20° before (— — —) and after (—x—x—) photoreduction by phenylhydrazine (10^{-3} *M*).

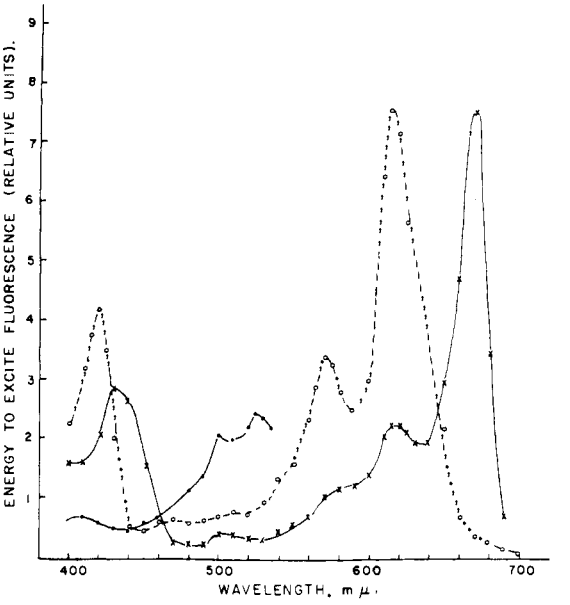

Fig. 3.—Action spectra for exciting fluorescence bands shown in Fig. 2 (670 mμ, —x—x—; 625 mμ, —O—O—; 545 mμ, —●—●—). Experiments were made at 20°.

phyll. (The absorption spectrum of reduced chlorophyll in diethyl ether is quite similar.) The relative heights of the maxima are dependent upon several variables, such as the number of light quanta absorbed and length of time in the dark after photoreduction.

The fluorescence from a solution of reduced chlorophyll possesses three pronounced maxima, at 670, 625 and 545 mμ (Fig. 2). Excitation of fluorescence at 670 mμ yields an action spectrum whose maxima are at 665, 615, 580 and 435 mμ (Fig. 3). These are the wave lengths of the principal and minor absorption maxima of unreduced chlorophyll *a* in EPA; the 670 mμ emission can, therefore, be identified as fluorescence from unreduced chlorophyll. The action spectrum for exciting fluorescence at 625 mμ (Fig. 3) possesses maxima at 615, 570 and 420 mμ. The action spectrum for exciting fluorescence at 545 mμ has maxima at 520, 500 and 410 mμ.

It is important to note that light absorbed at 520 mμ contributes only to the fluorescence at 545 mμ and not to that at 625 mμ. This is a good indication that there are two forms of reduced chlorophyll.

When chlorophyll is reduced in anhydrous diethyl ether, the resulting absorption spectrum is the same as that shown in Fig. 1 for EPA. Fluorescence maxima are observed at 665, 620 and 550 mμ. The intensity of the fluorescence maximum at 550 mμ is about one fourth that at 620 mμ. The action spectrum for exciting fluorescence at 620 mμ has maxima at 580 and 420 mμ and also a shoulder (suggestive of a third absorption band) at 550 mμ. A maximum at 615 mμ corresponding to the one observed in EPA is not resolvable.

Experiments at −193°.—The fluorescence and action spectra of reduced chlorophyll in EPA were obtained at −193°. The photoreduction is done at 20° and a rigid glass then is attained by cooling the solution to −193°. The fluorescence spectrum possesses maxima at 675, 625 and 530 mμ (Fig. 4), corresponding to those observed at room temperature at 670, 625 and 545 mμ, respectively. Upon cooling from 20 to −193° the yield of fluorescence at 670 and 625 mμ increases by less than a factor of two, whereas the yield at 530 mμ increases about fifteen fold.

The fluorescence at 670 mμ is again from unreduced chlorophyll. The action spectrum for excitation of fluorescence at 530 mμ has maxima at 525 and 410 mμ and a shoulder at 500 mμ (Fig. 5). The action spectrum for exciting fluorescence at 625 mμ showed maxima at 580 and 415 mμ; there is also a maximum at 520 and a shoulder at 500 mμ but these result from excitation of the very strong fluorescence band at 530 nμ, which slightly overlaps the weak fluorescence band at 625 mμ.

In Table I is a summary of absorption, fluorescence and action spectra maxima.

The fluorescence spectra observed here are essentially in agreement with those of Evstigneev and Gavrilova[3] for toluene solutions. However, it was found in the present work that in both diethyl ether and EPA, fluorescence is detectable from reduced forms of chlorophyll, even at room temperature, whereas the Russian authors detected the full complement of fluorescence bands only at −193°.

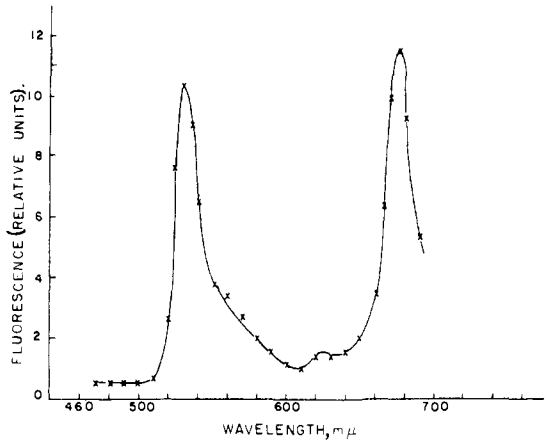

Fig. 4.—Fluorescence spectrum of chlorophyll *a* (10^{-6} *M*) in EPA at −193° after reduction by phenylhydrazine (10^{-3} *M*) at room temperature (20°).

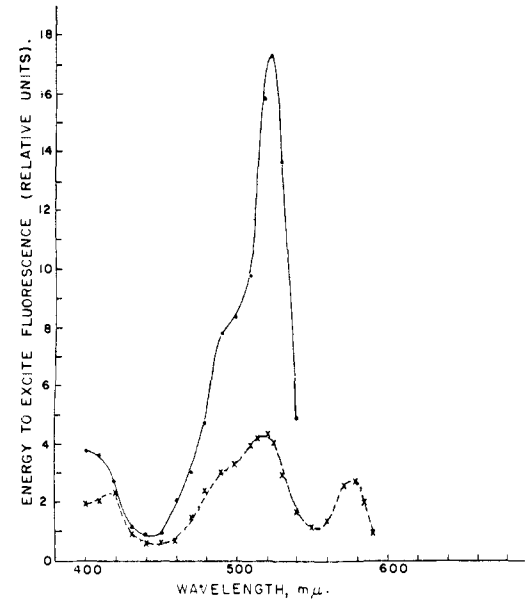

Fig. 5.—Action spectra for exciting the fluorescence bands of reduced chlorophyll *a* (10^{-6} *M*) in EPA at −193°; 620 mμ (—x—x—) and 540 mμ (—●—●—).

B. Bleaching of Chlorophyll in Rigid Solution.—A solution of chlorophyll *a* (10^{-6} *M*) and phenylhydrazine (10^{-4} *M*) in EPA is cooled to −193° to form a rigid glass. At this temperature, the fluorescence spectrum has a maximum at 675 mμ and a small band at around 520 mμ. (When the fluorescence of chlorophyll is quenched with excess phenylhydrazine the smaller band readily can be detected at 20°. This band probably originates from a carotene derivative present in chlorophyll as an impurity in amounts less than 1%.[5] It was found that when commercial carotene was exposed to the air the fluorescence yield at 520 mμ increased with time, suggesting that the fluorescence is associated with an oxidation product.)

TABLE I

SPECTRAL PROPERTIES OF REDUCED CHLOROPHYLL *a* IN EPA

Absorp. max., mμ	Fluoresc. max., mμ, 20°	Action spect.[a] max., mμ, 20°	Fluoresc. max., mμ, −193°	Action spect.[a] max., mμ, −193°
665	670	665, 615, 580, 435	675	
615 585	625	615, 570,420	625	580, 415
520 410	545	520, 500,410	530	525, 500, 410

[a] Action spectra for exciting fluorescence.

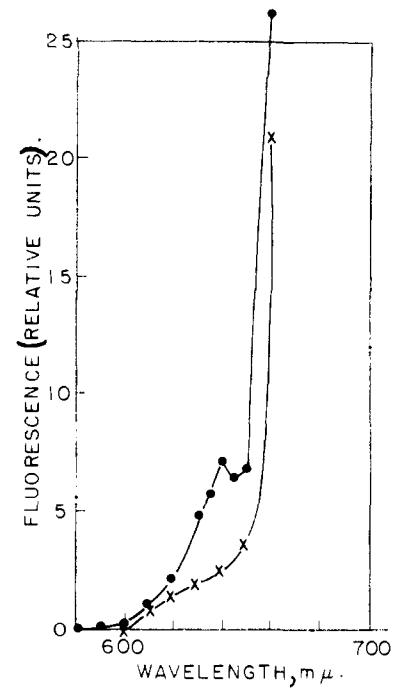

Fig. 6.—Fluorescence spectrum (in the region from 590 to 660 mμ) of a solution of chlorophyll *a* before (—X—X—) and after (—●—●—) 20 minutes illumination at −193°. This figure shows the fluorescence band at 640 mμ. Both fluorescence curves were made equal in magnitude (132 relative units) at their maxima. The portions of the fluorescence spectra not shown are similar to the dotted curve in Fig. 2.

When the rigid solution is illuminated, a new fluorescence band gradually appears at 640 mμ (Fig. 6). This band finally reaches a maximum value and cannot be increased by continued illumination. The action spectrum for exciting this fluorescence has an absorption maximum at 570 mμ.

The fluorescence intensity of the band at 640 mμ increases with increasing concentration of phenylhydrazine. However, complete data on concentration dependence cannot be obtained; when the concentration of phenylhydrazine is greater than 10^{-3} *M*, the solution becomes translucent upon freezing.

Since there is no evidence for the production of the reduced form of chlorophyll, which emits at 545 mμ, only partial photoreduction has occurred in this rigid system.

IV. Discussion

A. Spectral Properties of Reduced Chlorophyll.—The fluorescence and action spectra reported above suggest that the chlorophyll solution which has undergone reduction contains, in addition to residual unmodified pigment, two discrete forms of reduced chlorophyll. That there are two discrete forms is indicated by the existence of separate fluorescence bands, each of which has a different action spectrum for excitation. The form which emits at 625 mμ has absorption maxima at 615, 570 and 420 mμ, the one emitting at 545 mμ has absorption maxima at 520, 500 and 410 mμ (all at 20°).

The photoreduction reactions and the absorption maxima of the different forms of chlorophyll are

(5) S. S. Brody, unpublished.

postulated to be

$$C(665\ m\mu) + R \rightleftharpoons CR(665\ m\mu) \quad (2)$$

$$CR + h\nu \longrightarrow CR^*(585 \text{ and } 615\ m\mu) \quad (3)$$

$$CR^* \longrightarrow \ldots \longrightarrow CH(520\ m\mu) + R(\text{oxidized}) \quad (4)$$

The symbol CR* represents an electronically excited complex of chlorophyll, C, and phenylhydrazine, R. The reaction shown in eq. 4 may be simply a bimolecular dismutation or may consist of more than one step. Evidence in support of these wave length assignments is presented below in part C.

B. **Chlorophyll–Phenylhydrazine Complex.**—Experiments showing that a new fluorescence band can be induced, while chlorophyll is in a rigid solution, clearly indicate the existence of a chlorophyll-reductant complex. This follows from the fact that the production of new forms of chlorophyll requires a light-induced chemical reaction between chlorophyll and phenylhydrazine. This chemical reaction is possible in a rigid solution (where diffusion processes are negligible) only where chlorophyll and phenylhydrazine are complexed. Furthermore, if a complex were involved in the production of the new fluorescence band at 640 mμ, the final intensity of the band would be determined by the concentration of complex, which in turn depends upon the concentration of chlorophyll and phenylhydrazine (eq. 2). Such a dependence *was* noted between the intensity of the 640 mμ band and the concentration of phenylhydrazine (see section III B). That only a small fraction of the chlorophyll is converted into the form emitting at 640 mμ is evidenced by the fact that no pronounced decrease in the fluorescence of unreduced chlorophyll can be detected. This suggests that the concentration of complex is quite low.

While the concentration of the complex might be expected to change as the solution becomes rigid, no indication of such a change is noted in either absorption or fluorescence spectra.

When chlorophyll is bleached at $-193°$, the new fluorescence maximum is located at 640 mμ and not at 625 mμ (the latter position, as reported above, obtains when chlorophyll is reduced at 20° and then cooled to $-193°$). Perhaps the energy level of the excited complex (CR) is shifted from its normal position at 625 mμ to one at which it emits at 640 mμ because, in a solid state, the complex cannot dissociate.

Kinetic studies led earlier workers to suggest that a complex is involved in the photoreduction of chlorophyll. Livingston and Pariser[6] proposed that a complex was involved in photoreduction of methyl red in the presence of chlorophyll and phenylhydrazine. They visualized a complex between methyl red and tautomerized chlorophyll, with the complex being subsequently reduced by phenylhydrazine. However, Watson[7] suggested that the complex might be between the chlorophyll and phenylhydrazine. The low temperature experiments (Section IIIB) support Watson's contention regarding the composition of the complex and also his proposal that the complex is involved in the primary photochemical reduction. (Studies of the quenching and activation of chlorophyll fluorescence by phenylhydrazine also led other workers[8,9,10] to postulate the existence of a complex.)

The existence of complexes between chlorophyll and solvent molecules has been proposed by Linschitz[11] (to explain light induced changes in absorption at the temperature of liquid nitrogen) and by Freed and Sancier[12] (to explain changes in absorption as a function of temperature). While this type of dye-solvent complex may determine some of the photochemical properties of chlorophyll in various solvents, it does not play an important role in the experiments described here—since photochemically induced changes in fluorescence depend primarily upon the presence of phenylhydrazine.

Evstigneev, *et al.*,[8] and Bannister[13] reported that ascorbic acid produces practically no quenching of chlorophyll fluorescence; from this observation they argued against complex formation between chlorophyll and ascorbate. However, if quenching depends upon the concentration of complex, the change in fluorescence yield might well escape detection. For, if the concentration of the complex is of the order of 3% (see section IVD), or less, and the fluorescence yield of chlorophyll is about 30%, then the absolute change in yield will be less than 1%. With present methods of detection, such a small change cannot be measured reliably.

C. **First Stable Photochemical Product in Photoreduction of Chlorophyll.**—The experiments reported in section III B show that in rigid solution (where only partial photoreduction of chlorophyll occurs), the only species formed is the one emitting at 640 mμ. The product which emits at 545 mμ is not in evidence. These results suggest that the species emitting at 640 mμ is the first intermediate formed in the complete photoreduction of chlorophyll. That the production of the second intermediate (absorbing at 520 mμ) from the first intermediate (absorbing at 615 mμ) does not occur at $-193°$ indicates that this process requires dismutation or thermal reactions (eq. 4) which, of course, cannot occur in rigid systems.

Krasnovsky[2] suggested that the reduced form of chlorophyll absorbing at 520 mμ is a radical; that it is not was shown by Brody, Newell and Castner.[14] Of course, there still exists the possibility that the intermediate absorbing at 615 and 570 mμ is a radical.

D. **Estimate of Complex Concentration.**—For the series of reactions given in eqs. 2, 3 and 4, let us assume that the rate limiting factor determining the initial yield of photoreduction is the concentration of complex between chlorophyll and reductant (CR). If, for light absorbed by the complex, the quantum yield is 100%, then, it follows that the initial yield of photoreduction is given primarily by

(6) R. Livingston and R. L. Pariser, THIS JOURNAL, **70**, 1510 (1948).

(7) W. F. Watson, *Trans. Faraday Soc.*, **48**, 526 (1952).

(8) E. V. Evstigneev and A. A. Krasnovsky, *Compt. rend. (Doklady), Acad. Sci. U. S. S. R.*, **60**, 623 (1948); E. V. Evstigneev, V. A. Gavrilova and A. A. Krasnovsky, *ibid.*, **74**, 315 (1950).

(9) R. Livingston and Chum-Lin Ke, THIS JOURNAL, **72**, 909 (1950).

(10) R. Livingston, W. F. Watson and J. McArdle, *ibid.*, **71**, 1542 (1949).

(11) H. Linschitz, *Nature*, **169**, 193 (1952).

(12) S. Freed and K. Sancier, THIS JOURNAL, **76**, 198 (1954).

(13) T. T. Bannister, Thesis, Univ. of Illinois, 1958.

(14) S. S. Brody, G. Newell and T. Castner, *J. Phys. Chem.*, **64**, in press, (1960).

the ratio (CR)/(C) + (CR)—this is also the fraction of light quanta absorbed by the complex.

By further assuming that the yield of fluorescence of the "640 mμ-intermediate" is approximately the same as that of unreduced chlorophyll, an estimate can be made of the fraction of chlorophyll in the complexed state. This estimate is made by comparing the relative intensities of the fluorescence bands at 640 and 675 mμ, the result being 3% for the concentration of chlorophyll in the complexed state. This is in good agreement with the measured value[13] for the initial yield of photoreduction of chlorophyll by excess ascorbate in aqueous pyridine.

Acknowledgments.—The author wishes to express his appreciation to Dr. T. Bannister and Dr. E. Rabinowitch for several stimulating discussions on the photochemistry of chlorophyll, to Dr. M. Brody for her numerous suggestions which aided the course of this work, to Mr. Krishnan Kumar for his competent assistance in carrying out these experiments and to the members of the Photosynthesis Laboratory of the University of Illinois for their coöperation and interest.

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Catalyzed Hydrolysis of Benzyl Chloride

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The hydrolysis of benzyl chloride in aqueous acetone with and without mercuric chloride has been examined kinetically under various conditions and the results are discussed. The mercuric chloride-catalyzed reaction has been found to be approximately first order with respect to benzyl chloride and mercuric chloride. A complicating factor, the formation of a complex between mercuric chloride and benzyl chloride, has been noted.

The study of electrophilic catalysis in nucleophilic aliphatic substitution has hitherto been mainly on substances which substitute by the SN1 mechanism.[1,2] Although Hammett and his associates have examined the substitution reactions of some alkyl halides which undergo the normal reaction chiefly by the SN2 mechanism, the reactions in presence of the powerful catalysts they employed (various ionic species derived from mercuric salts) were purely SN1.[3] We have, therefore, decided to examine the hydrolysis of benzyl chloride, which is on the SN2–SN1 border line,[4] in the presence of catalyst mercuric chloride which is relatively weak, since it acts in its un-ionized form.[2]

Results and Discussion

Table I contains the first order rate constants for the normal and mercuric chloride-catalyzed hydrolysis of benzyl chloride, under various conditions. In Table II are given the energies and entropies of activation.

Although the rate constant for hydrolysis in the absence of mercuric chloride is not sensitive to reasonable variations in the benzyl chloride concentration, the rate of the catalyzed reaction decreases slightly with increase in the benzyl chloride concentration, particularly in the more aqueous solvents. This is, however, due, as shown later, to the special circumstance of complex formation between benzyl chloride and mercuric chloride and so it is concluded that the catalyzed reaction is approximately first order with respect to benzyl chloride. The rate of the catalyzed reaction varies linearly with the catalyst concentration in all solvents, over the concentration of catalyst examined. Further, the slopes of the lines do not vary much with the solvent, the respective values being 1.18, 1.00, 1.00, 1.02 and 1.20 10^{-4} l.mole/sec. for 45, 50, 60, 70 and 80% acetone. However, the lines so obtained do not pass through the origin but cut the rate-axis below the origin.[5] This is illustrated in Fig. 1 for 45 and 80% acetone. So, at low catalyst concentrations the linear relationship apparently no longer holds. It is concluded that the catalyzed reaction is strictly first order with respect to mercuric chloride only when the catalyst concentration is not too low.

The catalysed hydrolysis is complicated by the formation of an intermediate complex between benzyl chloride and mercuric chloride, so that the reaction path *via* the complex can be formulated as

$$PhCH_2Cl + HgCl_2 \xrightarrow{K} PhCH_2Cl\cdot HgCl_2 \xrightarrow[H_2O]{k}$$
$$PhCH_2OH + HgCl_3^- + H^+$$

Complex formation is observed in the solvent range 45–80% acetone, though not in 90% acetone. This is shown by the formation of a yellow color and a turbidity on dissolving the two in the solvent. (However, no heterogeneity is involved in the reaction, since the turbidity vanishes at the reaction temperature.) The complex dissolves on adding more benzyl chloride. Though the complex was isolated, an accurate analysis was not possible because of its instability. The results show, however, that it is probably a 1:1 complex. The intensity of color and turbidity decrease as the solvent becomes drier and vanishes in 90% acetone. Addition of

(1) E. D. Hughes, C. K. Ingold and co-workers, *J. Chem. Soc.*, 1236, 1243 (1937); 169 (1946); K. Bodendorf and H. Böhme, *Ann.*, **516**, 1 (1935).

(2) C. A. Bunton, E. D. Hughes and R. Anantaraman, unpublished work; see, however, C. K. Ingold, "Structure and Mechanism in Organic Chemistry," Cornell University Press, Ithaca, N. Y., 1953, pp. 359–360.

(3) (a) I. Roberts and L. P. Hammett, THIS JOURNAL, **59**, 1063 (1937); (b) O. T. Benfey, *ibid.*, **70**, 2165 (1948).

(4) S. C. J. Olivier and A. P. Weber, *Rec. trav. chim.*, **53**, 869 (1934); S. C. J. Olivier, *ibid.*, **53**, 891 (1934).

(5) This was kindly pointed out by one of the referees.