In each experiment the composition of the solution was calculated from the extinction coefficients five to ten minutes after the start of the experiment. The results of the calculations, *i. e.*, the concentrations of the various species, have been given in the body of this paper. In Table VII are given the original data presented in the form of stoichiometric molar extinction coefficient of plutonium which is defined here as the observed log I_0/I divided by the product of the cell length in centimeters and the stoichiometric molar concentration of plutonium. (Here one mole of the red or brown dimer is to be calculated as two moles of plutonium.) The calculated percentages of the four species in general added up to nearly 100% of the α counting analysis with deviations in only a few cases of more than 5% from this value. This degree of agreement is of the expected order of magnitude. In the calculations the spectrophotometric values were used while the total plutonium concentrations given in Table VII are based on the α analysis.

Summary

Two peroxy complexes of plutonium(IV) have been identified in aqueous solution. The equilibria of these ions with hydrogen peroxide and Pu^{+4} were measured spectrophotometrically as a function of the H_2O_2, plutonium and H^+ concentrations and formulas of the ions were deduced from these data.

The first complex, which is brown in color, contains two plutonium ions, one peroxide ion and probably one hydroxide group. The second complex, which is red, is made up of two plutonium ions and two peroxide ions. Alternatively the red complex may be a hydrate of this species consisting of two plutonium ions, one peroxide ion, one perhydroxide ion and an hydroxide ion.

The equilibrium quotients of the reactions in 0.5 *M* HCl were measured

$$2Pu^{+4} + H_2O_2 + H_2O = Pu(OO)(OH)Pu^{+5} + 3H^+$$

$$K_b = \frac{(Pu(OO)(OH)Pu^{+5})(H^+)^3}{(Pu^{+4})^2(H_2O_2)} = 8.8 \times 10^6$$

$$2Pu^{+4} + 2H_2O_2 = Pu(OO)_2Pu^{+4} + 4H^+$$

$$K_r = \frac{(Pu(OO)_2Pu^{+4})(H^+)^4}{(Pu^{+4})^2(H_2O_2)^2} = 6.3 \times 10^8$$

Possible structural formulas for the complexes are presented. Attention is called to the remarkable fact that both complexes contain two plutonium ions and that no evidence was obtained for peroxy complexes involving only one plutonium ion.

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[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY, INSTITUTE OF TECHNOLOGY, UNIVERSITY OF MINNESOTA]

Activation of the Fluorescence of Chlorophyll Solutions*

BY ROBERT LIVINGSTON, W. F. WATSON AND JAMIE MCARDLE

The fluorescence of chlorophyll in living plants has been studied in recent years in the hope of obtaining evidence regarding the process of photosynthesis. However, there has been a difference of opinion as to how these measurements should be interpreted. One group of investigators maintains[1] that photosynthesis and fluorescence are complementary processes. Others believe[2] that the relation between the two processes is an indirect one, the fluorescence intensity being controlled by the concentrations of certain photosynthetic intermediates and inhibitors. Studies *in vitro*[3] of dye-sensitized systems show in general that photochemical reactions and fluorescence are not complementary. However, there is little direct information available about the factors which determine the fluoresence intensity of chlorophyll solutions. The present experiments are part of a study designed to throw light on this problem.

It has been commonly accepted, on the basis of very scanty evidence, that the fluorescence of dissolved chlorophyll is substantially independent of the nature of the solvent. In contradiction to this view it was discovered recently[4] that chlorophyll dissolved in pure, dry hydrocarbons is practically non-fluorescent. Furthermore, the addition of a trace of water, alcohol or amine to such a solution raises the fluorescent yield to the "normal maximum" value of approximately 10%,[5] which is observed in pure alcohols and related solvents. Quantitative measurements of the intensity of fluorescence, at several temperatures and in a number of mixed solvents of known composition, indicate that the activation of the fluorescence is the result of the formation of an addition compound between a molecule of chlorophyll and one of the activator (*i. e.*, polar solvent).

Experimental Methods and Materials

The Fluorimeter.—The intensity of that part of the fluorescent light which is transmitted by a Wratten filter No. 88 was measured with a Photovolt Corporation (Model 512) photocell and amplifier. The photocell was placed

* This work was made possible by the support of the Office of Naval Research (Contract N6ori-212, Task Order I, NR 059 028) and by a grant-in-aid from the Graduate School of the University of Minnesota.

(1) E. C. Wassink, E. Katz and Dorrestein, *Enzymologia*, **10**, 285 (1942).

(2) J. Franck, C. S. French and T. T. Puck, *J. Phys. Chem.*, **45**, 1268 (1941).

(3) R. Livingston, *J. Phys. Coll. Chem.*, **52**, 527 (1948).

(4) R. Livingston, "The Photochemistry of Chlorophyll" in "Photosynthesis in Plants," Iowa State College Press, 1949.

(5) T. A. Prins, *Nature*, **134**, 457 (1934).

at an acute angle to the direction of the incident light, so as to receive a definite fraction of the fluorescent light which was emitted from the front surface of the fluorescence cell.[6] The exciting light was mainly of λ4358 and 4046 Å., isolated from a GE AH-4 arc by means of a Corning filter No. 585 and 3 cm. of 5% copper sulfate solution. The incident light was rendered parallel by suitable glass condensing lenses and illuminated an area of about 1 sq. cm. of the front face of the cell. The arc, lenses, filters, shutter, and fluorescent cell were mounted rigidly on an optical bench, and the entire apparatus, with the exception of the arc and condensing lenses, was contained in a light-tight box.

The Wratten filter (no. 88) absorbs all radiation of wave lengths less than 6800 Å.[7] It, therefore, transmits the fluorescent light corresponding to the second fluorescent maximum (λ7200 Å.) of chlorophyll a but absorbs light of the principal emission maximum.[8] This eliminates any effect upon the measurements of varying reabsorption of the fluorescent light. It has a further advantage, since the second maximum is practically independent of the solvent, while the first maximum varies by about 75 Å. with changes in solvent. In the few measurements made with chlorophyll b, a glass filter having a cut-off at about 6200 Å. was substituted for the Wratten filter.

The Fluorescence Cells.—The chlorophyll solutions were contained in cells of the Thunberg type,[9] which were further modified by the addition of a second bulb (see Fig. 1) to permit the rapid mixing of solutions and the efficient pumping out of dissolved gases and volatile solvents. Except where otherwise stated, the measurements were made at ambient temperatures, 25 to 35°. To study the influence of temperature, the cell (a Thunberg tube without side bulb) was placed in an unsilvered Dewar filled with water. The temperature was controlled manually with the aid of a thermometer, a small electric heater, and a stirrer.

Preparation of the Solutions.—The following routine procedure was used in preparing "dry" chlorophyll solutions. A measured, small sample of a stock solution of chlorophyll in ether was pipetted into a Thunberg tube, which had been washed out thoroughly with dry benzene, and an excess of freshly dried and purified hydrocarbon was added. The Thunberg tube was then attached by a ground glass joint to a vacuum line, and the solution was evaporated almost to dryness (at room temperature or below). The tube was opened, an additional sample of hydrocarbon was added, and then pumped off as before. This was repeated several times, until the fluorescence was seen to be of low intensity. In a few experiments, a special attempt was made to exclude water by distilling the several samples of hydrocarbon in the vacuum line through a tube filled with freshly cut sodium into the Thunberg tube, instead of pipeting the solvent into the tube. The technique was otherwise similar to that described previously. The concentrations of the stock chlorophyll solutions were determined spectrophotometrically with the aid of a Beckman spectrophotometer.[10]

Solutions containing known concentrations of activators were prepared by adding the appropriate volume of a stock solution of the activator (dissolved in the dry hydrocarbon) and an excess of solvent to the stock chlorophyll solution in the Thunberg tube, and then evaporating off the solvent until the standard volume was obtained. When volatile activators, such as water, were used, the final evaporation was dispensed with. Gaseous activators were added to the evacuated fluorescence cell, through a drying train, until the desired equilibrium pressure was obtained.

(6) Compare E. J. Bowen, *Trans. Farad. Soc.*, **35**, 15 (1939).

(7) Wratten Light Filters, Eastman Kodak Co., Rochester, N. Y., 1945.

(8) F. P. Zscheile and D. G. Harris, *J. Phys. Chem.*, **47**, 623 (1943).

(9) R. Livingston, D. Sickle and A. Uchiyama, *J. Phys. Coll. Chem.*, **51**, 777 (1947).

(10) C. L. Comar and F. P. Zscheile, *Plant. Physiol.*, **17**, 198 (1942).

Purification of Materials.—All reagents were of Reagent Grade except methanol, ether and dimethylaniline, which were of technical grade. Methanol was purified by the method described by Livingston and Pariser.[11] Cetyl alcohol was dissolved in benzene, evaporated to dryness three times, and kept at 100° for eight hours under high vacuum. *t*-Amyl and benzyl alcohols were stored for several days over Drierite and then distilled at atmospheric pressure, out of contact with the air. The phenol was kept for several weeks in a desiccator containing anhydrous magnesium perchlorate. Aniline was dried by potash, distilled at atmospheric pressure and then under vacuum. Dimethylaniline purified as for aniline was found to have a low efficiency of activation of fluorescence, which might be attributable to small amounts of impurities. It was further purified by two different methods. One sample was recrystallized three times and twice vacuum-distilled. The other was treated with acetic anhydride and extracted with water; the sparingly soluble oxalate was then recrystallized twice from water, potash added to liberate the free base which was twice vacuum-distilled after drying. The three purified samples possessed the same m. p. of 2.5°.[12] *n*-Heptylamine, benzylamine and piperidine were dried over Drierite and distilled at atmospheric pressure. Formamide was twice distilled *in vacuo*, after drying over Drierite.

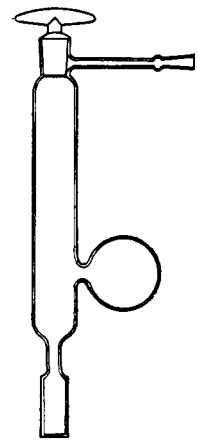


Fig. 1.—Cell used for fluorescence measurements.

The non-polar solvents were dried for several weeks over sodium and distilled freshly before use. Benzene, which was used for most of the experiments, was distilled in the vacuum line at low temperature. *n*-Heptane, isoöctane, *p*-chlorobenzene and nitrobenzene were distilled at atmospheric pressure. Styrene was distilled *in vacuo* from stabilizer, dried by magnesium perchlorate and distilled *in vacuo*.

Stock solutions of chlorophyll a and of chlorophyll b were prepared by a modification[9] of the method of Zscheile and Comar.[13]

Experimental Results

Non-fluorescence of Certain Chlorophyll Solutions.—It is common experience, and has been confirmed in the present researches, that the intensity of fluorescence of chlorophyll solutions appears to be substantially independent of the solvents used, for solvents varying as widely as methanol, ethyl ether and benzene. However, if extreme precautions are taken to remove water, alcohol and related substances from benzene solutions of chlorophyll, they are practically non-fluorescent. The intensity of fluorescence in carefully dried, pure benzene is less than 3% of that observed in ordinary "pure" benzene. Even this residual fluorescence may be due to the presence of traces of some impurity (probably water), since in all cases the fluorescence was further reduced by additional purification. The addition of as little as 0.01% of water is sufficient to raise the fluorescent intensity of a dry benzene solution

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

(12) A. Weissberger and E. Proskauer, "Organic Solvents," Oxford Press, 1935.

(13) F. P. Zscheile and C. L. Comar, *Botan. Gass.*, **102**, 463 (1941).

to its "normal" high value. If a dry, non-fluorescent solution is shaken in contact with laboratory air for a few seconds, it picks up enough water vapor to restore its fluorescent intensity to the usual high value.

Solutions of chlorophyll in any of the following pure, dry solvents are "non-fluorescent": benzene, *n*-heptane, isoöctane, styrene, chlorobenzene, carbon tetrachloride, and di-phenyl ether. Solutions in pure dry methanol, ethanol, octanol, acetone and probably ethyl ether[14] exhibit the normal high fluorescence.

Intensity of Fluorescence in Mixed Solvents.—Data illustrating the change of intensity of fluorescence with composition of the solution, for the system octyl alcohol–benzene over the whole range of concentration are plotted on Fig. 2. The intensity of fluorescence, expressed in arbitrary units, rises from a value of 2 in pure benzene to one of 56 in a solution whose mole fraction of alcohol is 0.0016. Over the remaining range, from X = 0.0016 to X = 1.0000, the increase in the intensity of fluorescence is not greater than 10%, and measurements on other analogous solvent systems suggest that possibly even this slight rise is due to experimental error.[15]

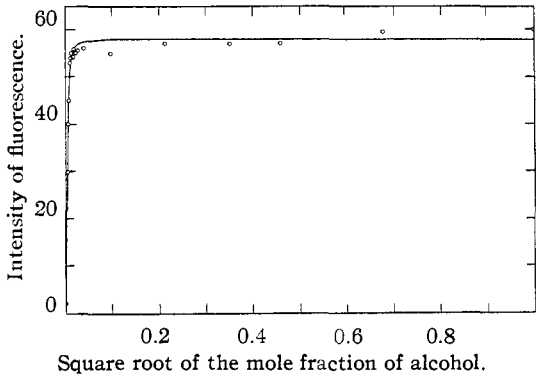


Fig. 2.—Intensity of fluorescence as a function of composition for the system octyl alcohol–benzene.

This behavior is qualitatively characteristic of all such pairs of solvents which were investigated over the entire range of composition. They include methanol–benzene and ethanol–benzene. In addition aniline–benzene was studied in solutions ranging from pure benzene to a mole fraction of 0.5.

The Activation of Fluorescence in Hydrocarbon Solvents.—The fluorescence of chlorophyll has also been measured in a number of other pairs of solvents in which the composition varied from the pure inert solvent to mixtures containing several times as much "activator" as was required to raise the fluorescence intensity to its maximum value. The initial rise of intensity due to addition of small amounts (0 to 0.0015*m*) of activator is illustrated by Fig. 3. The cases plotted were chosen as representative of the effect of strong (as piperidine) and moderate (as phenylhydrazine) activators.[16] It should be emphasized that in all cases of activation studied (including such weak activators as aniline), the intensity of fluorescence is raised, if sufficiently high concentrations of the activator are used, to a limiting value and that this value (at least to a first approximation) is independent of the chemical nature of the activator. In no case was the measured fluorescent intensity zero in the "pure" inert solvent. The lowest value which was attained was 3% of the limiting value (see Fig. 1). As this extreme purification was time-consuming and laborious, a routine technique of purification was adopted which reduced the intensity of the fluorescence in the inert solvent to about 10% of its limiting high value.

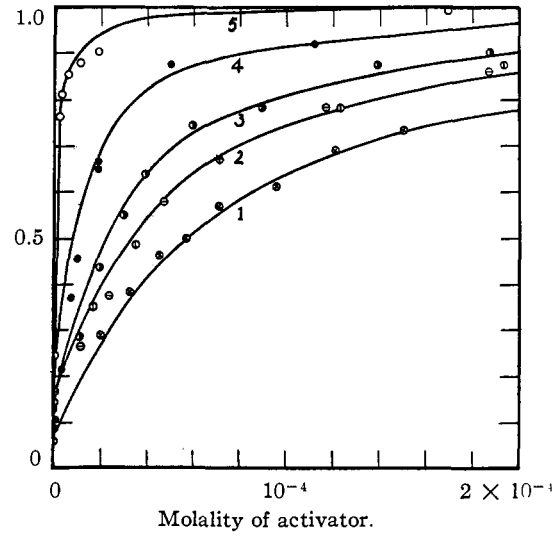


Fig. 3.—Intensity of fluorescence as a function of the concentration of activator:

Curve no.	Solvent	Activator	Chlorophyll
1	*n*-Heptane	Phenylhydrazine	a
2	Benzene	Benzyl alcohol	a or b
3	Benzene	Cetyl alcohol	a
4	Isoöctane	Methanol	a
5	Benzene	Piperidine	a

Table I summarizes the efficiency of the several activators which have been studied quantitatively. Except where noted, chlorophyll a at a concentration of 5×10^{-6} M was used. The first column lists the inert solvent; the second, the

(14) There is some uncertainty as to whether the sample of ether used was sufficiently pure.

(15) Preliminary measurements,[4] which were presented at the Chicago Symposium on Photosynthesis of the AAAS in December, 1947, indicated that the intensity of fluorescence in a solvent containing 99% heptane and 1% methanol is about 20% greater than the intensity in pure methanol. Additional work has shown that results of this type are not observed when the two solvents are completely miscible.

(16) At higher concentrations phenylhydrazine acts as a quencher of fluorescence. This interesting and unusual dual behavior will be discussed in a later paper which will be devoted to fluorescence quenching.

activator; and the third, the molarity of activator, $m_{1/2}$, required to raise the fluorescence to half of its limiting value. The quantities tabulated in the last two columns are discussed in a later section of this paper. In addition to the

TABLE I

EFFICIENCY OF ACTIVATION OF FLUORESCENCE

Solvent	Activator	$m_{1/2}$	K_1	$K_2(HB)_0$
Benzene	Dimethylaniline	6.8×10^{-2}	1.05×10	0.12
Benzene	Phenol	6.0×10^{-2}	1.55×10	.08
Benzene	Aniline	2.75×10^{-2}	4.55×10	.08
n-Heptane	Phenylhydrazine	8.0×10^{-4}	1.70×10^{3}	.13
Benzene	Phenylhydrazine	6.5×10^{-4}	1.78×10^{3}	.20
Benzene	Benzyl alcohol[a]	4.5×10^{-4}	2.60×10^{3}	.18
Benzene	Formamide	4.3×10^{-4}	2.70×10^{3}	.10
Benzene	Benzyl alcohol	4.2×10^{-4}	2.90×10^{3}	.09
Benzene	Benzyl alcohol[b]	4.2×10^{-4}	2.90×10^{3}	.14
Benzene	Benzoic acid	3.6×10^{-4}	3.15×10^{3}	.11
Benzene	Cetyl alcohol	2.9×10^{-4}	4.15×10^{3}	.14
Benzene	Octyl alcohol	2.0×10^{-4}	4.57×10^{3}	.14
iso-Octane	Methyl alcohol	1.1×10^{-4}	1.03×10^{4}	.10
Benzene	Benzylamine	3.9×10^{-5}	2.67×10^{4}	.09
Benzene	Benzylamine[b]	3.3×10^{-5}	3.00×10^{4}	.14
Benzene	Water	3.5×10^{-5}	2.95×10^{4}	.08
Benzene	Piperidine	7.5×10^{-6}	1.36×10^{5}	.15
Benzene	*n*-Heptylamine	6.5×10^{-6}	1.56×10^{5}	.11

[a] Solutions of $5 \times 10^{-6}M$ ethyl chlorophyllide *a* were used in these experiments. This material was kindly furnished by Professor J. Weiss, University of Durham. [b] Solutions of $5 \times 10^{-6}M$ chlorophyll *b* were used in these experiments.

substances listed in Table I, the following compounds have been studied and it has been demonstrated that solutions containing $(C_6H_5)_2NH$, $C_6H_5NHNHC_6H_5$ or $C_6H_5NO_2$ up to concentrations $0.1m$ are non-fluorescent; as are benzene solutions containing O_2, I_2, CO or C_2H_4 up to concentrations of $0.01m$. (The activating efficiency of ethanol is comparable to that of methanol, its $m_{1/2}$ value being $10^{-3}m$.)

Solutions of Chlorophyll b.—The effect of activators and inert solvents upon chlorophyll b are similar to those observed for chlorophyll a. Solutions of chlorophyll b in pure benzene exhibit only the slight residual fluorescence which is characteristic of chlorophyll a solutions. For chlorophyll b in benzene, the observed $m_{1/2}$ values of benzyl alcohol and benzylamine are, respectively, 4.2×10^{-4} and 3.3×10^{-5} moles/l. The ratios of these values to the corresponding ones for chlorophyll a solutions are 1.00 and 0.85, respectively.

Reversibility of Activation.—The activation of fluorescence is a strictly reversible phenomenon. A solution in benzene which has been activated by the addition of a small amount of methanol or ordinary ether can be rendered non-fluorescent by distilling off the added substance on a vacuum line.[17] The process may be repeated by adding a trace of methanol to the non-fluorescent solution and thereby reactivating it.

(17) As is discussed in the section on Experimental Methods, to obtain very low fluorescence it is necessary to add several portions of pure dry benzene to the solution and to pump off all but a small fraction of the solvent after each addition.

Fluorescence Spectra.—Within the limits of precision of our measurements, the spectra of the residual fluorescence in dry benzene, the fully activated fluorescence in "wet" benzene, and the fluorescence of partially activated benzene are identical. Table II lists the observed fluorescent maxima. The apparent uncertainty

TABLE II

THE MAXIMA IN THE FLUORESCENCE SPECTRA OF COMPLETELY AND PARTIALLY ACTIVATED CHLOROPHYLL A SOLUTIONS

Solvent	$I_t/I_{t,\max.}$	$\lambda_{\max.\ I}$, Å.	$\lambda_{\max.\ II}$, Å.	Observer
Benzene	0.20	6760	7300	L., W. and McA.
Benzene	0.80	6760	7250	L., W. and McA.
Benzene	1(?)	6730	7200–7300	Zscheile and Harris[8]
Ether	1(?)	6660	7300	L., W. and McA.
Ether	1(?)	6645	7200–7300	Zscheile and Harris[8]

of the wave lengths in the present measurements is about 25Å. The values for the wave lengths of the principal maxima, observed in the present measurements, are slightly longer than the corresponding values published by Zscheile and Harris.[8] This is very probably due to the effect of reabsorption in our measurements. For this reason the measured ratios of the intensities obtained by us are without absolute significance. It is important, however, that these ratios were the same for all solutions studied. The measurements were made with a Steinheil spectrograph, which was adjusted to take Raman spectra.[18] In order to reduce the effect of reabsorption on the fluorescence spectra, the conventional Raman tubes which held the solutions were masked except for a strip about 5 mm. wide adjacent to the exit window. Mercury arc lines were used as wave length standards. The plates were measured with a Leeds and Northrup recording densitometer.

Absorption Spectra.—The absorption spectra of chlorophyll in dry hydrocarbons differ distinctly from the corresponding spectra in ordinary (wet) solvents. Figure 4 illustrates the absorption spectra of chlorophyll b in wet and dry benzene. The spectra of chlorophyll a, in wet and dry benzene, show similar but slightly less marked differences.

The spectrum of chlorophyll a dissolved in benzene, containing a concentration of an activator sufficient to raise the intensity of fluorescence to its limiting value, is independent of the chemical nature of the activator. In other words, the absorption spectrum of activated chlorophyll in an inert solvent bears no relation to the spectra of chlorophyll dissolved in the pure activator. This is illustrated by Fig. 5, on which are plotted the absorption spectra of chlorophyll a dissolved in the following solvents: benzylamine, benzyl alcohol, dry benzene, and benzene activated

(18) We are indebted to Dr. Bryce Crawford for kindly placing this equipment at our disposal.

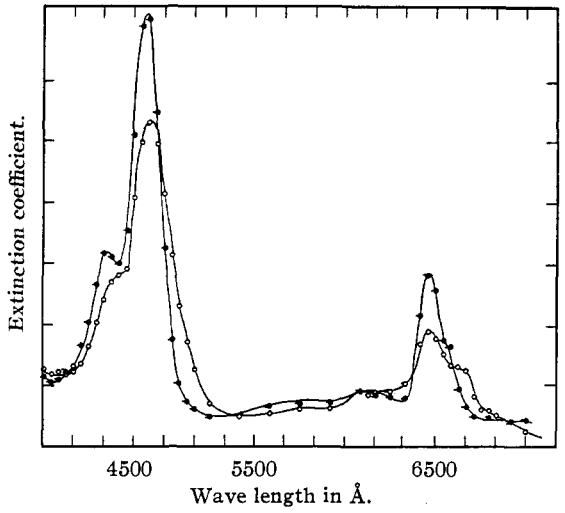


Fig. 4.—Absorption spectra of chlorophyll b in wet and dry benzene: O, dry; -●, wet.

severally with 0.0195 *m* benzyl alcohol and 0.0092 *m* benzylamine. It is especially striking that the spectra in benzene, activated by benzyl alcohol and by benzylamine, are identical, since the spectrum in pure benzylamine is shifted completely out of the range of wave lengths which Harris and Zscheile[19] report for a wide variety of solvents. Similar effects are obtained with chlorophyll b, and with chlorophyll a in other inert solvents.

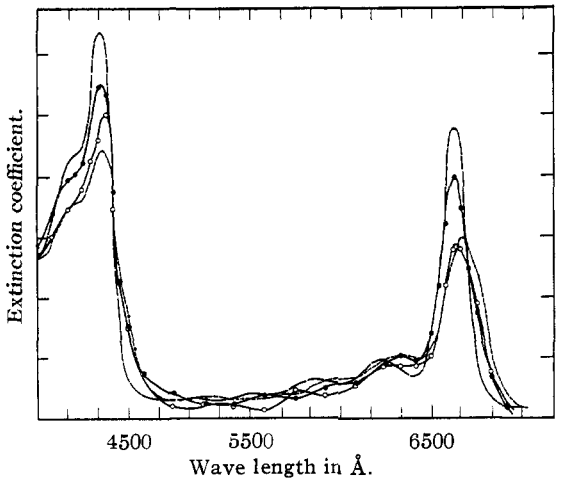


Fig. 5.—Absorption spectra of chlorophyll a in activating, inert and activated solvents: ----, dry benzene; – – –, benzylamine; -O—O-, benzyl alcohol; -●—●-, benzene activated with benzylamine or benzyl alcohol.

Temperature Effects.—The intensity of fluorescence, I_f, of chlorophyll dissolved in activating solvents decreases as the temperature increases. Over a fairly wide range (60° in the present experiments and 90° in Zscheile and Harris'[8] measurements, I_f is a linear function of temperature. In partially activated solutions in benzene the effect of temperature is more marked. In these latter solutions, within the range and precision of the measurements, I_f is a linear function of temperature when the activator is aniline or cetyl alcohol. However, plots of I_f against t for similar solutions in which heptylamine was the activator show definite curvature, particularly at low concentrations of activator, the slope decreasing as the temperature increases. The results of these measurements are summarized in Table III. In all of our measurements chlorophyll a at a concentration of 5×10^{-6} was used.

TABLE III

INTENSITY OF FLUORESCENCE AS A FUNCTION OF TEMPERATURE

Solvent	Activator	Temperature range, °C.	$\frac{1}{I_f}\left(\frac{\partial I_f}{\partial t}\right)_{HA}$	$\frac{I_f}{I_{f,\max.}}$
			at 25°	
CH_3OH	...	15 to 70	0.0027	1
$(C_2H_5)_2O$	...	−30 to 30	.0041	1
$(C_2H_5)_2O$	...	−62 to 28	.0038[a]	1
C_6H_6	1.2×10^{-2} *m*, $C_6H_5NH_2$	15 to 60	.0041	0.47[b]
C_6H_6	3.1×10^{-2} *m*, $C_6H_5NH_2$	15 to 60	.0022	.66[b]
C_6H_6	10.9×10^{-2} *m*, $C_6H_5NH_2$	25 to 65	.0028	.78[b]
C_6H_6	1.8×10^{-4} *m*, $C_{14}H_{29}OH$	10 to 60	.0094	.33[b]
C_6H_6	3.6×10^{-4} *m*, $C_{14}H_{29}OH$	15 to 70	.0100	.43[b]
C_6H_6	5.4×10^{-4} *m*, $C_{14}H_{29}OH$	15 to 60	.0073	.50[b]
C_6H_6	18.0×10^{-4} *m*, $C_{14}H_{29}OH$	15 to 55	.0055	.64[b]
			at 40°	
C_6H_6	7.3×10^{-6} *m*, $C_7H_{15}NH_2$	10 to 70	.0200	0.35
C_6H_6	12.2×10^{-6} *m*, $C_7H_{15}NH_2$	15 to 70	.0155	.48
C_6H_6	20.3×10^{-6} *m*, $C_7H_{15}NH_2$	15 to 70	.0158	.61
C_6H_6	36.5×10^{-6} *m*, $C_7H_{15}NH_2$	20 to 65	.0111	.79
C_6H_6	81.2×10^{-6} *m*, $C_7H_{15}NH_2$	15 to 65	.0075	.96
C_6H_6	1.63×10^{-2} *m*, $C_7H_{15}NH_2$	15 to 70	.0029	1.00

[a] From the data of Zscheile and Harris, ref. 8. [b] Based on estimated values of $I_{f,\max.}$

The absorption spectra of partially activated solutions of chlorophyll vary with temperature. At low temperatures the spectrum of such a solution approaches that of a completely activated solution, while at high temperatures it tends toward the spectrum characteristic of chlorophyll in an inert, dry solvent. This behavior is illustrated by the several absorption curves of Fig. 6.

Discussion

The fact that chlorophyll is fluorescent when dissolved in alcohols and similar solvents but is non-fluorescent in dry hydrocarbons suggests that the chlorophyll molecule exists in different chemical states in these two classes of solutions. The activation of the fluorescence by low concentrations of activators (such as water, alcohols, amines, etc.) is evidence for a stoichiometric reaction between chlorophyll and the activator. The alternate hypothesis, that the process of activation is a medium effect, appears very improbable when it is remembered that concentrations of heptylamine or piperidine as low as that

(19) D. G. Harris and F. P. Zscheile, *Bot. Gaz.*, **104**, 515 (1943).

(5×10^{-6} *m*) of chlorophyll produce a marked increase in the intensity of fluorescence.

If the hypothesis is accepted that the activation of fluorescence is due to a chemical interaction between chlorophyll and the activator, it should be possible to represent the data presented in this paper in terms of simple chemical equilibria. Possibly the simplest definite form of this general hypothesis is that chlorophyll exists as a non-fluorescent dimer in inert solvents but is dissociated into fluorescent monomer molecules in the presence of an activator.[20] There are three lines of evidence which cause us to reject this specific hypothesis. First, it would lead to the prediction that the fluorescence intensity of (at least some) partially activated solutions should be symbatic with temperature, which is contrary to observation (see Table IV). Second, it appears to be impossible to fit the observed relation (see Figs. 2 and 3) between the intensity of fluorescence and the concentration of the activator in terms of these specific equilibria. Third, when the concentration of chlorophyll is high enough to ensure complete absorption of the exciting light, the intensity of fluorescence of a partially activated solution is independent of the chlorophyll concentration, rather than decreasing with increasing concentration of chlorophyll as the dimer hypothesis would predict.[21]

Another simple specific form of the general hypothesis can be stated as follows. In an inert solvent, chlorophyll exists as a non-fluorescent, monomeric molecule, but in the presence of an activator a fluorescent addition compound between the activator and chlorophyll is formed. The concentration (GHA), of the fluorescent compound between chlorophyll, G, and the activator, HA, can be computed from the equilibrium relation

$$G + HA = GHA \text{ or } K_1 = (GHA)/(G)(HA) \quad (1)$$

Since in practically all of the cases studied, the equilibrium concentration (GHA), is negligible compared to the initial concentration, $(HA)_0$, we may write

$$\frac{(GHA)}{(G)_0} = \frac{K_1(HA)_0}{1 + K_1(HA)_0} \quad (2)$$

If we make the further minor simplifying assumption (compare Figs. 4 and 5) that the extinction coefficients, α_i, for the exciting light, of GHA and G are the same, we obtain the simple relation

$$\frac{I_f}{I_{abs.}\,\varphi} = \frac{I_f}{I_{f,\,max.}} = \frac{K_1(HA)}{1 + K_1(HA)_0}$$

where φ is the fluorescent yield for GHA, and $I_{f,\,max.}$ is the fluorescent intensity obtained in a fully activated solution. This equation may be written in the form

$$\log\left(\frac{I_{f,\,max.}}{I_f} - 1\right) = -\log (HA)_0 - \log K_1 \quad (3)$$

That equation (3) is generally consistent with the experimental data can be demonstrated by plotting log $(I_{f,\,max.}/I_f - 1)$ against log $(HA)_0$ as is illustrated by Fig. 7. The slopes of all of the straight lines on the figure are -1, as is required by equation 3. However, this equation fails in the low concentration range, since no account is taken in it of the presence of the adventitious activator, HB, which is (presumably) responsible for the residual fluorescence observed when no activator is added. Equation 3 may be corrected for this effect by introducing the second equilibrium

$$G + HB = GHB \quad K_2 = (GHB)/(G)(HB) \quad (4)$$

Combining equations 1 and 4, and introducing the safe approximation that $4(G)_0(HB) \ll [(G)_0 + (HB)_0]$, we obtain the relation

$$\frac{(GHA) + (GHB)}{(G)_0} = \frac{1}{1 + K_1(HA)_0}\left[K_1(HA)_0 + \frac{K_2(HB)_0}{1 + K_1(HA)_0 + K_2[(HB)_0 + (G)_0]}\right]$$

We can eliminate one arbitrary constant by making the minor simplifying assumption that $(HB)_0 + (G)_0 \simeq (HB)_0$. Then making the same assumptions as were used in the derivation of equation 3 (*i.e.*, $\alpha_{GHB} = \alpha_{GHA} = \alpha_G$, $\varphi_{GHB} = \varphi_{GHA}$ and $\varphi_G = 0$) we obtain equation 5, which is similar to equation 3 but has been corrected for the effect of the adventitious activator, HB.

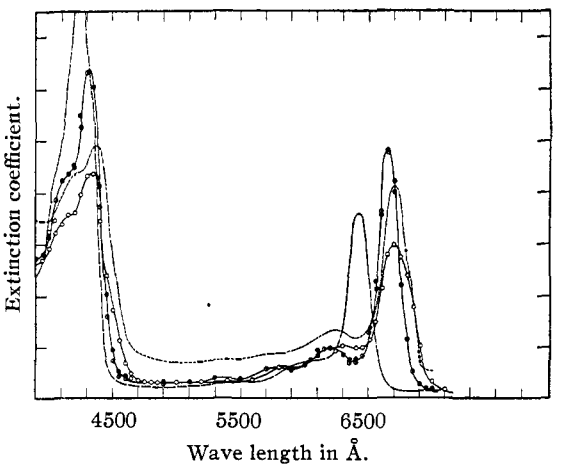


Fig. 6.—Absorption spectra of partially activated chlorophyll a in benzene as a function of temperature:

Solvent	Temp., °C.	Symbol
Dry	30	--------
Activated	30	- - -
Partially activated	11	-●—●—●-
Partially activated	67	-O—O—O-

(20) We are indebted to Professor James Franck for this suggestion.

(21) The following measurements were made with a solution of chlorophyll a in benzene, containing a trace of an adventitious activator (probably water). The absorption of the exciting light was practically complete even for the most dilute solutions:

Molality of chlorophyll	4.8×10^{-5}	1.5×10^{-4}	2.3×10^{-4}
$I_t/I_{t,\,max.}$	0.18	0.18	0.17

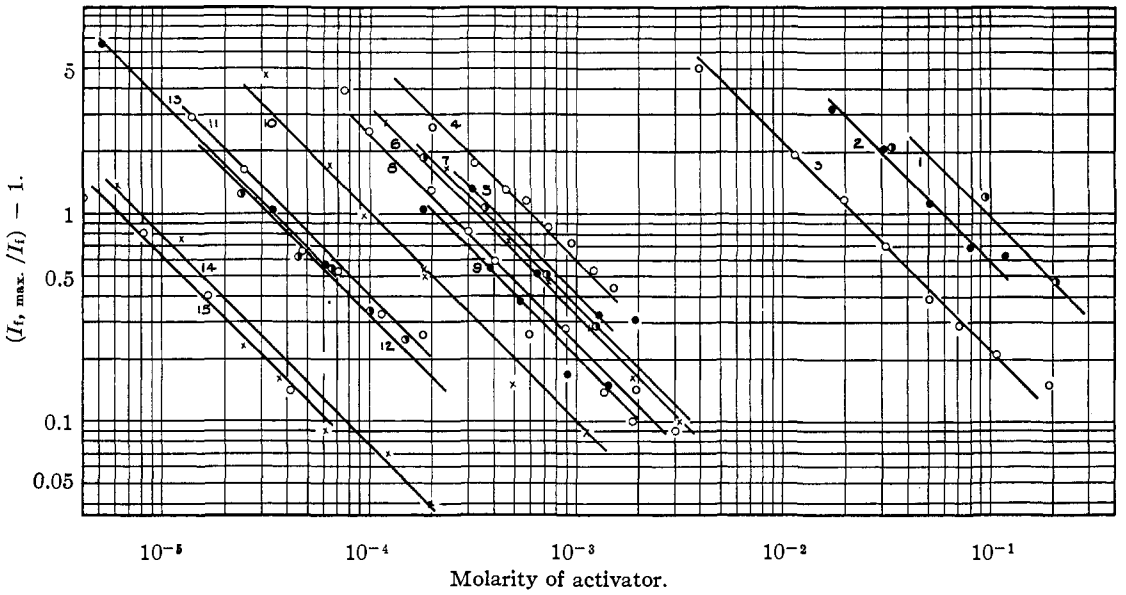


Fig. 7.—Functional relation between intensity of fluorescence and concentration of activator for several activators: 1, dimethylaniline; 2, phenol; 3, aniline; 4, phenylhydrazine in *n*-heptane; 5, formamide; 6, benzyl alcohol with chlorophyll a; 7, benzyl alcohol with chlorophyll b; 8, cetyl alcohol; 9, octyl alcohol; 10, methanol; 11, benzylamine with chlorophyll a; 12, benzylamine with chlorophyll b; 13, water; 14, piperidine; 15, *n*-heptylamine.

$$\frac{I_f}{I_{f,\,max.}} = \frac{1}{1 + K_1(HA)_0}\left[K_1(HA)_0 + \frac{K_2(HB)_0}{1 + K_1(HA)_0 + K_2(HB)_0}\right] \quad (5)$$

The agreement between equation 5 and the experimental data is illustrated by Figs. 2 and 3 where the points represent measured values and the curves are plots of equation 5. The data plotted are typical of all of the experiments, in that there is no systematic deviation between the computed curves and the observed points.

Of the parameters of this equation, $I_{f,\,max.}$ has the same value (within the limits of our measurements) for all activators and all solvents. The value of $K_2(HB)_0$, which is a relatively unimportant correction term, depends upon the care taken in purifying the solvent, etc. The present experimental values fall within the limits 0.07 to 0.15. The principle parameter, K_1, varies over a wide range; for those substances which have been observed to activate, from about 10^5 to 10. It is noteworthy, that (within the limits of our measurements) the value of K_1 is the same for chlorophyll b as for chlorophyll a, and that it is practically independent of the (inert) solvent used.

If, as seems very probable, the chief adventitious activator, HB, is water, K_2 in all cases may be identified with K_1 for water which has a numerical value of 3×10^4. It has been noted that it is difficult to purify these solutions sufficiently to reduce the value of $K_2(HB)_0$ much below 0.1. It is interesting and possibly significant[22] that the corresponding concentration of water, $(3 \times 10^4)^{-1} \times 10^{-1} = 3 \times 10^{-6}$, is approximately half the concentration of the chlorophyll.[23]

The heat of the reaction G + HA = GHA can be obtained from the measured temperature coefficient of the intensity of fluorescence (Table III). This can be done conveniently by using only those values of the intensity in which the contribution of the fluorescence of GHB is negligible, thereby justifying the substitution of equation 3 for equation 5. Differentiating equation 3 in respect to t, keeping $(HA)_0$ constant, there is obtained the equation

$$\frac{I_{f,\,max.} - I_f}{I_{f,\,max.}} \times \frac{d \ln K_1}{dt} = \left(\frac{\partial \ln I_f}{\partial t}\right)_{HA} - \frac{d \ln I_{f,\,max.}}{dt}$$

Differentiating this expression in respect to I_f at constant temperature, yields the relation

$$\frac{d \ln K_1}{dt} = -\frac{1}{I_{f,\,max.}}\left(\frac{\partial\left(\frac{\partial \ln I_f}{\partial t}\right)_{HA}}{\partial I_f}\right)_t$$

Therefore, $d \ln K_1/dt$ may be obtained from a plot of $(\partial \ln I_f/\partial t)_{HA}$ against I_f, using numerical values corresponding to 25°. The heat of the reaction is $\Delta H = RT^2(d \ln K/dt)$.

The values of the equilibrium constants at 25°, the heats of the reaction (calculated as outlined above), and the corresponding free energies and entropies of formation are given in Table IV.

(22) Rabinowitch, "Photosynthesis. I," Interscience Press, New York, N. Y., 1945, pp. 450–451.

(23) This computation is inconsistent with the assumption that $(HB)_0 \ll (G)_0$, which was used in the derivation of equation 5. However, it should be remembered that the quantity $K_2(HB)_0/[1 + K_2[(HB)_0 + (G)_0]]$ is approximately equal to 0.1, and for the purpose of the present computation, substituting $3K_2(HB)_0$ for $K_2(HB)_0$ in the denominator is relatively unimportant.

TABLE IV

SUMMARY OF THE HEATS OF FORMATION AND RELATED QUANTITIES FOR THE FORMATION OF SEVERAL COMPLEXES, GHA_i

Activator	K_1	$\Delta F°$, cal.	$\Delta H°$, cal.	$\Delta S°$, E. U.
Heptylamine	1.40×10^5	−7100	−6000[a]	3.3
Cetyl alcohol	4.2×10^3	−5000	−2500	5.0
Aniline	4.6×10	−2300	−1500	2.7

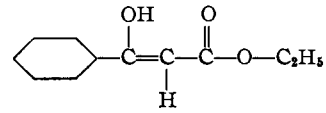
[a] Since in this case, $(\partial I_f/\partial t)_{HA}$ was dependent upon the temperature, the value of −6,000 cal. is an average value chosen to be representative of all the data, not the particular value for 40° given in Table III.

The estimated uncertainties of these quantities are about ±100 cal., ±600 cal., and ±2 E.U. for $\Delta F°$, $\Delta H°$ and $\Delta S°$, respectively.

The process of fluorescence activation of chlorophyll solutions is an "all-or-none" effect. The limiting fluorescent intensity is independent of the stability of the addition compound, as measured by K_1. The absorption spectra of the activated chlorophyll solutions are identical, regardless of the nature of the activator. In other words, if activation occurs at all, the optical properties of the activated chlorophyll are affected neither by the chemical nature nor by the molecular weight of the activator. This suggests strongly that the function of the activator is to produce an isomeric change in the chlorophyll molecule.

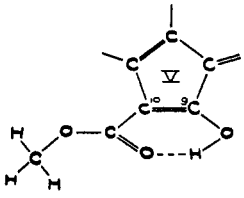
Although the list of compounds which has been studied is too restricted to justify any safe generalizations, there appear to be three factors which determine the stability of the addition compounds. (1) Only those substances which possess a hydrogen atom capable of forming hydrogen bonds are activators.[24] (2) The stability of the addition compound increases with the basicity of the activator. Amines are consistently better activators than are the corresponding alcohols. (3) Bulky groups attached directly to the active part (NH or OH) of the molecule, decrease its efficiency as an activator. This is, presumably, the explanation of why diphenylamine and hydrazobenzene fail to activate chlorophyll.

In light of these fairly well established generalizations it is interesting to speculate on possible definite mechanisms of activation. A postulate which appears to be consistent with the available data may be stated as follows. When chlorophyll is dissolved in an inert solvent, the oxygen attached to carbon 9 of ring V is enolized. This enol form[25] is stabilized both by chelate ring formation (hydrogen bonding between the enol hydrogen and the carbonyl oxygen of the methyl ester attached to carbon 10) and by resonance involving the main conjugated ring and the new double bond in ring V. An analog to this stabilized enol is discussed by Bhatnagar and Mathur.[26] An analysis, by Pascal's empirical method of the magnetic susceptibility data, indicates that ethyl benzoylacetate exists almost completely in the enolic form

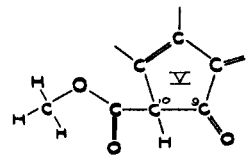


This compound has qualitatively the same possibilities of resonance and chelate stabilization as has ring V and its attached groups in chlorophyll. In chlorophyll, the additional resonance may offer a new mode of coupling between electronic energy and oscillational energy and thereby reduce the fluorescent yield.[27,28]

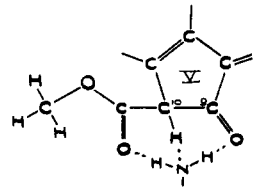
In the presence of water, amines or alcohols, hydrogen bonding between the hydrogen of the activator and in the keto oxygen competes with chelate formation and tends to favor the keto form. An examination of the steric possibilities indicates that for either water or a primary amine, the two hydrogens of the activator can interact simultaneously with the keto oxygen (carbon 9) and the carbonyl oxygen of the methyl ester group. A weak hydrogen bond between the H attached to carbon 10 and the strongly negative nitrogen (or oxygen) of the activator may contribute slightly to the stability of the addition complex.



Chelated enol form



Keto form



Stabilized keto form

Under all ordinary conditions (in solvents such as alcohol or even moist hydrocarbons) the keto form is dominant, and it is this form of the molecule which is responsible for the usual fluorescent yield of about 10%.[5]

This postulated keto–enol mechanism of fluorescence activation is in conflict with the commonly

(24) There are two apparent exceptions to this rule. Dimethylaniline is a weak activator, $K_1 = 11$. A possible explanation of its activity may be the ability of its parahydrogen to form weak hydrogen bonds. Ethyl ether, while not studied quantitatively, seems to be a fairly efficient activator. However, the interpretation of this latter result must remain in doubt, until it has been demonstrated that the activating efficiency of ether is not affected by further stringent purification.

(25) This nomenclature is adopted from Rabinowitch, "Photosynthesis," 1945, Vol. I, p. 442.

(26) Bhatnagar and Mathur, "Physical Principles and Applications of Magnetochemistry," The Macmillan Co., New York, N. Y., 1935, p. 89.

(27) G. N. Lewis and M. Calvin, *Chem. Rev.*, **25**, 273 (1939).

(28) J. Franck and R. Livingston, *J. Chem. Phys.*, **9**, 184 (1941).

assumed but unproved explanation of the allomerization of chlorophyll.[29] A preliminary experiment, performed with allomerized chlorophyll, indicates that fluorescence activation occurs equally well with allomerized and normal chlorophyll, which is contrary to predictions based upon the detailed hypotheses which have been used to explain allomerization and fluorescence activation, respectively. The available evidence is not sufficient to permit a definite decision which, if either, of these detailed, incompatible mechanisms is correct. There is no conflict between the experimental facts or between the general explanation of allomerization and of fluorescence activation.

Acknowledgment.—The authors wish to acknowledge the many valuable suggestions, chiefly in regard to structural considerations, of Dr. R. Arnold and Dr. W. Lipscomb, of this department.

Summary

Chlorophyll dissolved in pure dry hydrocarbons is practically non-fluorescent. The addition to such a solution of 0.01% or more of water, or certain alcohols or amines, raises the intensity of the fluorescence to the value which is characteristic of chlorophyll dissolved in alcohols or similar solvents. The absorption spectrum of chlorophylls in dry hydrocarbons differs from the spectrum of chlorophyll in hydrocarbon solvents containing a trace of water or other "activator." Moreover the spectrum in the presence of a small amount of activator is independent of the chemical nature of the activator and bears no resemblance to the spectrum of chlorophyll dissolved in the pure activator.

For all activators studied, the intensity of fluorescence, I_f, fits a relation of the form

$$\frac{I_{f,\,max.}}{I_f} = \frac{1}{K_1(HA)} + 1$$

where $I_{f,\,max.}$ is the upper limiting value of the fluorescence, (HA) is the concentration of the activator, and K_1 is an adjustable constant which is a measure of the efficiency of the activator.

These results are consistent with the postulate that a molecule of chlorophyll and one of the activator form an addition compound which is the fluorescent entity, and that the simple chlorophyll molecule in an inert solvent is non-fluorescent. This postulate has been interpreted in terms of hydrogen bonding, probably involving a keto oxygen on ring V of the chlorophyll molecule.

(29) Compare Rabinowitch, "Photosynthesis," Vol. I, 1945, pp. 459-462 and 492-493.

MINNEAPOLIS, MINN. RECEIVED AUGUST 6, 1948

[CONTRIBUTION FROM THE NOYES CHEMICAL LABORATORY, UNIVERSITY OF ILLINOIS]

Polarography of Copper Complexes. I. Ethylenediamine, Propylenediamine, Diethylenetriamine and Glycine Complexes

BY H. A. LAITINEN, E. I. ONSTOTT, J. C. BAILAR, JR., AND SHERLOCK SWANN, JR.

Several complex ions of copper were studied by means of the dropping mercury electrode in order to determine the effect of specific coördinating agents on the stability of the cuprous and cupric oxidation states. Results on complexes of ethylenediamine, propylenediamine, diethylenetriamine, and glycine are reported in this paper.

The stability and composition of the ethylenediamine and propylenediamine complexes of copper have been determined by Carlson, McReynolds and Verhoek,[1] who used the method of Bjerrum.[2] Job and Brigando[3] and Haendler[4] have employed the method of continuous variations for the study of the diethylenetriamine complex of copper. Keefer[5] previously has studied the glycine·complex using the dropping mercury electrode, but it was desired to extend the range of his investigation to lower *p*H values by using suitable buffer solutions.

All of these complexes are reduced directly to the amalgam, in contrast to the cupric complexes of ammonia,[6,7] pyridine,[8] thiocyanate ion,[9] and chloride ion,[10] which are reduced first to the respective cuprous complexes before being further reduced to the amalgam.

Experimental

Reagent grade chemicals were used without further purification except for the glycine which was once recrystallized. Solutions of the amines, obtained from the Carbide and Carbon Chemicals Corporation, were standardized potentiometrically with hydrochloric acid using a Beckman *p*H meter. A solution of cupric nitrate was standardized iodometrically.

The solutions for analysis were made up from stock solutions of the reagents. Potassium nitrate was used as indifferent electrolyte for the amine complexes, and for the glycine complex the buffer of potassium dihydrogen phosphate plus sodium hydroxide was used as indifferent electrolyte. Gelatin and methyl cellulose were found to be

(1) Carlson, McReynolds and Verhoek, THIS JOURNAL, **67**, 1334 (1945).

(2) Bjerrum, "Metal Ammine Formation in Aqueous Solutions," P. Haase and Son, Copenhagen, 1941; *C. A.*, **35**, 6527 (1941).

(3) Job and Brigando, *Compt. rend.*, **210**, 438 (1940).

(4) Haendler, THIS JOURNAL, **64**, 686 (1942).

(5) Keefer, *ibid.*, **68**, 2329 (1946).

(6) Stäckelberg and Freyhold, *Z. Elektrochem.*, **46**, 120 (1940).

(7) Lingane, *Chem. Rev.*, **29**, 1 (1941).

(8) Lingane and Kerlinger, *Ind. Eng. Chem., Anal. Ed.*, **13**, 77 (1941).

(9) Kolthoff and Lingane "Polarography," Interscience Publishers, Inc., New York, N. Y., 1946.

(10) Lingane, *Ind. Eng. Chem., Anal. Ed.*, **15**, 583 (1943).