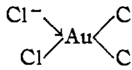
which consumes hydrogen ion.[15] We believe the final flocculated ovalbumin–chloroaurate to be merely insoluble and chemically altered but undenatured protein.

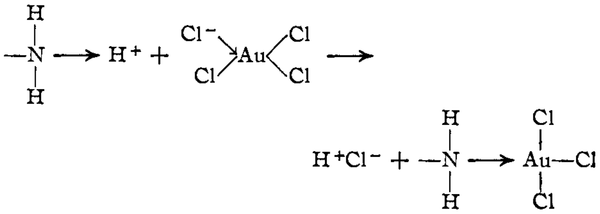
It was mentioned earlier that the *p*H was observed to decrease in unbuffered solutions. Figure 6 shows that hydrogen ion is liberated concurrent with the combination of the protein with the chloroauric acid. From this change in *p*H one can calculate the number of $AuCl_4^-$ ions which combine per molecule of ovalbumin, on the basis of the reasonable assumption that one hydrogen ion is released per $AuCl_4^-$ ion reacted. This calculation produced values ranging from 40 to 45 $AuCl_4^-$ ions per molecule of ovalbumin, corresponding to selected molecular weights of the protein ranging from 40,000 to 45,000. It is of interest to compare these values with the number of basic groups contributed by arginine, histidine, and lysine, which are given variously from 40 to 41 per ovalbumin molecule.[16]

The observation that the induction period is of longer duration at low *p*H values is now explained since hydrogen ion is produced in the reaction.

We turn now to the probable chemical reaction between amino groups and the $AuCl_4^-$ ion. The chloroaurate ion is itself a complex formed by $AuCl_3$ and Cl^- ion, *i.e.*



This ion is presumably first attracted to the positive $-NH_3^+$ centers on the surface of the protein by coulombic forces, and when it is sufficiently near, there is a splitting out of H^+Cl^- making the two electrons of nitrogen which were formerly coördinated with hydrogen ion now available for coördination with the gold of the $AuCl_3$. This produces $-NH_2\cdot AuCl_3$ according to the scheme



This reaction is being investigated further in our laboratories in view of the possibility that chloroauric acid may serve as a reagent for the determination of the number of basic amino groups in a given protein molecule.

BATON ROUGE, LOUISIANA

(15) D. Lloyd and A. Shore, "The Chemistry of the Proteins," P. Blakiston' Sons and Co., Inc., Philadelphia, Pa., 1938, pp. 429–431.

(16) H. L. Fevold, *Advances in Protein Chem.*, **6**, 202 (1951).

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

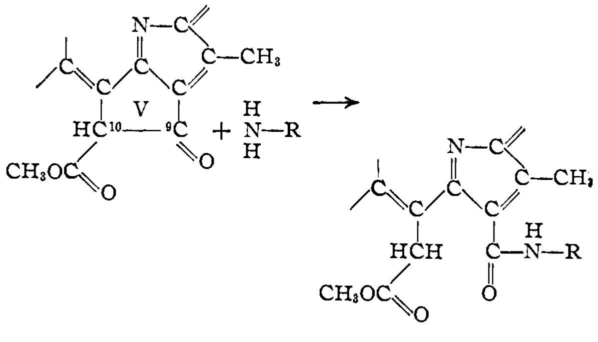
The Reaction of Chlorophyll in Amines[1]

BY ALBERT WELLER[2] AND ROBERT LIVINGSTON

RECEIVED OCTOBER 29, 1953

Chlorophyll dissolved in amines undergoes an irreversible reaction which involves cleavage of ring V. Reaction rates in different amines and at different temperatures were measured spectrophotometrically. The specific rates parallel the basicities of the amines. The energy of activation is very low, $\sim$3 kcal. A mechanism analogous to the aminolysis of esters is suggested and discussed. It is suggested that the reported[3] phosphorescence of chlorophyll dissolved in isoamylamine, is very probably due to the chlorin-6-acidamide formed in the amine reaction.

In the course of investigations which have been carried out in this Laboratory on the photochemical behavior of chlorophyll and related compounds, it has been observed[4] that the absorption spectrum of chlorophyll is shifted to much shorter wave lengths (about 200 Å.) in amines. It cannot be ascribed to medium effects, but is due to a reaction product of chlorophyll[5] which is formed irreversibly in a reaction between chlorophyll and amine. Fischer and Göbel[6] using methylpheophorbide and different amines showed that it is the chlorin-6-acidamide which is formed by cleavage of ring V by the amine.



This reaction is of special interest, since Kautsky and co-workers[3] observed phosphorescence of solvents of chlorophyll ($-a$ + $-b$) in isoamylamine but not when other (non-amine) solvents were used. It seems probable, therefore, that the phosphorescence which those authors observed was due to chlorin-6-acidamide rather than to chlorophyll.

Krasnovskii and Brin[7] attribute the difference,

(1) This work was made possible by the support of the Office of Naval Research (NR 051,028, Contract N6ori-212, T. O. I.) to whom the authors are indebted.

(2) Post-doctoral Fellow 1951–1952. Present address: Laboratorium für physikalische Chemie, Widerholdstr. 15, Stuttgart, Germany.

(3) H. Kautsky, A. Hirsch and W. Flesch, *Ber.*, **68**, 152 (1935).

(4) R. Livingston, W. F. Watson and J. McArdle, THIS JOURNAL, **71**, 1542 (1949).

(5) J. W. Weigl and R. Livingston, *ibid.*, **74**, 3452 (1952).

(6) H. Fischer and S. Göbel, *Ann.*, **524**, 279 (1936).

(7) A. Krasnovskii and G. Brin, *Doklady Akad. Nauk S.S.S.R.*, **89**, 527 (1952); *C. A.*, **47**, 8195 (1953).

which they observed between the absorption spectra of chlorophyll-*a* in ether and in a series of liquid amines, to the reversible formation of a complex between a molecule of the amine and the (magnesium atom of the) chlorophyll molecule. While, in part, this may be true for solutions of chlorophylls in weakly basic amines, the large shift produced by piperidine, etc., must be the consequence of an irreversible reaction. If chlorophyll-*a*, dissolved in piperidine, is dried by evaporation at room temperature and the resulting solid dissolved in ether, the absorption maxima of this solution occur at 640 and 411 mμ, rather than at 661 and 427 mμ, as shown by an ethereal solution of native chlorophyll-*a*. The principal maxima of ethereal solutions of the products of all of the amine–chlorophyll-*a* reactions which are reported in the present paper are at 640 ± 1 and 411 ± 1 mμ.

Experimental Methods and Materials

Procedure.—The reaction rates were measured spectrophotometrically. The spectra were taken with a Cary recording spectrophotometer using a tungsten lamp and 2-cm. glass cells. The cells were filled with about 6 cc. of the amine. At the time $t = 0$ about 0.15 cc. of an ethereal chlorophyll solution was added to one cell from a syringe. The cell was shaken for a few seconds to complete mixing and was inserted in the sample compartment of the Cary. It was also found that 6% or less of ether did not appreciably affect the rate. The rate was the same in an evacuated cell as when oxygen present. Accordingly, no precautions were taken to eliminate air from the solutions which were used in those experiments reported in this paper. Measurements at low temperatures were carried out with a dewar cell of special design (see Fig. 1). The cell proper was filled with an amine methylcyclohexane mixture which was kept at 0° or −78° by filling the dewar with ice-water or a Dry Ice–methanol mixture.

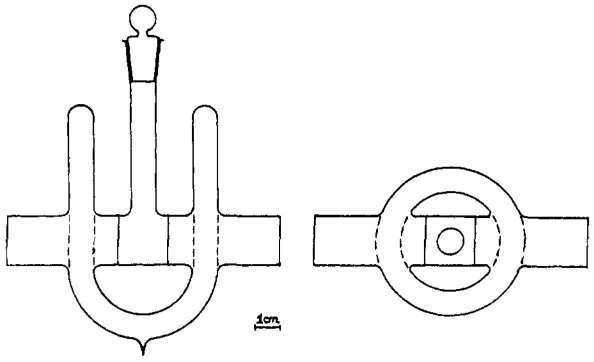


Fig. 1.—Dewar cell used for measurements at low temperatures.

Materials.—The following amines were used: piperidine, isoamylamine, isobutylamine, *sec*-butylamine, phenylhydrazine, triethylamine and aniline. Except for phenylhydrazine all the amines were dried with solid potassium hydroxide and distilled in a stream of dry helium. The method of purification of phenylhydrazine has been described elsewhere.[8] Methylcyclohexane was distilled from sodium. Stock solutions of chlorophyll-*a* and of chlorophyll-*b* were prepared by a modification[9] of the method of Zscheile and Comar.[10]

Experimental Results

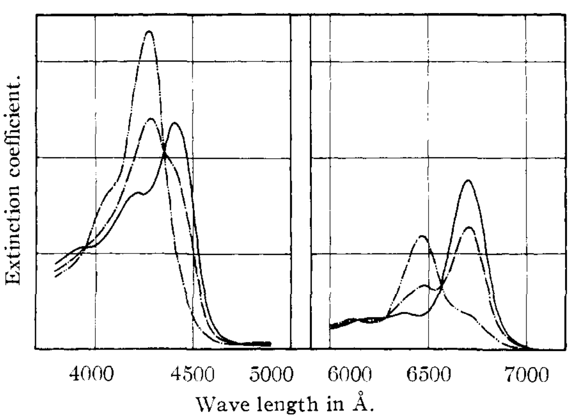


Fig. 2.—Absorption spectra of chlorophyll-*a* in phenylhydrazine as a function of time: ———, 4 min. after mixing (0.1% reaction product); —·—, 1250 min. after mixing (26.6% reaction product); —··—, 5720 min. after mixing (75.7% reaction product). (Other curves have been omitted in order to avoid overcrowding.)

Determination of the Reaction Rate Constants.

In amine solutions the reaction is first order in respect to chlorophyll. It is the disappearance of the chlorophyll spectrum and the appearance of the chlorin-6-acidamide spectrum which make it possible to follow the reaction spectrophotometrically. Figure 2 shows, as an example, the spectra of chlorophyll-*a* in phenylhydrazine solution at the beginning of and during the reaction. As indicated by the isosbestic points, no intermediate is formed in spectrophotometrically detectable amounts during the course of the reaction. Therefore

$$E = \frac{C_{\text{chl}}}{C_0}E_0 + \frac{C_0 - C_{\text{chl}}}{C_0}E_\infty \qquad (1)$$

where E is the extinction measured during the course of the reaction, E_0 the initial extinction (chlorophyll alone) and E_∞ the final extinction (chlorin-6-acidamide alone). From 1 follows

$$\frac{C_{\text{chl}}}{C_0} = \frac{E - E_\infty}{E_0 - E_\infty} \qquad (2)$$

Since it takes about 5 min. to take one spectrum (from 700 to 380 mμ), it was necessary for the faster reactions in piperidine, isoamylamine and isobutylamine to measure the extinction during the course of the reaction at one particular wave length (preferentially at the maximum of the long wave length band of the chlorophyll). Plotting log $(E - E_\infty/E_0 - E_\infty)$ *vs.* time gives straight lines. Some of those plots are shown in Fig. 3 for chlorophyll-*a* and for chlorophyll-*b*. From the slopes of these plots and analogous plots for the other amines, k' can be calculated. Table I summarizes the velocity constants of the reaction in several amines.

The last column lists the pK_a values of the amines in water as given by Hall and Sprinkle.[11] The log k' is independent of the chlorophyll concentration, which was between 5 and 8 × 10^{-6} *M*. There is a clear parallelism between log k' and pK_a although steric requirements are an important factor, as can be seen by comparing iso- and *sec*-butylamine. In addition to the substances listed in Table I the following compounds have been studied: it was found that allomerized chlorophyll-*a*

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[8] R. Livingston and Chun-Lin Ke, THIS JOURNAL, **72**, 909 (1950).

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

[10] F. Zscheile and C. Comar, *Botan. Gaz.*, **102**, 463 (1941).

[11] N. F. Hall and M. R. Sprinkle, THIS JOURNAL, **54**, 3469 (1932).

TABLE I

LOGARITHM OF VELOCITY CONSTANTS FOR THE REACTION OF CHLOROPHYLL-*a* AND OF CHLOROPHYLL-*b* IN AMINES AT 26° AND THE pK_a VALUES FOR THE AMINES

Amine	log k' (sec. $^{-1}$) chl.-*a*	log k' (sec. $^{-1}$) chl.-*b*	pKa
Piperidine	-1.88	-1.40	11.13
Isoamylamine	-2.59	-1.89	10.64
Isobutylamine	-2.82	-2.31	10.42
sec-Butylamine	-4.41	-3.68	10.56
Phenylhydrazine	-5.38		8.8
Aniline	<-8.0	<-8.0	4.6

in isobutylamine and chlorophyll-*b* in triethylamine did not react at all in three days, k' being $<10^{-8}$ sec.$^{-1}$; for pheophytin-*b* in isobutylamine, $\log k' = -3.9$.

Temperature Effects.—The rate of the reaction between chlorophyll-*a* and isobutylamine has been measured in methylcyclohexane + 1.71 moles/l. isobutylamine at 26°, 0° and −78°. The chlorophyll concentration was 5×10^{-6} *M*. The results of these measurements are summarized in Table II.

TABLE II

LOGARITHM OF VELOCITY CONSTANT, $k = k'/c_{\text{amine}}$ FOR THE REACTION OF CHLOROPHYLL-*a* IN ISOBUTYLAMINE–METHYLCYCLOHEXANE AT DIFFERENT TEMPERATURES

T, °K.	$\log k \left(\frac{1}{\text{sec. ml.}}\right)$	d	
299	-3.040	0.751	
273	-3.146	.775	$\Delta H^{\ddagger} \leqq 3$ kcal.
195	-4.636	.842	

The second column lists the logarithm of the bimolecular reaction rate constant $k = k'/c_{\text{amine}}$; the third the density d of the solvent from which the change in concentration is calculated. A plot of log k *vs.* $(1/T)$ shows, however, definite curvature, so the value for the energy of activation is not very accurate, but is certainly extremely small (<6 kcal.) which implies that the entropy of activation must be strongly negative (~ −50 e.u.).

Discussion

It is obvious that the amine reaction differs in many respects from the phase test reaction.[12] In the amine reaction no intermediate appears and no oxygen is involved. Furthermore, it was found in some preliminary experiments with chlorophyll-*a* in pyridine, that the addition of KOH in methanol at room temperature resulted in the appearance of the intermediate within less than 1 sec. and in its disappearance after some 20 to 30 sec. At Dry Ice temperature, no reaction at all occurred within two hours indicating that the energy of activation is at least 20 kcal., in contrast to the 3 kcal. found for the amine reaction. It was also found that, in order to get the phase test intermediate a trace of alcohol is necessary. The same condition must be fulfilled for the allomerization process[13] (which probably is a slow phase test reaction without detectable appearance of the intermediate). No alcohol is necessary for the amine reaction. Because of these differences, it appears probable that the mechanism of the amine reaction differs from those of the allomerization and phase test reactions. It is reasonable to assume that the keto form cleaves, rather than the carbanion or enol form. In analogy to the aminolysis of esters,[14] the mechanism on the following page is suggested.

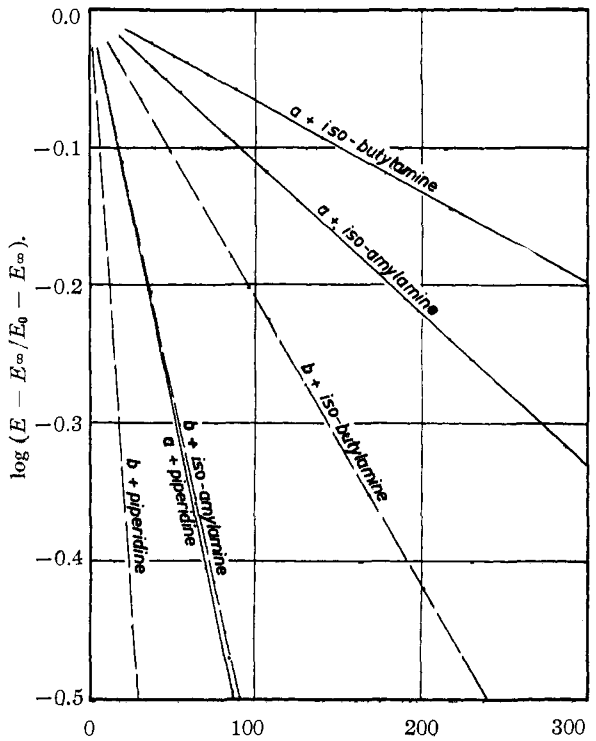

Fig. 3.—Plots of log $(E - E_\infty/E_0 - E_\infty)$ *vs.* time for the reaction of chlorophyll-*a* and of chlorophyll-*b* with piperidine, isoamylamine and isobutylamine.

The mechanism corresponds to the mechanism suggested by Pearson and co-workers[15] for the solvolytic cleavage of the carbon–carbon bond of β-diketones. Furthermore, it is consistent with the fact that the rate of the reaction is determined by the basicity of the amine. It is the electron density on the nitrogen which affects both the basicity of

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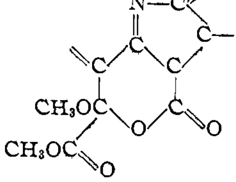
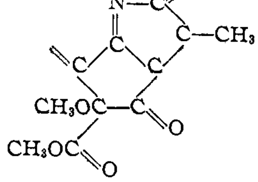
[12] H. Molisch, *Ber. dtsch. bot. Ges.*, **14**, 16 (1896).

[13] H. Fischer and H. Pfeiffer, *Ann.*, **555**, 94 (1944). In this paper the authors show that the allomerization with oxygen yields a lactone (I), whereas with quinone or iodine under nitrogen a 10-methoxy compound (II) is formed. For both reactions (at least a small amount) alcohol is necessary.



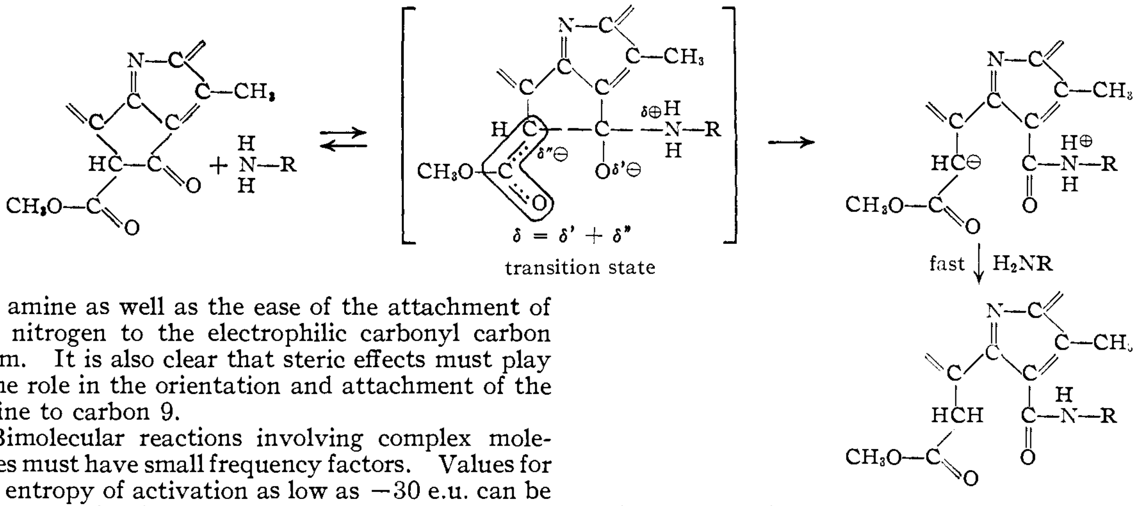
I



II

[14] See *e.g.* the work of E. McC. Arnett, A. R. Day, M. Gordon and J. G. Miller, THIS JOURNAL, **70**, 1946 (1948); **71**, 1245 (1949); **72**, 5635 (1950).

[15] R. G. Pearson and E. A. Mayerle, *ibid.*, **73**, 926 (1951); R. G. Pearson and A. C. Sandy, *ibid.*, **73**, 931 (1951).



the amine as well as the ease of the attachment of the nitrogen to the electrophilic carbonyl carbon atom. It is also clear that steric effects must play some role in the orientation and attachment of the amine to carbon 9.

Bimolecular reactions involving complex molecules must have small frequency factors. Values for the entropy of activation as low as −30 e.u. can be accounted for in this manner. In this case an additional loss of entropy is imposed on the transition state, because of a considerable loss of mobility due to the partly double bond character of the ester side chain.

The increase of the reaction rate in going from chlorophyll-*a* to chlorophyll-*b* (*i.e.*, by substituting CHO for CH_3 on carbon 3, which is more than 10 Å. apart from the reacting carbon 9) must be ascribed mainly to a decrease of the energy of activation. This decrease, of about 0.8 kcal. may be due either to a reduction in the repulsion between carbon 9 and amino nitrogen and/or to an increase in the ionizability of the bond to be broken. The same holds for the increase of the reaction rate by going from pheophytin-*b* to chlorophyll-*b*, where the decrease of energy of activation is about 2.2 kcal. This is another illustration of the fact that the magnesium makes ring V more reactive.

OXFORD, ENGLAND

NOTES

A Proposed Approach to the Chelate Effect[1]

BY ARTHUR W. ADAMSON

RECEIVED SEPTEMBER 8, 1953

It has generally been observed that complex ions formed by the coördination of groups around a central metal ion tend to be more stable, *i.e.*, possess smaller dissociation constants, the larger the number of points of attachment of the central metal ion to each ligand. Thus bis-(ethylenediamine) complexes are more stable than the corresponding tetramines and tris-(ethylenediamine) ones more so than hexamines. Similarly with the tridentate diethylenetriamine complexes, and, finally, the hexadentate ligand ethylenediaminetetraacetic acid has become known for the unusually stable complexes which it forms with a large variety of ions. This increase in stability with dentate character has been termed the "chelate effect." It has been stated, in a general way, to be an entropy effect[2,3] while Schwarzenbach[4] has, in addition, presented a detailed kinetic treatment supposing a two-stage addition of a bidentate ligand with the rate of addition of the second functional group proportional to an effective concentration estimated from its free volume. This leads to a chain length effect roughly in accord with the meager existing data.

While specific structural aspects are undoubtedly relevant, it is suggested here that the chelate effect is largely a consequence of the arbitrary asymmetry in the usual choice of standard states. Consider the general equation for the formation of a complex ion from a hydrated metal ion M and the ligand X.

$$M(H_2O)_n^a + pX^b = [M(H_2O)_{n-p}X_p]^{(a+pb)} + pH_2O \quad (1)$$

The hypothetical one molal state is employed for solutes while for the solvent the pure substance is chosen. In comparing a series of processes of type (1), of varying n and p values, not only are the magnitudes of ΔS^0 and ΔF^0 dependent upon the choice of standard state, but also their *relative* values or positions in a sequence. If the effect of standard state choice is regarded as primarily one of varying the translational entropy contributions, a brief can be made for the use of the hypothetical mole fraction unity state for the solutes. Such a state is one of minimum translational entropy for a solute retaining the properties it possesses in dilute solution. The conversion

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[1] This work was carried out under contract N6onr23809 between the Office of Naval Research and the University of Southern California.

[2] A. E. Martell and M. Calvin, "The Chemistry of the Metal Chelate Compounds," Prentice-Hall Inc., New York, N. Y., 1952, p. 149f.

[3] C. G. Spike and R. W. Parry, THIS JOURNAL, **75**, 2726, 3770 (1953).

[4] G. Schwarzenbach, *Helv. Chim. Acta*, **35**, 2344 (1952).