mit der ATP-Bildung. Die Reaktionsraten des Austausches sind zwar wesentlich kleiner als bei der Phosphorylierung im Licht und den bisher bekannt gewordenen ATP-ase-Reaktionen in Chloroplasten (JAGENDORF und URIBE¹⁵). Die Austauschreaktion verläuft unter ähnlichen Bedingungen wie der lichtinduzierte ATP — P_a-Austausch (CARMELI und AVRON⁸) und wie die chemiosmotische Phosphorylierung. Die Induktion der Austauschreaktion durch den Säure-Base-Übergang scheint analog zu ihrer Induktion durch Licht zu sein. Von besonderem Interesse ist die Tatsache, daß auch ohne p_H-Wechsel oder Lichtinduktion eine nur durch DTT bewirkte Austauschreaktion erscheint.

Zusammenfassend kann gesagt werden, daß Chloroplasten die Fähigkeit haben, einen ATP — P_a-

Austausch zu katalysieren, welcher entweder durch Licht, durch einen Säure-Base-Wechsel oder durch ein starkes Reduktionsmittel induziert wird. In allen Fällen könnte angenommen werden, daß die Bildung eines primären, energiereichen Zustandes oder Zwischenproduktes in Chloroplasten die Voraussetzung für den Beginn der Austauschreaktion darstellt. Die Ähnlichkeit im Kofaktorenbedürfnis und in der Wirkung von Ammoniumionen als Entkoppler lassen vermuten, daß zwischen der durch Licht, durch ein Reduktionsmittel bzw. durch einen p_H-Wechsel induzierten Austauschreaktion und der Photophosphorylierung bzw. der chemiosmotischen Phosphorylierung enge Zusammenhänge bestehen.

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¹⁵ A. T. JAGENDORF u. E. URIBE, in: Energy conversion by the photosynthetic apparatus, Brookhaven Symposium 19, 1967, p. 215.

Copper Replacement of Magnesium in the Chlorophylls and Bacteriochlorophyll

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Copper chelates of chlorophylls "a" and "b" and an oxidized form of bacteriochlorophyll "a" were prepared and separated by an improved method of column and thin-layer chromatography, and their physical properties and thermodynamics involved in the primary metal replacement reaction were studied. In glacial acetic acid the Mg(II) ions of the photosynthetic pigments were replaced rapidly by Cu(II) ions at 40—100° and profound physical changes were noted in the chelation products. Copper chelates were not fluorescent while their parent pigments and pheophytins were. A general lowering of absorbance and a blue shift of absorption maxima were observed with the copper complexes. The molar absorptivity values of copper chelates were determined by the metallic microtitration method and the direct analysis of chelated copper by the oxalyldihydrazide (ODH) of copper method. In the present assay, the primary reaction of copper replacement of Mg(II) in the 3 photosynthetic pigments was the bimolecular S_E2 type. The primary reaction lasted only a short time (1—5 min) at temperatures of 40—90°, and the higher the temperature, the larger the constants of the bimolecular reaction became. On longer treatment, the metal replacement reaction was complicated by the increasing content of pheophytin. The reaction rate constants became progressively smaller in the order of chlorophyll "a" — "b" — bacteriochlorophyll "a". At 70° the half lives of 20 μM chlorophylls "a" and "b" and bacteriochlorophyll "a" for the copper replacement were 1.2, 15.2, and 117.4 minutes, respectively. Based on transition state theory, some thermodynamic constants relevant to this primary metal substitution reaction at various temperatures were calculated, and the possible mechanism involved were discussed.

Copper chelate of chlorophyll "a" (Cu-CHL "a")* and "b" (Cu-CHL "b") was recently prepared from purified pheophytins (ΦΦs), and physicochemical studies of these derivatives have already been re-

ported¹⁻⁴. However, detailed methods for preparing the purifying these complexes are lacking, as are comparative physical data, such as absorptivity and thermodynamics involved in the replacement reaction

* Abbreviations will be used in the text; CHL, chlorophyll; BCHL, bacteriochlorophyll "a"; Cu-CHL, copper-chelated chlorophyll; Cu-BCHL, copper-chelate of an oxidized form of BCHL; ΦΦ(s), pheophytin(s).

¹ A. N. SIDOROV and A. N. TEREININ, Opt. Spectr. (USSR) (English Transl.) **8**, 254 [1960].

² A. G. TWEET, G. L. GAINES, JR., and W. D. BELLAMY, Nature [London] **202**, 696 [1964].

³ I. L. KUKHKEVICH and A. I. BULYAK, Utr. Khim. Zh. **31**, 943 [1965]; C. A. **64**, 2326 d.

⁴ B. D. BEREZIN and N. I. SOSNIKOVA, Zh. Fiz. Khim. **39**, 1348 [1965]; C. A. **63**, 8651 c.

of these pigments. While most copper replacement studies have been made with the relatively stable porphyrins⁵⁻⁷, little work has been done with intact photosynthetic pigments, especially with bacteriochlorophyll (BCHL).

It is well known that the Mg(II) ion of a CHL molecule is easily replaced by two hydrogen ions in dilute acid, yielding a $\Phi\Phi$, while the Cu(II) ion is readily chelated in glacial acetic acid¹. It will then be interesting to study the kinetics of copper replacement of Mg(II) of the photosynthetic pigments in glacial acetic acid to learn (1) whether the metal replacement takes place by a direct bimolecular reaction, $\text{PMg} + \text{Cu}^{2+} \rightleftharpoons \text{PCu} + \text{Mg}^{2+}$, following a S_{E}^2 mechanism, or by the two step reaction,

following a S_{E}^1 mechanism (here, PMg and PCu indicate, respectively, the photosynthetic pigments chelated with Mg(II) and Cu(II) and $\text{PH}_2 = \Phi\Phi$) and (2) whether different photosynthetic pigments react differently in terms of thermodynamics.

This paper presents (1) detailed methods of preparing and separating Cu-CHL "a", Cu-CHL "b" and the copper chelate of an oxidized form of BCHL (Cu-BCHL), (2) chromatography and absorbance of the copper complexes in relation to the parent pigments in various solvent systems, (3) some thermodynamic quantities involved in the transition state of the metal replacement, and (4) the possible mechanism of the primary reaction of the metal replacement with 3 common photosynthetic pigments.

Materials and Methods

Extraction and chromatography of photosynthetic pigments

Chlorophylls "a" and "b" were extracted with acetone from frozen spinach leaves ground in a blender. The photosynthetic bacteria, *Rhodospirillum rubrum* (ATCC No. 277), were cultured in tripticase soy broth and harvested, and BCHL was extracted as previously described⁸. The acetone solutions of crude plant or bacterial extracts were reduced to a small volume for fractional separation by column or thin-layer chromatography (TLC).

A dry-packed column (2.5 × 20 cm) was used for the column chromatography. About 100 g of Kieselguhr G (Brinkman Instr. Inc., N.Y.) was well suspended in 100 ml of petroleum ether (30–60°, bp) containing 5 ml of triolein. It was then filtered, dried in the air, and powdered in a ball mill. Gentle suction was applied after a small amount (2–3 ml) of sample was placed on the column. A mixed solvent system containing methanol, acetone, and water in a 6:2:1 ratio (v/v) was then passed into the column. Exactly the same system was used in preparing the 7 × 7 inch thin-layer plates for the preparation of smaller amounts of photosynthetic pigments.

For the preparation and further analyses of the pigments and derivatives, thin-layer plates of diatomaceous earth, either powdered Gas Chrom P or Adsorbosil mixed with 25% of CaSO_4 (both from Applied Sci. Lab. Inc., Pa.), were used with various solvent systems (Table 1).

Fluorescence and absorbance analysis

For fluorometric analysis, a Baird Atomic Fluorometer of expanded wavelength, equipped with a red-sensitive tube (RCA 7102), was used in addition to the regular detector (IP 28). The wavelengths of excitation and emission were calibrated using a Neon Pen-ray lamp supplied by Baird Atomic. All the solvents used for the analysis were fluorometric grade purchased from the Harleco Sci. Products, Calif.

Absorption spectra of the photosynthetic pigments, $\Phi\Phi$ s, and copper chelates were obtained with either a Cary 14 or a HITACHI 139 spectrophotometer. The wavelengths as well as absorbance of the two instruments agreed closely.

Copper chelates — preparation, isolation, and quantitation

To examine the effect of heat upon degradation, intact photosynthetic pigments were heated at 100° in glacial acetic acid. After 15 minutes at 100° all 3 pigments were converted to $\Phi\Phi$ s without noticeable degradation as determined by TLC.

Copper chelation of these pigments was easily achieved by a 10–15 minute heating (100°) of the mixture of a pigment and copper acetate in glacial acetic acid with an approximate molar ratio of 1:5. Single species of copper chelates with a trace amount of unreacted $\Phi\Phi$ appeared on a thin-layer plate. For routine analyses, 2–3 mg of purified pigments were used for the chelation. Heated samples were vacuum-dried, taken up in a small amount of cold ether and passed through a dry-packed Adsorbosil column to remove the copper residues. The filtrate was reduced in volume and the copper complex was further purified

⁵ W. S. CAUGHEY and A. H. CORWIN, J. Amer. chem. Soc. **77**, 1509 [1955].

⁶ J. E. FALK and R. S. NYHOLM, in: A. ALBERT, G. M. BADGER, and C. W. SHOPPEE (Eds.), Current Trends in Heterocyclic Chemistry, Butterworths, London 1958, p. 130.

⁷ J. W. BARNES and G. D. DOROUGH, J. Amer. chem. Soc. **72**, 4045 [1950].

⁸ W. S. KIM, Biochim. biophysica Acta [Amsterdam] **112**, 392 [1966].

TLC system	CHL-a	$\Phi\Phi$ -a	Cu-CHL „a“	Migration relative to $\Phi\Phi$ -a	$\Phi\Phi$ -b	Cu-CHL „b“	BCHL	$\Phi\Phi$ -BCHL	Cu-BCHL
1	4.00	1.00	1.50	6.10	1.10	2.00	6.70	1.90	2.30
2	0.71	1.00	0.37	0.22	0.57	0.46	0	0.40	0.28
3	1.60	1.00	1.55	1.62	1.33	1.51	1.58	1.45	1.55

Table 1. Three TLC systems applied to the separation of the photosynthetic pigments, pheophytins, and copper pheophytins. *System 1*: Plate was coated with Kieselguhr G (0.5 ml thickness) impregnated with 5% (v/v) triolein and the TCL was run with a mixed solvent of methanol, acetone, and water in a ratio of 6 : 2 : 1 (v/v); *System 2*: Plate was coated with Adsorbosil (0.5 mm thickness) mixed with 25% (w/v) of CaSO_4 and TCL run with a mixed solvent of petroleum ether (30–60° bp), ethyl ether, and glacial acetic acid in a 100 : 50 : 1 (v/v) ratio; *System 3*: Plate was covered with powdered 0.5 ml thick diatomaceous earth (Gas Chrom P) and TCL run with the same solvent as System 1.

by preparative TLC on various plates (Table 1). The bluish-green copper chelate band was eluted with ether or other solvents for further analysis. Two methods for quantitation of the copper chelates were applied; the direct analysis of the copper content by oxalyldihydrazide (ODH) and the indirect metallic microtitration of pheophytins, a method originally described by OLIVER and RAWLINSON⁹ and modified recently by KIM¹⁰.

Kinetics and thermodynamics of the metallic substitution reaction

To measure the reaction rate, tubes containing equimolar (20 μM) pigment and copper acetate and their acetate blanks were placed in a constant temperature bath for a definite time. A graduated 15 ml pyrex tube containing 20 nmole of copper acetate in 1.0 ml of glacial acetic acid and a tube containing only acetic acid were placed in the bath with their tops open. After 2–3 minutes of equilibration, 20 nmole of pigments dissolved in 10 μl acetone was added to each of the tubes. A timer was started simultaneously with the addition of the pigment, and after a definite period of heating (Fig. 6), tubes were transferred quickly to an ice bath. The volume of the tubes was brought to 3.0 ml at 25° by adding glacial acetic acid, and the difference spectra were run. The increase of absorbance of the newly developing visible band of complex (650 $\text{m}\mu$ for Cu-CHL „a“, 630 $\text{m}\mu$ for Cu-CHL „b“, and 663 $\text{m}\mu$ for Cu-BCHL) was traced and the reaction rates and order of the reaction were determined from data obtained at various times and temperatures. Thermodynamic constants relevant to the transition state of the metallic substitution were calculated using the ARRHENIUS equation¹¹, EYRING's equation¹², and the second law of thermodynamics^{11, 13}.

⁹ I. T. OLIVER and W. A. RAWLINSON, *Biochem. J.* **49**, 157 [1951].

¹⁰ W. S. KIM, *Analytic Chem.* Unpublished data.

¹¹ F. DANIELS and R. A. ALBERTY, *Physical Chemistry*, John Wiley, New York 1962, p. 271, 294.

¹² H. EYRING, *J. chem. Physics* **3**, 107 [1935].

¹³ A. A. FROST and R. G. PEARSON, *Kinetics and Mechanism*, John Wiley, New York 1962, p. 77, 123.

Results

Extraction and separation of pigments by chromatography

Detailed procedures for extracting and separating the photosynthetic pigments from spinach and bacteria are shown in Fig. 1, and the separation

Fig. 1. Scheme of extraction and separation of the photosynthetic pigments and carotenoids from spinach and bacterial cells of *Rhodospirillum rubrum*. C, carotenoid; $\Phi\Phi$, pheophytin; BCHL, bacteriochlorophyll; a, chlorophyll „a“; b, chlorophyll „b“; x, xanthophylls; KG, Kieselguhr G; GP, Gas Chrom P; ASB, Adsorbosil; PV, Polyvinyl thin-layer materials.

patterns of parent pigments, $\Phi\Phi$ s, and copper chelates on three different TLC systems are illustrated in Table 1. On the Kieselguhr column 3 discrete bands were separated from the bacterial extract and 5 main bands from spinach. On TLC of the same system, further separation was noted; the bacterial extract was separated into 2 carotenoids (pink), a $\Phi\Phi$ (violet pink) and a thick BCHL (blue) respective to the origins, while the spinach extract was separated into $\Phi\Phi$ „a“ (gray-green), $\Phi\Phi$ „b“ (yellow), CHL

„a” (deep green), CHL „b” (light green), and 3 bands of xanthophylls (yellow). Further purification could be achieved by various TLC methods (Table 1). Single pigment bands from the TLC were eluted with cold ethyl ether.

Fluorescence and absorbance of photosynthetic pigments and their copper complexes

The spectra and maximal wavelengths of fluorescence and excitation of all the photosynthetic pigments and their $\Phi\Phi$ s in various solvents agreed closely with data published earlier^{8,14}. None of the copper derivatives of the 3 photosynthetic pigments were fluorescent in any of the 15 common organic solvents tested. In methanol only Cu-CHL „a” was soluble, while Cu-CHL „b” and Cu-BCHL were insoluble.

Figs. 2, 3 and 4 show the absorption spectra of 3 photosynthetic pigments, $\Phi\Phi$ s and the copper chelates in ether. The absorptivities of the parent pigments and $\Phi\Phi$ s were determined by the fluorometric Mg analysis⁸ and copper titration method⁹, respectively, in conjunction with their absorption spectra. Table 2 presents the maximum absorption wavelength ($A_{\lambda\max}$) and the millimolar absorptivity (ξmM) of the major absorption bands of these pigments dissolved in ether. From Figs. 2, 3 and 4 and Table 2 it should be noted that the $A_{\lambda\max}$ of visible absorption peaks of all the copper complexes were

Fig. 2. Absorption spectra of purified chlorophyll „a” and its derivatives in ether. Note the blue shift of main absorption bands and reduction of absorptivity of red band of Cu-CHL „a” relative to the parent pigment, CHL „a”.

¹⁴ C. S. FRENCH, J. H. C. SMITH, M. I. VIRGIN, and R. L. AIRTH, *Plant Physiol.* **31**, 369 [1956].

Fig. 3. Absorption spectra of chlorophyll „b” and its derivatives in ether. Note the blue shift of main absorption bands and reduction of absorptivity of the main bands of Cu-CHL „b” relative to the parent pigment, CHL „b”.

Fig. 4. Absorption spectra of bacteriochlorophyll „a” and its derivatives in ether. Note the shifts of SORER band of Cu-BCHL toward red and red band toward blue and the marked reduction of absorptivity of main bands of the copper chelate relative to the parent BCHL „a” and its pheophytin. The ligand of Cu-BCHL is an oxidized form of bacteriopheophytin.

shifted toward shorter wavelengths with respect to their Mg analogues, while the $\Phi\Phi$ peaks showed a bathochromic shift. The SORER bands of Cu-CHL „a” and „b” also shifted to shorter wavelengths while the SORER band of Cu-BCHL was shifted toward the red side. Table 3 lists the main absorption bands of copper chelates and their absorptivities in some common solvents. The solvent effects were observed by the changes in intensities of absorbance and the spectral position of absorption maxima; in glacial acetic acid the relative absorptivity values of major peaks were the smallest, and in pyridine the “red-shifts” of the absorption maxima were the greatest.

CHL-a		$\Phi\Phi$ -a		Cu-CHL „a“		CHL-b		$\Phi\Phi$ -b		Cu-CHL „b“		BCHL		$\Phi\Phi$ -BCHL		Cu-BCHL	
$A_{\lambda\max}$	ξ m M	$A_{\lambda\max}$	ξ m M	$A_{\lambda\max}$	ξ m M	$A_{\lambda\max}$	ξ m M	$A_{\lambda\max}$	ξ m M	$A_{\lambda\max}$	ξ m M	$A_{\lambda\max}$	ξ m M	$A_{\lambda\max}$	ξ m M	$A_{\lambda\max}$	ξ m M
430	147.1	409	151.5	414	108.3	453	192.6	434	244.0	430	112.5	358	75.2	357	120.8	425	40.5
662	112.9	667	73.1	648	58.5	645	68.3	653	47.5	626	35.6	770	98.7	750	73.1	666	35.5

Table 2. Main absorption wavelength maxima ($A_{\lambda\max}$) and the millimolar absorptivity (ξ m M) of 3 photosynthetic pigments, pheophytins, and copper pheophytinates in ether.

Copper chelate	Ether		Acetone		Acetic acid		Benzene		Pyridine		MeOH	
	$A_{\lambda\max}$	ξ m M**	$A_{\lambda\max}$	ξ m M	$A_{\lambda\max}$	ξ m M	$A_{\lambda\max}$	ξ m M	$A_{\lambda\max}$	ξ m M	$A_{\lambda\max}$	ξ m M
Cu-CHL „a“	414	108.3	412	108.8	412	92.5	416	104.9	422	98.7	412	96.6
	648	58.5	649	58.4	650	48.5	654	58.6	655	56.6	650	55.5
Cu-CHL „b“	430	112.6	432	117.2	437	93.2	438	108.3	450	100.8	—***	—
	626	35.6	627	36.7	627	29.8	630	36.7	635	34.1	—	—
Cu-BCHL „a“	425	40.5	428	42.0	428	31.1	432	38.0	435	39.0	—	—
	666	35.5	665	36.1	672	30.8	672	36.4	675	36.3	—	—

Table 3. Absorptivity of main absorption maxima of copper-chelated photosynthetic pigments in various solvents. * Wavelength of maximum absorption. ** Millimolar absorptivity. *** Both Cu-CHL „b“ and Cu-BCHL „a“ are insoluble.

Determination of reaction rates

Fig. 5 shows the difference spectra obtained from 3 photosynthetic pigments reacted with equimolar (20 μ M) copper acetate in glacial acetic acid at 100° for a period of 10 minutes. The spectra were

Fig. 5. Difference absorption spectra between copper complexes and photosynthetic pigment blanks in glacial acetic acid. Equimolar (20 μ M) mixture of a pigment and copper acetate in 3.0 ml of glacial acetic acid was heated for 10 min. at 100°. New outgrowth of absorption bands of SORET and red peaks is evident. Note also pheophytination of CHL „a“ and „b“ showing negative peaks at 672 and 655 $m\mu$.

drawn using the reference blank which contained only the pigment and no copper. A prominent new visible band and a SORET band were developed as

the copper chelates formed. The development of visible red absorption peaks, 650 $m\mu$ for Cu-CHL „a“, 630 $m\mu$ of Cu-CHL „b“, and 663 $m\mu$ Cu-BCHL, was traced for the kinetic analysis. It is quite evident from the figure that the difference spectra of Cu-CHLs „a“ and „b“ indicate the demetallization showing negative peaks of 672 and 655 $m\mu$, respectively. Although it is not shown in the figure the difference spectrum of Cu-BCHL developed a negative peak around 770 $m\mu$ indicating that BCHL was still predominant over its $\Phi\Phi$.

Fig. 6. Kinetics of copper chelation with 3 photosynthetic pigments. An equimolar (20 μ M) mixture of pigment and copper acetate was heated at temperatures and for times indicated; a, initial concentration of reactants; x, concentration of pigment reacted.

When replacement reaction was followed for shorter time intervals (1–5 min.) at temperatures between 40–90° instead of 100°, the mode of metal substitution reaction was bimolecular (S_E^2) and the pseudo second order rate constants were calculated from the slope of the reciprocal of unreacted pigment versus reaction time at various temperatures (Fig. 6). Marked differences in reaction rate were noted among the three pigments. The rate decreased in the order: CHL "a" > "b" > BCHL. Although it is not indicated in Fig. 6, it was found that a longer treatment of the reaction mixtures at higher temperatures complicated the reaction owing to the formation of $\Phi\Phi_s$, and the reaction no longer followed the second order.

The half lives of 20 μ M reactants at various temperatures were calculated and Fig. 7 shows the curves obtained from 3 complexing reactions. The rate constants and half lives of the equimolar reactants calculated are presented in Table 4.

Fig. 7. Half life periods of 20 μ M photosynthetic pigments reacted with equimolar copper in acetic acid at different temperatures.

Determination of thermodynamic quantities of the primary metal replacement reaction

On the basis of the transition state theory of EYRING¹¹ and using the empirical formula of ARRHENIUS¹², the activation energy, E_a , involved in the primary bimolecular reaction between the photosynthetic pigments and copper was determined. Fig. 8 is the plot between the logarithms of the pseudo second order rate constants, k_2' , and reciprocals of absolute temperature, $1/T$. From the negative slopes, E_a values were calculated and the activation enthalpy changes, $\Delta H^\ddagger$, were determined from the simplified

Constant *	Dimension	40°	50°	60°	70°	80°	90°
k_2'	M ⁻¹ sec ⁻¹	90	180	350	720	152	185
$t_{1/2}$	minute	9.3	4.6	2.4	1.2	9.1	4.5
E_a	kcal mole ⁻¹	14.0	14.6	13.9	14.9	14.8	14.8
$\Delta H^\ddagger$	kcal mole ⁻¹	3.3	6.6	13.2	24.7	12.5	22.7
n_E/n	$\times 10^4$	—6.2	—8.9	—11.5	—14.2	—6.4	—8.7
$\Delta S^\ddagger$	cal deg ⁻¹ mole ⁻¹	15.9	16.8	17.8	18.8	17.0	18.9
$\Delta F^\ddagger$	kcal mole ⁻¹	7.3	3.7	2.0	1.0	13.0	8.0
$K^\ddagger$	$\times 10^{12}$	0.07	0.02	0.005	0.001	0.07	0.02
s	M ⁻¹ sec ⁻¹ , $\times 10^{-13}$				0.30	0.07	0.01
						34.0	8.5
						190.0	100.0
						14.8	15.6
						5.7	16.6
						0.2	0.1
						16.7	16.6
						117.4	15.0
						7.1	26.9
						3.6	31.0
						231.5	55.6
						17.3	17.3
						16.7	16.6
						0.4	0.9
						2.9	0.1
						14.8	17.6
						100.0	20.0
						34.0	2.0
						0.01	0.5

Table 4. Copper replacement of Mg of 3 photosynthetic pigments and thermodynamic constants of transition state of reaction. Equimolar (20 μ M) mixture of pigments and copper acetate in 1.0 ml of glacial acetic acid was heated at various temperatures for 15–300 seconds. Data are the summary of 5 different experiments. * k_2' , Pseudo second order rate constant, $t_{1/2}$, Half life, E_a , Activation energy, $\Delta H^\ddagger$, Activation enthalpy change, n_E/n , Fraction of activated molecules, $\Delta S^\ddagger$, Activation entropy change, $\Delta F^\ddagger$, Activation free energy change, s , Frequency factor.

Fig. 8. Graphical determination of activation energy, E_a for reaction of equimolar ($20 \mu\text{M}$) mixtures of photosynthetic pigments and copper.

formula, $E_a = \Delta H^\ddagger + RT$ where R is the gas constant. The MAXWELL-BOLTZMANN distribution law¹³ was used to calculate the fraction of activated molecules, n_E/n . The values of E_a and $\Delta H^\ddagger$ were the smallest in the metal replacement of chlorophyll "a" and increased in the order: CHL "b" < BCHL. The activation entropy change, $\Delta S^\ddagger$, and the activation equilibrium constant, $K^\ddagger$, increased progressively in the order: CHL "a" < "b" < BCHL, and decreased as the temperature increased, while the reverse was true with the activation free energy change, $\Delta F^\ddagger$. The magnitude of constant "s" is often equated with the effective number of collisions that cause the bimolecular reactions¹²; in the present substitution experiments, "s" values increased progressively in the order: CHL "a" < "b" < BCHL, and decreased as temperature increased. Similar experiments were repeated 5 times and data obtained were essentially similar. Typical data obtained from an experiment are summarized in Table 4.

Discussion

An earlier report¹⁵ that Cu-BCHL was fluorescent was not repeatable. The revocation is consistent with earlier findings¹⁶ that no metallic porphyrins chelated with the first transition metals were fluorescent.

Metalloporphyrins chelated with closed-shell metal ions, such as Mg(II), Ca(II), Zn(II), Cd(II), Ba(II), etc., were fluorescent in the presence of a polar ligand, such as water¹⁷. The immediate quenching of the fluorescence of the photosynthetic pigments by copper replacement may then be due to the unpaired 3d electron of the copper ion. The photo-excited π electron may be paired off with the 3d electron reaching the ground state without fluorescing.

BELLAMY and MURTY^{**} suggested that the two species of Cu-CHL "a" isolated from their preparation might be caused by allomerization; the normal species absorbed maximally at 420 and 650 $m\mu$ in ether with an absorptivity ratio of 1.30 while the allomerized form absorbed at 409 and 644 $m\mu$ with the ratio of 1.43. In the present experiment where intact photosynthetic pigments and copper acetate were heated in glacial acetic acid at 100° for 10 to 15 minutes, only a single copper chelating band was isolated from 3 different TLC systems. In ether, the Cu-CHL "a" absorbed maximally at 414 and 648 $m\mu$ (Table 3) and the ratio of absorptivity was as high as 1.85. The discrepancy between the two sets of data cannot be explained at the present time since critical structural analysis has not been carried out. It is highly unlikely the Cu-CHL "a" prepared in this experiment was totally allomerized since the complex gave a positive reaction in phase test with 30% KOH.

The general tendency of reduction in intensity and shortening in wavelength of the major absorption bands of copper chelates relative to the Mg analogues may be related to the electronegativity of copper being higher (1.9) than that of Mg (1.2) as suggested by GOUTERMAN¹⁸⁻²⁰. Although no proof of the reaction mechanism is available, the tetrahydro form of BCHL may become oxidized to become the G-2 form⁸ which appears to be the 2-acetyl 3,4-hydroxy chlorophyll "a" rather than the 2-acetyl chlorophyll "a" suggested earlier²¹. The G-2 component of BCHL absorbed maximally at 435 and 675 $m\mu$ in ether while the unoxidized BCHL absorbed at 358 and 770 $m\mu$. The absorption spec-

¹⁵ W. S. KIM and D. D. FELLER, *Federat. Proc.* **25**, 578 [1966].

¹⁶ J. B. ALLISON and R. S. BECKER, *J. chem. Physics* **32**, 1410 [1960].

¹⁷ R. W. LIVINGSTON, *Handbuch der Pflanzenphysiologie*, W. RUHLAND (Ed.), **1**, 1960, p. 830.

^{**} W. D. BELLAMY and N. R. MURTY, Personal communication.

¹⁸ M. J. GOUTERMAN, *J. chem. Physics* **30**, 1139 [1959].

¹⁹ M. J. GOUTERMAN, *J. chem. Physics* **33**, 1523 [1960].

²⁰ M. J. GOUTERMAN, *J. molecular Spectroscopy* **6**, 138 [1961].

²¹ H. FISCHER and A. STERN, *Die Chemie des Pyrrols*, Vol. II, 2, Akademische Verlagsgesellschaft, Leipzig 1940 b, p. 311.

tra of Cu-BCHL in ether showed the Soret at 425 and the red maximum at 666 $m\mu$ (Table 3). In view of the general shift of the absorption peaks to the blue side with both Cu-CHLs "a" and "b", it would be logical to assume that the ligand pigment of Cu-BCHL could be the G-2 component rather than the intact BCHL. Experiments are in progress to confirm this possibility.

The Mg ion of CHL "a" does not replace the isotope ^{28}Mg in aqueous acetone²². The replacement of Mg with Cu ions in photosynthetic pigments was easily achieved in acetic acid especially at higher temperatures (40–100°). While the parent pigments were easily dissociated in dilute acid, the copper chelates were not dissociated in glacial acetic acid, conc. HCl or even conc. H_2SO_4 in room temperature. Only after a prolonged reflux in the latter acid or wet oxidation with perchloric acid was the copper ion released. This stability difference in the two classes of metalloporphyrins may be due primarily to the fact that Cu-N bond is more covalent (79%) than Mg-N bond (53%) as calculated from the electronegativity values of PAULING²³.

The possibility of adding more ligands in the direction of the "z" axis of the metal ion to the square d^9 copper complexes of the photosynthetic pigments may well be ruled out from the reported fact that a repulsive force of the filled d_{z^2} orbital is exerted through the "z" axis, while the interaction of the porphyrin N atoms with the half-filled $d_{x^2-y^2}$ orbital in the planar direction becomes much stronger²⁴. This 1:1 stoichiometry of the metal-ligand binding ratio was further confirmed by the present experimental results that two sets of molar absorptivity values determined by metallic microtitration and the ODH copper analysis method were in good agreement.

The metal replacement of porphyrin molecules in solution may primarily depend upon the power of

electrostatic attraction. Cu-CHL "a" has only one electron-seeking side chain, the 2-vinyl group, while "b" has two, the 2-vinyl and 3-formyl groups. Consequently the "b" molecule would cause a greater decrease in π electron densities surrounding N atoms, thus reducing the electrostatic attraction of the copper ion²⁵. This, in turn, would influence the reduction of the reaction rate, the increase of $\Delta S^\ddagger$, E_a , $\Delta H^\ddagger$, $K^\ddagger$, and "s" values, and the decrease of $\Delta F^\ddagger$ which is not spontaneous (Table 4). The lowest rate of copper replacement with BCHL cannot be explained on the basis of an electrophilic 2-acetyl group. As postulated earlier, the resulting Cu-BCHL may in reality be the Cu-G-2 which may bear two hydroxyl groups at the 3 and 4 β -carbon positions of the porphyrin nucleus, thus giving the complex 3 electron-withdrawing groups in contrast to 1 in Cu-CHL "a" and 2 in Cu-CHL "b". However, there is no reason to believe that the electrostatic effect is the only cause of rate-limiting in the replacement reaction. Others, such as the geometry of planar space of the porphyrin molecules, steric hindrance, or the resonance energy encircling the 18-membered Simpson loop, many also affect the copper in such a way that the binding of Cu-N could be influenced in different degrees. The relatively higher values of $\Delta S^\ddagger$ obtained with the formation of Cu-BCHL appear to suggest a greater degree of structural distortion of the molecule.

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²² S. ARONOFF, *Biochim. biophysica Acta* [Amsterdam] **60**, 193 [1962].

²³ L. PAULING, *The Nature of the Chemical Bond*, Cornell Univ. Press, Ithaca 1945, N.Y.

²⁴ L. E. ORGEL, *An Introduction to Transition-Metal Chemistry; Ligand-Field-Theorem*, Methuen, London 1960.

²⁵ J. E. FALK, *Porphyrins and Metalloporphyrins*, Elsevier Publ. Co., New York 1964, p. 30.