6. The frequency dependence of the dielectric constant increment is the same for all the albumins, deviating only slightly from a theoretical Debye curve with a critical frequency of 0.85 megacycle. The dispersion of the dielectric increment of all the pseudoglobulin solutions is given by a single curve which has a small but significant deviation from a Debye curve with a critical frequency of 0.24 megacycle.

7. The relaxation times calculated from these critical frequencies are several times larger than would be calculated from the molecular weights on the basis of present theories.

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The Rate of Conversion of Chlorophyll to Pheophytin

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The conversion of chlorophyll to pheophytin, in dilute acid solutions, resulting in the replacement of magnesium with hydrogen, is a reaction which occurs at a measurable rate in acetone solutions. Information on the rate of this reaction is of interest because of possible contributions to an understanding of the complex linkage of magnesium in the phorbin nucleus. Willstätter and Stoll[1] have shown that colloidal solutions of chlorophyll in water may be decomposed by carbon dioxide, and further that chlorophyll *a* is more rapidly converted to pheophytin *a* than the *b* component to the corresponding pheophytin *b*. Under comparable conditions for measuring the uptake of carbon dioxide, they observed, in forty-eight hours, 25 per cent. decomposition of chlorophyll *b*, as against 80% for chlorophyll *a* in twenty-four hours. These results were obtained by analysis of the magnesium contents of the partially decomposed chlorophylls.

Dorries[2] and Röben and Dorries[3] have utilized the appearance in the pheophytin spectrum of a prominent band (which has a maximum absorption close to 5350 Å. in acetone solution) as a qualitative means of detecting injury to leaves exposed to various gases. By observing spectrophotometrically the increased absorption at this wave length, we have estimated the proportion of chlorophyll converted to pheophytin in solutions of various acids in 90% aqueous acetone, at given time intervals.

Our data substantiate the validity of this calculation, where oxalic acid was used, because the initial rate was maintained for several hours, and we were able to demonstrate one chlorophyll and one pheophytin component by the Tswett column technique, in spite of the difficulties attendant upon the separation of components in solutions necessarily dilute for spectroscopic analysis. No other products were detected, so that the course of this reaction was reasonably established. Measurements were also made with sulfurous, sulfuric, and hydrochloric acids. The reaction rate was too slow with acetic acid for accurate measurement. The data may be interpreted on the basis of a first order reaction with respect to equivalent concentration of acid (*i. e.*, normality). The data are less conclusive with regard to chlorophyll concentration. They favor a second order reaction for chlorophyll in oxalic acid solution, but with the other acids the initial rate is not maintained for a sufficient time for a satisfactory decision.

Experimental

Stock solutions of chlorophyll were prepared by dissolving known amounts of chlorophyll (5X grade, American Chlorophyll Company) in acetone. Only preparations which responded satisfactorily to the tests listed by Willstätter and Stoll,[4] including the Molisch phase test, were used. Aliquots were pipetted into 250-ml. volumetric flasks, diluted to *ca.* 200 ml. with acetone, the appropriate amount of water and standardized acid added, and the solutions quickly brought to volume with acetone. The solvent was 90% aqueous acetone. The acid concentrations were varied from 0.05 to 0.0001 *N*, and the chlorophyll concentrations from 13.3 to 66.4 mg. per liter. The sulfurous acid solution was freshly prepared from liquid sulfur dioxide. Solutions were stored in the dark at room temperature (20–25°), and periodic measurements were made.

A Bausch and Lomb Universal Spectrophotometer was used to estimate visually the transmissions at the position

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[1] Willstätter and Stoll, "Assimilation der Kohlensäure," Verlag von Julius Springer, Berlin, 1918.

[2] Dorries, *Ber. bot. Ges.*, **50b**, 47 (1932).

[3] Röben and Dorries, *ibid.*, **50b**, 52 (1932).

[4] Willstätter and Stoll, "Untersuchungen über Chlorophyll," Verlag von Julius Springer, Berlin, 1913, p. 144.

of maximum absorption for the pheophytin band, close to 5350 Å. Errors in measurement can be discussed only briefly. No pre-dispersing instrument was used and stray light could not be avoided completely. This causes greater errors where a steep portion of the curve is being measured than where the curve is flatter. Furthermore, with this type of spectrometer, a finite band must be isolated, for visual photometry of the two fields. Thus in the neighborhood of 5350 Å., a spectral region of 20–25 Å. must be covered. All instrumental factors were kept constant during a given run; if it became necessary to make any changes, the absorption coefficients for pure chlorophyll and pheophytin standards were redetermined, so that results were on a comparable basis. To cancel the effect of temperature on concentration, it was found desirable to examine a pure chlorophyll solution at each time interval. Measurements of the absorption coefficients for three levels of chlorophyll were made over a period of sixty hours. For chlorophyll, the average value for nine determinations was 4.31 ± 0.11, at 5350 Å. (specific absorption coefficient, logarithm to base ten). For ash-free pheophytin prepared from the chlorophyll by the method of Willstätter and Stoll[4] (p. 254), the value was 9.69. In later experiments, with narrower slits, this value was raised approximately 7%, to 10.35, while the value for chlorophyll was raised less than 2%, indicating the relative flatness of the absorption curve for chlorophyll in this region, when compared with that of pheophytin. The percentage of pheophytin formed after a given time in an unknown solution was calculated from the expression: $(\alpha_x - \alpha_c)/(\alpha_p - \alpha_c) \times 100$ where α_p, α_c are the coefficients for the pure pheophytin and chlorophyll standards, valid for the given set of conditions at the time the unknown was measured.

Data—Discussion

The course of the reaction is illustrated in Fig. 1, for sulfurous acid at nine levels ranging from 0.05 to 0.0001 *N*, for one level of chlorophyll, at 13.3 mg. per liter. The percentage of pheophytin formed is plotted as a function of time. A consideration of Fig. 1 shows that the reaction is too rapid at normalities above 0.005 *N*, and too slow below 0.0002 *N*, with respect to sulfurous acid, for satisfactory rate measurements.

It is possible from the data in this form to obtain an independent though crude check on the course of the reaction as affected by acid concentration, by multiplying the half time of the reaction (*i. e.*, the time obtained by interpolation when 40% of the chlorophyll has been decomposed) by the normality, which should be constant for a reaction unimolecular with respect to acid concentration. This is shown in Table I for sulfurous acid.

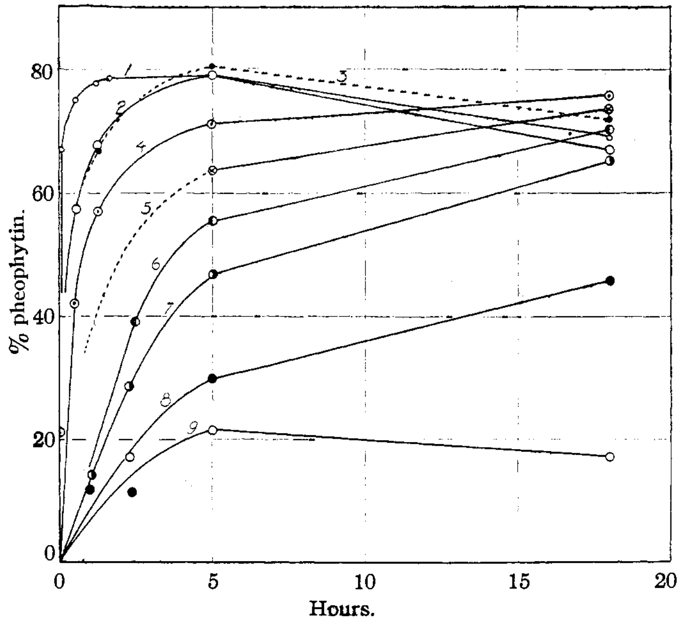

Fig. 1.—Rate of pheophytin formation at various concentrations of sulfurous acid with chlorophyll concn. 13.3 mg./liter and normality of acid: 1, 0.05; 2, 0.02; 3, 0.01; 4, 0.005; 5, 0.002; 6, 0.001; 7, 0.0005; 8, 0.0002, and 9, 0.0001.

With all the acids examined (with the exception of acetic), there is only a limited range, extending over approximately one order of magnitude, of acid concentrations at which satisfactory rate measurements are practicable. Further, there is evidence of complicating reactions after a level of 70 to 80% transformation has been attained, with no assurance that these do not occur considerably earlier for the stronger and more concentrated acids. An instrumental factor prevented the use of wide variations in the chlorophyll concentrations. Visual spectrophotometric determinations should be limited to transmissions between 15 and 35%. This means that the factor (concentration × depth of solution) cannot vary

TABLE I

HALF TIME × NORMALITY, FOR SULFUROUS ACID, AT THREE LEVELS OF CHLOROPHYLL CONCENTRATIONS

Normality	Chlorophyll, in mg. per liter 13.3	26.6	66.4
0.05	0.0020	0.0015	0.0055
.02	.0078	.0020	.0042
.01	.0037	.0023	.0049
.005	.0024	.0050	.0035
.002	.0022	.0020	.0074
.001	.0027	.0016	.0076
.0005	.0020	.0015	.0050
.0002	.0026	.0012	.0072
.0001	...	...	...
Average	.0032	.0021	.0056

greatly. The open type cup cell permitted depths of 1 ± 0.01 cm. to 6 ± 0.01 cm. Too large a percentage error is introduced if shorter depths are used, and this limited chlorophyll concentrations to a range of approximately 10 to 70 mg. per liter.

As shown in Fig. 2 the reaction is essentially similar for hydrochloric, sulfuric, oxalic and sulfurous acids. The concentrations of acid were selected to illustrate approximately equal rates.

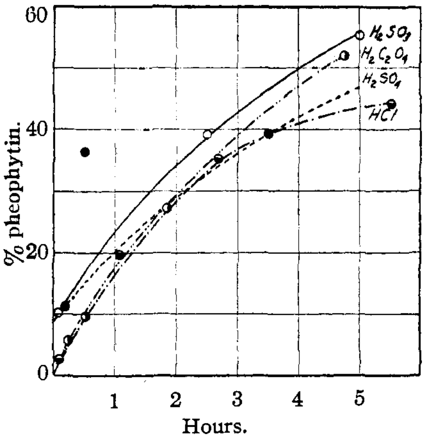

Fig. 2.—Similarity in rate of pheophytin formation in various acids: 0.001 N H_2SO_3 at 13.3 mg. chlorophyll per liter; 0.002 N $H_2C_2O_4$ at 26.6 mg. chlorophyll per liter; 0.0002 N H_2SO_4 at 26.6 mg./liter; and 0.000125 N HCl at 48.1 mg. chlorophyll per liter.

The data presented in Table II indicate that for oxalic acid solutions the reaction is definitely bimolecular with respect to chlorophyll concentration. For the other acids the rate constants decrease too rapidly with time to show any definite preference, although the second order rate constant, k_2, is more constant than the corresponding first order k_1, particularly when plotted against the acid concentration. The decrease in value of k_2 with time is smaller with the more dilute acid solutions but only with oxalic is a constant rate maintained for over seven hours. The three chlorophyll levels (a, b, c, respectively) are as 1:2:5, with k_2 values therefore of $1:{}^1/_2:{}^1/_5$. For comparison of our large number of observations with sulfurous acid, we have therefore plotted k_a, $2k_b$, $5k_c$ as a function of time. The points so plotted fall on a smooth curve for the more concentrated acid solutions, or on a straight line for the more dilute. This is shown in Fig. 3, for three concentrations of acid, and three of chlorophyll. From these plots, we extrapolated to obtain an initial k_2 (k_0) at zero time. The subscript for k for the other acids indicates the comparison time used, in hours. These are listed in Table III where, in column 3, are given the values for k/N.

Table II[a]

Rate Constants for First and Second Order Reactions with Respect to Chlorophyll for Various Acids

Time, hours	Pheophytin, %	K_1	$K_2 \times 100$[b]
	0.002 N Oxalic		
0.233	5.7	0.250	0.965
.517	9.6	.202	.772
1.85	27.3	.172	.762
4.75	52.0	.154	.892
7.0	56.4	.118	.694
22.0	81.2	.076	.737
	0.002 N Sulfurous		
0.25	55.4	4.03	0.186
.5	61.4	1.90	.119
1.85	67.5	0.60	.041
4.75	80.6	.344	.033
7.0	77.0	...	...
22.0	81.2	...	...
	0.002 N Sulfuric		
0.233	67.3	4.80	0.332
.5	73.0	2.61	.202
1.85	81.2	0.902	.088
4.75	90.5	.494	.077
7.0	83.0	...	...
22.0	85.6	...	...
	0.000125 N Hydrochloric		
0.083	2.8	0.321	0.696
.5	9.1	.189	.324
1.07	19.6	.207	.470
2.67	35.2	.163	.424
5.5	44.2	.106	.300
7.0	49.1	.097	.286
23.0	60.4	.040	.138

[a] Chlorophyll concn. at 26.6 mg./liter for all except hydrochloric acid; for latter 48.1 mg./liter. [b] Average for twenty-two hours at 26.6 mg./liter chlorophyll, 0.804 × 10^{-2}. Average for twenty-two hours, at 53.2 mg./liter chlorophyll, 0.381 × 10^{-2}. As k_2 has units of $1/c$, the rate constant should be halved.

Thus on the assumption that the reaction is of second order with respect to chlorophyll, it is apparently of first order with respect to equivalent acid concentration. Even on the assumption of first order reaction with respect to chlorophyll the initial values of k_1, obtained by extrapolation of the plot of k_1 against time, vary inversely with normality thus indicating again a first order with respect to acid concentration. The following data for sulfurous acid solution at all three levels of chlorophyll concentration are typical.

Normality of acid	k_1	k_1/N
0.005	0.715	143
.002	.338	169
.001	.127	127
.0005	.101	202

TABLE III

RATE CONSTANTS FOR PHEOPHYTIN FORMATION AT VARIOUS ACID CONCENTRATIONS

Normality of acid, N	k_0	k/N
Sulfurous acid: chlorophyll levels, 13.3, 26.6, 66.4 mg. per liter, reduced to 13.3 level		
0.05	3.2	64
.02	1.2	60
.01	0.3	30
.005	.13	26
.002	.046	23
.001	.021	21
.0005	.010	20
.0002	.007	35
.0001	.005	50
Sulfuric acid: chlorophyll levels, 13.3, 26.6, 66.4 mg. per liter, reduced to 13.3 level		
	$k_{0.167}$	
0.005	2.32	464
.0005	0.23	460
.0002	.031	155
.0001	.029	290
Oxalic acid: chlorophyll level, 48.1 mg. per liter		
	k[a]	
0.005	0.0059	1.18
.002	.0041	2.05
.001	.00081	0.81
(From Table I) Chlorophyll levels, 26.6, 53.2 mg., reduced to 48.1 level		
0.002	0.0045[b]	2.25
.002	.0042	2.10
Hydrochloric acid: chlorophyll level, 48.1 mg. per liter		
	$k_{0.083}$	
0.005	0.448	89.6
.002	.288	144
.001	.272	272
.0005	.061	122
.00025	.013	52
.000125	.007	56

[a] Five hour average, five determinations. [b] Twenty-two hour average.

No color change was detected when acetic acid in concentrations up to 0.01 N was added as above, even after several days. When 5 cc. of glacial acetic and 5 cc. of water were added to 90 cc. of acetone solution of chlorophyll, only a slight change in the color of the solution was detected in twenty-four hours. The reaction proceeds more rapidly if the glacial acetic acid is added to 90% *aqueous* acetone.

The tests outlined by Willstätter and Stoll[4] (p. 144) were performed in detail on all solutions. Evidence for the formation of chlorophyllides, of free carboxyl groups, and other decomposition products was completely lacking. The Molisch phase test, applied to the controls at the beginning and end of the experiments, indicated no allomerization. Decomposition of chlorophyll by acid normally follows one of two courses, either to pheophytin or to pheophorbide, depending upon whether a weak or strong acid is used. At the dilutions used, there was no apparent pheophorbide formation. Since we found, for the stronger acids, a failure to maintain the initial rate, and a falling off in the calculated percentage of pheophytin, on standing, we assume that other reactions must be taking place, presumably of an oxidative nature, with, however, substantially slower rates. Thus, at forty-four hours, the apparent percentage of pheophytin for solutions 1–8 (Fig. 1) was still 60–65. We have, therefore, considered only the initial rates, in the solutions containing sulfurous, sulfuric or hydrochloric acid.

The rate remains constant for more than twenty-two hours in the oxalic solutions, and we have been guided by this in suggesting that the course of the reaction is initially substantially identical for the various acids examined.

Leaf chlorophyll consists of an approximately 3:1 mixture of components *a* and *b*. Our results (Fig. 1) indicate that a steady state is attained after a 70–80% conversion of chlorophyll; in view of the work of Willstätter and Stoll[1] cited at the beginning, we examined the possibility that our reaction is preponderantly that of chlorophyll *a* and its conversion to pheophytin *a*. The photographs of pheophytin spectra[4] indicate that pheophytin *b* contributes to the 5350 Å. absorption band, and we have substantiated this by converting the chlorophyll components, separated on inulin,[5] to their respective pheophytins, and examining their absorption spectra in this region. Both components contribute substantially to the absorption at this wave length.

Estimation of the extent to which a given component in the mixture has been converted to its respective pheophytin is therefore of peculiar difficulty, the more so because the solu-

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(5) G. Mackinney, *Plant Physiol.*, **13**, 123 (1938)

tions are necessarily dilute for spectroscopic analysis. However, the reaction was satisfactorily followed with oxalic and the most dilute hydrochloric acid solutions. After forty-eight hours, the pigments, in 90% acetone, were transferred to hexane, and adsorbed on inulin in a Tswett column. Development of the chromatogram gave only two zones, the lower, a dark brown, containing the pheophytin, and an upper green zone. There was no evidence of a blue zone. Experience with this method applied to a study of leaf extracts[5] indicates that this may be interpreted as showing that chlorophyll *b* (green) is still present when equilibrium has been reached, and that all the chlorophyll *a* (blue) has been converted to pheophytin, which may be eluted readily from the adsorbent.

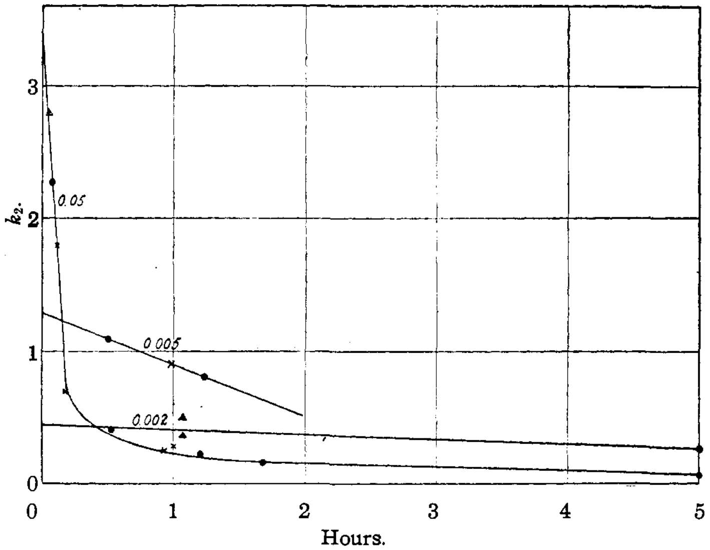

Fig. 3.—Rate of decrease of second order rate constant for pheophytin formation in sulfurous acid solutions. The ordinates should be divided by 10 for the 0.005 and 0.002 *N* levels: ●, k_2 at 13.3 mg. chlorophyll per liter; △, 2 × k_2 at 26.6 mg. per liter; ×, 5 × k_2 at 66.4 mg./liter.

With the acid concentrations used in this work, it seems probable that the conversion of chlorophyll *a* to pheophytin *a* has been the main reaction measured. While further work obviously involves a study of the pure components, it is doubtful whether the presence of chlorophyll *b* obscures the results nearly so much as the complicating reactions which, as we have shown, cause a marked decrease in the reaction rate with time.

As pointed out in the review by J. H. C. Smith[6] the magnesium cannot be given a definite berth in the molecule. The number of "active" hydrogens observed in the magnesium-free derivatives is always one less than in the magnesium-containing compounds. Smith comments that if the magnesium had replaced the two imide hydrogens, a difference of two active hydrogens would be expected, and that as yet this is unexplained.

If the reaction is unimolecular with respect to acid, the two hydrogens replacing the magnesium must enter at different rates, and we suggest a chain mechanism such that the final rate law is $V = k(\text{chlorophyll})^n(\text{H}^+)$, where n is probably 2, rather than a reaction involving a collision between two molecules of chlorophyll with one hydrogen ion.

Summary and Conclusions

The conversion of chlorophyll to pheophytin by acids in 90% aqueous acetone solutions was found to occur at a measurable rate at concentrations of 0.01 to 0.0002 *N*, with about 70% conversion in twenty-four hours. Upon longer storage a decrease in the apparent pheophytin content occurred, probably owing to other reactions. Both the first and second order rate constants decreased with time, the decrease being more pronounced at the higher concentrations of acid, particularly after the reaction had proceeded to about 40% completion, except for dilute solutions of oxalic acid, where the second order rate constant remained essentially stationary. The reaction is apparently of second order with respect to concentration of chlorophyll in oxalic acid solutions, probably also in the other acids. It is definitely of first order with respect to the equivalent concentration of all acids tested.

It is suggested that the incomplete conversion into pheophytin may be ascribed to the different rates at which chlorophylls *a* and *b* react with acid. Under the conditions studied, a complete conversion of *a* is obtained, with but little conversion of *b*. It is also suggested that the dependence of the reaction upon the first power of acid concentration is additional evidence that the two magnesium bonds replaceable by hydrogen are not equivalent.

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(6) J. H. C. Smith, *Ann. Rev. Biochem.*, **6**, 500 *et seq.* (1936).