here as an x' band without independent proof such as might be obtained by extremely difficult polarization experiments.[18b] Therefore, these two bands are identified in Table II in general terms as merely λ_2 and λ_3.

When both cationic and anionic species in an ionizable compound are interfacial modifying,[7] lower[20] C.M.C.'s, as found here, are to be expected. Gegenion concentrations in the pH 1.00 and 6.50 buffer solutions are not far different, and thus the C.M.C.'s in these two buffers should be nearly comparable[11] as seems to be the case here.

(20) Scott, Tartar and Lingafelter, THIS JOURNAL, **65**, 698 (1943).

TABLE II

INFLUENCE OF LONG CHAIN QUATERNARY AMMONIUM SALTS ON COLOR AND ABSORPTION MAXIMA OF B.P.B.

Species	Band	Aqueous maxima, mμ	Micellar maxima, mμ
HD$^-$	λ_x	435 (yellow)^a	425 (yellow-green)
HD$^-$	λ_y	280	285
D$^=$	λ_x	590^a & 592^b (purple)	605 (blue)
D$^=$	λ_2	380	380
D$^=$	λ_3	310	325

^{a} Results here check with value reported by Haring and Heller, reference 15. ^{b} Value reported previously by Brode, reference 14.

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A Spectrophotometric Study of the Molisch Phase Test of Chlorophyll*

BY BOLESLAW DUNICZ, THOMASINE THOMAS, MARCEL VAN PEE AND ROBERT LIVINGSTON

The visible absorption spectra of the intermediate compounds, responsible for the "brown" phase of the Molisch phase test of chlorophylls a and b, have been determined. The compound derived from chlorophyll a has a broad region of absorption starting at about 5500 Å. and extending at least to 3800 Å. In addition there is a minor maximum centered at 6750 Å. The compound corresponding to chlorophyll b has its major maximum at 5300 Å. This maximum has a distinct shoulder at about 5000 Å. There appears to be a region of general absorption starting about 4600 and extending at least to 3800 Å. The formation of the intermediate occurs within a second or less after the chlorophyll and base are mixed. It is postulated that the formation of the brown color is due to the neutralization of the proton on carbon 10 of ring V of the chlorophyll molecule.

It has been known for many years that an ethereal solution of freshly prepared chlorophyll exhibits a transitory brownish color when it is treated with a strong base (such as concentrated alcoholic potash). Conant and his co-workers[1] have demonstrated that the green product of this reaction is a chlorin formed by the oxidative hydrolysis of ring V. Measurements of the absorption spectrum of the intermediate were made in the hope that this might help in the identification of the reaction intermediate.

Fig. 1.—Absorption cell.

Experimental Materials and Methods

Materials.—The chlorophylls a and b were prepared and purified by a modification of the method of Zscheile and Comar.[2a,b] The samples used were free from spectrophotometrically detectable quantities of pheophytin[3] and allomerized chlorophyll.[4] The solvents were purified anhydrous samples. The trimethylbenzyl ammonium hydroxide was prepared by prolonged drying of a commercial sample (Triton B) over phosphorus pentoxide in a vacuum desiccator.

(1) J. B. Conant, S. E. Kamerling and C. C. Steele, THIS JOURNAL, **53**, 1615 (1931); J. B. Conant, J. F. Hyde, W. W. Moyer and E. M. Dietz, ibid., **53**, 359 (1931); Steele, ibid., **53**, 1371 (1931).

(2) (a) R. Livingston, D. Sickle and A. Uchiyama, J. Phys. Colloid Chem., **51**, 775 (1947); (b) F. Zscheile and C. Comar, Botan. Gaz., **102**, 463 (1941).

(3) C. Comar and F. Zscheile, Plant. Physiol., **17**, 198 (1942).

(4) Unpublished work, W. F. Watson.

Apparatus.—The absorption measurements were made with a Beckmann spectrophotometer. An ethereal chlorophyll solution was mixed continuously with a relatively concentrated solution of a base, and the resulting solution flowed through the absorption cell (see Fig. 1). The cell proper (a) was made from 10 mm. precision square Pyrex tubing. The flow was controlled by applying gas pressure to the reservoirs containing the stock solutions. It was limited by the capillaries (b,b'). The small bulbs (c,c') were introduced to ensure complete mixing of the solutions before they reached the cell (a). The efficiency of mixing was checked by direct observation using a colored and a colorless solution under the conditions of the experiments. Connections between the solution reservoirs and the inlet tubes (d,d') were of glass, with the exception of short lengths of flexible tygon tubing. The flow rate and the composition of the mixture were determined directly during each measurement.

The stock solutions were prepared on a vacuum line. Known quantities of chlorophyll in ether and of base in methanol were added to separate reservoirs. The solutions were frozen and the reservoirs evacuated. The solvents were degassed by boiling at low temperature, and the appropriate volumes were distilled under vacuum into the reservoirs. Purified nitrogen was then admitted to the reservoirs, their stopcocks were closed, and they were transferred to the flow system. This system was flushed out with commercial "oxygen-free" nitrogen.

Experimental Results

Typical measured absorption spectra of the flowing mixtures are represented by the dotted curves of Figs. 2 and 3 for chlorophyll b and chlorophyll a, respectively.[5] The ordinates are the optical densities of solutions when the total chlorophyll or chlorophyll derivative concentration is approximately 8.3×10^{-6} m. In addition to the results for the flowing mixtures, absorption curves are plotted for the chlorophylls and for the relatively stable products. Inspection of the curves for the flowing mixtures, and of other similar data

(5) These curves have been corrected for a slight absorption in the violet due to Triton B.
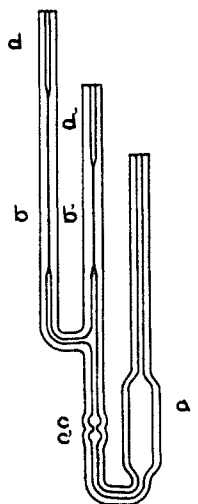
which are not reproduced in this paper, indicates that these solutions contain either the starting chlorophyll, the end product, or both as well as the unstable intermediate.

It is impossible to compute exactly the extinction curve of the intermediate compound from the known absorption spectra of chlorophyll and of the product and any number of curves for mixtures of unknown composition.[6] However, the unknown extinction curve may be fixed within limits, whose precision depends upon the form of the several extinction curves. In the present case this is more easily illustrated with the chlorophyll b system, since these mixtures contain only the final product and the intermediate compound. This is apparent from Fig. 2, since the mixture curves and the curve for the product have several crossing points in common, while the crossings with the chlorophyll curve are entirely at random. Obvious outside limits for the desired extinction curve can be obtained by assuming, first that the solution contains only the intermediate compound, and second that it contains as much of the product as is consistent with the requirement that no negative values for the extinction constant occur. In many cases the limits may be greatly narrowed if we make the very probable assumption that the maxima (or minima) of the extinction curves for the several pure compounds do not occur at the same wave lengths.[7] The solid curves of Figs. 2 and 3, which represent the extinction values for the intermediates were obtained by a method of successive approximations based upon these assumptions. The data for the chlorophyll a system are somewhat more complex, since the mixtures appear to contain all three compounds. However, the absorption curve for the intermediate can be obtained by a method which is essentially similar to that used for chlorophyll b.

The precision with which the computed extinction values for the intermediate compounds are known, varies with the wave length. For the intermediate derived from chlorophyll b, the uncertainty of the relative extinction constants between 4700 and 6200 Å. is not much greater than that corresponding to the stable compounds. The form of the curve is accurately defined in this region, and the numerical values (relative to chlorophyll) are probably known within ±10%.[8] The existence of the broad maximum at 4000 Å. is also definite. Whether there is a broad minor maximum near 6400 Å. is questionable. The region between 4200 and 4500 Å. is that of greatest uncertainty, but even here it is established that there is no pronounced maximum or minimum. In the case of the chlorophyll a derivative the curve is well determined between 4600 and 6200 Å. There is a broad region of absorption, starting at 5400 Å. and extending to the short wave length limit of the spectrum. In addition there is a definite, although minor, maximum near 6800 Å., and there

(6) E. Q. Adams and L. Rosenstein, THIS JOURNAL. **36**, 1452 (1914).

(7) A. C. Hardy and F. M. Young, *J. Opt. Soc. Am.*, **38**, 854 (1948).

(8) The strong maximum at 5300 Å. and its shoulder at approximately 4950 Å. which are shown in Fig. 2, were reported by Willstätter and Stoll, "Untersuchungen über Chlorophyll," Springer, 1913, p. 168.
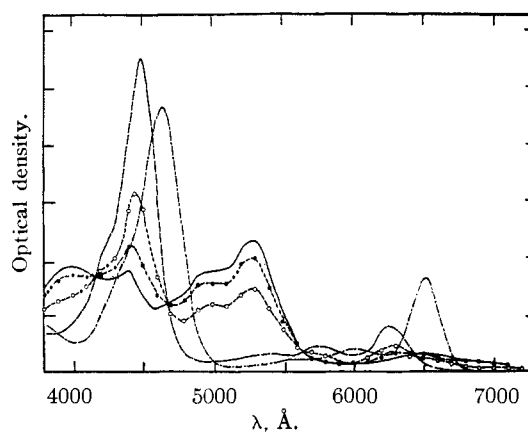

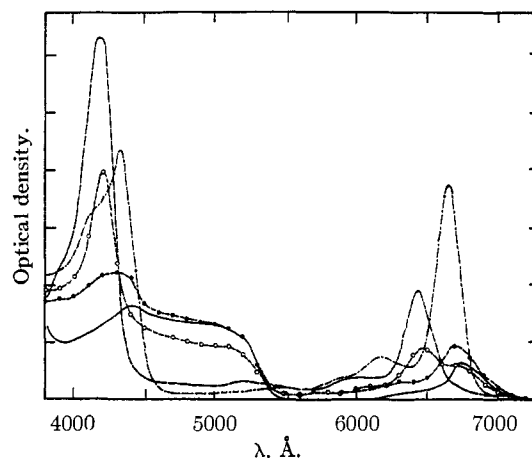Fig. 2.—Absorption curves for chlorophyll *b*.

Fig. 3.—Absorption curves for chlorophyll *a*.

is some indication that another maximum exists at a wave length somewhat shorter than 3800 Å.

The results obtained with the flow system are summarized in Table I. The percentage compositions of the mixtures were computed with the aid of the extinction constants plotted in Figs. 2 and 3.

The disappearance of chlorophyll b was too rapid to be observed in our experiments. The concentration of the product increased with time, but no simple kinetic explanation could be given to account for the rate of increase. Probably, in spite of all precautions to eliminate air, our solutions still contained some oxygen, and its depletion reduced the rate as the reaction progressed.[9]

Flow experiments, with solutions of chlorophyll b saturated with air, gave results qualitatively similar to those in Table I, but the rate of formation of the final product was distinctly faster.

Preliminary static experiments, made with evacuated solutions at approximately 0°, resulted in absorption spectra for the "brown phase" which closely resembled those obtained with the flow system. However, this method has the disadvantage that the reaction is occurring at a noticeable rate while the spectrum is being measured.

Qualitative experiments were performed in a static system, sealed to a vacuum line, from which oxygen could be more rigorously excluded. Under

(9) Compare C. Steele, THIS JOURNAL. **53**, 317 (1931).

TABLE I

SUMMARY OF EXPERIMENTS IN A FLOW SYSTEM

Expt.	[Chph] × 10⁶ moles/l.	[Base] × 10³ moles/l.	Vol. % of MeOH after mixing	t, °C.	Reciprocal of rate of flow (sec./cc.)ᵃ	Percentage composition		
						Chph	Product	Intermediate
			Chlorophyll b					
1	8.13	8.07 T.B.	4.3	27	11.3	0	11	89
2	8.13	8.07 T.B.	4.3	27	1.5	0	5	95
3	8.33	7.69 T.B.	4.1	27	10.0	0	22	78
4	8.01	7.99 T.B.	4.3	27	1.1	0	17	83
5	8.90	7.03 KOH	9.5	25	11.1	0	46	54
6	8.24	7.90 KOH	10.5	25	0.9	0	12	88
			Chlorophyll a					
7	8.93	70.0 T.B.	9.5	25	11.1	13	30	57
8	8.30	78.1 T.B.	10.5	25	0.8	23	0	77
9	8.27	7.86 KOH	4.0	25	10.0	29	44	27
10	8.04	8.17 KOH	4.1	5	1.4	52	7	41

ᵃ Proportional to the interval between mixing and measurement.

these conditions the reddish-orange color of the chlorophyll b intermediate could be detected for more than one-half hour, and the final product was light yellow instead of the usual green. When air was admitted 5 or 10 minutes after mixing, the reddish color of the intermediate was quickly replaced by the green of the ordinary product. Once formed, the pale-yellow anaerobic product did not turn green even on prolonged exposure to air.

Chlorophyll a disappears at least one-tenth as rapidly as chlorophyll b, under the present experimental conditions. In contrast, the rates of appearance of the green products appear to be comparable, at least in the initial stages.

Discussion

The present results support, in general, the conclusions which have been based upon chemical evidence. Since the formation of the intermediate compound does not require oxygen, it must consist of an interaction of chlorophyll with the base. In the presence of oxygen, the intermediate is oxidized to green chlorins (1). In the absence of oxygen, a slower irreversible reaction takes place, leading to the formation of a straw colored product.

Of the several hypotheses advanced to explain the formation[10] of the intermediate phase, the two following appear the most probable. Either, ring V could be split hydrolytically, or the keto group

(10) E. Rabinowitch, "Photosynthesis," Interscience Publishers, Inc., New York, N. Y., 1945, pp. 456-466.

on carbon 9 of ring V could be enolized and ionized. A third possibility is direct acid ionization of the CH⁻ group in position 10. Since the half life of chlorophyll in strongly basic solution is less than 1 second (much less in the case of chlorophyll b), the first hypothesis is practically excluded. This leaves the second or the third mechanism. Both would introduce a negative charge into a conjugated chain directly attached to the main resonating ring. This charge can migrate into the main ring, and thus would explain the observed major change in the absorption spectrum. In the generally accepted formula for allomerized chlorophyll, the hydrogen on carbon 10 is replaced by an hydroxyl, methoxyl, etc. In this case, too, the hydrogen ion from the hydroxyl should certainly be[11] neutralized in basic solution. However, the resultant charge on the oxygen atom would be isolated from the main conjugated system by two single bonds, and would, therefore, not be expected to have any pronounced effect upon the absorption spectrum, thus explaining the failure of allomerized chlorophyll to give the phase test.

(11) The argument advanced against this structure by Livingston and Watson (THIS JOURNAL, 71, 1452 (1949)) has been shown by the recent work of Krasnovskii and Evstigneev, Doklady Akad. Nauk., 70, 261 (1950) to be very probably incorrect.