251.   *The Allomerization of Chlorophyll.*

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The autoxidation of chlorophyll in methanol ("allomerization") and in other solvents is influenced by the solvent, the concentration of the chlorophyll, and dissolved salts.   The absorption spectra of the products are reported and the mechanism of allomerization is briefly discussed.

"ALLOMERIZATION" is the term applied to the autoxidation of chlorophyll in alcoholic solution which precludes it from showing a positive Molisch phase test, the transient brown coloration on addition of alcoholic potassium hydroxide. Allomerization is associated with an uptake of oxygen equimolar with the chlorophyll present.[1]   Conditions for promotion of the reaction have not previously been systematically investigated, except for evidence that the alcoholic solvent plays a part.[2]   The reaction has been envisaged as the formation of an unstable hydroperoxide on $C_{(10)}$ (of Fischer's formula), and its conversion into an alkyl ether by the solvent.[2]

EXPERIMENTAL

Chlorophyll-*a* and -*b* were prepared from fresh spinach leaves by Zscheile and Comar's method[3] except that 0·5% propan-2-ol in light petroleum was used for chromatographic

[1] Conant, Bailey, and Kammerling, *J. Amer. Chem. Soc.*, 1931, **53**, 2838.
[2] Fischer and Strell, "FIAT Review of German Science.   Biochemistry, Part 1," 1947, p. 141.
[3] Zscheile and Comar, *Bot. Gaz.*, 1941, **102**, 463.

separation.[4] The pigments were re-chromatographed to attain the accepted spectral standards of purity [5] and were stored at 0° in purified ether.

Methanol was dried by refluxing it with sodium and dimethyl phthalate and distilling it.[6] Drying by magnesium turnings activated by iodine, and distillation without a fractionating column, yielded a solvent in which chlorophyll rapidly allomerized; the acceleration was apparently caused by traces of magnesium methoxide. A stock of such a solvent provided a convenient method for preparing the allomerized product.

Anhydrous halides were of the purest grades commercially available and did not require drying since up to 1% of water had no detectable influence on the reaction. A decline in allomerizing activity occurred on storage of stock solutions of halides in methanol (Table 1), perhaps by conversion of halide into less reactive methoxide. The effect of this complication was minimized by preparation of stock salt solutions just before use.

TABLE 1. *Accelerating activity of fresh and 24 hr.-old solutions of halides in* $6 \cdot 6 \times 10^{-6}$M-*solutions of chlorophyll-a in methanol.*

Salt	Concn. (10^{-5} M)	Allomerization (%) after 3 hr. for :		Salt	Concn. (10^{-5}M)	Allomerization (%) after 3 hr. for :	
		fresh solns.	24 hr.-old solns.			fresh solns.	24 hr.-old solns.
LaCl$_3$ ...	0·61	92	76	MgCl$_2$ ...	11·5	67	43
	0·57	40	24		7·4	43	23
	0·41	12	9		4·9	30	14

Ethoxides and methoxides were prepared by direct reaction of metal and alcohol, followed by vacuum-distillation and recrystallization.

The fluorimeter and fluorescence cells have been described previously.[7] A Wratten No. 88 filter effectively cut off radiation of less than 7100 Å from the photocell. Absorption spectra were measured by Beckman and Unicam photoelectric spectrophotometers.

Extents of allomerization of dilute solutions of chlorophyll-*a* were obtained from absorption measurements at 4200 and 3900 Å. Wavelength 4200 Å was chosen since a large change of absorption occurred there during allomerization. Conversely, 3900 Å is a crossing-point of the spectra of reactant and product, no significant change in absorption during allomerization being shown by the following typical data for a $5 \cdot 6 \times 10^{-6}$M-solution of chlorophyll-*a* in methanol :

Allomerization (%)	5	10	68	98
$(\log I_0/I)_{3900}$	0·337	0·340	0·345	0·340

From the two measurements at each extent of allomerization, the fraction of chlorophyll remaining, a/a_0, was obtained from :

$$a/a_0 = (s - s_X)/(s_A - s_X) \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$

where s, s_A, and s_X are the ratios of absorption at 4200 and 3900 Å observed for the mixture, for chlorophyll-*a*, and for the allomer, respectively. Since the measurement was independent of dilution and different fillings of the photometer cells, errors from these causes were eliminated. From 10% to 90% of allomerization the error in the calculation of the fraction of chlorophyll remaining was $\pm 1\%$, and outside this region it was $\pm 2\%$.

Extents of allomerization of more concentrated solutions of chlorophyll-*a* were deduced from similar spectral ratios, s (see p. 1209). With the concentrated solutions also, the absorption at 3900 Å remained almost unchanged during allomerization, as illustrated by the following measurements :

Chlorophyll-*a* concn. (10^{-4} mole/l.)	0·8	3·2	8·0	32·0
$(\log I_0/I)_{3900}$ initially	0·429	0·422	0·438	0·465
$(\log I_0/I)_{3900}$ after complete allomn.	0·420	0·428	0·458	0·460

RESULTS

Dilute Chlorophyll Solutions.—It is found that allomerization to give one product is predominant below 10^{-4}M-chlorophyll concentration. Reaction giving two more products becomes important at higher concentrations. For this reason, it is convenient to designate chlorophyll concentrations less than 10^{-4}M as the dilute solution range.

[4] Strain, *Ind. Eng. Chem. Anal.*, 1946, **18**, 605.
[5] Comar and Zscheile, *Plant Physiol.*, 1942, **17**, 198.
[6] Livingston and Pariser, *J. Amer. Chem. Soc.*, 1948, **70**, 1510.
[7] Livingston, Watson, and McArdle, *ibid.*, 1949, **71**, 1542.

FIG. 1. *Decomposition of chlorophyll-a and -b in acetone.*

—— Chlorophyll-*a*; – – – chlorophyll-*b*.

A, 1 hr. at 25°. E, 14 hr. at 25°.
B, 24 hr. at 56°. F, 64 hr. at 56°.
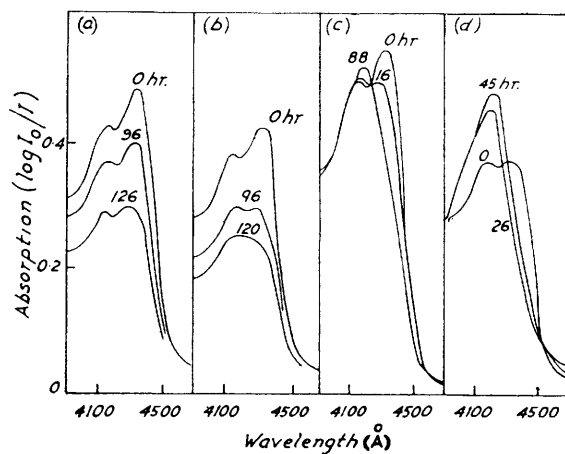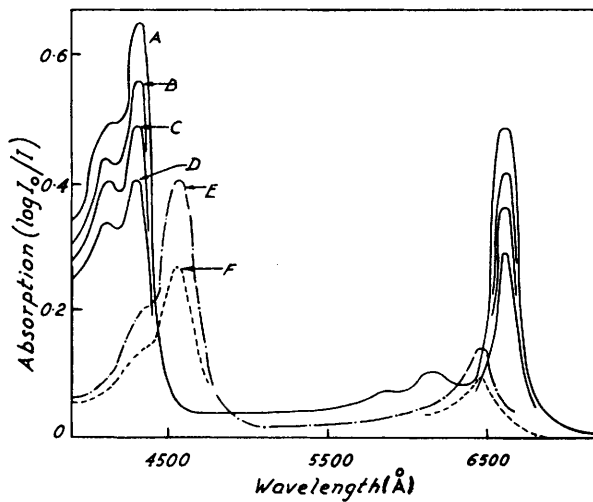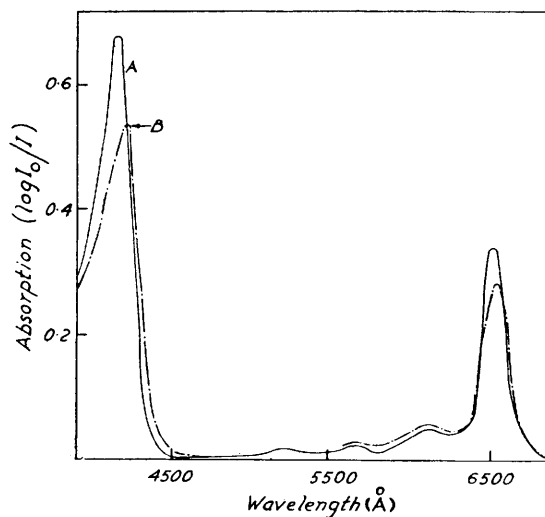C, 48 hr. at 56°.
D, 120 hr. at 56°.

FIG. 2. *Decomposition and allomerization of chlorophyll-a in (a) octanol, (b) n-butanol, (c) ethanol, and (d) methanol at 65°.*

FIG. 3. *Spectra of allomerized chlorophyll-a in (A) ether and (B) in methanol and ethanol.*

Effect of solvent. In acetone, the shape of absorption spectra of chlorophyll-*a* and -*b* remained unaltered on prolonged exposure to oxygen at room temperature and when the solvent was re-fluxed (Fig. 1). The general decrease in absorption demonstrates the absence of coloured products.

Different results were obtained with chlorophyll-*a* in alcoholic solvents. The size of the alkyl group had a marked influence on the rate of the competitive processes, (i) the general degradation noted in non-alcoholic solvents and (ii) the specific change occurring in alcohols, *viz.*, allomerization. General degradation was dominant in octyl alcohol, but decreased relatively to allomerization in the series, butyl, ethyl, and methyl alcohol (Fig. 2). The spectral changes on allomerization were, most noticeably, elevation of the minor blue maximum at the expense of the major blue maximum till the latter disappeared, and a slight decrease of the red maximum with a displacement of 80 Å to shorter wavelength. The absorption spectra in methanol at different stages of allomerization possessed six intersection points in the visible region with the spectrum curve of the isolated allomerized product, showing that general
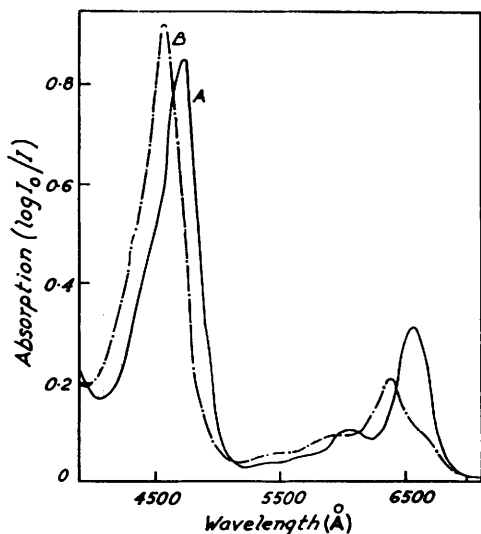
FIG. 4. *Spectra of (A) chlorophyll-b and (B) allomerized chlorophyll-b in methanol.*

degradation could be neglected and that a product of constant spectral properties was being produced.

Spectra of allomerized chlorophylls- and chlorophyllides-a and -b. After several weeks' allomerization, the pigments present in methanol and ethanol solutions of chlorophyll-*a* were separated chromatographically. The alcoholic solvents were replaced by light petroleum, and the solutions chromatographed as in the preparation of chlorophyll. One major band was obtained in addition to that for non-allomerized chlorophyll. The spectra in methanol and in ether of the main product of allomerization are recorded in Fig. 3. Two minor components also separated on the column, of interest later in considering allomerization in more concentrated solutions. Spectra of identical shape were obtained on allomerizing ethyl chlorophyllide-*a*.

More complex spectral changes occurred in alcoholic solutions of chlorophyll-*b* kept at room temperature or at 65°. Several pigments separated on a chromatographic column, none of which could be distinguished as the primary coloured product. The reaction at room temperature was therefore accelerated by a trace of magnesium chloride and also by lanthanum chloride, and the products were chromatographed within 24 hr. One major and two minor bands separated. The major band was the same whichever accelerator was used (Fig. 4). In view of the identity of the product on acceleration of chlorophyll-*a* by these salts with the product on non-accelerated allomerization. it appears justifiable to consider the spectrum of Fig. 4 as that of allomerized chlorophyll-*b*. A main product with this spectrum was also obtained after accelerated allomerization of ethyl chlorophyllide-*b*.

Acceleration by salts in methanol solutions. The rate of allomerization was greatly increased by traces of certain metal salts, to give for chlorophyll-*a* a product stable for several days in the presence of excess of salt. No allomerization occurred on mixing rigorously degassed solutions of

chlorophyll and salt in an H-tube. However, allomerization appeared relatively insensitive to oxygen concentration, since evacuation to *ca.* 1 mm. immediately after mixing retarded the rate of accelerated allomerization only to about one-third.

First-order rate dependence on chlorophyll concentration was observed for accelerated allomerization (see Fig. 5). Relative values of apparent rate constants at equimolecular salt concentrations are recorded in Table 2. The nature of the cation is clearly the most important factor, although a small effect of the anion can be observed.
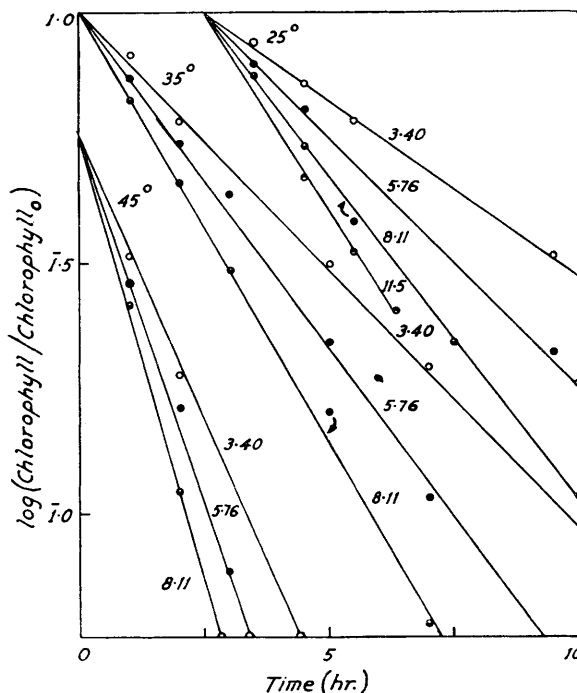
FIG. 5. *Acceleration of allomerization of chlorophyll-a by magnesium chloride.*

Chlorophyll concn., $6 \cdot 6 \times 10^{-6}$ mole/l.

The numbers on the curves refer to salt concentrations $\times 10^5$ mole/l.

(The curves have been separated by displacement of origin.)

The order of reaction with respect to accelerator concentration [equation (2) being assumed to hold] was obtained for magnesium chloride by varying salt concentration in $6 \cdot 6 \times 10^{-6}$M-solutions of chlorophyll-*a*. The slopes of Fig. 5 give k', from which a and k have values at 25°,

TABLE 2. *Effects of salts on the allomerization of chlorophyll-a in methanol.*

Concns. (mole/l.) : chlorophyll-*a*, $6 \cdot 4 \times 10^{-6}$; salt, $6 \cdot 0 \times 10^{-5}$.

Salt	Allomerization (%) after (hr.)					Relative rates	Salt	Allomerization (%) after (hr.)					Relative rates
	1	3½	6	8	27			1	3½	6	8	27	
None	—	—	1·0	1·2	6·7	1	MgBr₂	33	80·0	96·5	100	100	92
NaCl	—	1·2	2·4	—	10·6	1·6	MgI₂	37	84·7	92·2	97·0	100	98
NaBr	—	1·2	1·6	—	15·9	2·4	BaCl₂	—	2·0	4·3	7·5	32·2	4·8
NaI	—	1·2	—	—	17·6	2·6	BaBr₂	—	2·0	7·1	8·2	35·2	5·2
CsBr	—	2·0	2·3	2·7	8·2	1·2	BaI₂	—	3·9	11·8	17·6	50·2	7·5
CsI	—	1·0	1·2	2·0	11·8	1·8	LaCl₃	100	—	—	—	—	>4C0
MgCl₂	43	86·0	96·8	100	100	99							

35°, and 45° of 0·65, 0·65, and 0·65, and $3 \cdot 63 \times 10^{-2}$, $5 \cdot 08 \times 10^{-2}$, and $1 \cdot 06 \times 10^{-1}$ mole$^{-0 \cdot 65}$ l.$^{0 \cdot 65}$ sec.$^{-1}$ respectively. The calculated activation energy is 10 kcal./mole.

$$-\text{d[Chlor.]}/\text{d}t = k[\text{Accel.}]^a[\text{Chlor.}] = k'[\text{Chlor.}] \quad . \quad . \quad . \quad (2)$$

Similar experiments with lanthanum chloride gave no realistic value for order of reaction, there being a narrow range of catalyst concentration over which the rate changed from very slow to too rapid to be measured spectrophotometrically. The lower limit was reached with lanthanum chloride concentrations of the order of the chlorophyll concentration.

Acceleration by salts in ethereal solutions. Addition of salts to ethereal solutions produced

more extensive changes in absorption spectra of chlorophyll-*a* (Fig. 6). Methanol was required for dissolution of the salt, and also appeared to have a protective action against extensive decomposition. Visually, the solutions became straw-yellow and non-fluorescent. The change was not reversed by replacing the ether by methanol in absence of air throughout the operation.

Fluorescence changes. The measured fluorescence intensity decreased on allomerization of chlorophyll-*a*. For 5×10^{-6}M-solutions in methanol, the intensity decreased to a constant value of 55% of the original in absence of added catalyst, and with various concentrations of lanthanum
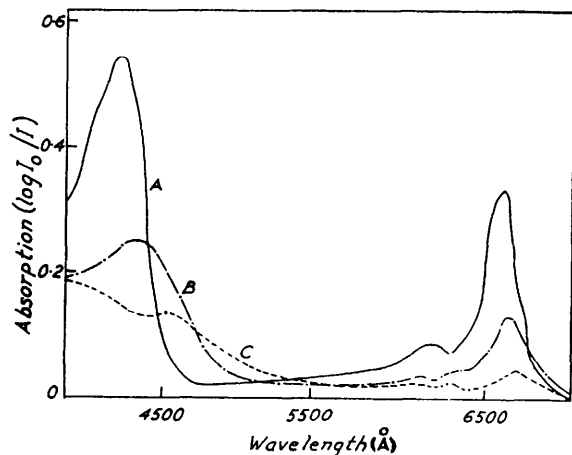
Fig. 6. *Spectra 24 hr. after addition of lanthanum chloride to ether–methanol solutions of chlorophyll-*a* (5×10^{-6} mole/l.).*

A, in 10% methanol–ether.

B, in 20% methanol–ether, with 10^{-3} mole/l. of LaCl$_3$.

C, in 10% methanol–ether, with 10^{-3} mole/l. of LaCl$_3$.

Fig. 7. *Decrease in fluorescence on allomeriz- ation of chlorophyll-*a* (5×10^{-6} mole/l.).*
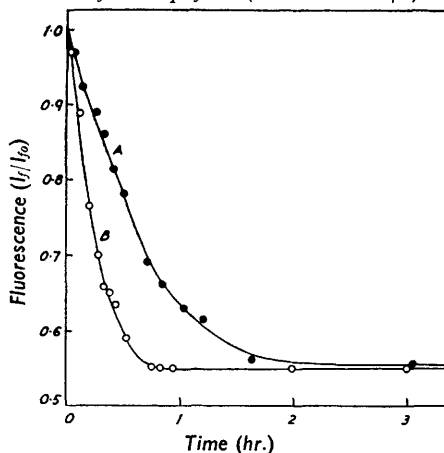
A, with 10^{-5} mole/l. of LaCl$_3$.
B, with 5×10^{-5} mole/l. of LaCl$_3$.

Fig. 8. *Increase in fluorescence on allomerization of chlorophyll-*b* (5×10^{-6} mole/l.).*
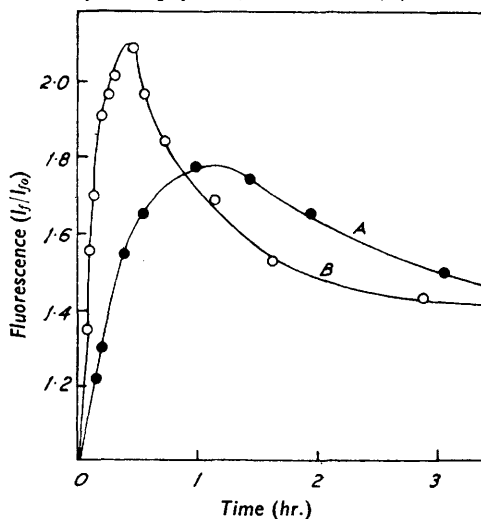
A, with 10^{-5} mole/l. of LaCl$_3$.
B, with 10^{-4} mole/l. of LaCl$_3$.

chloride (Fig. 7) and other salts. Correction for the fractions of 0·553 and 0·427 of the incident light absorbed by chlorophyll-*a* and its allomer, respectively, gives a relative fluorescence intensity of the allomer of 71%. Three further corrections are required for a determination of relative yield owing to : (i) re-absorption of the measured fluorescence of the allomer, (ii) change of wavelengths affecting photocell sensitivity, and (iii) different fractions of the fluorescence spectrum being cut off by the photocell filter. The red absorption maximum moved 100 Å to shorter wavelength. An approximately mirror-image fluorescence spectrum with a similar shift being assumed, factors (i) and (ii), but not (iii), are likely to be unimportant; concerning (iii), about 30% less area under the fluorescence spectrum curve is estimated to be transmitted

by the filter. These measurements therefore indicate that allomerized chlorophyll-*a* possesses a fluorescence of an intensity of the order of that of chlorophyll-*a*.

In contrast with chlorophyll-*a*, the measured fluorescence intensity increased on the allomerization of chlorophyll-*b*. In absence of added catalyst or with accelerated allomerization, a maximum in fluorescence was obtained (Fig. 8), after which more extensive decomposition was indicated by absorption spectra. On correction for the fractions of 0·227 and 0·338 of the incident light absorbed by 5×10^{-6} M-chlorophyll-*b* and its allomer, respectively, the maximum of fluorescence was 158% of the initial value. Arguments on probable wavelength changes of

Fig. 9. *Allomerization of different concentrations of chlorophyll-a.*

(The numbers on the curves refer to initial chlorophyll concentrations $\times 10^3$ mole/l.)
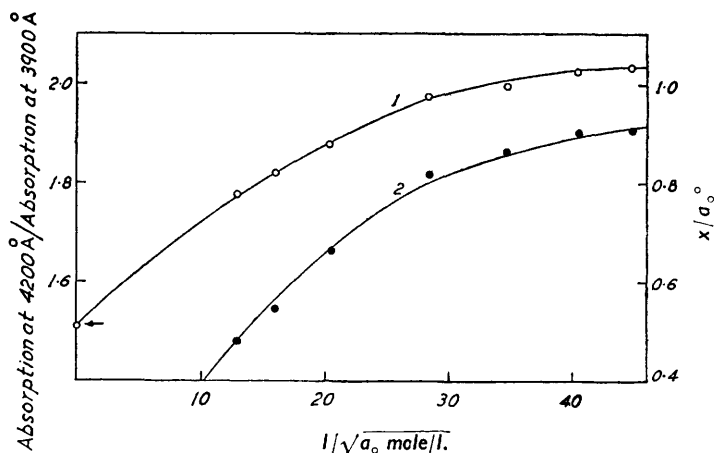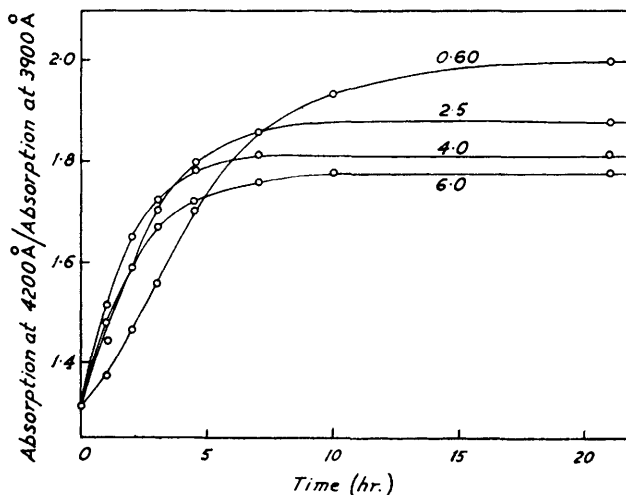
Fig. 10.

Curve 1. *Ratios of absorption at 4200 Å/3900 Å on allomerization, s, for different initial chlorophyll-a concentrations.*

Curve 2. *Calculated molar fractions of substance* X.

The arrow refers to the point $(s_{\mathrm{Y}'} + s_{\mathrm{Y}''})/2$

fluorescence indicate that this figure would be increased on making the further corrections necessary for relative yields.

Concentrated Chlorophyll-a Solutions.—With concentrations of chlorophyll-*a* from 5×10^{-6} to 5×10^{-5}M, the spectra on complete allomerization were identical and the rates were dependent on the chlorophyll concentration to the first power. However, at higher concentrations different behaviour was noted : (i) the initial rate of reaction as measured by the spectral method increased markedly with chlorophyll concentration (Fig. 9) and (ii) the spectral changes became static at 4200 Å/3900 Å ratios well below that for complete allomerization in more dilute solution (Figs. 9 and 10, curve 1).

In accord with these indications of more complex reaction, concentrated chlorophyll solutions after allomerization yielded two major products on chromatography in addition to the main product obtained with dilute solutions; the bands for unaltered chlorophyll and for these three products clearly separated on elution. Their spectra are recorded in Fig. 11.

Selective elution was attempted, although with too short columns to be more than semi-quantitative (the main objective being a first chromatography for spectra determination). After application of the approximate equality of extinction coefficients at 3900 Å, the proportions of the other two products compared with that from dilute solutions increased with initial chlorophyll concentration (Table 3). This is empirically the explanation of the decrease in final 4200/3900 Å ratio with chlorophyll concentration.

TABLE 3. *Approximate molar fractions of products after allomerization of chlorophyll-a solutions of different concentrations in methanol.*

Initial chlorophyll-a (mole/l. $\times 10^3$)	Molar fractions of		
	Substance X	Substance Y'	Substance Y''
0·67	0·77	0·13	0·10
3·96	0·43	0·34	0·22
6·70	0·31	0·35	0·34

The allomerization of concentrated solutions was catalysed by salts, but the increase in rate of reaction did not alter the spectral properties of the final product. For example, a 4×10^{-3}
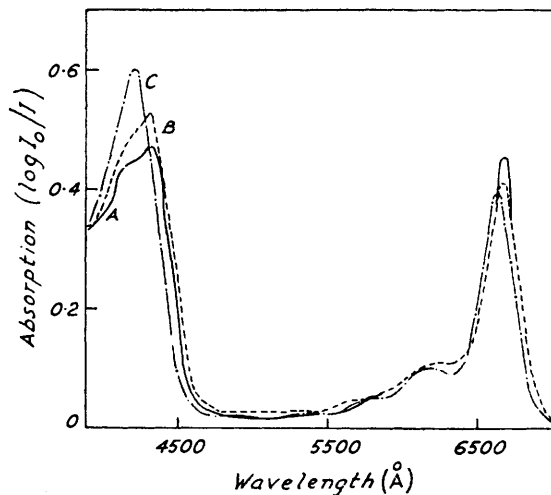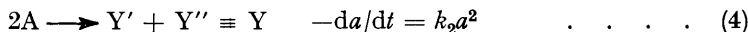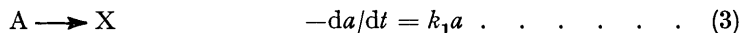

FIG. 11. *Absorption spectra of (A) chlorophyll-a, (B) substance Y', and (C) substance Y'' in methanol.*

M-solution of chlorophyll-*a* in methanol was completely allomerized within 5 min. by magnesium methoxide to give a spectral ratio, s_f, of 1·846, whereas an uncatalysed control reached the same ratio after 30 hr.

DISCUSSION

Uncatalyzed Allomerization.—The most obvious interpretation of the rate and product results is that the predominant mechanism in dilute solution involves a rate-determining step dependent on chlorophyll concentration, a, to the *first* power to produce a single product, X, and that a second mechanism of significance only in more concentrated solutions involves a rate-determining step or steps dependent on chlorophyll concentration to the *second* power to produce the two further products, Y' and Y'' :

$$\text{A} \longrightarrow \text{X} \qquad -\mathrm{d}a/\mathrm{d}t = k_1 a \quad . \quad . \quad . \quad . \quad . \quad (3)$$

$$2\text{A} \longrightarrow \text{Y}' + \text{Y}'' \equiv \text{Y} \qquad -\mathrm{d}a/\mathrm{d}t = k_2 a^2 \quad . \quad . \quad . \quad . \quad (4)$$

The following analysis shows that the product and rate data are consistent with the schematic mechanism of (3) and (4). The composite product symbol Y is introduced merely to condense later algebraic equations; Y is of concentration equivalent to the sum of Y' and Y'', and of a 4200/3900 Å spectral ratio, s_Y equal to the arithmetic mean of the ratios for Y' and Y''.

First to be considered are the concentrations, x and y, of substances X and Y expected at the end of reaction for various initial chlorophyll concentrations, a_0, according to equations (1) and (2).

The final spectral ratio, s_f, is related to x, s_X, and s_Y by :

$$x/a_0 = (s_f - s_Y)/(s_X - s_Y) \qquad \ldots \ldots \ldots \text{(5)}$$

Also, $\qquad (\mathrm{d}x/\mathrm{d}t)/(-\mathrm{d}a/\mathrm{d}t) = k_1 a/(k_1 a + k_2 a^2)$

from which, on complete reaction,

$$x = 2{\cdot}303(k_1/k_2) \log_{10}(1 + k_2 a_0/k_1) \qquad \ldots \ldots \text{(6)}$$

Equation (6) provides values of k_1/k_2 from x determined by equation (5). Initial rate data are next used to provide a second equation in k_1 and k_2. This requires chlorophyll
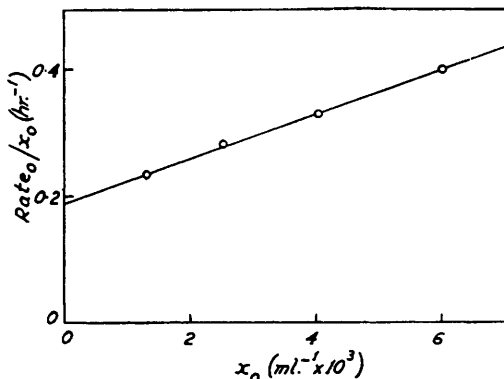
FIG. 12. *Initial rates of allomerization for different initial chlorophyll-*a *concentrations.*

concentrations at measured early periods of reaction to be calculated from the observed spectral ratios, s. For small extents of reaction,

$$x/y = k_1/k_2 a_0 \qquad \ldots \ldots \ldots \ldots \text{(7)}$$

and $\qquad s = s_A(a/a_0) + s_X(x/a_0) + s_Y(y/a_0) \qquad \ldots \ldots \ldots \text{(8)}$

Substituting (7) in (8) and rearranging, we have

$$a = \frac{a_0[s - s_X + (s - s_Y)\, k_2 a_0/k_1]}{[s_A - s_X + (s_A - s_Y)\, k_2 a_0/k_1]} \qquad \ldots \ldots \ldots \text{(9)}$$

Finally, $\qquad (-\mathrm{d}a/\mathrm{d}t)_0/a_0 = k_1 + k_2 a_0 \qquad \ldots \ldots \ldots \text{(10)}$

Application to Data.—Average values of s for chlorophyll-a, X, Y', and Y'' were respectively 1·334, 2·070, 1·418, and 1·597 from 18, 7, 9, and 6 samples separately isolated by chromatography. Introducing theses in equation (5), with the values of s_f from Fig. 10, curve 1, we have the yields of x for various a_0 recorded in Fig. 10, Curve 2. The curve is of eqn. (6) with $k_1/k_2 = 0{\cdot}0024$ mole/l. Values of s at different times from Figs. 9 and similar data provide the initial rates of conversion of Fig. 12. In accord with eqn. (10), linearity of $-(\mathrm{d}a/\mathrm{d}t)_0/a_0$ versus a_0 is obtained, to give values of k_1 and k_2 at 35° of $5{\cdot}1 \times 10^{-5}$ sec.$^{-1}$ and $1{\cdot}0 \times 10^{-2}$ sec.$^{-1}$ mole^{-1} l., respectively.

Yields of X and Y on complete allomerization are given by eqn. (6), *i.e.*, curve 2 of Fig. 10. The experimental value of $k_1/k_2 = 0{\cdot}0024$ mole/l. gives 90% and 50% yields of X for initial chlorophyll concentrations of 0·57 and $5{\cdot}3 \times 10^{-3}$ mole/l., respectively. The approximate yields of X, Y' and Y'' on chromatographic separation (Table 3) are of the order calculated from the initial chlorophyll concentrations.
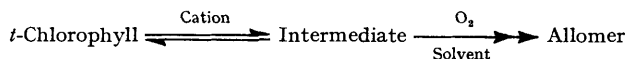
Catalyzed Allomerization.—Variation of cation and anion among series of salts clearly shows that the former is the catalyzing species. The order of reaction with respect to salt concentration for the one salt investigated, 0·65 for magnesium chloride, appears somewhat unrevealing but is consistent with catalytic activity of the free cation of a partially dissociated salt.

This leads to some general considerations of chlorophyll reactivity and structure

influenced by small concentrations of cation. In contrast with the homopolar chemistry usually envisaged for chlorophyll, catalyzed allomerization is more similar to ionic or ion–dipolar molecule reactions. Two formal structures for a dipolar chlorophyll mloecule are suggested by the classical formula, a positive central magnesium and an anionic porphyrin ring, and an enol form of oxygen of ring v.[8] The cation may function as catalyst by formation of the metal enolate.

The spectra of chlorophyll-*a* in different solvents also indicate a polar tautomeric species (see Fig. 2). Methanol solutions show almost equal heights of the two peaks of the blue band and of the red peak, for chlorophyll-*a*. Ascent of the alcohol series greatly reduces the relative height of the minor blue peak. The relative absorption of the red peak shows less solvent effect. Solvent effects on the spectrum of the allomerized product, X, are less pronounced (Fig. 2), as would be expected if a tautomeric species were no longer present.

Although not detected directly, an intermediate between the reactive form of chlorophyll and the allomer requires to be invoked :

$$t\text{-Chlorophyll} \underset{}{\overset{\text{Cation}}{\rightleftharpoons}} \text{Intermediate} \underset{\text{Solvent}}{\overset{O_2}{\longrightarrow}} \text{Allomer}$$

With the autoxidative step the more rapid, insensitivity to oxygen pressure is understandable. Lack of detectable reaction with cation in absence of oxygen is most plausibly explained by reversible formation of the intermediate as the catalysed reaction. The decomposition in ether can be similarly attributed to catalyzed production of an intermediate and its instability in this medium. Finally, since the products of reaction in concentrated solution were unaltered by salt catalysis, a chlorophyll molecule itself seems capable of reacting with the intermediate. Thus the apparently independent allomerization processes in dilute and concentrated solution may be related.

[8] Rabinowitch, " Photosynthesis," Interscience Publ., New York, 1945, Vol. I, p. 459.