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THE KINETICS OF PHOTOCONVERSION OF PROTOCHLOROPHYLLIDE IN ETIOLATED BEAN LEAVES

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SUMMARY

1. The kinetics of photoconversion of the active protochlorophyllide of etiolated bean leaves was studied in near monochromatic light at 630, 637 and 647 nm.

2. Two independent methods of measurement showed that the photoconversion was not first order, but closely second order or the sum of two first-order terms. The order of the kinetics was independent of the wavelength of the actinic light.

3. The second-order kinetics or the sum of two first-order terms can be explained in terms of the occurrence of protochlorophyllide in molecular groups with energy transfer between protochlorophyllide and chlorophyllide *a* within each group. Energy transfer is competitive with the photoconversion process at room temperatures. With due allowance for this energy transfer, the true kinetics of photoconversion is first order, showing the 'restricted collision' view of photoconversion to be tenable.

INTRODUCTION

Smith and Benitez[1] studied the photoconversion of protochlorophyllide in etiolated barley leaves, and found that the kinetics of transformation were consistent with a second-order reaction. Some photoconversion was found to take place even at —70 °C, which appeared inconsistent with the implied bimolecular collision process of a second-order reaction. Later Smith and French[2] investigated the photoconversion of etiolated bean leaves, and of protochlorophyllide holochrome extracts, and in each case found a second-order rate law of the form $dc/dt = -kc^2$, c being the instantaneous protochlorophyllide concentration. Boardman[3] found that the photoconversion of holochrome preparations from etiolated bean leaves did not follow simple first-order kinetics, and expressed the transformation as the sum of two first-order reactions, $T\% = 50(2 - e^{-k_1 t} - e^{-k_2 t})$ with T the percentage transformation at time t. This type of law held down to —55 °C in temperature, with reduced total transformation as the temperature was lowered, the values of k_1 and k_2 being dependent on the temperature.

Sironval *et al.*[4], on the other hand, using near monochromatic actinic light, found that at 647 nm whole bean leaves gave a first order photoconversion law, but at 630 nm the photoconversion appeared to follow the sum of two first-order processes. Schultz[5] investigated the photoconversion of protochlorophyllide holochrome in light

at 630, 640 and 650 nm. The results plotted on a log/linear basis did not give straight lines, but were much closer to a second-order rate law in each case.

It is of importance to establish the true nature of the photoconversion process to permit interpretation at the molecular level. For this reason we have reinvestigated the photoconversion of etiolated bean leaves with near monochromatic light at 630, 637 and 647 nm, by two independent methods of analysis. The kinetics of photoconversion are interpreted in terms of our recent findings[6,7] that protochlorophyllide molecules are organized in groups.

MATERIALS AND METHODS

Brown Beauty beans (*Phaseolus vulgaris* L.) were grown in darkness as previously described[7] for 14 days. Single leaves were mounted diagonally in 1 cm quartz spectrofluorimeter cells and held between thin clear sheets of perspex (0.5 mm thick) for photoconversion measurements.

Two independent experimental methods were then used to determine the kinetics of photoconversion in monochromatic actinic light. In the first method, the formation of chlorophyllide *a* was determined by the change in absorbance at 690 nm in a dual wavelength spectrophotometer. In the second method, the loss of protochlorophyllide due to photoconversion was measured following actinic light exposures for fixed times, by means of fluorescence analysis.

The light-induced change in absorbance at 690 nm was measured in an Aminco–Chance dual wavelength spectrophotometer (American Instrument Corp. Md., U.S.A.), fitted with a side actinic illumination attachment. The reference wavelength was 735 nm. Actinic light was provided by Xenon Arc Source, 150 W and passed through a 3-cm layer of water, a broad band red filter, and then to the input of a Bausch and Lomb 500 mm grating monochromator. The monochromator could be set either at 630, 637 or 647 nm, with a band width of $\pm$ 3 nm. The output was directed by a light-pipe to be incident on the etiolated leaf mounted in the spectrophotometer. The incident actinic light was set at 1000 ergs$\cdot$cm$^{-2}\cdot$s^{-1} at the leaf surface. The photomultiplier was moved 5 cm back from the normal close operating position to minimise possible fluorescence artefacts. A Corning glass filter 4-77 protected the photomultiplier from the actinic light incident on the leaf sample. Recordings of absorbance changes at 690 nm were made, the start of the recorder being synchronised with the exposure of the leaf to the actinic light. The recording speed was 0.1 inch$\cdot$s^{-1} with the amplitude sensitivity set at 5 % T, to give the absorbance change at 690 nm, referenced to 735 nm as photoconversion occurred. Photoconversion was complete within the recording time of 150 s but the leaf was exposed for a further 60 s to ensure maximal photoconversion.

In the second method, individual leaves were mounted in a spectrofluorimeter, some details of which were given earlier[8], and photoconversions made using the instrument light source as actinic light. Partial photoconversions were made at selected exposure times at 647 nm, band width $\pm$ 5 nm, and uniform incident light intensity of 2000 ergs$\cdot$cm$^{-2}\cdot$s^{-1}. After each exposure, the leaf was removed in the dark, ground in 5 ml of ethanol and the extract was clarified by centrifugation. During the operations, leaves were handled either in the dark or in green 'safelight'. Five independent experiments were made at each exposure time. Fluorescence analyses of the ethanol

extracts were made at 77°K (ref. 9), with excitation at 440 nm and band width ± 1.5 nm, and emission band width ± 1 nm. The low temperature served to increase both the sensitivity and selectivity of the method. Fully corrected emission spectra were recorded for each sample, the protochlorophyllide peak being at 628 nm. The samples were all at high dilution, with linear fluorescence response to concentration.

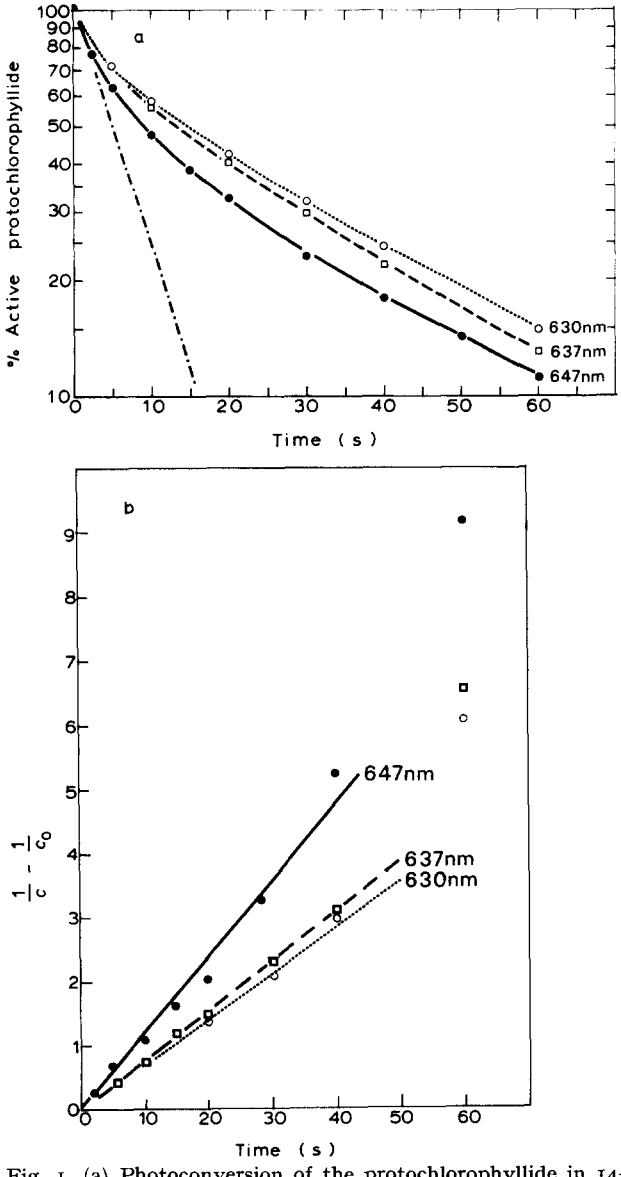

Fig. 1. (a) Photoconversion of the protochlorophyllide in 14-day etiolated bean leaves. Photoconversions were made at three wavelengths: ○—○, 630 nm; □—□, 637 nm; ●—●, 647 nm. Bandwidth ± 3 nm and incident light intensity 1000 ergs·cm^{-2}·s^{-1} in each case. Measurements are the average values of 5 photoconversions at each wavelength as determined in an Aminco–Chance spectrophotometer as described in the text, and plotted on a log/linear basis. (b) Results (a) replotted on the basis of a second-order rate law.

RESULTS

Method I

The results of photoconversion measurements made in the dual wavelength absorption spectrophotometer for actinic monochromatic light at 630, 637 and 647 nm are shown as log/linear plots in Fig. 1a. Values of percentage active protochlorophyllide were derived from the absorbance change *versus* time curve using the simple complementary relation, $c_1/c_0 = 1 - a_1/a_0$, where c_0 is the active protochlorophyllide concentration at $t = 0$, c_1 the concentration at any later time t_1, a_0 is the concentration of chlorophyllide *a* at full conversion and a_1 the concentration at the same time t_1. At any given time *t*, each point of Fig. 1a is the mean value obtained from 5 independent photoconversion experiments.

The results show that photoconversion does not follow a first-order law of the form $dc/dt \propto c$ at any of the three wavelengths of actinic light. A second-order dependence ($dc/dt \propto c^2$) gives an equation of the form $1/c - 1/c_0 = k't$. In Fig. 1b, the results shown in Fig. 1a are replotted as the function $1/c - 1/c_0$ against time. The results closely follow a second-order law for $1 > c/c_0 > 0.25$ *i.e.* for photoconversions up to about 75 %.

We have used 690 nm as the absorbance wavelength for measuring the formation of chlorophyllide *a*. It would be preferable to use 680 nm in order to avoid complications due to the rapid shift (Shift III) in the absorption maximum from 678 to 682 nm following photoconversion[7,10,11]. However, we found that actinic light at 647 nm breaks through the filters to a small extent at 680 nm, forcing the use of 690 nm, where no breakthrough was experienced.

The results of Figs 1a and 1b therefore include a small effect due to Shift III and this is discussed later.

Method II

The kinetics of photoconversion of protochlorophyllide in 647 nm actinic light, as determined by fluorescence analysis are shown in Fig. 2 on a log/linear plot.

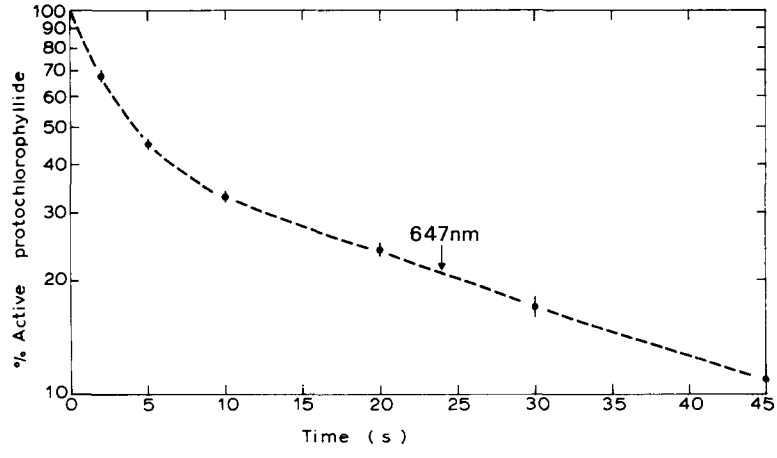

Fig. 2. Photoconversion of the protochlorophyllide in 14-day etiolated bean leaves, determined by fluorescence analysis as described in the text. Photoconversion at 647 nm, bandwidth ± 5 nm, incident intensity 2000 ergs·cm^{-2}·s^{-1}. Each point is the average of 5 independent measurements.

The inactive protochlorophyll was found to be about 14 % of the total, and due allowance was made for this in calculating the values of c/c_0 at each exposure time. The results follow a second-order law for $1 > c/c_0 > 0.25$, but over a wider range they may also be expressed as the sum of two first-order components. These results do not incorporate the effect due to Shift III as do the results of *Method I*, and the curve obtained by *Method II* (Fig. 2) has a slightly greater initial slope and a slightly smaller slope at later times as $c/c_0 \rightarrow 0.1$, by comparison with the corresponding curve in Fig. 1a. The time scale in Fig. 2 is adjusted to compensate for the higher actinic light intensity used in *Method II*. Aside from these differences the results of the two methods are in close agreement, and both methods show that at 647 nm the photoconversion of protochlorophyllide does not follow the first-order law. The kinetics of photoconversion of protochlorophyllide in 630 nm light was also investigated by this method and were found to be similar to those at 647 nm (details not shown).

DISCUSSION

The results which show substantial agreement with those of earlier workers[1,2] indicate that the photoconversion of protochlorophyllide in etiolated leaves is not first order, but can be fitted to second-order kinetics for the greater part of the time course. Alternatively, as shown previously[3], the results may be fitted to the sum of two first-order processes. Boardman[3] has suggested that the photoconversion of protochlorophyllide should be regarded as a restricted collision process between the excited protochlorophyllide molecules and the molecule which donates the hydrogen atoms or electrons. In Boardman's model, protochlorophyllide and the donor molecule are considered an integral part of the protochlorophyllide holochrome. However, such a reaction should follow first-order kinetics.

From studies of the fluorescence properties of extracts of the protochlorophyll holochrome, Kahn *et al.*[6] concluded that the protochlorophyllide molecules were organised into groups of at least 4 molecules per group. Thorne[7], investigating the photoconversion in etiolated bean leaves concluded that protochlorophyllide *in vivo* was located in discrete groups of up to 20 molecules. In both holochrome extracts and in leaves, energy transfer was observed at 77 °K between protochlorophyllide molecules, and also between protochlorophyllide and chlorophyllide *a* after photoconversion of a fraction of the protochlorophyllide[6,7].

With photoconversion at room temperature, we must also consider energy transfer from protochlorophyllide to chlorophyllide *a*. The overlap spectra integral[12] of protochlorophyllide and chlorophyllide *a* will allow energy transfer from protochlorophyllide to chlorophyllide *a* but the reverse process has very low probability. Therefore, energy transferred to chlorophyllide *a* is lost to the photoconversion process.

In a closely located group of molecules, the relative probability of photoconversion (P_c) and the relative probability of energy loss by transfer (P_t) are essentially fast compared with the emission of fluorescence or internal loss within a protochlorophyllide molecule. Then for every photon absorbed $(P_c + P_t) \rightarrow 1$. In each molecular group, to a first approximation, $P_t \propto (c_0 - c)$ and $P_c \propto c$, where c_0 and c are protochlorophyllide concentrations as before. The rate of photoconversion at time t under constant illumination, $dc/dt \propto A\, P_c/(P_c + P_t)$, where A is the instantaneous absorbance of the protochlorophyllide, and $P_c/(P_c + P_t)$ is a correction for the increase in energy

transfer as photoconversion progresses. Since at any instant $A \propto c$ and $P_c/(P_c + P_t) \propto c$, then $dc/dt \propto c^2$ and an apparent second-order law results.

The results of Fig. 2 have been analysed on the basis of this treatment (Table I).

TABLE I

THE PHOTOCONVERSION OF ETIOLATED BEAN LEAVES AT 647 nm AND 20 °C

Analysis of the results of Fig. 2. c/c_0 is the fraction of active protochlorophyllide at each time, $(dc/dt)/(dc_0/dt)$, the relative rate of conversion at time t to the initial rate determined graphically, P_t the relative probability of energy transfer from protochlorophyllide to chlorophyllide *a* at a given time, P_c the relative probability of photoconversion at the same time, at 20 °C. At each time $(P_t + P_c)$ is approx. 1.

Time (s)	c/c_0	$(dc/dt)/(dc_0/dt)$	$1/(1+P_t/P_c)$	P_t/P_c
0	1.00	1.00	1.00	0
2	0.68	0.50	0.73	0.37
5	0.45	0.20	0.44	1.26
10	0.33	0.078	0.24	3.2
20	0.24	0.04	0.167	5.0
30	0.17	0.03	0.176	4.7
45	0.11	0.018	0.164	5.1

Values of dc/dt and dc_0/dt were determined graphically from a plot of c/c_0 against time. Since at the time $t = 0$, $dc/dt \propto c_0$ and $P_t = 0$, then from the measured values of dc_0/dt and c_0 at $t = 0$ and from dc/dt and c at any instant later, we have the relation $(dc/dt)/(dc_0/dt) = (c/c_0) \cdot 1/(1 + P_t/P_c)$. From this is calculated the values of $1/(1+P_t/P_c)$ and P_t/P_c as shown in the final two columns of Table I. In the later stages of photoconversion *i.e.* $c/c_0 < 0.25$, the value of P_t/P_c reaches an approximately constant value. It is reasonable to suppose that at the stage of photoconversion where three in every four molecules have been transformed, then on the average each remaining protochlorophyllide will have chlorophyllide *a* as neighbours, so that further photoconversion does not change the probability of energy transfer. Over the range $1 > c/c_0 > 0.25$, $dc/dt \propto c^2$, but over the full range of conversion the photoconversion may be expressed also as the sum of two apparent first-order processes. Boardman[3], expressing the photoconversion in extracts of protochlorophyll holochrome as the sum of first-order reactions, obtained rate constants of $k_1 = 0.50$ and $k_2 = 0.09$. Since k_1 is considerably greater than k_2, then to a first aproximation the ratio k_1/k_2 determines the relative rates of photoconversion at the two extremes, *i.e.* in the range of low photoconversion where the probability of energy transfer to chlorophyllide *a* is low, and at high photoconversions where the probability of transfer reaches a constant value. The ratio of the rate constants obtained by Boardman ($k_1/k_2 = 5.5$) is similar to the value of $1 + P_t/P_c = 6$, for $c/c_0 < 0.25$, which results from our present treatment. The apparent first-order kinetics which Sironval *et al.*[4] reported for the photoconversion of protochlorophyllide at 647 nm were based on fluorescence emission spectra at 77 °K. However, the height of the fluorescence emission band at 655 nm at 77 °K may not be used to estimate the amount of photoconversion because of energy transfer to chlorophyllide *a*[6,7].

The photoconversion of protochlorophyllide, which may be fitted either to second-order kinetics or to the sum of two first-order processes is, in essence, attri-

butable to the occurrence of protochlorophyllide in energy-transferring molecular groups, both *in vivo* and in the isolated holochrome. With due allowance for this energy transfer term, the photoconversion itself is first order which is compatible with the restricted collision concept[3].

Support for these conclusions comes from the recent studies of Henningsen and Kahn[13]. They used a mixture of saponins to extract and purify photoactive subunits of protochlorophyllide holochrome from barley leaves. The protochlorophyllide holochrome prepared in the presence of the detergents appeared to contain only one protochlorophyllide molecule per photoactive complex. There was no evidence of energy transfer from protochlorophyllide to chlorophyllide *a*[13] in the saponin holochrome and the kinetics of transformation were consistent with a first-order reaction (O. F. Nielsen and A. Kahn, private communication). This behaviour of the saponin holochrome is in contrast with the protochlorophyllide holochrome in barley leaves, where energy transfer is observed at 77°K (S.W. Thorne and N. K. Boardman, unpublished observations) and the kinetics of transformation are second order[1]. It appears therefore that protochlorophyllide in etiolated barley leaves also occurs in molecular groups which seem to be disrupted, however, on extraction into buffers containing saponin[13].

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