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The Reduction of Protochlorophyllide into Chlorophyllide

II. The Temperature Dependence of the $P_{657-647} \rightarrow P_{688-676}$ Phototransformation*

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Abstract

The temperature dependence of the $P_{657-647} \rightarrow P_{688-676}$ phototransformation in etiolated bean leaves was studied using either 647 or 630 nm actinic light. When irradiating with 647 nm photons the phototransformation rate increased from +37 °C to −18 °C; the rate decreased below −18 °C; it became zero at about −75 °C. When irradiating with 630 nm photons one of the two first-order rate constants of the phototransformation behaved similarly to the rate constant found in the presence of 647 nm photons; the other 630 nm rate constant behaved in another way. Denaturation of the protochlorophyllide-lipoprotein complex occurred in the dark at temperatures higher than +45 °C. A strong decrease of the phototransformation rate was found at these temperatures.

The $P_{657-647} \rightarrow P_{688-676}$ phototransformation was defined as an irreversible modification of the spectral properties of etiolated leaves when illuminated with a suitable light intensity for periods of the order of a second. The kinetics as well as some other features of this phototransformation have been discussed elsewhere (SIRONVAL *et al.* 1968). The present paper deals with its temperature dependence.

The temperature dependence of the production of chlorophyllide from protochlorophyllide when etiolated leaves are illuminated was previously described by SMITH and BENITEZ (1954). BOARDMAN (1962) studied this dependence using preparations of the protochlorophyllide-lipoprotein complex and obtained results which were not significantly different from the data of SMITH and BENITEZ. We intend to show that the temperature dependence of the $P_{657-647} \rightarrow$ $\rightarrow P_{688-676}$ phototransformation behaves in a way which differs somewhat from that observed when studying the production of chlorophyllide from protochlorophyllide in the light.

MATERIAL AND METHODS

We used beans (*Phaseolus vulgaris* L.) of the variety "Commodore". The methods for growing the plants in complete darkness, for illuminating them with light of given wavelength and intensity for short periods, for registering the low temperature fluorescence spectra, and for estimating

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the amount of the $P_{657-647} \rightarrow P_{688-676}$ phototransformation from the fluorescence spectra have been described elsewhere (SIRONVAL *et al.* 1968).

The procedure for experimenting the temperature effects involved essentially the following steps:

a) etiolated, primary bean leaves were cut in darkness;

b) each leaf blade was separately stretched on the back of a copper holder seen in Fig. 1. This holder was especially designed to fit in the devices described in Figs. 1 and 2 of SIRONVAL *et al.* (1968) for sample illumination and for registration of the low temperature fluorescence;

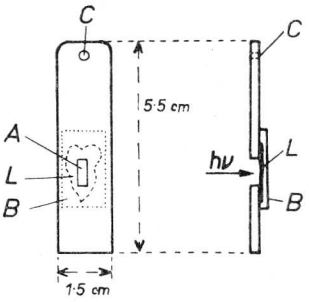

Fig. 1. Copper holder used for holding the leaf in the light beam of the devices described in Figs. 1 and 2 of the paper by SIRONVAL *et al.* (1968). The holder was hung in the beam through the hole *C*. *L* = leaf. The direction of the light is seen on the right side of the figure (arrow); the light reached the leaf through the window *A*. *B* was a sheet of black cardboard.

c) the holder was kept for 10 to 15 min at a known temperature (see below);

d) it was transferred, as rapidly as possible, to the device of Fig. 1 in SIRONVAL *et al.* (1968) for leaf illumination;

e) it fell into liquid nitrogen at the very end of a short time illumination (less than 1 s), — the amount of the $P_{657-647} \rightarrow P_{688-676}$ phototransformation being thereafter estimated from the low temperature fluorescence spectrum.

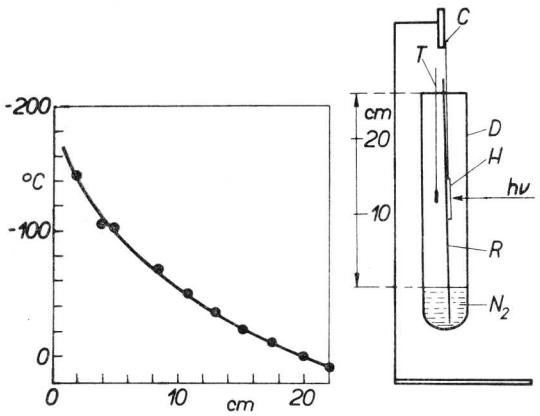

Fig. 2. The device used for cooling below −10 °C. A wooden rule *R* was placed in a *Dewar D*. The holder *H* could slide along the rule at the desired height above the liquid nitrogen N_2. The holder was hung by a nylon wire at *C*. *C* could be moved away by a magnet liberating the sample holder *H*. The fall of *H* in liquid nitrogen was regulated in such a way that it occurred at the very end of the illumination. *T* = thermometer. An example of a temperature gradient is given in the left part of the figure.

The same procedure was followed at every temperature, including room temperature. During the manipulations, from cutting to cooling the samples in liquid nitrogen, the only light consisted of an *Osram 4543* lamp surrounded by a neutral filter plus a green cellophane filter with a maximum transmission at 520 nm. The manipulations were never interrupted; for each sample, they

lasted about 15—20 min between cutting and cooling in liquid nitrogen. All the leaves used were of the same age (20 d after germination) and, as far as possible, of the same length (midrib of about 1·5 cm) and weight (about 30 mg).

Getting the leaves to a given temperature during illumination: Temperatures between room temperature and +60 °C were obtained by placing the holder (with the leaf) for 10 to 15 min in the air in a closed cylindrical copper box (11 cm long, 5 cm diameter) dipped in a water bath kept at the required temperature.

Below room temperature to −30 °C, the copper box was dipped in a mixture of glycerine–water (70% glycerine in weight; freezing temperature: −38·9 °C). The mixture was automatically kept at the required constant temperature inside a *Dewar*. The holder remained in the box for 10 to 15 min.

The time between taking the holder out of the box and the end of the illumination never exceeded 4—5 s. It was shown that, by this procedure, the sample was at the required temperature during its illumination.

In the temperature range between −10 and −100 °C, a special *Dewar* was used in which a temperature gradient was established by maintaining a constant amount of liquid nitrogen at the bottom as shown in Fig. 2. The holder was kept in the *Dewar* along a wooden rule, at a definite level, the temperature of which was estimated. The sample was left at this level for 10 to 15 min before being directly illuminated inside the Dewar through a longitudinal window (2 cm wide) by a suitable perpendicular light beam. The sample fell as usual into liquid nitrogen at the very end of the illumination.

It was verified that, within the temperature range from −10 to −30 °C, the form of the temperature dependence of the rate of the $P_{657-647} \rightarrow P_{688-676}$ phototransformation was the same when using the *Dewar* method as when using the copper box method.

Light intensity estimation: The estimation of the intensity of the light was made as described earlier (SIRONVAL *et al.* 1968). When illuminating inside the special *Dewar*, any direct estimation was impossible. In this case, the intensity of the light for a given position of the *Dewar* was estimated from the formula:

$$\frac{K_D}{K_c} = \frac{I_D}{I_c}$$

where K_D is the rate constant found at a given temperature T (for instance −10 or −15 °C) using the *Dewar* method; K_c is the rate constant found at the same temperature T using the copper box method; I_c is the intensity measured when using the copper box method and I_D the unknown intensity when using the *Dewar* method.

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RESULTS

A. Effects of temperature on the rate of the $P_{657-647} \rightarrow P_{688-676}$ phototransformation

The effects of temperature on the rate of the $P_{657-647} \rightarrow P_{688-676}$ phototransformation were studied at two wavelengths: 647 and 630 nm. These wavelengths were chosen considering that:

1. etiolated leaves have distinct absorption bands at these wavelengths;
2. it was previously shown that the phototransformation tended to first order kinetics in 647 nm light, while in 630 nm light the kinetics were never of the first order (SIRONVAL *et al.* 1968).

1. Phototransformation by 647 nm photons

The measurements were made in the temperature range from +37 to −75°C, the actinic light being filtered through a 647 nm interference filter (half-band width 32 Å).

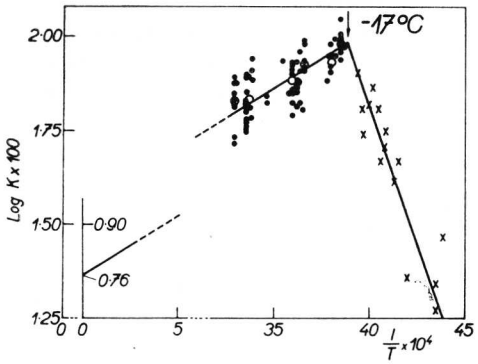

Fig. 3. Temperature dependence of the rate of the phototransformation $P_{657-647} \rightarrow \rightarrow P_{688-676}$; the etiolated bean leaves were irradiated for 1 s with 647 nm photons. Intensity of the light of the order of 35×10^{-11} einstein s^{-1} cm^{-2}. The values of log $(100 \times K)$ were plotted in function of the inverse of absolute temperature, K being the first order rate constant. Black points: individual values; white points: mean values. The crosses correspond to values obtained with the *Dewar* method (see "Methods" and Fig. 2). The extrapolation of the experimental data for K between +30 and −18 °C to infinite temperature is given in the left part of the figure.

The phototransformation was first found to tend to 1st order kinetics at four temperatures: +44°, +38°, +25°, +8 °C. No attempt was made to estimate the order of the kinetics below −20 °C. In comparing the results at these temperatures, the rate constant appeared to increase when the temperature was lowered.

Series of estimations of K ($K_{S-1} = 2{\cdot}3/t \log 100/100 - T\%$, t being the time in s and $T\%$ the corresponding percentage of phototransformation) were thereafter systematically made at different temperatures keeping the illumination time at a constant value of 0·881 s and the light intensity at the leaf surface at $35{\cdot}3 \times 10^{-11}$ einstein s^{-1} cm^{-2} (±10%). The results are shown in Fig. 3.

It was observed that:

a) the increase of K was about 50% from +37 °C down to −18 °C. A maximum rate was reached at about −17 to −18 °C. The rate decreased below −17 °C. It approached zero at −75 °C.

b) between +37 °C and −18 °C, K varied more or less exponentially with the inverse of the absolute temperature. Consequently, K being equal to:

$$K = n\varepsilon_{vivo}I \qquad (1)$$

where n is the quantum yield of the phototransformation, ε_{vivo} the *in vivo* absorption coefficient of the pigment to be transformed (the $P_{657-647}$ pigment) and I the intensity of the light (see SIRONVAL *et al.* 1968); n also appeared to vary roughly as an exponential function of the inverse of the absolute temperature.

2. Phototransformation by 630 nm photons

The measurements were made in the same temperature range and using the same experimental procedure as for 647 nm photons. The light was filtered through a 630 nm interference filter (half-band width: 32 Å).

Kinetics were followed at six temperatures between +36 and −19 °C. They all agreed without any exception with BOARDMAN's equation (BOARDMAN 1962) assuming the occurrence of 2 distinct classes of pigment-lipoprotein complexes transformed at 2 different rates K_1 and K_2:

$$T\% = 100 - Ae^{-K_1 t} - (100 - A)\, e^{-K_2 t} \qquad (2)$$

where $T\%$ is the percentage of the phototransformation at time t and A the percentage of the molecules belonging to the class transformed at rate K_1. Computation showed that A was equal to 50% in the experimented temperature range.

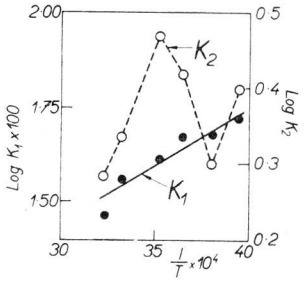

Fig. 4. Temperature dependence of the rate of the phototransformation $P_{657-647} \rightarrow P_{688-676}$; the etiolated bean leaves were irradiated with 630 nm photons. The intensity of the light was about 95×10^{-11} einstein s^{-1} cm^{-2}. The values of $\log(100 \times K_1)$ (black points) and of $\log K_2$ (white points) were plotted in function of the inverse of the absolute temperature, K_1 and K_2 being the two first order rate constants obtained from equation (2) for $A = 50$ (see SIRONVAL *et al.* 1968). The straight, full line has the slope of the line in Fig. 3 between +30 and −18 °C. Each pair of points for K_1 and K_2 are values calculated, using equation (2), from the mean of 25 separate evaluations of $T\%$ at one given temperature (0·881 s illuminations).

Fig. 4 gives the values of $\log K_1$ and $\log K_2$ $(K_1 < K_2)$ as a function of the inverse of the absolute temperature for $A = 50\%$ in equation (2). It essentially shows that:

(*a*) $\log K_1$ seemed to increase linearly when decreasing the temperature from + 27° to −18 °C. A somewhat more marked increase of the rate was observed from +37 to +27 °C. The slope of the line between +27 and −18 °C was the same as the slope of $\log K$ in Fig. 3 when illuminating with 647 nm photons;

(*b*) the behaviour of K_2 was more complicated than that of K_1. The values of K_2 increased from +37 °C to about +20° and decreased after that. A transient drop appeared generally at −10 °C.

Table 1 gives values of $T\%$ in a series of measurements made at −10 °C and compares them to corresponding values found at +1° and −19 °C. It was observed that the −10 °C drop was rather variable, being sometimes very evident, but also sometimes imperceptible. The −10 °C drop was previously described by SMITH and BENITEZ (1954).

Fig. 5 is an attempt to compare the effect of temperature on the extent of the $P_{657-647} \rightarrow$ $\rightarrow P_{688-676}$ phototransformation when 630 nm photons are used to some results of SMITH and BENITEZ (1954). SMITH and BENITEZ (1954) measured the formation of chlorophyllide in

Table 1

Values of $T\%$ calculated for a 1 s illumination with 630 nm photons from the experimental values obtained after illuminating the leaves during 0·881, 0·461, 0·241, and 0·121 s (the 10 °C drop is essentially due to the behaviour of K_2; see Fig. 4).

Temperature		$T\%$				Mean
+ 1 °C		62·6	63·5	61·7	63·0	62·7
	(1)*	54·4	54·4	55·5	55·0	54·8
−10 °C	(2)	56·7	58·8	57·6	63·2	59·1
	(3)	61·0	58·8	58·8	58·0	59·2
−19 °C		63·2	63·5	61·4	60·7	62·2
Actual duration of the illumination(s)		0·881	0·461	0·241	0·121	

* (1) (2) and (3) refer to three kinetics which have been followed separately.

etiolated barley using a low intensity light from a tungsten source; they estimated the percentage of pigment reduced after 10 min illumination at different temperatures. The amount of the $P_{657-647} \rightarrow P_{688-676}$ phototransformation at some different temperatures was expressed as the value of $T\%$ found after illuminating etiolated bean leaves by a same amount of 630 nm photons. Although the experimental circumstances are dissimilar, the similarity of both curves of Fig. 5 is evident, especially in the region between room temperature and −20 °C.

Below −20 °C, the rate of the $P_{657-647} \rightarrow P_{688-676}$ phototransformation by 630 nm photons appeared to decrease rapidly. The phototransformation was very

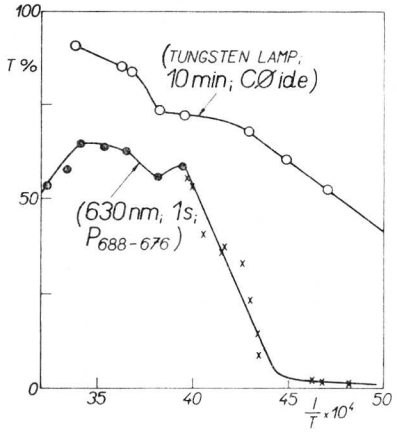

Fig. 5. Comparison of some of our results on the effect of temperature on the rate of the $P_{657-647} \rightarrow P_{688-676}$ phototransformation when 630 nm photons are used, with corresponding data of SMITH and BENITEZ (1952) for chlorophyllide production. The data of SMITH and BENITEZ (○—○) are taken from Fig. 1 of their paper in which $T\%$ is the percentage of chlorophyllide (Cøide) found after illuminating etiolated barley for 10 min with a 40 W tungsten lamp (in Fig. 1 of SMITH and BENITEZ' paper, some values of $T\%$ lower than those reproduced here are shown in the region between $1/T \times 10^4 = 38$ and 39·5). The percentage of the $P_{657-647} \rightarrow P_{688-676}$ phototransformation $T\%$ (●—●) was estimated from the low temperature fluorescence spectra found after a 1 s illumination of etiolated leaves by a constant amount of 630 nm photons; the crosses refer to values obtained using the *Dewar* method (see "Methods" and Fig. 2).

44 slow at -50 °C; it seemed to be zero at about -80 °C. No attempt was made to follow kinetics below -20 °C.

B. Denaturation of the protochlorophyllide-lipoprotein complex at temperatures higher than +45 °C

When leaves were kept in the dark at temperatures higher than +45 °C, the form of the low temperature fluorescence spectrum was changed; an increase of the emission at 630 nm was observed. Simultaneously the relative height of the 657 nm emission decreased and the band was somewhat

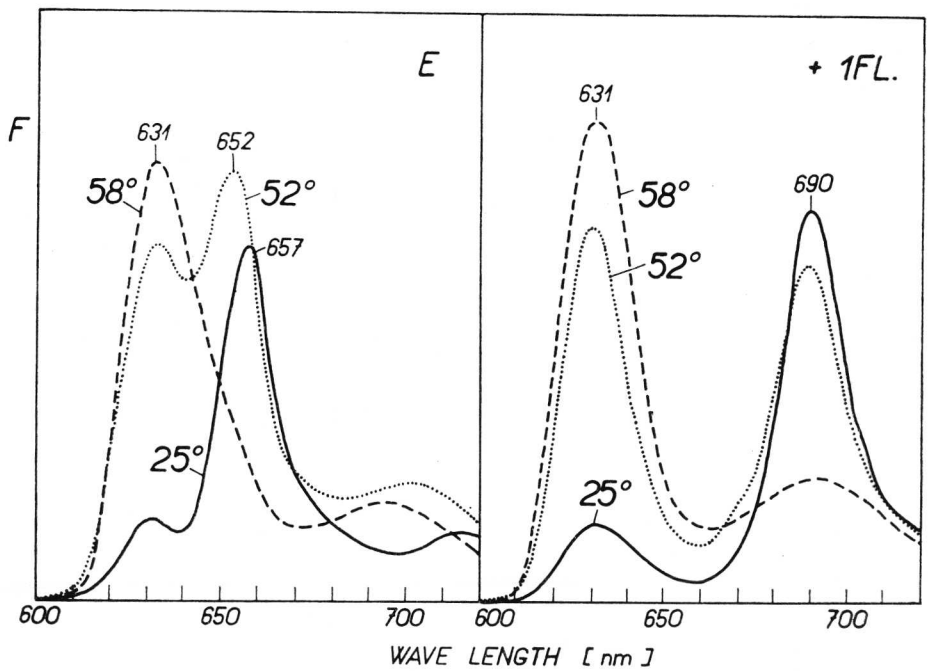

Fig. 6. Influence of keeping the leaves at temperatures higher than 45 °C on the low temperature fluorescence of etiolated leaves before (left) and after (right) one saturating flash. $E =$ the etiolated leaves were kept in darkness for 10 min at the indicated temperatures before freezing at -196 °C in the dark. $+1$ *Fl.* = the leaves were kept in darkness for 10 min at the indicated temperatures, then received a 1 ms flash of white light and were immediately frozen at -196 °C. $F =$ relative fluorescence intensity.

shifted to shorter wavelengths, probably as a consequence of the rise of the 630 nm emission (Fig. 6). It was found that the 630 nm emitting pigment formed by the heat treatment was not phototransformable. The inactivation appears to result from the denaturation of some properties of the protein moiety (see DUJARDIN and SIRONVAL 1969). On the other hand, the unchanged 657 nm pigment still showed the $P_{657-647} \rightarrow P_{688-676}$ phototransformation at temperatures as high as +55 °C.

We tried to determine the proportion of the denaturated, non-phototransformable pigment present after a given heat treatment as follows: etiolated leaves were warmed up for 10 min in darkness at several temperatures chosen between +20 and +60 °C as indicated in "Methods". The relative amount of the 630 nm emitting pigment was estimated from the low temperature fluorescence spectra as follows:

$$D\% = 100 \times \frac{H\,630}{H\,630 + \overline{H}\,657} \tag{3}$$

where $H\,630$ is the height of the 630 nm emission and $\overline{H}\,657$ is equal to: $[H\,657 - (R\,657 \times H\,630)]$, $H\,657$ being the height of the 657 emission, and $R\,657$ a correction coefficient (SIRONVAL *et al.* 1968). $D\%$ is an index of the proportion of the 630 nm pigment relative to the sum of the 657 nm emitting (phototransformable) plus the 630 nm emitting (non-phototransformable) pigments. Fig. 7 reproduces the results (curve D) and compares them with the data of SMITH and BENITEZ (1954; curve S). It appeared that the denaturation of the $P_{657-647}$ complex started in the temperature range around +45 °C. The shift observed when comparing the results of SMITH and BENITEZ with our results in Fig. 7 appears rather feeble in view of the methodological differences.

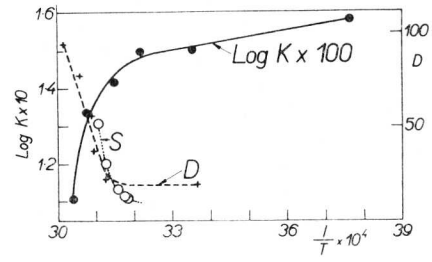

Fig. 7. Heat denaturation of the $P_{657-647}$ complex in the etiolated leaf and influence of the denaturation on the rate of the $P_{657-647} \rightarrow$ $\rightarrow P_{688-676}$ phototransformation by 647 nm photons (illumination periods less than 1 s). The leaves were kept for 10 min in the dark at the indicated temperatures (abscissa), the illumination occurring just after this time interval. ●—● (curve log $K \times 10$): values of log $K \times 10$ (ordinate on the left), K being the first order rate constant of the transformation. +---+ (curve D): values of the amount of denaturation expressed in term of $D\%$ (D: ordinate on the right) calculated from equation (3). ○····○ (curve S): data taken from Fig. 3 of SMITH and BENITEZ (1952); the points refer to the percentage transformation of protochlorophyllide into chlorophyllide (ordinate on the right) in etiolated barley leaves which had been immersed for 10 min at the indicated temperatures (abscissa) and illuminated afterwards for 10 min at room temperature with white light from a tungsten source (100 fc).

The denaturation rate increased when increasing the temperature. After 10 min at 58 °C the denaturation was concerned with nearly all molecules of the complex. It also reached 100% when the leaves were kept at any temperatures above 45 °C for sufficiently long periods.

Using 647 nm photons, the first order rate constant K was estimated for the phototransformation of the $P_{657-647}$ pigment which remained transformable after a given heat treatment. It was found that the rate constant for the phototransformation of this pigment dropped when the leaves were kept for 10 min at temperatures higher than +45 °C (Fig. 7; curve log $K \times 10$).

DISCUSSION AND CONCLUSIONS

Fig. 5 shows that, when using 630 nm photons which produced a non-first order $P_{657-647} \rightarrow$ $\rightarrow P_{688-676}$ phototransformation, the temperature dependence of the overall process appeared

46 similar to the dependence found in white light for chlorophyllide formation. But this did not hold true when the temperature dependence was studied in the presence of 647 nm photons which tend to produce first order kinetics (Fig. 3). Moreover it is seen from Fig. 4 that, when the phototransformation was not of the first order (*i.e.* in the presence of 630 nm photons), the two first-order rate constants K_1 and K_2 behaved independently. One of the constants (K_1) showed a temperature dependence similar to that of the rate constant (K) found with 647 nm photons. These results support the model presented by SIRONVAL *et al.* (1968) which assumes two classes of the protochlorophyllide-lipoprotein (complexes *a* and *b*), one of which involves energy transfer from an accessory pigment (complex *b*) when 630 nm photons are given. Assuming the occurrence of these classes, the similarity of the behaviours of K_1 and K shows that these constants should be related to a same class of complex (complex *a*). This class should not involve any energy transfer from an accessory pigment (see the discussion of the model in SIRONVAL *et al.* 1968).

On the other hand, in the presence of 630 nm photons, the overall temperature dependence which involves K_1 and K_2 simultaneously is likely to be similar, — and is actually similar—, to the behaviour found in white light (Fig. 5), since in both cases the phototransformation should include at the same time processes lacking energy transfer (in complex *a*) and processes involving energy transfer (in complex *b*). It is therefore justifiable to discuss separately the temperature dependences of K_1 (or K) and of K_2.

We first turn to the temperature dependence of K (or K_1). If the value of ε_{vivo} in equation (1) is taken to be equal to $\varepsilon_{\text{ether}}$, — the molar absorption coefficient of protochlorophyllide in ether (about 88,000 $\text{M}^{-1}\,\text{l}\,\text{cm}^{-1}$) —, the quantum yield n calculated using equation (1) from the data of Fig. 3 should amount to more than 10 molecules of pigment phototransformed per incident photon absorbed at room temperature. This means that, for the quantum yield to be 1 at room temperature, ε_{vivo} should have a value many times larger than $\varepsilon_{\text{ether}}$. It is doubtful that this could be achieved by some suitable *in vivo* orientation of the pigment: a strong dichroism should be expected under this assumption, which is not actually found.

If we do not postulate any orientation of the pigment, another mechanism must be found which could account for the unexpected high value of n. This mechanism should explain how the absorption of 1 photon by 1 molecule of pigment may induce changes in much more than 1 pigment molecule. When trying to describe the situation, it seems useful to consider (*1*) that the pigments are linked to lipoproteins, (*2*) that the molecules of the lipoprotein-pigment complexes are included in the lamellae of the prolamellar body, and (*3*) that the high degree of organization of the lamellae is accounted for by forces linking the proteins in definite ways. These forces make it possible to visualize an intralamellar cooperativity (see for instance the model of CHANGEUX *et al.* 1966). Indeed, cooperativity seems to be suggested by the form of the temperature dependence of K (or K_1), although we are not yet able to discuss this form correctly.

In any case, at infinite temperature each lipoprotein should behave independently from its neighbours. Admitting the exponential dependence of K, and hence of n, on the inverse of the absolute temperature, the extrapolation to infinite temperature of the experimental data for K between $+37$ and -17 °C in Fig. 3 leads to a value of ε_{vivo} in equation (1) of 166,000 $\text{M}^{-1}\,\text{l}\,\text{cm}^{-1}$. This is about twice the value of $\varepsilon_{\text{ether}}$, suggesting that, in fact, n should approach a minimum value of 2 at infinite temperature. In other words: if ε_{vivo} is equal to $\varepsilon_{\text{ether}}$, we should admit that each molecule of the pigment-protein complex involves at least 2 pigment molecules necessarily phototransformed simultaneously at infinite temperature per absorbed photon. The 2 pigment molecule picture could help in understanding some optical properties of the complex including the optical changes which appear upon heat denaturation (Fig. 7; see also DUJARDIN and SIRONVAL 1969). It moreover agrees with chemical analyses (SCHOPFER and SIEGELMAN 1968).

On the other hand the denaturation of some pigment-lipoprotein molecules is likely to change the strength of the links between proteins in the lamellae, and hence to act on the value of K. Actually partial denaturation is correlated to a reduction of the rate of the phototransformation of the non-denaturated, still phototransformable complex molecules (Fig. 7).

The temperature dependence of K_2 does appear to involve factors which do not play any role in the temperature dependence of K (or K_1). The explanation of the facts possibly needs the assumption (SIRONVAL *et al.* 1968) following which the molecules of complex (*b*), — phototransformed at rate K_2 —, receive energy from an accessory pigment. This energy transfer should constitute an additional event complicating the picture of the temperature dependence.

It might also be pertinent to consider the coincidence between the temperature range of the denaturation of the protochlorophyllide-lipoprotein complex and the range of the thermal denaturation of the photochemical centres of system II as recently reported by LAVOREL (1969).

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