

riboflavin, thiamine, and carotene content of leaf blades was not statistically significant over a period of six weeks. In midribs and roots, the concentration of each of the vitamins decreased as the plants grew larger and the decrease was statistically significant for each vitamin except carotene, in midribs, and thiamine, in roots.

Distribution of the whole plant content of carotene, riboflavin, and thiamine among plant organs did not change appreciably as plant dry weight increased, but there was a marked change in the distribution of ascorbic acid which rather closely followed the distribution of plant dry matter.

In all experiments the total amount of each vitamin per plant was positively and significantly correlated with the total amount of plant dry matter. Increase in the total amount of vitamin per plant per unit increase in plant dry matter was characteristic for each vitamin and was not influenced by the environmental conditions under which the plants were grown. Regression equations derived for the estimation of the amount of each of the vitamins per plant from plant dry weight were applicable to plants weighing from 2 grams to 69 grams.

THE EFFECT OF TEMPERATURE ON THE CONVERSION OF PROTOCHLOROPHYLL TO CHLOROPHYLL A IN ETIOLATED BARLEY LEAVES¹

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Protochlorophyll is converted to chlorophyll when dark-grown leaves containing it are illuminated, but it is not converted to chlorophyll when dissolved in organic solvents and illuminated. Conditions exist in the leaf, therefore, which facilitate this conversion. In order to analyze the mechanism of the transformation, the effects of various factors on the reaction have been examined. Among the factors examined is temperature, and it is chiefly with this factor that this paper deals.

Only fragmentary data concerning the effect of temperature on this conversion have been published. Liro (12) found that irradiation of etiolated seedlings at -15°C produced chlorophyll. Scharfnagel (15) detected chlorophyll formation in dark-grown corn seedlings when they were illuminated at -6°C . Lubimenko (13) observed that temperature had no sensible influence on the photochemical transformation of protochlorophyll to chlorophyll. Koski and Smith (8) showed that in dark-grown barley leaves the conversion was completely inhibited by temperatures around 90°C , and that the rate of photochemical conversion was little if at all affected by changing the temperature from 5 to 18°C (6, 9, 18).

The observations that the transformation takes place at freezing temperatures, that the rate is little affected by change of temperature in the room-tem-

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1. CONNER, R. T. and STRAUB, G. J. Determination of thiamine by the thiochrome reaction. *Ind. Eng. Chem. Anal. Ed.* 13: 380-384. 1941.
 2. HEINZE, P. H., KANAPAU, M. S., WADE, B. L., GRIMBALL, P. C., and FOSTER, R. L. Ascorbic acid content of 39 varieties of snap beans. *Food Research* 9: 19-26. 1944.
 3. MOORE, L. A. and ELY, RAY. Extraction of carotene from plant material. *Ind. Eng. Chem. Anal. Ed.* 13: 600-601. 1941.
 4. PETERSON, W. J., BRADY, D. E., and SHAW, A. O. Fluorometric determination of riboflavin in pork products. *Ind. Eng. Chem. Anal. Ed.* 15: 634-636. 1943.
 5. SNEDECOR, GEORGE W. *Statistical Methods*. Iowa State College Press, Ames, Iowa. 4th ed. 1948.
 6. SOUTHERN COOPERATIVE GROUP. Studies of sampling techniques and chemical analyses of vegetables. *Southern Coop. Ser. Bull.* 10. March, 1951.
 7. WATSON, S. A. and NOGGLE, G. R. Effect of mineral deficiencies upon the synthesis of riboflavin and ascorbic acid by the oat plant. *Plant Physiol.* 22: 228-243. 1947.
 8. WYND, F. L. Relationship between dry weight, protein and vitamins in leaves of fresh cereals. *Food Research* 11: 195-202. 1946.

perature range, and that high temperature stops the transformation suggested that a systematic investigation of the effect of a wide range of temperatures on the transformation of protochlorophyll *in situ* would greatly extend our understanding of this process. Accordingly, the transformation of protochlorophyll to chlorophyll a in intact dark-grown barley leaves has been studied in the temperature range from -195 to $+55^{\circ}\text{C}$.

MATERIALS AND METHODS

LEAF MATERIAL: Leaves from etiolated barley seedlings (*Hordeum vulgare*) were used in these experiments. The seedlings were grown in pots of sand and were watered with tap water. The temperature of the darkroom in which they were grown was about 22°C . The leaves were harvested from seedlings which had been grown from 9 to 13 days after planting. They were cut about 5 cm below the tip. During the care and handling of the plants, the only light to which they were exposed was from a flashlight screened with a dark-green cellophane filter. The exposures from the flashlight to which the leaves were subjected were ineffective for the transformation.

ANALYTICAL METHODS: The percentage transformation was measured by the method employed by Koski, French, and Smith (7) in determining the action spectrum for the conversion of protochlorophyll to chlorophyll a. In brief, it consisted of grind-

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ing the leaves in a mortar with sand and acetone, transferring the pigments to ether, and measuring the relative concentration of the pigments spectrophotometrically. Spectrophotometric measurements were made with a Beckman Model DU spectrophotometer at wave lengths 700, 663, and 624 $m\mu$. At these wave lengths the specific absorption coefficients of the pigments are known (9) and from the absorption values observed the relative concentrations of the protochlorophyll and chlorophyll were calculated. The absorption of the pigments at 700 $m\mu$ is negligible so that the optical density observed at this wave length served for correcting the optical densities obtained at 663 and 624 $m\mu$. For convenience, a curve relating the ratio of the readings at 624 and 663 $m\mu$ to the percentage transformation was used for calculating the percentage conversion. The equation used for constructing the curve was

$$T = \frac{39.9 \times 100}{(D_{624}/D_{663}) 95 + 26}$$

in which T is the percentage transformation, D_{624} and D_{663} are the optical densities observed, 39.9 and 95 are the specific absorption coefficients of protochlorophyll and chlorophyll a at 624 and 663 $m\mu$ respectively, and 26 is the difference between the specific absorption coefficients of protochlorophyll (39.9) and chlorophyll a (13.9) at 624 $m\mu$ (equation 1 (7)). In the development of this equation, it is assumed that the specific absorption coefficient of protochlorophyll is 0 at 663 $m\mu$, and that no chlorophyll existed in the leaf previous to illumination.

From the optical density measurements, D_{624} and D_{663} , the optical density at 624 $m\mu$ due to protochlorophyll if no conversion had occurred (designated as pchl) can be calculated from the equation:

$$\text{pchl} = D_{624} + 0.275 D_{663}$$

The constant, 0.275, is the difference of the specific absorption coefficients of protochlorophyll and chlorophyll a at 624 $m\mu$, divided by the specific absorption coefficient of chlorophyll a at 663 $m\mu$. This optical density can be related to the amount of protochlorophyll in the sample before undergoing any treatment and in this way the degree of loss of pigment brought about by the treatment can be estimated. In our experience, the value of D_{624} for protochlorophyll in 1 gm of untreated dark-grown barley leaves is approximately 0.1, and this value has frequently been used by us to judge the destructiveness of any treatment to which the leaves have been subjected.

APPARATUS FOR IRRADIATION: Various light sources were used in the different experiments. In the experiments with polychromatic light, a 40-watt tungsten lamp was used. It was placed at the correct distance from the leaves to furnish the desired intensity of irradiation.

Monochromatic light was obtained from either a sodium or a mercury lamp. The sodium vapor lamp used was a Lab-Arc, manufactured by the General Electric Vapor Lamp Co. Light predominantly of wave length 589 $m\mu$ was obtained from the sodium

lamp. To further monochromatize this light either a copper sulfate solution or a Zeiss Monochromat Filter A was placed in the light path. The intensity of the irradiation was controlled by varying the distance of the leaves from the lamp or by inserting wire screens in the light path.

The mercury lamp used was of the type Mazda 100-watt A-H4. The different spectral lines used were isolated from the general radiation by means of various filters.

The wave length 436 $m\mu$ was isolated by means of Corning glass filters No. 5113 and 3389; the wave lengths 577/579 $m\mu$ by Zeiss Monochromat Filter A; and the wave length 546 $m\mu$ by Corning glass filter no. 4303 with Zeiss Monochromat Filter B.

The intensities of irradiation used in the different experiments were measured by means of a General Electric light meter (foot candle meter, 8DW 40 Y16). This instrument was not used to measure intensities in absolute units but only in comparative units. Also it was used, comparatively, only with light from the same source and of the same quality.

The relative number of quanta per second necessary to produce a given response of the light meter at different wave lengths were:

wave length in $m\mu$	589	579	546	436
relative no. quanta	1.046	1.000	1.060	1.196

TEMPERATURE TREATMENTS: In the experiments carried out at temperatures from -195 to $+5^\circ\text{C}$, the following procedure was used: The harvested leaves (1 gm) were placed in a vial or test tube, about 20×150 mm, surrounded with a metal sheath and immersed for from 30 to 60 minutes in a bath at the desired temperature. The vial or test tube was fitted with a stopper carrying a long glass tube which was open to the air but protected the leaves from the vapor of the bath. The container for the bath was an unsilvered Dewar flask of either 350 or 430 ml capacity. The lowest temperature, -195°C , was obtained by using a bath of boiling liquid nitrogen. The temperature -77°C was attained by adding solid carbon dioxide directly to an acetone bath. Higher temperatures, up to $+5^\circ\text{C}$, were obtained by putting solid carbon dioxide into a long test tube which dipped into the acetone. The desired intermediate temperatures were maintained constant by manually introducing solid carbon dioxide into the test tube as required and by adjusting the carbon dioxide container to the proper depth of immersion.

After the leaves had attained temperature equilibrium with the bath in the dark, the metal shield was removed and the leaves were illuminated in the bath for the desired time. The leaf container was rotated continually, either manually or mechanically, during the exposure so that all the leaves would receive as nearly equal illumination as was possible with this set up. The light source used will be indicated in the description of the individual experiments.

In these experiments, the geometry of the apparatus was such that the exact intensity incident on the leaves could not be measured; however, the geometry

was kept constant so that in comparative experiments the intensities of irradiation should have been strictly comparable.

After the leaves had been irradiated, they were placed in the dark and flooded with 12 ml of acetone and the pigments extracted and measured in the manner already described.

In the experiments in which the leaves were treated at temperatures from +35 to +55°C, the leaves, usually 1 gm, were harvested, placed in a wire basket, and immersed in a two-liter bath of distilled water. The leaves were held in the dark at the proper temperature for the desired time, from 5 to 120 minutes, and then were quickly removed from the bath and plunged into cold distilled water. After that, they were spread out on a glass plate parallel to each other and as close together as possible and then were illuminated for 10 minutes at room temperature with light from a 40-watt tungsten lamp at 100 fc intensity. Although the transformation limits may not have been reached by this exposure, they were approached closely enough to give a true qualitative picture of the inactivating effect of temperature. The leaves were then transferred to acetone and the quantities of pigments determined.

KINETIC STUDIES: The progress of the transformation was followed in etiolated barley leaves by irradiating a series of one-gram samples with monochromatic light of constant intensity (15 fc) for various periods of time. Fixed periods of illumination were used in all experiments, namely, 0.5, 1.5, 3.0, 5.0, 7.5, 10.0, 15.0, and 25.0 minutes. The irradiations were carried out at a temperature of $6 \pm 2^\circ\text{C}$ in a coldroom. Since the reaction rate is little affected by temperature, this relatively crude temperature regulation was satisfactory. In order to study the conversion without interference by formation of additional protochlorophyll, a temperature considerably below room temperature was necessary.

In these experiments, the cut leaves were stored for about an hour in the dark coldroom, then laid out close together and parallel to each other on a glass plate and irradiated for the desired time at the designated intensity. After irradiation, the extent of the transformation was determined by the analytical methods already described. The wave lengths used were 589, 579/577, 546, and 436 μ .

RESULTS

The percentage transformation of protochlorophyll to chlorophyll a at various temperatures, from -77°C to about 23°C , is shown in figure 1. The results designated with a plus sign inside a circle (\oplus) were obtained with leaves which had been maintained for 60 minutes in the dark at the temperature indicated before being illuminated for 10 minutes at 100 fc intensity with light from a tungsten lamp. These results demonstrate that even at low temperatures a remarkably large photochemical transformation of protochlorophyll to chlorophyll a takes place in the leaf. However at the lowest temperature used, -195°C

(not shown in the figure) there was no conversion of protochlorophyll to chlorophyll.

At -10 and -20°C , the transformation obtained by the procedure just described fell far below the curve projected from the results obtained at other temperatures. If, however, the leaves had been frozen at about -77°C and maintained at this temperature for a half hour before being raised to -10 or -20°C and equilibrated at these temperatures for 25 minutes before being illuminated, the percentage transformation was increased as the points marked with the double circle (\odot) indicate. This result suggests that slow freezing damages the tissue. This is compatible with similar observations obtained with other biological material. The effect is variable, however, because in some experiments under other circumstances the conversions obtained at -10 and -20°C were consist-

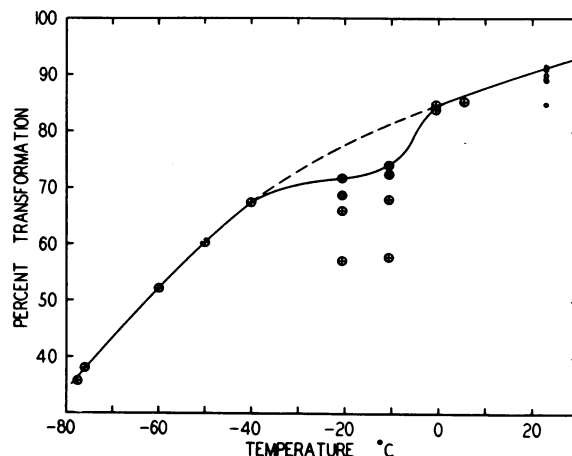


FIG. 1. The percentage transformation of protochlorophyll to chlorophyll a in etiolated barley leaves when illuminated at temperatures from -77° to 23°C for 10 min with light of 100 fc intensity supplied by a 40-watt tungsten lamp.

ent with those obtained over the whole temperature range.

When the leaves were frozen at a low temperature and then thawed, they lost entirely their capacity for bringing about the protochlorophyll-chlorophyll transformation. This effect is well illustrated in the following examples: After the leaves had been frozen at the temperature of solid carbon dioxide, they were thawed in the dark and illuminated with the tungsten lamp at about 100 fc intensity. When the thawing and illumination took place in air, the transformation observed was 0%. Under these conditions, the retention of the pigment was low, about 20%. In order to avoid photooxidation, the thawing and illumination was carried out in an inert atmosphere; in carbon dioxide, 5% transformation was observed and 83% retention; in hydrogen, 0% transformation and 100% retention. These results demonstrate that even though oxidation of the pigments is prevented by using an oxygen-free atmosphere, practically no photochemical

conversion to chlorophyll occurs in leaves which have been thawed. Separate experiments demonstrated the conversion to be nearly complete when fresh etiolated leaves were illuminated in a hydrogen atmosphere.

The inhibition of the transformation at very low temperatures may be reversed at higher temperatures provided the leaves are not thawed or brought even to incipient thawing. This was demonstrated by subjecting etiolated barley leaves to various irradiation treatments at the two temperatures -69 and -40°C . The upper temperature limit was set at -40°C so as to avoid the variable effects of incipient thawing. The results of several experiments are given in table I. These results show that if there is no illumination at the lower temperature, the reversal of the temperature inhibition is complete (series no. 6 and 7). If there is illumination at the lower temperature there

TABLE I

THE REVERSAL OF THE LOW-TEMPERATURE INHIBITION OF THE TRANSFORMATION OF PROTOCHLOROPHYLL TO CHLOROPHYLL A. IRRADIATION WITH WAVE LENGTH 577/579 $\text{m}\mu$ AT 200 FC INTENSITY

SERIES	TEMPERATURE	TREATMENT		TRANSFORMATION
		DARK	LIGHT	
no.	$^{\circ}\text{C}$	min.	min.	%
1	-69	60	30	49.1
2	-69	30	30	49.2
3	-40	60	30	68.5
4	-69	60	30	61.9
	-40	30	30	
5	-69	60	15	62.8
	-40	30	15	
6	-69	60	0	66.5
	-40	30	30	
7	-69	30	0	68.2
	-40	30	30	
8	-69	60	15	52.3
	-69	30	15	

seems to be a slight permanent damage to the transformation mechanism (series no. 4 and 5).

A divided period of illumination increases the degree of transformation relatively little over a continuous period of illumination of the same duration. (Compare no. 8 with 1, 2.) This was also demonstrated in another experiment at -69°C in which two 5-minute periods of illumination were divided by 60 minutes of darkness; the percentage transformation was 34.4. With another lot of leaves, ten minutes' continuous illumination gave a transformation of 33.5%. In both cases the intensity used was 200 fc and wave length 579/577 $\text{m}\mu$ (mercury lines).

The chief conclusion from all these experiments is that a very low temperature does not of itself permanently damage the photochemical transformation system.

The transformation obeys the reciprocity law at low temperatures. At about -76°C , illumination with 100 fc intensity (tungsten lamp) for 10 minutes yielded 38.0% conversion while 33.3 fc intensity for

30 minutes gave 37.5% conversion. And 100 fc intensity for 30 minutes gave 49.7% conversion as against 49.9% conversion brought about by 10 minutes' irradiation with 300 fc intensity. The adherence to the reciprocity law is also shown by the data in figure 2 in which the percentage transformation is plotted against the product of time and intensity. Although neither was held constant in the different runs, the points obtained fall close to a smooth curve.

The protochlorophyll-chlorophyll conversion obeys formally a second-order rate law in which the proportionality factor, k , includes the intensity of irradiation (cf equation 1). Integration of equation 1 leads to equations 2 or 3, when T is zero at zero time. By substituting T' and t' , and T'' and t'' in equation 2, two equalities are obtained from which k can be eliminated and equation 4 obtained.

$$\frac{dT}{dt} = k(T_{\infty} - T)^2 = k'I(T_{\infty} - T)^2 \quad (1)$$

$$k = \frac{1}{t} \left[\frac{1}{T_{\infty} - T} - \frac{1}{T_{\infty}} \right] \quad (2)$$

$$T = \frac{kT_{\infty}^2 t}{1 + kT_{\infty} t} \quad (3)$$

$$T_{\infty} = \frac{T' \frac{T''}{t''} (t'' - t')}{T' - \frac{T''}{t''} t'} \quad (4)$$

From equation 4, the transformation at infinite time, T_{∞} , can be obtained from the transformations T' and T'' observed at times t' and t'' . Substitution of T_{∞} in equation 2 permits k to be evaluated and placing T_{∞} and k in equation 3 allows the transformation, T , to be computed for any time t .

In figures 4 and 5 the close fit of the calculated curves to the experimental points (circles) demonstrates the suitability of the second-order rate expression for calculating the progress of the reaction. The constants used for computing curves 1 and 2, figure 4, are

curve 1: T_{∞} , 94.1; k , 0.0190; I , 50 fc
curve 2: T_{∞} , 95.7; k , 0.0066; I , 17 fc

The ratio of the light intensities used was 2.94 and the ratio of the velocity constants observed was 2.88. The close agreement of these two values indicates how nearly the velocity of the transformation depends on the first power of the light intensity.

At all temperatures, the transformation follows a second-order rate law when the progress of the reaction is related to the total incident energy. This is shown in figure 2 where the percentage transformation, T (ordinate), is plotted against the product of intensity \times time, i.e., foot candle-minutes (abscissa). The points represent observed values gotten at the three temperatures indicated, and the smooth curves values computed according to the second-order law. The values of T_{∞} and k for the three temperatures are shown in table II. The velocity constant increases with the temperature. The energy of activation corresponding to the increase in temperature from -70.0

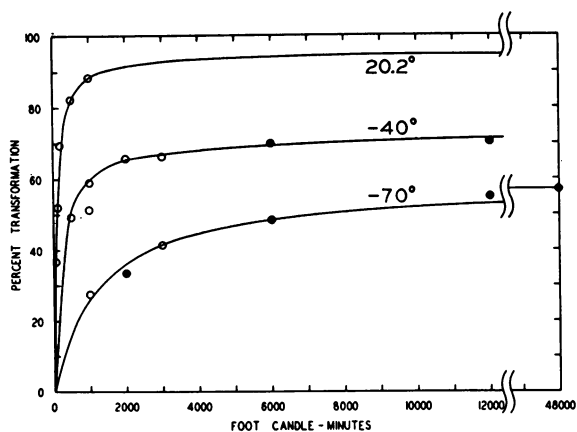


Fig. 2. The progress of the transformation of protochlorophyll to chlorophyll a in etiolated barley leaves when illuminated with light of wave length 579/577 $m\mu$. The intensity used was either 100 or 200 fc and the time of illumination was varied from 0.5 to 240 minutes. The circles represent the values determined experimentally either at 100 fc (\circ) or 200 fc (\bullet); the lines depict values calculated from the second-order rate law, equations 1 to 4, using the constants listed in table II.

to -40.0°C is about 5270 cal and from -40 to $+20.2^{\circ}\text{C}$ about 1330 cal. These values are more consistent with photochemical activation of the process than with thermochemical activation (2). The limit of the transformation, T_{∞} , also increases with the temperature. The data in table II show that $\ln T_{\infty}$ decreases linearly with increase of $1/\theta$.

Preliminary results were obtained on the effect of wave length on the transformation of protochlorophyll to chlorophyll a at low temperatures. At the wave lengths studied, 579/577, 546, and 436 $m\mu$, the degree of transformation for a constant period of illumination increased with increase in temperature. The increase in the percentage conversion with change of temperature was practically the same for wave lengths 436 and 579/577 $m\mu$, but was slightly, probably not significantly, larger at 546 $m\mu$. If the positions of the absorption bands shift with temperature (5), there is no a priori reason why the ratios of the rates at two temperatures should be equal for different wave lengths.

The transformation of protochlorophyll to chlorophyll was studied also in leaves which had been heated

TABLE II

THE INFLUENCE OF TEMPERATURE ON THE VELOCITY CONSTANT AND THE LIMIT OF PHOTOTRANSFORMATION OF PROTOCHLOROPHYLL TO CHLOROPHYLL A

t	k	T_{∞}	$1/\theta^*$	$\ln k$	$\ln T_{\infty}$
+20.2	0.000132	95.3	0.00341	- 8.934	4.558
-40.0	0.0000735	71.3	0.00429	- 9.519	4.267
-70.0	0.0000138	58.4	0.00493	- 11.195	4.067

* θ is the absolute temperature.

to various temperatures above room temperature. The results of these experiments are shown in figure 3. The values obtained at the same temperature are designated by the same symbol and are connected by a smooth curve.

A temperature of 35°C applied for 60 minutes before illumination had no detrimental effect on the transformation. At 39°C the effect of heating the leaves was slight, yet noticeable. At 40°C the temperature effect was definitely evident, and the damage to the transformation was about proportional to the length of time of heating. As the temperature of heating was raised, the rate of inactivation was greatly increased so that after heating for 30 minutes at 48.85°C only 6% of the pigment was transformed by illumination. After heating at 55.3°C for 5 minutes, less than 4% was transformed by illumination. The heat of activation for the inhibition of the trans-

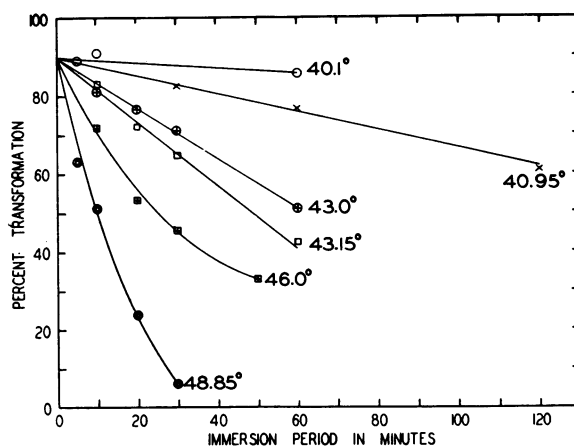


Fig. 3. The percentage transformation of protochlorophyll to chlorophyll a in etiolated barley leaves which had been immersed at the various temperatures shown in the figure for various periods of time and then illuminated for 10 minutes at room temperature with light of 100 fc intensity from a tungsten lamp.

formation varies from approximately 280,000 to 60,000 cal. in going from the lower temperatures to the higher. These results suggest that denaturation of a protein is the cause for the decreased transformation (10, 14).

Both the rate and the limit of the transformation are decreased by heating the leaves, as the results shown in figure 4 and in table III demonstrate.

In figure 4, the results obtained with heated (curve 3) and unheated (curve 1) leaves are compared. Both samples of leaves were irradiated at room temperature with light of wave length 589 $m\mu$ and 50 fc intensity. The heated leaves had been held at a temperature of 43.2°C for a period of 60 minutes before being illuminated. The circles designate values determined experimentally and the smooth curves values calculated according to the second-order rate law, equation 3, by use of the constants estimated from equations 2 and 4, namely,

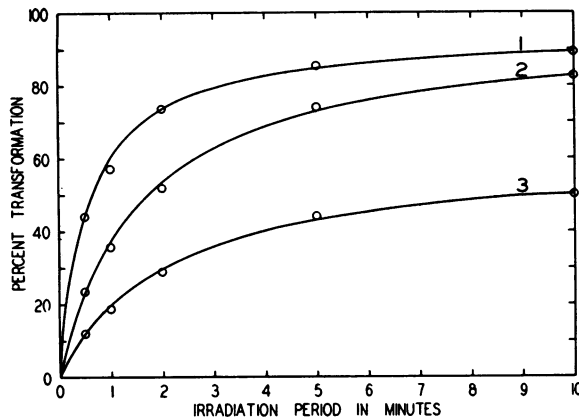


FIG. 4. The effect of light intensity and previous heating on the transformation of protochlorophyll to chlorophyll a in etiolated barley leaves illuminated with light of wave length 589 $m\mu$. Curves 1 and 2 depict results obtained with unheated leaves illuminated with light intensities of 50 and 17 fc respectively; curve 3, results obtained with leaves heated for 60 minutes at 43.2°C previous to being illuminated with light of 50 fc intensity. The circles represent experimental data; the lines, data calculated from the second-order rate equation employing the constants listed on pages 138 and 140.

curve 1: T_{∞} , 94.1; k , 0.0190; intensity, 50 fc
 curve 3: T_{∞} , 61.0; k , 0.0078; intensity, 50 fc

Both the rate and limit of the transformation are lowered by heating the leaf, the rate being reduced to 41.3 % that in the unheated leaf and, correspondingly, the limit to 64.8 %.

The conclusion that heating the leaf lowers both the rate and extent of the transformation can be drawn from further experiments in which duplicate samples of leaves were heated for definite periods of time at 43.2°C. One sample of the pair was illuminated for 0.5 minute and the other for 15 minutes with wave length 589 $m\mu$ at 50 fc intensity. The results of these experiments are given in table III. The fact that heating lowers the extent of the transformation is evidence that part of the holochrome is destroyed by heat. In this experiment, the destruction progresses nearly logarithmically with time.

TABLE III

THE EFFECT OF HEATING BARLEY LEAVES AT 43.2°C, FOR VARIOUS PERIODS OF TIME, ON THE RATE AND LIMIT OF TRANSFORMATION OF PROTOCHLOROPHYLL TO CHLOROPHYLL A. SODIUM LIGHT OF 50 FC INTENSITY WAS USED

PERIOD OF HEATING MIN.	PERCENT TRANSFORMATION OBTAINED BY ILLUMINATION FOR		LIMIT OF TRANSFORMATION CALC. T_{∞}	SECOND-ORDER VELOCITY CONSTANT k
	0.5 MIN.	15 MIN.		
0	44.9	89.2	92.4	0.0205
20	20.9	71.0	77.3	0.0096
40	16.2	60.5	66.8	0.0096
80	9.7	45.9	52.7	0.0086

Heating of the leaves preceding illumination lowers the rate of the reaction more in the early stages of heating than in the later stages. In the first 20 minutes of heating the rate was reduced to 46.8 % that of the initial rate, whereas an additional 60 minutes of heating lowered the rate only an additional 5 %.

In the previous sections of this article, the second-order rate law (equation 1, p. 138) has been used to correlate the data of various experiments. How applicable is this equation to the transformation reaction?

This question was answered by following the progress of the transformation in leaves which had been illuminated for various periods of time with light of various wave lengths as described on page 137. The results of these experiments are given in figure 5 and in table IV. In figure 5 the data obtained in individual determinations are designated with dots; the average values of the percentage transformation for each period of illumination are represented by circles; and the percentages of transformation calculated from the second-order rate equation are represented by the smooth curves.

In table IV are listed the calculated and observed values of the transformation for each wave length, along with the constants used for calculation. At wave lengths 589, 579/577, and 546 $m\mu$ the calculated and observed values agree very well, but at 436 $m\mu$ there is considerable discrepancy between the two values. The cause of this discrepancy is unknown.

The excellent agreement between the observed and calculated results at the three longer wave lengths justifies the correlation of experimental data by the second-order rate equation at these wave lengths as has been done in the previous sections of this article.

DISCUSSION

The primary objective of the work reported in this paper was to analyze the mechanism of the photochemical transformation of protochlorophyll to chlorophyll a. Because the photochemical transformation of protochlorophyll occurs only when the pigment is in its natural state and not when it is dissolved in organic solvents, it is concluded that the pigment exists in the leaf in some sort of active complex, called the protochlorophyll holochrome (18).

What is the chemical nature of this holochrome? One approach for ascertaining this is to determine the effect of heating the leaf at different temperatures on the protochlorophyll-chlorophyll transformation. Heating at temperatures from 40 to 55°C lowered the limit to which the transformation proceeded. The lowering of the limit was greater the longer the time of heating, and the rate of lowering was faster the higher the temperature (fig 3). Heating the leaf in this temperature range brought about changes in the limit, and in the rate of lowering the transformation limit, similar to the effect of heating on the limits and rates of the heat denaturation of certain proteins (10, 14). Because of this, it was inferred that the protochlorophyll holochrome is a pigment-protein complex. This was also indicated by the fact that freezing and

TABLE IV

COMPARISON OF DATA OBSERVED WITH VALUES CALCULATED FROM THE SECOND-ORDER RATE EQUATION AT DIFFERENT WAVE LENGTHS. INTENSITY = 15 FC IN EVERY CASE

WAVE LENGTH	589 m μ	579/577 m μ	546 m μ	436 m μ
T _∞	95.6	98.7	99.5	92.5 *
k	0.00616	0.00367	0.00264	0.00449

Time min.	T calc.	T obs.	T calc.	T obs.	T calc.	T obs.	T calc.	T obs.
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.5	21.7	22.0	15.1	15.4	11.6	11.3	15.9	22.2
1.5	44.8	44.7	34.7	34.7	28.2	28.3	35.5	39.5
3.0	61.0	61.6	51.4	51.6	43.9	43.4	51.3	51.1
5.0	71.3	70.9	63.6	63.4	56.5	55.8	62.4	59.6
7.5	77.9	77.7	72.1	72.4	66.0	65.1	70.0	66.3
10.0	81.7	81.8	77.3	77.4	72.1	72.8	74.5	72.7
15.0	85.9	85.9	83.4	83.0	79.4	80.7	79.7	77.0
25.0	89.5	89.4	88.9	88.9	86.4	86.2	84.3	83.7

* An average value gotten from values increasing regularly from 88.7 to 96.3.

thawing completely destroyed the capacity for transforming protochlorophyll to chlorophyll a.

Not only did heating decrease the limit of the transformation but it also decreased the rate at which the transformation took place (fig 4; table III). The rate was lowered much more rapidly in the early

stages of heating than in the later stages. In the later stages of heating, the rate was not greatly affected even though the limit of the transformation was considerably reduced. Whether heating affects an enzyme operative in the reaction, as well as the protochlorophyll holochrome, or whether it disturbs

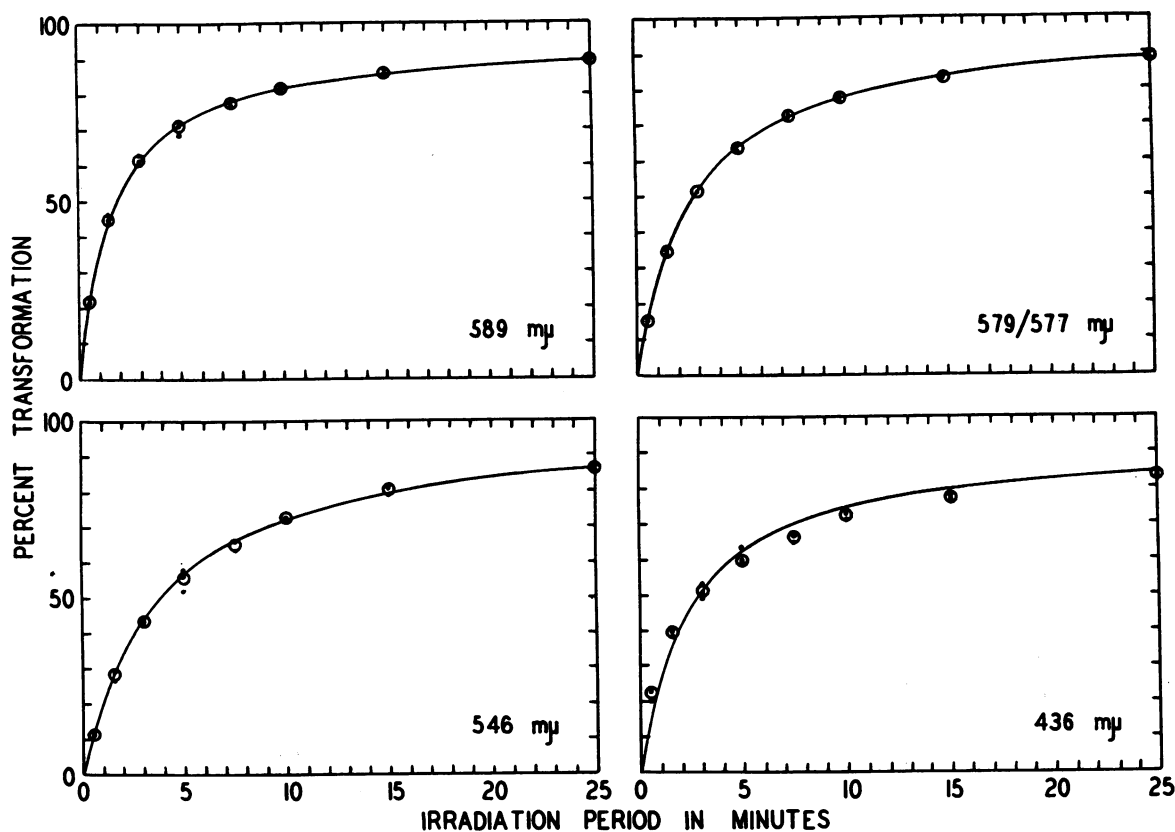


FIG. 5. The progress of the transformation of protochlorophyll to chlorophyll a in etiolated barley leaves when illuminated with various wave lengths of light (intensity 15 fc) at a temperature of $6 \pm 2^\circ\text{C}$. The dots represent experimental results; the circles represent the average of the observed values; and the lines depict values calculated from the second-order rate equation by substitution of the constants shown in table IV.

the optical absorption properties of the holochrome and thus the rate, is still a matter of conjecture.

According to the commonly accepted chemistry of the conversion of protochlorophyll to chlorophyll *a*, the reaction is a hydrogenation (1). The question then arises concerning the mechanism of hydrogen transfer to the protochlorophyll molecule.

The fact that the reaction proceeds at low temperatures has been taken as evidence against the enzymatic nature of the reaction. This may not be strictly valid, inasmuch as enzymes are known to possess appreciable activity at low temperatures (4, 16, 17). The temperature coefficients of the enzymatic reactions are, however, of an entirely different order of magnitude from those found for the protochlorophyll transformation. For example, in comparing the activities of lipase, trypsin, and invertase at 20 and -15°C , factors of 200, 3000, and 600 times were obtained, whereas for the protochlorophyll transformation a factor of about 10 was found for the temperature interval from 20 to -70°C . Furthermore, at -70°C , no activity was observed for any of the enzymes named during a 27-day period, while the photochemical transformation of protochlorophyll proceeded quite rapidly at this temperature. Because of these facts, it is concluded that the reaction is controlled by photochemical activation rather than by enzyme activation (2, 3).

Although the reaction appears to be strictly photochemical, it does not proceed at -195°C . This lack of transformation presents something of an anomaly.

If the reaction were strictly a photochemical, intramolecular hydrogen transfer, the progress of the reaction at constant light intensity and temperature probably should conform to the first-order rate law (11). Instead of this, the reaction follows the second-order rate law under most conditions (figure 5). It is difficult to square this rate law with strictly physical processes; more likely, it relates to chemical processes. Consequently, it seems highly improbable that the photochemical action is a simple intramolecular hydrogen transfer within the holochrome. The second-order rate law is in accord with the assumption of hydrogen transfer through molecular collision.

Results obtained at low temperatures bear on this point. The transformation limit depends upon the temperature: the lower the temperature, the lower the limit (table II), and at -195°C the limit of the transformation is zero. If the conversion depends on intramolecular hydrogen transfer, it is difficult to see how the limit would be so drastically affected by the temperature. If it depends on collision between molecules, then at low temperatures in the frozen state of the leaf the sphere of action with the light-excited reactant would become more circumscribed and the transformation limit would tend to become smaller.

Inasmuch as the kinetics indicate the reaction to be bimolecular with respect to protochlorophyll, the molecular species which reacts with the protochlorophyll must occur in the same concentration as the protochlorophyll. This is probable only if the second

molecular species is protochlorophyll or is in equilibrium with the amount of protochlorophyll present. This points either to the reaction between excited and unexcited protochlorophyll molecules or to the reaction between protochlorophyll and a photodissociated product of the protochlorophyll holochrome. For the reaction to accord with a second-order process, the loss of excitation energy should be rapid as compared to the intermolecular reaction.

Since the limit of the transformation is decreased when the temperature is lowered and is increased again when the temperature is raised, the effect of temperature on the limit of the transformation is reversible (table I). This is consistent with the assumption of a collision mechanism for the reaction in which the sphere of action in the frozen leaf material is reduced reversibly by lowering the temperature.

The rate of the reaction depends on the first power of the light intensity. This indicates that the bimolecular process is not dependent on the collision of two photochemically excited molecules.

From this analysis, it seems highly probable that the formation of chlorophyll from protochlorophyll is not strictly a photochemical, intramolecular transfer of hydrogen within the holochrome but requires collision processes. Whether the second reactant is another molecule of protochlorophyll or whether it is another substance that is excited by the light absorbed by protochlorophyll is still to be determined.

SUMMARY

The effect of temperatures from -195 to $+55^{\circ}\text{C}$ on the photochemical transformation of protochlorophyll to chlorophyll *a* in etiolated barley leaves has been studied. At -195°C no transformation occurs. At -70°C fairly rapid and extensive conversion takes place. The conversion increases in rate and extent with increase in temperature up to 40°C . Freezing the leaves at -10 or -20°C appears to damage the transformation system. This damage can be partially avoided by freezing the leaves quickly at -77°C before raising them to -10 or -20°C for irradiation. The extent of the transformation, which is limited by a given low temperature, can be increased by illumination of the frozen leaf at a higher temperature, provided no thawing occurs. Thawing of the leaf completely prevents the transformation.

Leaves heated to 40°C begin to lose their capacity for bringing about the phototransformation of protochlorophyll to chlorophyll *a*. The longer the period of heating the greater becomes the loss of transformation capacity, and the higher the temperature, the more rapidly the loss takes place. Heating the leaves at 55°C for 5 minutes almost completely destroys their transformation capacity. These experiments indicate that the protochlorophyll holochrome is a pigment-protein complex.

Kinetic studies on the transformation in etiolated barley leaves at wave lengths 589, 579/577, and 546 $m\mu$ show the progress of the reaction to be rigorously a second-order process with respect to the protochlorophyll; but at 436 $m\mu$ the second-order rate law

is not strictly obeyed. The cause of this is not known. This kinetic behavior coupled with the progressive lowering of the transformation limit with lowering of the temperature suggests that the reaction is not strictly a photochemical, intramolecular process but involves intermolecular interactions. Because the reaction depends on the first power of the light intensity, it is unlikely that two photoactivated holochromes have to react.

LITERATURE CITED

1. FISCHER, H. Fortschritte der Chlorophyllchemie. Naturwissenschaften. 28: 401-405. 1940.
2. GRIFFITH, R. O. and McKEOWN, A. Photoprocesses in gaseous and liquid systems. Pp. 647 ff. Longmans, Green and Co., New York. 1929.
3. HEINMETS, F., VINEGAR, R., and TAYLOR, W. W. Studies on the mechanism of the photosensitized inactivation of *E. coli* and reactivation phenomenon. Jour. Gen. Physiol. 36: 207-225. 1952.
4. JOSLYN, M. A. and DIEHL, H. C. Physiological aspects of low temperature preservation of plant products. Ann. Rev. Plant Physiol. 3: 149-170. 1952.
5. KEILIN, D. and HARTREE, E. F. Effect of low temperature on the absorption spectra of haemoproteins; with observations on the absorption spectrum of oxygen. Nature 164: 254-259. 1949.
6. KOSKI, VIOLET M. Gene action in relation to the development of chloroplast pigments in *Zea Mays* L. Pp. 1-41. Ph.D. Thesis, University of Minnesota. 1949.
7. KOSKI, VIOLET M., FRENCH, C. S., and SMITH, JAMES H. C. The action spectrum for the transformation of protochlorophyll to chlorophyll a in normal and albino corn seedlings. Arch. Biochem. Biophys. 31: 1-17. 1951.
8. KOSKI, VIOLET M. and SMITH, JAMES H. C. The isolation and spectral absorption properties of protochlorophyll from etiolated barley seedlings. Jour. Amer. Chem. Soc. 70: 3558-3562. 1948.
9. KOSKI, VIOLET M. and SMITH, J. H. C. The nature of the transformation of protochlorophyll to chlorophyll. Year Book 48: 90-91. Carnegie Inst. Washington. 1949.
10. KUNITZ, M. The kinetics and thermodynamics of reversible denaturation of crystalline soybean trypsin inhibitor. Jour. Gen. Physiol. 32: 241-263. 1948.
11. LEIGHTON, P. A. and LUCY, F. A. The photoisomerization of the *o*-nitrobenzaldehydes. Jour. Chem. Phys. 2: 756-759. 1934.
12. LIRO, J. I. Ueber die photochemische Chlorophyllbildung bei den Phanerogamen. Ann. Acad. Sci. Fennicae, Ser. A. 1: 1-147. 1909.
13. LUBIMENKO, V. Les pigments des plastes et leur transformation dans les tissus vivants de la plante. Rev. Gen. Bot. 38: 1-115. 1927.
14. NEURATH, H., GREENSTEIN, J. P., PUTNAM, F. W., and ERICKSON, J. O. The chemistry of protein denaturation. Chem. Rev. 34: 157-265. 1944.
15. SCHARFNAGEL, W. Biologische Untersuchungen zur Chlorophyllbildung. Planta 13: 716-744. 1931.
16. SIZER, I. W. Effects of temperature on enzyme kinetics. Advances in Enzymol. 3: 35-62. 1943.
17. SIZER, I. W. and JOSEPHSON, E. S. Kinetics as a function of temperature of lipase, trypsin, and invertase activity from -70° to 50°C (-94 to 122°F). Food Research 7: 201-219. 1942.
18. SMITH, JAMES H. C. and YOUNG, VIOLET M. K. Chlorophyll formation and accumulation in plants. Radiation Biology. Alexander Hollaender, Editor. (In press.)

THE DEVELOPMENT OF CHLOROPHYLL AND OXYGEN-EVOLVING
POWER IN ETIOLATED BARLEY LEAVES
WHEN ILLUMINATED¹

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When etiolated leaves are illuminated they produce chlorophyll and acquire the ability to carry on photosynthesis. By following simultaneously the development of chlorophyll and photosynthetic capacity in such leaves much may be learned concerning the participation of chlorophyll in photosynthesis. This approach has been used by several investigators, who have reached different conclusions concerning the simultaneity of chlorophyll formation and the onset of photosynthetic capacity as a brief survey of previous investigations will show.

Engelmann (4) found that all the chlorophyll-containing cells which he examined were capable of evolving oxygen when they were illuminated. Furthermore, he claimed that chlorophyll-free but etiolin-con-

taining (protochlorophyll-containing (22)) cells of dark-grown seedlings gave off oxygen immediately upon being illuminated, which claim was verified by Ewart (5).

Irving (8) and later Willstätter and Stoll (23) examined the development of photosynthetic capacity in relation to chlorophyll formation by following the photosynthetic utilization of carbon dioxide during the greening of seedlings. Irving stated that "Etiolated leaves do not possess the power to photosynthesize CO₂ even when they have developed a large part of their chlorophyll. When the leaves have developed their full green color photosynthetic ability develops rapidly. Photosynthetic activity is not directly connected to the amount of chlorophyll produced. It must be some other component of the photosynthetic apparatus which controls the beginning of the com-

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