CATALASE–CHLOROPHYLL RELATIONSHIP IN BARLEY SEEDLINGS

DAVID APPLEMAN

(WITH TWO FIGURES)

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Introduction

For over two decades H. VON EULER (3) and his coworkers have studied the relationship of catalase to chlorophyll. In their work they utilized various chlorophyll defective plants including genetic albinos, variegated, chlorotic and etiolated plants. In general, they found a parallelism between chlorophyll content and catalase activity. NAKAMURA (11) working with a number of higher and lower forms of photosynthesizing organisms came to the same conclusions. Our observations on this problem agree in a general way with the above authors with one important exception, namely, the catalase–chlorophyll relationships in etiolated seedlings. EULER and his coworkers (4) have reported that catalase activity in etiolated seedlings of barley, rye, wheat and oats are generally lower than in green seedlings. We found that the catalase activity in etiolated seedlings of the above species is consistently and significantly higher than in green seedlings. It is primarily with this phase of the problem that the present paper is concerned.

Material and methods

Barley, variety Sacramento, was used in all the experiments. The details of the method and equipment used for determination of catalase activity are adequately described in a previous publication (1). Oxygen liberated from H_2O_2 by the enzyme preparation was measured manometrically. The rate of evolution of oxygen was recorded continuously by means of a simple device. A satisfactory rate measurement was obtained within 30 seconds from the beginning of the reaction.

In the preparation of the samples, only the leaf blades were used. Also, whenever a comparison was made between green and etiolated seedlings, not only were the seedlings grown under exactly the same conditions, with the exception of light, but also corresponding parts of leaves were used. The sampling is important since the catalase activity increases markedly from the base to the tip of the leaf (5). A sample (0.5 to 1.5 gm.) of fresh leaves was quickly weighed, on a torsion balance. The leaves were cut with scissors into short sections and placed in a mortar which contained a small, measured, amount of M/10 phosphate buffer, pH 7. The tissue was ground until the suspension was fine enough to be pipetted with a pipette having a large opening. The suspension was transferred to a glass stoppered flask with sufficient buffer solution to make the final suspension contain 10 to 50 mg. of fresh tissue per ml., depending on the expected activity. One ml. of this final suspension was used for catalase determination.

The catalase activities, AF (**1**), are reported as the initial rates AF $= \frac{\text{ml. } O_2 \times 10^3}{\text{mg. of dry sample} \times \text{sec.}}$. Unless otherwise stated, all determinations were made at 0° C and pH 7.

Three other leaf samples similar to the one taken for catalase determination were taken at the same time. One sample was used for determination of per cent. dry matter by drying the sample to constant weight. The second sample was used for determination of total nitrogen by the semimicro Kjeldahl method. The third sample was used for determination of chlorophyll by extracting the leaf sample with methanol and measuring the per cent. transmission of the extract at $\lambda = 6650$ Å on a Beckman spectrophotometer. The specific absorption coefficient (κ) was determined on a purified chlorophyll (a + b) sample and the value, 0.045, was used in all determinations. The chlorophyll is calculated in mg./liter in the extract.

Results

In many preliminary trials performed over a period of several years, we found that the catalase activity in etiolated seedlings of barley, wheat and corn was always higher than in the analogous green seedlings. The etiolated seedlings gave an activity two to ten times as high as the green, depending on age, part of blade used, conditions of culture, and the concentration of H_2O_2 used for the determinations. While the other conditions mentioned generally affect the green and etiolated seedlings in the same direction, beyond a certain point the concentration of H_2O_2 affects the green differently from the etiolated. Figure 1 shows a typical experiment. It is evident that the etiolated plant has its maximum catalase activity at a higher H_2O_2 concentration than the green. To determine whether the difference is due to the enzyme/substrate ratio, the sample of green material used per determination was increased fivefold, to give a rate of oxygen evolution close to that of the etiolated sample. The larger sample, as is evident from the graph, caused no appreciable change in the H_2O_2 concentration at which the maximum activity was obtained. It appears that the catalase in the etiolated seedling may be qualitatively different from that in the green seedling. In order to obtain further support for this possibility, activation energies of the green and etiolated preparations were compared.

BLAGOVESCHENSKI (**2**) reports that catalases from different plants have different activation energies, as determined by their Arrhenius constants (μ). OWTSCHINNIKOW (**13**) found that the μ value of catalase from etiolated wheat seedlings is different from that of the analogous green seedlings. In table I are given some representative Arrhenius constants for barley and wheat catalase which we have determined. Each of the values is an average of three obtained at the three temperature intervals, 0 to 10°, 10 to 20°, 20 to 30° C. The difference between the highest and lowest value within each set never exceeded 600. The values 20 to 30° C showed the greatest variability. In all cases, the etiolated leaves have a lower activation energy

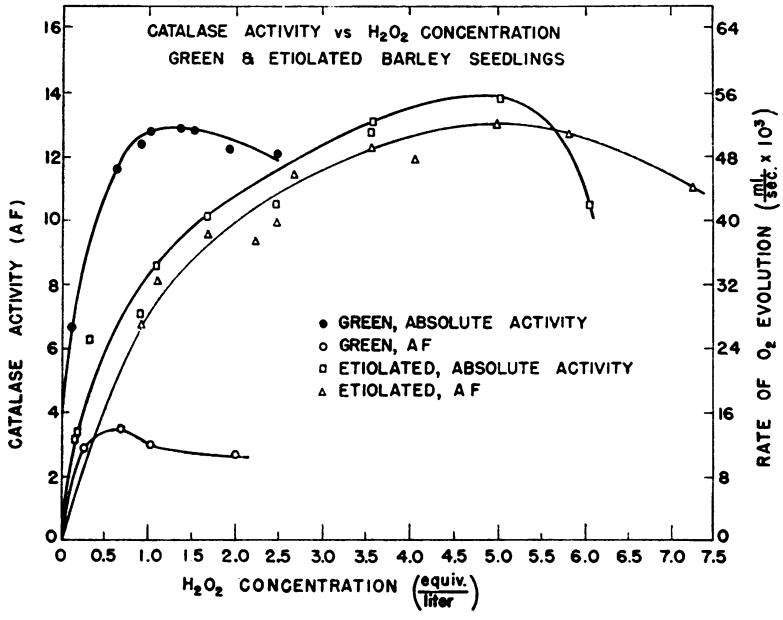

FIG. 1. The effect of H_2O_2 concentration on the catalase activity of green and etiolated barley.

than the green. The difference in activation energy is another indication that the catalase in the etiolated seedling may differ qualitatively from that in the green seedling.

The activation energies given in table I were calculated from the Arrhenius equation,

$$\mu = 4.6 \frac{(\log k_2 - \log k_1)}{(1/T_1 - 1/T_2)}$$

where k_1 and k_2 are the rates (AF) at the absolute temperatures T_1 and T_2. The temperatures used were 0, 10, 20, and 30° C. Activation energies are generally given as calories per mole. Since we do not know the actual amount of catalase in our preparations, the values are comparative only.

TABLE I

ACTIVATION ENERGIES (μ) FOR CATALASE IN ETIOLATED AND GREEN SEEDLINGS OF BARLEY AND WHEAT.

Plant	Age in days from planting	Days of exposure to light	μ
Barley	6	0	3020
Barley	6	2	8085
Barley	7	0	5140
Barley	7	4	11330
Barley	10	0	5240
Barley	10	7	11800
Wheat	7	0	3580
Wheat	7	4	9770

Since, upon illumination, an etiolated plant becomes green and acquires all the characteristics of the normally green plant, including its catalase activity, it was of interest to follow the changes in catalase activity during greening. Figure 2 shows the results of such a study. In this experiment, barley seeds were germinated in a dark chamber on screen trays according to the method of HOAGLAND and BROYER (9) in Hoagland solution. On the evening of the third day after planting, when shoots were about 1 cm. long, half the trays were removed from the dark chamber and placed in front of a north window where they remained under normal day and night conditions. The rest were left in the dark chamber. Samples of both sets of plants were taken every evening thereafter for catalase determination. On

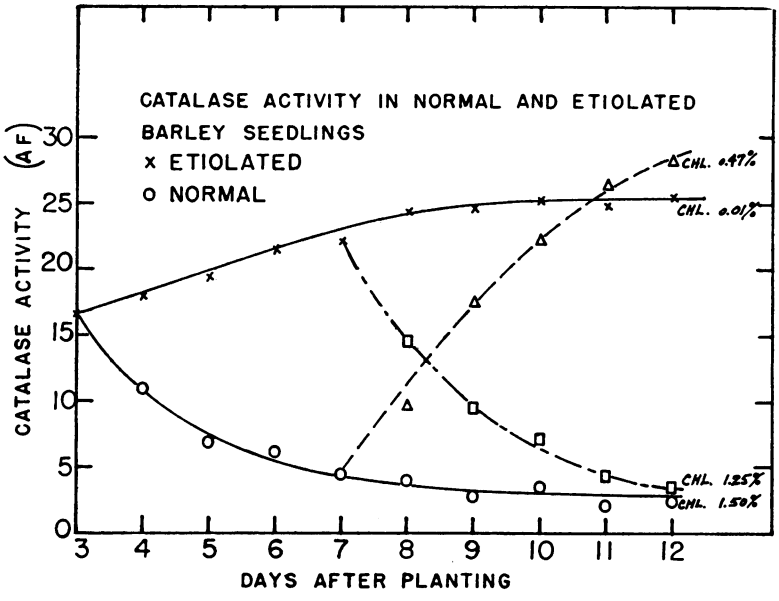

FIG. 2. Changes in catalase activity in barley seedlings induced by light and darkness.

the seventh day, two trays of plants from the dark chamber were moved to the light; and two trays of plants that had been in the light were moved back to the dark chamber.

It is evident from the data that the etiolated plants attained a maximum catalase activity between the eighth and tenth day while at the same time the illuminated plants attained a minimum in catalase activity as well as the normal amount of chlorophyll. An observation not shown on the graph is of interest. Seedlings from the light series which were permitted to continue growth after the twelfth day increased slowly in catalase activity. On the twentieth day they attained a maximum activity of AF 13 which is approximately the value ordinarily found in normally grown barley of this age. In the partially green plants, which were returned to the dark chamber after four days of illumination, catalase activity increased fivefold in

five days of darkness. These plants still retained most of the chlorophyll they accumulated while in the light. When exposed to light the seven-day-old etiolated seedlings lost their catalase activity at a faster rate than the initial, three-day-old seedlings. They also became green more rapidly.

It appears that when chlorophyll is being synthesized rapidly in the plastids, catalase activity decreases; conversely, when chlorophyll synthesis is blocked, catalase activity increases. In order to explore this generalization further, experiments were designed in which chlorophyll synthesis was blocked by means other than etiolation.

LIRO (**10**) in his classic paper has indicated that the terminal process in the chlorophyll synthesis is purely photochemical. The formation of the precursor, however, is markedly influenced by temperature and takes place very slowly if at all, at temperatures below 1° C. SMITH (**15**) has shown conclusively that etiolated seedlings, when illuminated at 1° C, form no more chlorophyll than the amount equivalent to the protochlorophyll pres-

TABLE II

CATALASE ACTIVITY AND CHLOROPHYLL CONTENT IN BARLEY SEEDLINGS GROWN IN THE DARK AT 25°C AND EXPOSED TO ILLUMINATION OF A MAZDA LAMP (250 fc) AT 1 AND 25° C. EXPERIMENTS A AND B WERE DONE AT DIFFERENT TIMES WITH DIFFERENT LOTS OF PLANTS

	Age in days after planting	Days of exposure	Temp. °C	Catalase A F	Chlorophyll as % of dry matter
A	6	0	25	14.0	0.006
	7	1	1	12.8	0.003
	7	1	25	11.0	0.95
B	8	0	25	22.9	trace
	10	2	1	18.9	0.003
	10	2	25	3.70	0.80

ent in them. Presumably, at the low temperature protochlorophyll is not synthesized. We therefore set up an experiment in which chlorophyll formation was blocked by low temperature.

Barley seedlings, grown in the dark for six to eight days at 25° C, were divided into two samples and exposed to light at 25 and 1° C respectively, and the changes in catalase activity were determined. The results, as presented in table II, indicate that when chlorophyll synthesis is blocked, in this case by low temperature, catalase activity suffers only a very small decrease. This observation is not in agreement with EYSTER'S (**6**) belief that when etiolated plants are illuminated the catalase in them is subject to photooxidation. It is possible, however, that in this experiment the light intensity was too low to cause an appreciable inactivation of catalase.

It is well known that certain mineral deficiencies interfere with chlorophyll synthesis. Experience with chlorotic crop plants indicates that they always show a decrease in catalase activity roughly parallel to the decrease in chlorophyll. It was of interest, however, to study the catalase activity

TABLE III

CATALASE ACTIVITY IN THE FIRST LEAF OF BARLEY SEEDLINGS FROM WHICH THE SEED WAS REMOVED AND THE SEEDLINGS GROWN IN SOLUTIONS WITH AND WITHOUT MAGNESIUM.

Age in days after planting	Days on culture solution	Catalase A F	
		+ Mg	− Mg
6	3	8.7	11.8
8	5	7.8	8.4
13	10	6.0	4.8

in the initial stages of a mineral deficiency, before chlorophyll content is reduced. Since Mg is required in relatively high concentration for normal plant growth, we hoped to produce a Mg deficiency in young seedlings. We found, however, that barley seeds have sufficient Mg for the first leaf to develop normally. We resorted to two ways of growing seedlings with a Mg deficiency sufficient to influence chlorophyll content. In the first method, the seeds were germinated on filter paper moistened with distilled water, and the seeds were cut away from the embryos when the coleoptile was about one cm. in length. The seedlings were divided in two lots and planted in Mg-deficient and complete culture solutions. Seedlings, so treated, grew very slowly and were weak and spindly whether grown in a complete or a Mg-deficient medium. In the second method, the barley seeds were germinated and grown in a nutrient solution deficient in Mg. The second leaf was used for catalase determination when it was approximately 10 cm. long.

The results of these experiments are summarized in tables III and IV. These results are interpreted to indicate that just before the concentration of Mg becomes deficient for chlorophyll synthesis but is still sufficient for its other functions, there is a rise in catalase activity. When Mg concentration drops still lower, catalase activity decreases along with chlorophyll content. It would have been desirable to have more frequent sampling. FINKLE (7) working with *Chlorella vulgaris*, cultured in low Mg media, has obtained results which show this phenomenon more strikingly.

Discussion

The experimental observations indicate that etiolated seedlings have a very much higher catalase activity than green seedlings. When etiolated

TABLE IV

CATALASE ACTIVITY AND CHLOROPHYLL CONTENT IN THE SECOND LEAF OF BARLEY SEEDLINGS GROWN IN SOLUTIONS WITH AND WITHOUT MAGNESIUM.

Age in days after planting	Catalase A F		Chlorophyll as % dry matter	
	+ Mg	− Mg	+ Mg	− Mg
12	4.3	8.4	1.39	1.35
14	13.8	12.7	1.61	1.34
16	13.8	7.7	1.53	1.13

seedlings are illuminated, under favorable condition for the synthesis and accumulation of chlorophyll, catalase activity decreases. When etiolated seedlings, high in catalase, are illuminated under conditions in which chlorophyll synthesis does not take place, such as low temperature, there is no appreciable decrease in their catalase activity. When chlorophyll synthesis is blocked by low Mg in the culture medium, there is an increase in catalase activity. Although this increase is of short duration, the fact that it was observed under three different experimental conditions definitely establishes its existence. When green seedlings are placed in the dark their catalase activity increases rapidly. Etiolated grain seedlings have a catalase which is, apparently, qualitatively different from that in the normally green seedlings. While the catalases were not isolated, it is doubtful that the differences observed could be ascribed to substances in the preparation other than catalase. Both the green and etiolated preparations are completely inactivated by heating at 80° C for five minutes or by 10^{-3} molar KCN. Also when a heat inactivated sample of one is added to an active sample of the other, it does not affect the activity in any way. Cell-free extracts of green and etiolated seedlings give results similar to that of the suspensions.

In view of our results, we do not think that a parallelism between chlorophyll and catalase describes the situation adequately. A dynamic equilibrium probably exists between all porphyrin containing substances in the chloroplasts. Under normal conditions a definite quantitative relationship exists between chlorophyll, catalase and probably other substances containing porphyrin. By interfering with the normal functioning of the cell, it is possible to change this quantitative relationship drastically. In the case where chlorophyll synthesis is inhibited by etiolation, the catalase in the etiolated seedlings is more active than in the green, as is indicated by its lower activation energy. Whether this is a general occurrence whenever chlorophyll synthesis is blocked by changes in environmental conditions has not been investigated. Should it prove to be general, it will be of interest to look for a possible functional relationship of this highly active catalase to the biosynthesis of chlorophyll.

GRANICK (**8**) postulated a scheme of heme and chlorophyll synthesis which assumes a common path until the formation of protoporphyrin **9**. From the protoporphyrin the synthesis may proceed along two branches, one leading to the hemes, the other to the chlorophylls. In the normal chloroplast both of these substances are present. It is, however, reasonable to expect to find some mutant plants deficient in chlorophyll in which an enzyme, or enzymes, specifically concerned only with the branch of synthesis involving magnesium are inteferred with. In such a mutant one may expect to find catalase activity as high as or higher than in the normal, green plant even though it may be low in or completely devoid of chlorophyll.

We have examined a large number of albino seedlings for catalase activity and, so far, have found one, an avocado seedling which was completely devoid of chlorophyll but had a catalase activity three times as high as the normally green seedlings. This observation is of particular interest

in view of the fact that eight other albino avocado seedlings had catalase activity only 0.2 to 0.3 as high as that in the normal green seedlings, indicative of an interference in the porphyrin synthesis earlier in the path of synthesis probably before the separation into the two branches. Another case is that of an artificially produced *Chlorella vulgaris* mutant which is very pale green. This mutant was supplied to us by Dr. S. Granick of the Rockefeller Institute for Medical Research under his no. 504. In this organism catalase activity is approximately twice as high as in the normal green parent cell. Further work on this and other mutants is now in progress.

The two major pigments of protoplasm, hemoglobin and chlorophyll, occur in highly specialized structures, the red blood cell and the chloroplast. It may be significant that practically all the catalase activity in the blood and in the green plant cell is localized in the red blood cell (**14**) and chloroplast (**12**) respectively. The close association between chlorophyll and catalase in the plastids, coupled with the possibility of inducing experimentally, changes in their relationship offers a fruitful field of research leading to a better understanding of the biosynthesis and relationships of the porphyrin-containing proteins in nature.

Summary

Catalase activity of barley seedlings grown under several experimental conditions was determined. The catalase activity was higher in etiolated seedlings than in green seedlings. When etiolated seedlings were illuminated catalase activity decreased rapidly. Conversely, when seedlings were transferred from light to darkness there was a rapid increase in catalase activity. Etiolated plants, exposed to light at 1° C lose very little catalase activity, but also do not accumulate chlorophyll. Barley plants in the early stages of Mg deficiency have a higher catalase activity than similar plants grown in a complete culture solution. Catalase of etiolated seedlings has a lower activation energy than the catalase in the green seedlings. Arguments are presented to show that, on theoretical grounds, it may be expected to find genetic albinos high in catalase. Two actual examples are cited in support of the arguments.

It is concluded that there is a dynamic equilibrium between the porphyrin-proteins in the chloroplast. When rapid chlorophyll synthesis takes place, catalase activity suffers a decrease. When chlorophyll synthesis is blocked, catalase activity rises rapidly if it is at a low level, or it does not decrease if it is at a high level.

Department of Agriculture
University of California
Los Angeles, California

LITERATURE CITED

1. Appleman, D. Manometric determination of catalase activity apparatus and method. Anal. Chem. **23**: 1627–1632. 1951.

2. BLAGOVESCHENSKI, A. V. On the qualitative differences of enzymes. Enzymologia **2**: 203–214. 1937.
3. EULER, H. VON. Zur kenntnis der enzymatischen Hydroperoxyd-Spaltung durch Pflanzen extrakte. I Genetische und chemische Einflüsse auf die Enzymbildung. Arkiv. Kemi, Mineral, Geol. A**24**(13): 1–15. 1947.
4. EULER, H. VON, EDELO, S., and GERNOW, I. Zur Kenntnis der enzymatischen Hydroperoxyd-Spaltung durch Pflanzen extrakte. II. Arkiv. Kemi, Mineral, Geol. A**26**(5): 1–10. 1948.
5. EULER, H. VON. Uber die Keimung von Samen unter Einvirkung antibiotischer Stoffe. Arkiv. Kemi, Mineral, Geol. A**25**(17): 1–9. 1948.
6. EYSTER, H. C. Catalase activity in chloroplast pigment deficient types of corn. Plant Physiol. **25**: 630–638. 1950.
7. FINKLE, B. J. Studies of chlorophyll formation and growth of green algae. Ph.D. thesis. University of California, Los Angeles. 1949.
8. GRANICK, S. Biosynthesis of chlorophyll and related pigments. Ann. Rev. of Plant Physiol. **2**: 115–144. 1951.
9. HOAGLAND, D. R. and BROYER, T. C. General nature of the process of salt accumulation by roots with description of experimental methods. Plant Physiol. **11**: 471–507. 1936.
10. LIRO, I. J. Uber die Photchemische chlorophyllbildung bei den Phanerogamen. Ann. Acad. Sci. Fennicae Ser. A. **1**: 1–147. 1909.
11. NAKAMURA, V. H. Über die quantitativen Beziehunger zwischen der Katalase in Chloroplasten und dem Chlorophyll, nebst einigen Bemerkungen uber die Rolle der Katalase in Assimilationsvorgang. Japanese Jour. of Bot. **11**: 221–236. 1941.
12. NEISH, A. C. Studies on chloroplasts. II. Their chemical composition and the distribution of certain metabolites between the chloroplasts and the remainder of the leaf. Biochem. Jour. **33**: 300–308. 1939.
13. OWTSCHINNIKOW, N. N. Zur Frage des chemismus der Photosynthese. Compt. Rend. Acad. Sci. U.R.S.S. **31**: 163–164. 1941.
14. PERLMANN, G. E. and LIPMANN, F. Catalase in serum and plasma. Arch. Biochem. **7**: 159–166. 1945.
15. SMITH, J. H. C. Processes accompanying chlorophyll formation. In: Photosynthesis in Plants, J. Franck and W. E. Loomis, Editors. Iowa State College Press, Ames, p. 209–217. 1949.