# CHLOROPHYLLASE ACTIVITY OF SPINACH CHLOROPLASTIN[1, 2]

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When spinach chloroplasts are treated with digitonin, a chlorophyll-lipoprotein complex is solubilized. Careful addition of ethanol to the clear green solution gives a green precipitate which is one of the components (factor 1) of cytochrome c photooxidase (1, 9, 10). Since factor 1 is a chlorophyll-lipoprotein complex, it may properly be called chloroplastin. The present paper is concerned with the determination of the chlorophyllase activity of this chloroplastin.

## MATERIALS AND METHODS

Chlorophyll a was prepared according to Zscheile and Comar (14). It was dissolved in ethyl ether and stored at −15° C. All organic solvents were redistilled. The spectrophotometric observations were made with a Beckman recording spectrophotometer. Chloroplastin was the precipitate designated factor 1 prepared from spinach leaves as previously described (9). Two digitonin extractions were always made.

PREPARATION AND ASSAY OF CHLOROPHYLLASE FROM CHLOROPLASTIN. Chlorophyllase is the enzyme, first described by Willstätter and Stoll (13), which catalyzes the hydrolytic (or alcoholytic) cleavage of the phytol moiety from chlorophyll, giving free phytol and chlorophyllide. The assay procedure here described is a modification of the method of Weast and Mackinney (12), and is based on the change in solubility of pigment that occurs when the phytol moiety is removed from the chlorophyll molecule. Chlorophyll can be extracted from aqueous acetone by petroleum ether, but chlorophyllide remains in the acetone layer. The hydrolysis reaction is carried out in 66 % acetone in water. The chlorophyll naturally present in the leaf preparation may serve as enzyme substrate or the enzyme may be freed of chlorophyll by exhaustive extraction with aqueous acetone, after which chlorophyll is added back. In the present studies the latter alternative was preferred because of its higher accuracy.

For removal of chlorophyll, the moist precipitate of chloroplastin was suspended in about ten volumes of 80 % aqueous acetone and the suspension was filtered with suction. The acetone extraction was repeated 10 times or more if necessary. The residue was suspended in water (50 ml for material from 6 kg spinach), and the insoluble enzyme was recovered by centrifugation (5 min at 2500 × G). No trace of chlorophyllase activity was present in the aqueous extract. The enzyme was obtained in the form of a paste (about 1 g per kg spinach) containing about 80 per cent water. A bright red pigment which collected on the surface of the precipitate after centrifugation from water had no effect on enzyme activity. The amount of chlorophyllase obtained was the same whether the acetone extraction was carried out at room temperature or at 0° C. Almost complete loss of enzyme activity often occurred if the paste was thoroughly air-dried, and a moderate (20–50 %) loss occurred on freezing.

An aliquot of a solution of chlorophyll a in ethyl ether was measured into the bottom of a reaction flask, and the ether was removed by evacuation. (About 0.02–0.04 mg of chlorophyll was a convenient amount.) The chlorophyll residue was dissolved in 8 ml of 80 % acetone, and a weighed amount of enzyme paste was added with 2 ml of $H_2O$. The mixture was ground to a fine suspension with a glass rod. The stoppered flask was incubated in the dark with shaking for 3 hours at 22° C. After filtration of the suspension, a 5 ml aliquot of the filtrate was transferred to a glass-stoppered 10 ml graduated cylinder, and 5 ml of petroleum ether were added. The liquid layers were mixed by shaking and allowed to separate into a petroleum ether, acetone layer (usually 6.2 ml) on top and an aqueous acetone layer (usually 3.8 ml) below. Occasionally the acetone layers were turbid and had to be cleared by centrifugation. The visible spectrum of each layer was determined in a 1 cm cell. The amount of chlorophyll added to a particular reaction mixture was determined by making an identical reaction mixture without added enzyme, and carrying through the incubation and extraction exactly as in the case of the reaction with enzyme. Correction was thus automatically made for chlorophyll destroyed in the absence of enzyme during the incubation period. The record from a typical set of measurements is shown in figure 1. In the present studies, the complete spectrum from 400 to 700 mμ was recorded, to ensure the identity of the substances measured; but readings may be limited to the maxima in the red. To calculate the amount of chlorophyll a in the petroleum ether, the unit absorbancy used was 102 $cm^2$ $mg^{-1}$ at the maximum of about 660 mμ. The unit

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absorbancy represents the optical density or absorbancy of a solution containing 1 mg per ml of chlorophyll, observed with a light path of 1 cm. To calculate the chlorophyllide a in the acetone, the unit absorbancy used was 89 $cm^2$ $mg^{-1}$ at the maximum of about 663 mμ. Chlorophyllide was calculated as mg chlorophyll. The assumption was made that the extinction coefficient of chlorophyllide in aqueous acetone was the same as that of chlorophyll in the same solvent. The unit absorbancy values of 102 and 89 for chlorophyll a in petroleum ether and in aqueous acetone were determined experimentally. The reference value employed was the unit absorbancy of chlorophyll a in pure ether = 102 at 660 mμ, as reported by Zscheile and Comar (14). Mackinney (7) reported a value of 90 for anhydrous ether, 93.4 for U.S.P. ether, and 82 for aqueous acetone. Harris and Zscheile (3) reported that the red absorption maximum of chlorophyll a in pure acetone is 83 % of the red absorption maximum in ether.

Not shown in figure 1 are the absorbancies of the solvent layers from a blank determination in which the incubation was carried out with enzyme alone without added chlorophyll. If the chlorophyll had been completely removed from the enzyme, there was no contribution to light absorbancy at 660 mμ. It was important to check this point with every enzyme preparation, because prolonged incubation with aqueous acetone may remove pigment resistant to removal in a brief extraction period. Other substances which absorb light of longer wave lengths may also be extracted into the acetone, particularily if the method is applied to leaf preparations different from chloroplastin. Though these other substances have no well-defined absorption peaks, they may take a considerable contribution to the total absorbancy at 660 mμ. If precautions are taken to carry out appropriate blank determinations, however, the method is applicable to a wide range of preparations.

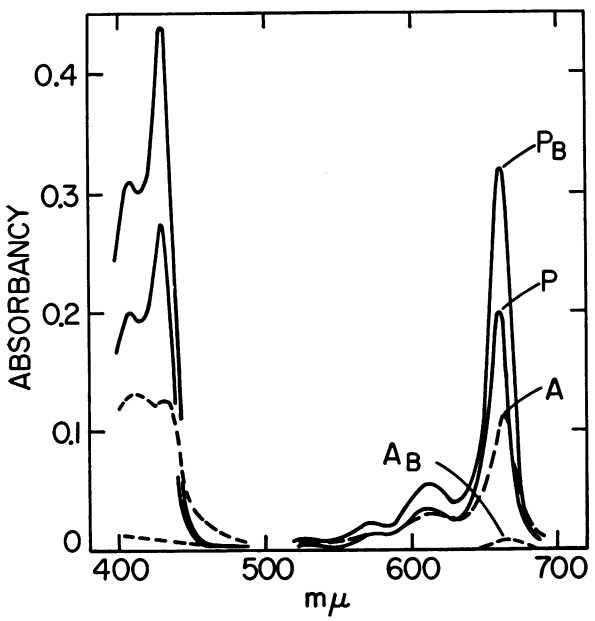


FIG. 1. Record of light absorbancy of petroleum ether and acetone layers from chlorophyllase assay procedure. Absorbancy = log $I_0/I$, where $I_0$ = intensity of incident light, and I = intensity of transmitted light.

Incubation was with 100 mg wet paste from chloroplastin for 3 hours at 22° C.

P = absorbancy of petroleum ether layer after incubation with enzyme. $P_B$ = absorbancy of petroleum ether layer after incubation without enzyme. A = absorbancy of acetone layer after incubation with enzyme. $A_B$ = absorbancy of acetone layer after incubation without enzyme.

Blank determinations carried out with the enzyme preparation alone without added chlorophyll did not give any light absorption between 600 and 700 mμ.

## Results and Discussion

The numerical values calculated from the spectrophotometric data of figure 1 are given in the first line of table I. This table also includes the results of two sets of measurements made with varying chlorophyll concentrations, and another set of duplicate determinations carried out at two different temperatures. The data given in the table were selected from more than 100 separate experiments to illustrate typical assay results and the degree of duplicability. Recovery of total pigments ranged from 70 to 97 %, with an average of about 80 %. This was considerably better than the recovery we could achieve with the recently described chromatographic procedures for assaying chlorophyllase (2, 11).

Because of the variable and incomplete recovery of pigment, it is not a straightforward matter to express the enzyme activity numerically. In table I, the per cent hydrolysis has been calculated both on the basis (A) of the decrease of pigment in the petroleum ether layer, and (B) of the increase of pigment in the acetone layer. The difference between these two numbers is of course larger when the per cent recovery is smaller. The per cent hydrolysis calculated in either fashion was linearly proportional to the amount of enzyme assayed within the range from 100 to 200 mg of enzyme paste from chloroplastin. This proportionality is a convenient rule of thumb, but only reflects the fact that the deviations from linearity in this activity range are not greater than the fluctuations of duplicate determinations. The data in table I show that the per cent hydrolysis observed in a given time period is almost independent of initial chlorophyll concentration in the range tested. This suggests first order kinetics. When the reaction was followed with time, however, the results confirmed the conclusion of Weast and Mackinney (12) that the enzyme reaction rate decreased with time, but the kinetics were neither first nor second order. The kinetics are obviously complex, and may reflect a basically first order reaction which is inhibited by a

TABLE I

RECOVERY OF PIGMENT IN ASSAY

| EXPERIMENT | CHLOROPHYLL | | CHLOROPHYLLIDE* | RECOVERY** | % HYDROLYSIS*** | |
|---|---|---|---|---|---|---|
| | ADDED MG | RECOVERED MG | RECOVERED MG | % | A | B |
| Data from fig 1 | 0.038 | 0.024 | 0.0094 | 88 | 37 | 25 |
| Effect of chlorophyll Concentration 1) | 0.033 | 0.017 | 0.013 | 91 | 48 | 39 |
| | 0.298 | 0.182 | 0.064 | 83 | 39 | 21 |
| 2) | 0.032 | 0.011 | 0.014 | 78 | 67 | 44 |
| | 0.065 | 0.028 | 0.020 | 74 | 57 | 31 |
| | 0.103 | 0.053 | 0.027 | 78 | 49 | 26 |
| Effect of temperature 20° | 0.033 | 0.015 | 0.011 | 79 | 55 | 33 |
| | 0.032 | 0.013 | 0.013 | 81 | 59 | 41 |
| 40° | 0.032 | 0.007 | 0.024 | 97 | 78 | 75 |
| | 0.033 | 0.008 | 0.022 | 91 | 76 | 67 |

* Calculated as chlorophyll

** $100 \times \frac{\text{chlorophyll recovered} + \text{chlorophyllide recovered}}{\text{chlorophyll added}}$

*** % Hydrolysis. A. Calculated on basis of decrease in chlorophyll. B. Calculated on basis of formation of chlorophyllide. All reactions carried out with 100 mg wet paste from chloroplastin.

reaction product and/or accompanied by progressive enzyme inactivation.

The claim of Mayer (8) that chlorophyllase is inactivated by washing with water and reactivated by adding salts could not be confirmed. The only effect of extraction with water, as applied in preparing the enzyme for assay, was the removal of soluble materials with no enzyme activity. Adding phosphate buffer of pH close to neutrality did not increase enzyme activity but gave higher blank values, so buffer was generally omitted from the assay system.

No alcoholysis of chlorophyll could be obtained with our chloroplastin preparations. This is in agreement with the observations of Weast and Mackinney who found only a trace of alcoholysis reaction in spinach preparations (12).

The present studies were prompted by the possibility that the activity of factor 1 in the cytochrome photooxidase system might be dependent on the formation of a relatively unstable chlorophyllide from the chlorophyll present in the preparation. Krossing (6) had concluded previously that the chlorophyllase activity of leaves was localized in the chloroplasts, so it seemed quite likely that the chlorophyllase might be present in factor 1 itself. The present studies confirmed this supposition. Careful measurements showed, however, that factor 1 contained no detectable chlorophyllide, nor was there any formation of chlorophyllide during incubation of factor 1 for 24 hours at room temperature, either in digitonin solution or in aqueous suspension under conditions used for the assay of cytochrome photooxidase. The presence of digitonin was shown to have no effect on the chlorophyllase assay. Thus, though the chlorophyllase is solubilized with the chlorophyll by the digitonin, chlorophyll and enzyme remain firmly attached under circumstances which permit no trace of enzyme action. Only after the chlorophyll has been removed from the lipoprotein by the action of suitable organic solvents is the enzyme capable of causing chlorophyll hydrolysis. The dependence of chlorophyllase action on the presence of organic solvents resembles the similar dependence of phosphatidase c. The organic solvent may act to promote appropriate contact between enzyme and substrate (4, 5). In the intact chloroplastin preparation itself one must conclude either that the enzyme is inactive or that the chlorophyll is bound in such a way as to be protected from enzyme action. It is impossible at present to distinguish between these two possibilities, because the solvents known to permit enzyme action also cause at least partial extraction of the endogenous chlorophyll. Aqueous suspensions of externally added chlorophyll are not attacked by chloroplastin, but this can be attributed to absence of contact between enzyme and substrate.

A final question to which an answer was sought was whether all the chlorophyllase of the leaf could be accounted for in the chloroplastin fraction. Because of the difficulty of assaying chlorophyllase quantitatively, particularly in crude leaf homogenates, a conclusive answer to this question could not be obtained. The most reliable procedure for determining the relative activity of two different enzyme preparations appeared to involve the determination, by trial and error, of those amounts of enzyme giving

TABLE II

RELATIVE CHLOROPHYLLASE ACTIVITY

| PREPARATION | % HYDROLYSIS IN 3 HRS** |
|---|---|
| Acetone powder from whole leaf, 1 g dry wt | 33 |
| Acetone powder from washed chloroplasts, 100 mg dry wt | 44 |
| Residue* from washed chloroplasts, 100 mg wet wt | 30 |
| Residue* from factor 1, 100 mg wet wt | 41 |

* Extracted with aqueous acetone until free of chlorophyll, then extracted with water. Water content, 80 %.
** Calculated on the basis of disappearance of pigment from the petroleum ether layer.

about the same per cent hydrolysis of the same amount of chlorophyll. Table II contains the results of some assays of different preparations from the same batch of leaves. These enzyme measurements were performed with the object of getting accurate values for comparative purposes. Assays of the dried acetone powders of whole leaves and of washed chloroplasts should be comparable, and showed that the powder from chloroplasts was about thirteen times as active as the powder from whole leaves. The ratio of the chlorophyll content of the whole leaves to that of the chloroplasts, calculated on a dry weight basis, was about 1 to 15. The wet paste from chloroplastin was somewhat higher in activity on a dry weight basis than the wet paste from chloroplasts. These data are compatible with the possibility that in the mature spinach leaf, chlorophyllase is localized entirely in the chlorophyll-lipoprotein complex of the chloroplasts, since most of the chlorophyll of the chloroplasts can be solubilized in the form of chloroplastin, and since the chloroplastin contains most though not all the dry weight of the chloroplasts. Because of the inaccuracy of the assays, however, more work would be required to establish this site as the exclusive location of the enzyme.

## SUMMARY

A method for the assay of chlorophyllase has been described. The enzyme has been shown to be present in the chlorophyll-containing component of cytochrome c photooxidase of spinach leaves in sufficient quantity to suggest that all the chlorophyllase of the mature leaf may be present in a chlorophyll-lipoprotein complex. The chlorophyllase of digitonin-solubilized chloroplastin has no hydrolytic effect on the chlorophyll bound to lipoprotein in the preparation, but acts only after the chlorophyll has been separated by suitable organic solvents.

## LITERATURE CITED

1. BISHOP, N. I., H. NAKAMURA, J. BLATT, and B. VENNESLAND. 1959. Kinetics and properties ot cytochrome c photooxidase of spinach. Plant Physiol. 34: 551–557.
2. GAGE, R. S. and S. ARONOFF. 1956. Chlorophyllase in soybean. Plant Physiol. 31: 477–478.
3. HARRIS, D. G. and F. P. ZSCHEILE. 1943. Effects of solvent upon absorption spectra of chlorophylls a and b; their ultraviolet absorption spectra in ether solution. Bot. Gaz. 104: 515–527.
4. KATES, M. 1957. Effects of solvents and surface-active agents on plastid phosphatidase c activity. Canadian Jour. Biochem. Physiol. 35: 127–142.
5. KATES, M. and P. R. GORHAM. 1957. Coalescence as a factor in solvent stimulation of plastid phosphatidase c activity. Canadian Jour. Biochem. Physiol. 35: 119–126.
6. KROSSING, G. 1940. Versuche zur Localisation einiger Fermente in den verschiedenen Zellbestandteilen der Spinatblätter. Biochem. Zeits. 305: 359–373.
7. MACKINNEY, G. 1941. Absorption of light by chlorophyll solutions. Jour. Biol. Chem. 140: 315–322.
8. MAYER, H. 1930. Untersuchungen über die Chlorophyllase. Planta 11: 294–330.
9. NIEMAN, R. H., H. NAKAMURA, and B. VENNESLAND. 1959. Fractionation and purification of cytochrome c photooxidase of spinach. Plant Physiol. 34: 262–267.
10. NIEMAN, R. H. and B. VENNESLAND. 1959. Photoreduction and photooxidation of cytochrome c by spinach chloroplast preparations. Plant Physiol. 34: 255–262.
11. SIRONVAL, C. 1954. Mesure de l'activité de la chlorophyllase par chromatographie sur papier. Physiol. Plantarum. 7: 523–530.
12. WEAST, C. A. and G. MACKINNEY. 1940. Chlorophyllase. Jour. Biol. Chem. 133: 551–558.
13. WILLSTÄTTER, R. and A. STOLL. 1913. Untersuchungen über Chlorophyll. Berlin.
14. ZSCHEILE, F. P. and C. L. COMAR. 1941. Influence of preparative procedure on the purity of chlorophyll components as shown by absorption spectra. Bot. Gaz. 102: 463–481.