Phytochemistry, 1965, Vol. 4, pp. 435 to 443. Pergamon Press Ltd. Printed in England

CHLOROPHYLLASE OF *CHLORELLA VULGARIS* BEIJERINCK

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(*Received* 9 *October* 1964)

Abstract—Some properties of chlorophyllase (E.C.3.1.1.14 = chlorophyll chlorophyllido-hydrolase) in homogenates from *Chlorella vulgaris* Beijerinck 211–11h are described. Chlorophyllase is localized in the chloroplast; the enzyme is easily solubilized and can be strongly activated after a short incubation with trypsin. Detergents do not influence activity. The possible function of the enzyme is discussed with relation to both the chlorophyll-bearing chloroplast protein (structural protein) and the formation and destruction of chlorophyll.

INTRODUCTION

CHLOROPHYLLASE catalyses the reaction: chlorophyll $\rightleftarrows$ chlorophyllide + phytol. The usual reaction conditions favour cleavage of the pigment; however, Willstätter and Stoll[1] and Shimizu and Tamaki,[2] showed the reversibility of the reaction *in vitro*. According to several investigators,[3–5] the enzyme is insoluble and is localized in the chloroplast, suggesting an association with the pigment-bearing structural protein of this organelle.[6]

Earlier work, emphasizing physiological aspects, dealt with the correlation between enzyme activity and chlorophyll content of the plants. The enzyme activity in roots,[4,7] etiolated seedlings,[8,9] and albino leaves[10] was less than in chlorophyll-containing tissues. During breakdown of the pigment in autumn, chlorophyllase activity was not usually observed to increase, but even decreased.[4] Since activity increased when plants turned green, some authors have concluded that the enzyme catalyses the synthesis of chlorophyll from chlorophyllide and phytol *in vivo*.[2,10] The work in this paper was carried out to investigate whether changes in chlorophyllase activity are due to the formation or destruction of chloroplast protein or to changes in chlorophyll content. The experiments also should indicate the localization and possible physiological role of the enzyme. For this purpose green algae were chosen because: (*a*) the quantitative relation of chlorophyll and structural protein of the chloroplast in algae under various physiological conditions has already been studied,[11] and (*b*) algae can quickly alter their chlorophyll content.[11] Most of the results refer to *Chlorella vulgaris*.

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[1] R. WILLSTÄTTER and A. STOLL, *Untersuchungen über Chlorophyll*, Springer Verlag, Berlin (1913).
[2] S. SHIMIZU and E. TAMAKI, *Arch Biochem. Biophys.* **102**, 152 (1963).
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[4] H. MAYER, *Planta* **11**, 294 (1930).
[5] C. ARDAO and B. VENNESLAND, *Plant Physiol.* **35**, 368 (1960).
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[7] P. D. PETERSON and H. H. MCKINNEY, *Phytopathol.* **28**, 329 (1938).
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[9] M. HOLDEN, *Photochem. Photobiol.* **2**, 175 (1963).
[10] E. G. SUDYINA, *Photochem. Photobiol.* **2**, 181 (1963).
[11] P. BÖGER, *Flora (Jena)* **154**, 174 (1964); A. PIRSON and P. BÖGER, *Nature*. In press (1965).

RESULTS

Some Enzyme Characteristics

Willstätter and Stoll found that chlorophyllase acts in aqueous acetone or alcohol. Almost all the succeeding workers[3–5,7,8,12,13] used between 45 and 80 per cent aqueous acetone to test the enzyme. Our experiments indicate that the optimal acetone concentration was 40 per cent (Fig. 1*a*). Less acetone caused the formation of colloidal solutions so that the accessibility of the substrate to the enzyme was probably impeded. In aqueous systems[14] this difficulty can be overcome by using the detergent Triton X-100.

The pH-optimum was reported to be 6 for the enzyme from *Heracleum*,[4] and between 7·5 and 8 for sugar beet.[8] According to our data with two different buffers, the pH optimum for the *Chlorella vulgaris* was 7·2–7·3 (Fig. 1*b*). Addition of citrate (0·04 M) as used by Holden,[8] proved to be inhibitory in our system. Phosphate-, collidine-, triethanolamine-, imidazole-buffers were tested; the latter was the least inhibitory. The activity was strongly

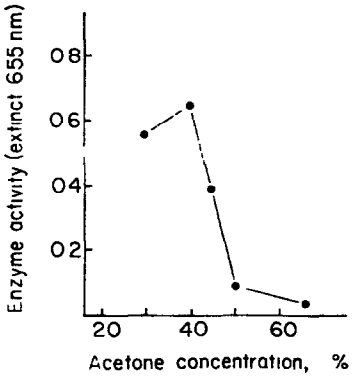

FIG. 1*a*.

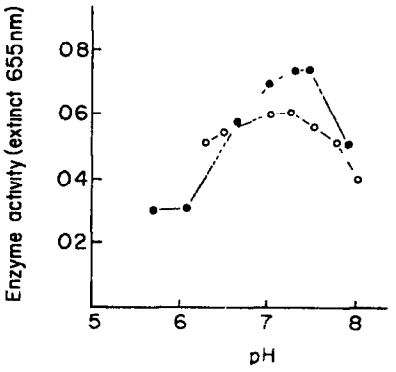

FIG. 1*b*.

FIG. 1(*a*, *b*). CHLOROPHYLLASE OF *Chlorella vulgaris*.

Dependence of activity on pH and acetone concentration. 1*a*—Acetone concentration (pH 6–6·3, reaction mixture without buffer); 1*b*—pH: determination of the optimum with two buffer systems (40% acetone), (●) 0·05 M imidazole buffer, ( ) 0·05 M triethanolamine buffer.

reduced by buffers more concentrated than 0·05 M. Addition of Mg^{++}, Zn^{++}, cysteine, ethylmercaptan and EDTA (10^{-3} to 10^{-2} M) had no influence. Dialysis in the cold for 24 hr also did not change the activity.

The algal enzyme was easily solubilized in contrast to chlorophyllase of higher plants.[5,8,14] When cells were homogenized (for 1·5 min) at pH 7·3 and centrifuged, about 50 per cent of the enzyme was found in the supernatant solution. However, at pH 6·3 only 10 per cent was solubilized, and to obtain chlorophyllase in good yield it was necessary to homogenize at a pH value above 7. It was not sufficient to adjust the homogenate to such a pH value later. When digitonin or Na-dodecylsulphate (0·5%) was added to the homogenate, the enzyme was totally solubilized after the mixture had been allowed to stand at room temperature for at least 1 day (see also Fig. 3). The term "soluble" indicates that the protein remains in the supernatant solution after 35 min centrifugation at 100,000 *g*.

[12] C. A. WEAST and G. MACKINNEY, *J. Biol. Chem.* **133**, 551 (1940).
[13] J. BARRETT and S. W. JEFFREY, *Plant Physiol.* **39**, 44 (1964).
[14] A. O. KLEIN and W. VISHNIAC, *J. Biol. Chem.* **236**, 2544 (1961).

Although chlorophyllase is insoluble at pH values below 6 it is active in homogenates down to pH 4, when kept at room temperature. The data of Fig. 2 were obtained by heating homogenates, which were adjusted to different pH values, for 5 min at 75°. The enzyme showed maximum stability at pH 5·5 where the activity was as high as that of the unheated sample. The occurrence of a maximum can be explained by assuming that heat denaturation is accelerated both at low pH values and by increasing solubility of the enzyme (see Fig. 2). Extraction of the homogenate with acetone at 0° reduced chlorophyllase activity by only 10–20 per cent on a nitrogen basis.

Homogenates which had been extracted with acetone as well as unextracted homogenates showed a slow increase of activity when kept at room temperature. The maximum activity was observed after a week's storage at room temperature at pH 7. This slow activation process could not be accelerated by detergents. Figure 3 shows that it could be delayed to some extent

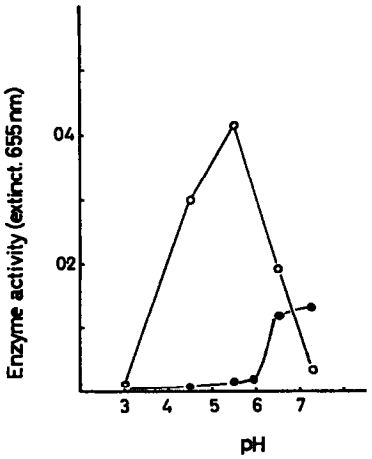

FIG. 2. CHLOROPHYLLASE OF *Chlorella vulgaris*.

(○) Stability of enzyme in homogenates with different pH values after heating for 5 min at 75°. All controls, kept 5 min at room temperature, had about the same activity (extinction value approximately 0·46). (●) Enzyme activity in the supernatant solution of homogenates with different pH values. Samples not heated. All assays were performed at pH 7·3.

by some of the detergents during the first day of the activation process. All the samples, including that without detergent, yielded approximately the same activity after 6 days, though they differed in the amount of solubilized enzyme (white columns). When the supernatant solution of the homogenate was tested and stored by itself at room temperature, an increase of activity of the *solubilized* enzyme was also observed (black columns). Therefore, activation was not caused by a release of the enzyme from cellular structures as described by Trebst and Wagner[15] for a latent polyphenoloxidase in chloroplasts.

The activation of the enzyme had a pH-optimum which was shifted during the activation process. It went from a low pH value at the start of the process to pH 7 after some days (see Fig. 4). This is explained by assuming that at lower pH values, the activation is faster than at pH 7, and that the enzyme (or its precursor) is less stable in acid than in neutral media. This slow activation process could be accelerated by the addition of trypsin and papain. As Fig. 5 shows, the activation is then produced in a matter of minutes. Activity obtained with trypsin

[15] A. TREBST and S. WAGNER, *Z. Naturforsch.* **17b**, 396 (1962).

addition is slightly higher than that without trypsin. This difference is probably due to a partial inactivation of the enzyme in the sample without trypsin being stored for days at room

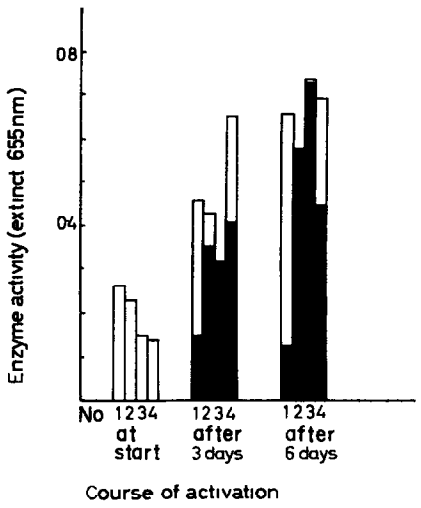

FIG. 3. CHLOROPHYLLASE OF *Chlorella vulgaris*.

Course of enzyme activation while keeping the homogenate at 25°, pH 7 with the addition of detergents (0·5%). Cells were homogenized at pH 6 8: No. 1 = without detergent; No. 2 = Na-dodecylsulphate; No. 3 = digitonin; No. 4 = Tween 20. White column: total activity of the homogenate; black column: activity of the supernatant solution alone.

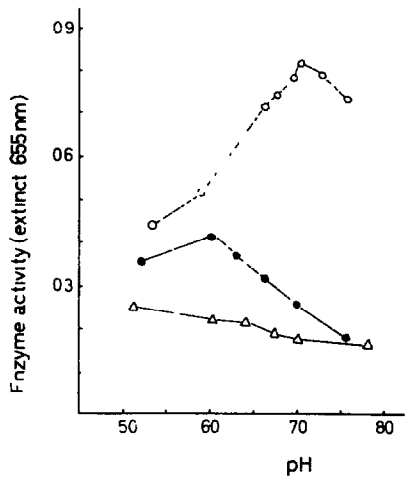

FIG. 4. CHLOROPHYLLASE OF *Chlorella vulgaris*.

Change of the pH-optimum of enzyme activation while keeping the homogenate at 25° and different pH values. ○ = at start; ● = after 1 day; △ = after 4 days.

temperature. With the trypsin concentrations used, tryptic degradation of the enzyme was insignificant during the experimental time. If trypsin concentration was increased, however, the enzyme was degraded. One can assume that the very slow activation process during storage of the homogenate may result from the action of an endogenous peptidase.

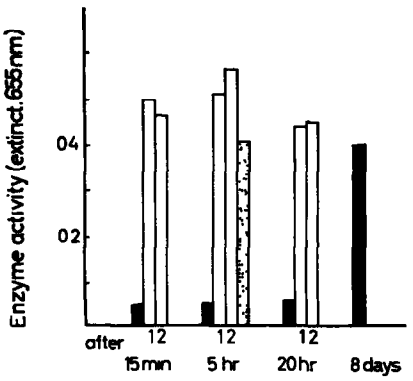

FIG. 5. CHLOROPHYLLASE OF *Chlorella vulgaris*.

Enzyme activation with trypsin and papain. Black column: without peptidase added; white column: with trypsin (1) 0·05%, and (2) 0·005%; dotted column: with 0·05% papain.

For the activated enzyme the Michaelis constant was determined to be approx. 7×10^{-5} M chlorophyll with our test conditions (see Experimental). We also found chlorophyllase activity in homogenates of *Chlorella pyrenoidosa* and *Chlamydomonas reinhardi*. None of the treatments described above, however, resulted in an increase of activity.

Localization of Chlorophyllase

The enzyme activity—after trypsin addition—was next examined in relation to the amount of insoluble structural protein of the chloroplast. Earlier work had shown that the latter can be varied in *Chlorella* by different light intensities.[11] Cells grown in strong light contain less chloroplast protein (and less chlorophyll) than those grown in weak light.

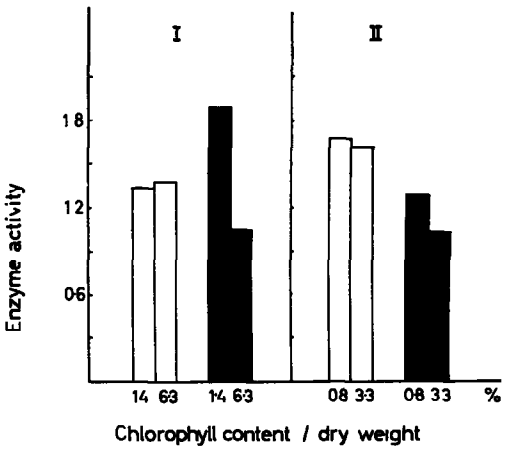

FIG. 6. CHLOROPHYLLASE OF *Chlorella vulgaris*.

Enzyme activity of cells with two different chlorophyll contents (enzyme activation with trypsin).

I: cells grown in complete medium; II: cells grown in N-deficient medium.

White column: chlorophyll split/nitrogen of structural protein of the chloroplast/hr. Black column: chlorophyll split/chlorophyll of structural protein of the chloroplast/hr.

Figure 6 (left side) shows typical data of chlorophyllase activity (*with* tryptic activation) from algae with a low and a high chlorophyll content. The activity related to the amount of insoluble chloroplast protein was the same in spite of the different chlorophyll contents. These data infer that chlorophyllase correlates with the structural protein of the chloroplast. We further can conclude that chlorophyllase is localized in the chloroplast; the earlier findings of the enzyme's localization in higher plants are hereby confirmed for *Chlorella vulgaris*.

When the enzyme was related to chlorophyll, however, we obtained a high activity with cell material of low chlorophyll content and vice versa (Fig. 6). In this case chlorophyll is obviously not a convenient reference for enzyme activity.

Figure 6 (right side) shows chlorophyllase activity of cells grown 1–2 days in the absence of nitrogen in the light. The activity was 15–20 per cent higher than that from cells of a complete medium when related to the structural protein of the chloroplast. When related to chlorophyll, activity did not change much in spite of different chlorophyll contents of the cells, contrary to the findings with cells from a complete medium. This can be explained by the earlier results, that in N-deficient cells the ratio of chlorophyll to structural protein remains almost the same, although the pigment content of the cells changes.

Physiological Role of Chlorophyllase

Cells grown in potassium-deficient medium in the light have a very low chlorophyll content. After addition of potassium, these cells were transferred to darkness, and an increase of their chlorophyll content up to 50 per cent and more within 15 hr was observed. By omitting the nitrogen of the culture medium the protein content remained constant during chlorophyll formation (cf. ref. 11). Under these conditions the chlorophyll was always totally phytylated. Chlorophyllase was measured (*with* tryptic activation) before and after chlorophyll increase (Table 1). It can be seen that based on chlorophyll content, the enzyme activity decreased corresponding to the increase of the pigment. This signifies that the enzyme activity has not changed, when related to the amount of structural protein of the chloroplast.

In a similar experiment, we tested chlorophyllase activity *without* activation with trypsin. We used potassium-deficient cells with different chlorophyll levels, thereby also obtaining different chlorophyll increases after the addition of potassium. When chlorophyll had reached its new level in the dark, activity was always found to have increased considerably.

TABLE 1. CHLOROPHYLLASE ACTIVITY OF POTASSIUM-DEFICIENT CELLS OF *Chlorella vulgaris* (ENZYME ACTIVATED WITH TRYPSIN)

Chlorophyll		Enzyme activity*		
Original content of cell: % per dry weight	Increase 15 hr after addn. of K (in N-free soln.) %	Original	After	Decrease %
1·00	63	6·22	3·62	58
1·00	63	6·14	3·75	61
1·01	60	5·26	3·01	57
1·49	46	4·30	2·58	60
1·18	67	5·81	2·85	50
1·63	55	5·09	2·86	57

* Chlorophyll split/chlorophyll of structural protein of the chloroplast/hr.

As we were interested in the correlation between activity and accumulation of pigment, we plotted the *mean* values of the chlorophyllase activity before and after chlorophyll formation against the increase of chlorophyll and obtained a close correlation between these figures (Fig. 7).

When potassium-deficient cells were placed in darkness and no potassium added, no chlorophyll was built up, and the activity remained low. In the dark these cells could neither increase chlorophyll nor chlorophyllase activity (measured without trypsin addition).

When cells were cultivated without nitrogen in the light, their chlorophyll was slowly destroyed.[11] In spite of pigment destruction, chlorophyllase activity remained at the normal level. Our data, therefore, do not prove a relation between decomposition of the pigment and chlorophyllase activity. However, it seems more probable that the enzyme is involved in the synthetic process.

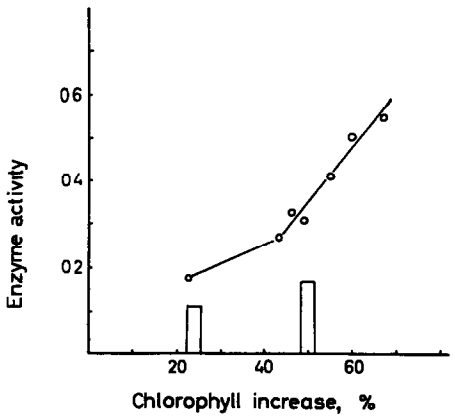

FIG. 7. CHLOROPHYLLASE OF *Chlorella vulgaris*.

Correlation of enzyme activity in potassium-deficient cells and chlorophyllase increase after addition of potassium. Enzyme *not* activated with trypsin. Mean values of activity before and after chlorophyll increase were plotted against chlorophyll increase in %.

Reference for activity: chlorophyll split/nitrogen of structural protein of the chloroplast/hr. Columns data from two samples to which no potassium was added and therefore no additional chlorophyll was formed.

DISCUSSION

The chloroplast is destroyed and the chlorophyllase partially released from the chloroplast debris by homogenization of the cell. The localization of the enzyme in the chloroplast, however, was inferred from the close relation between chlorophyllase activity and the amount of structural protein of the plastid in the cell. This close relation was observed in cells of complete, nitrogen- and potassium-deficient media. Hence the previous preparative data of the amount of chloroplast protein in the cell[11] could be supported by enzymological tests.

The increase of chlorophyllase activity produced by action of a peptidase is probably the same as described for other enzymes: Szarkowski[16] found cresolase activity of tyrosinase only after tryptic treatment; Freedland and Waisman[17] described an increase of fructose-1,6-diphosphatase from rat liver after incubation with papain—an activation which Heise and

[16] J. W. SZARKOWSKI, *Bull. Acad. Polon. Sci. Classe II*, **5**, 1 (1957).
[17] R. A. FREEDLAND and H. A. WAISMAN, *Cancer Res.* **20**, 1317 (1960).

Kleitke[18] could improve by application of trypsin. Kenten[19] succeeded in activating a latent polyphenoloxidase from leaves of *Vicia faba* by both trypsin and Na-dodecylsulphate.

These findings and our data point to an unspecific mode of activation which frees chlorophyllase from inhibiting proteins or peptides. These are attached to the enzyme: since the activation also occurs after solubilization, the inactive enzyme complex must be rather stable. Even detergents cannot split it. It is, however, of interest to note that chlorophyllase can be completely separated from chlorophyll by solubilizing the enzyme.

Probably the high activity after trypsin addition does not occur in the living cell; we could not find it immediately after homogenization without the addition of protease enzymes. Furthermore we never observed the activation of the enzyme in closely related strains of unicellular algae. This may be due to slight differences in composition and structure of chloroplast proteins. For example Kenten demonstrated an activation of polyphenoloxidase of *Vicia faba* but not clover or pea leaves following the trypsin treatment.[20] It should be considered that differences in enzyme protein may also influence the conditions of the optimal course of the enzymatic test. Possibly the great differences in enzymatic activity of higher plants[1, 4, 12] and within the same algal group[13] can be explained by this.

Under the physiological conditions mentioned above, the chlorophyll content of cells increases or decreases in a remarkably short time. Chlorophyllase increased in the first case, but remained unchanged in the latter. This may indicate that the enzyme functions in the synthesis of the pigment. But more data are needed before any theory can be advocated.

EXPERIMENTAL

Material

Chlorella vulgaris Beijerinck 211-11h, *Chlorella pyrenoidosa* Chick 211-8b and *Chlamydomonas reinhardi* Dang. 11-32 were from the algae collection of E. G. Pringsheim, Göttingen. They were grown in mineral medium as described previously.[11]

Homogenization

Algae were washed once with distilled water and then suspended in an aqueous medium which contained the following components in a final volume of 30 ml: 10 ml of polyethylenglycol-300 (Fluka, Switzerland), 1·5 mmoles of citrate buffer (pH 7·0), 20 μmoles of cysteine, 20 μmoles of ascorbic acid and 3 μmoles diisopropylfluorophosphate (DIPFP). The suspension was shaken vigorously for 1·5 min with glass beads (0·5 mm in diameter) in a homogenizer (Braun, Germany). Then the algal material was diluted to 40–50 ml with water containing 0·1 mM DIPFF. The peptidase inhibitor was not added when the enzyme was later activated with trypsin. Other alterations in the conditions of homogenization are mentioned in the appropriate experiments.

Chlorophyll

Chlorophyll was determined in methanol or 80% acetone according to Mackinney[21]. Substrate for the enzyme assay was a crude chlorophyll extract prepared from *Chlorella vulgaris*, which had a chlorophyll content of ~ 7% per dry weight. The extract was freed from

[18] E. HEISE and CH. KLEITKE, *Z. Naturforsch.* **17b**, 284 (1962).
[19] R. H. KENTEN, *Congr. Intern. Biochem.* (3e *Congr. Brussels*) p. 102, *Résumés communs.* (1955).
[20] R. H. KENTEN, *Biochem. J* **67**, 300 (1957).
[21] G. MACKINNEY, *J. Biol. Chem.* **140**, 315 (1941).

any chlorophyllide as follows: After the cells had been extracted three times with acetone in the cold, the pooled extracts were adjusted to 70% acetone and 1% NaCl and shaken with an aliquot of petrol ether (b.p. 40–60°). The green petrol ether phase was then shaken four times with aliquots of an acetone–NaCl mixture (350 ml acetone, 200 ml 2% NaCl-solution). The petrol ether was removed in a vacuum circulation evaporator (Büchi, Switzerland) at 0°, and the residue dissolved in acetone and stored in the cold.

Enzyme Test

Chlorophyllide, formed by enzyme action, was determined. The enzyme preparation in 0·05 M imidazole-buffer, pH 7·3 made in 40% acetone and chlorophyll (0·35 mg/ml) were made to a volume of 5 ml. Reaction vessels were shaken in a water bath for 1 hr at 25°. The reaction was stopped by adding 2 ml of the mixture to 3 ml acetone. The whole was centrifuged, and 3 ml of the supernatant shaken with 5 ml petrol ether and 2 ml 2% NaCl-solution. After centrifugation, the chlorophyllide in the acetone phase ($\sim$ 45% acetone) was measured at 655 nm. The extinction values were used directly in Figs. 1. to 5 to express enzyme activity. To calculate the concentration of the chlorophyllide, a factor was determined from the extinction values of chlorophyll concentrations in 80% acetone against the equivalent values in 45% acetone.

Activation

The homogenate was incubated with 0·05% trypsin (Merck, Germany) at 30° for 1 hr. During the long activation process without peptidase added, bacterial decomposition was prevented by chloramphenicol (100 μg/ml).

Acknowledgements—The author is grateful to Professor Dr. A. Pirson, by whose support this work could be done. The Deutsche Forschungsgemeinschaft gave technical help and provided the author with a grant for some months.