BBA 75503

VARIATIONS OF NADH OXIDASE ACTIVITY AND BACTERIOCHLOROPHYLL CONTENTS DURING MEMBRANE DIFFERENTIATION IN *RHODOSPIRILLUM RUBRUM*

J. OELZE AND G. DREWS

Lehrstuhl für Mikrobiologie, Institut für Biologie II der Universität, Freiburg i.Br. (Deutschland)

(Received April 15th, 1970)

SUMMARY

1. Alterations in the protein patterns depending on culture conditions were investigated in purified thylakoids isolated from *Rhodospirillum rubrum*. The corresponding bacteriochlorophyll contents and NADH oxidase activities of the thylakoids were determined.

2. High oxygen tension both in light and in darkness caused preferential synthesis of specific membrane proteins. These proteins are representative of cytoplasmic membrane fractions from anaerobically light-grown and aerobically dark-grown cells. Under these conditions the bacteriochlorophyll contents of thylakoids decreased whereas the activity of NADH oxidase increased 4–5-fold.

3. When such cells were incubated anaerobically at low light intensity (400 lux), proteins were newly synthesized that were typical of thylakoids from anaerobic illuminated cells. These proteins had not been formed previously in the presence of oxygen. The amount of bacteriochlorophyll increased. However, NADH oxidase activity on a bacteriochlorophyll basis decreased.

4. In cultures growing anaerobically at moderate light intensity (4000 lux) all of the thylakoid proteins were synthesized in typical ratios. There was only a very slight increase of NADH oxidase activity. Bacteriochlorophyll contents remained nearly constant.

5. The results strongly suggest a correlation between variations in catalytic functions and protein patterns of thylakoids.

INTRODUCTION

Investigations on membrane functions in the non-sulfur purple bacteria have shown that the cells adjust to varied culture conditions by an increase of specific membrane-bound physiological activities. The development of the photosynthetic apparatus, under conditions of anaerobiosis in the light or growth under reduced oxygen pressure in the dark, has been described by several authors[1–4]. There are also many reports concerning respiratory activities of crude membranes derived from aerobically dark-grown cells as well as from anaerobically light-grown organisms[5–14]. Respiratory values on a protein basis were lower in anaerobically light-grown cells than in aero-

bically dark-grown cells. Most of the authors cited discuss a coupling between the photochemical and the respiratory electron transport systems.

Recently KEISTER AND MINTON[9], using anaerobically light-grown cells that doubled 4 times under subsequent aerobic dark conditions, reported that the activity of the NADH oxidase system of a crude thylakoid fraction increased about fivefold. These conditions had no effect on the ratio of bulk bacteriochlorophyll to reaction center bacteriochlorophyll (P 870). This indicates that in this special case, when pigment synthesis is inhibited[15], the amount of total bacteriochlorophyll related to the other constituents of the photosynthetic apparatus is constant. This is contrary to results under conditions when synthesis of pigment occurs[2].

Our investigations on membrane differentiation have shown that high oxygen partial pressure together with an increase of light intensity induced the cells to change the protein pattern of thylakoids. This was due to a preferential synthesis of those proteins that characterize the protein pattern of membranes from aerobically dark-grown cells[16,17]. In a foregoing publication we have shown that NADH oxidase activity in anaerobically dark-grown cells of *Rhodospirillum rubrum* was not equally distributed between the cytoplasmic membrane and thylakoids[14]. Therefore it seems necessary to study membrane differentiation with purified membrane preparations. For methodological reasons purified thylakoids were used throughout the experiments. The total purification of the cytoplasmic membrane of *R. rubrum* has not been described.

The present paper reports changes in protein composition and function of purified thylakoids depending on variations of culture conditions, *e.g.* oxygen and light. The results generally support previous observations which indicated that membrane differentiation involves preferential synthesis and incorporation of specific proteins[17]. The correlation between function and protein pattern of thylakoid membranes is discussed.

MATERIALS AND METHODS

Rhodospirillum rubrum, strain FR1, was cultivated aerobically in the dark. 20–24-h-old cultures were used for inoculation of 50-ml bottles with metal screw caps. The bottles were completely filled with R8ÄH medium which was used throughout the experiments[18]. The bottles were incubated at 30° and 4000 lux for 14–18 h. The resulting cultures were used as inoculum for two types of culture condition. First, bottles were kept under alternating light intensities as described previously[17,19].

For the second set of experiments 1.5-l New Brunswick fermenter was used at 30° under the following conditions.

A. Before inoculation pure nitrogen was bubbled through the culture medium for 4–5 h. After 5 h of cultivation under nitrogen at 4000–5000 lux at the surface of the fermenter the organisms were harvested. Under these culture conditions growth as well as bacteriochlorophyll synthesis continued. For this reason the bacteriochlorophyll content per mg of cell protein remained constant (Fig. 1).

B. In another group of experiments the inoculated culture medium was gassed with air, resulting in oxygen pressures between 130 and 150 mm Hg within the suspension (high aeration). One part of these experiments was done in the dark whereas the other part was done in the light. As stated above, the light intensity on the surface of the fermenter was adjusted to 4000–5000 lux. The cells were also culti-

vated under these conditions for 5 h. As shown in Fig. 2, no bacteriochlorophyll was formed but growth continued. This caused a decrease of the cellular bacteriochlorophyll content.

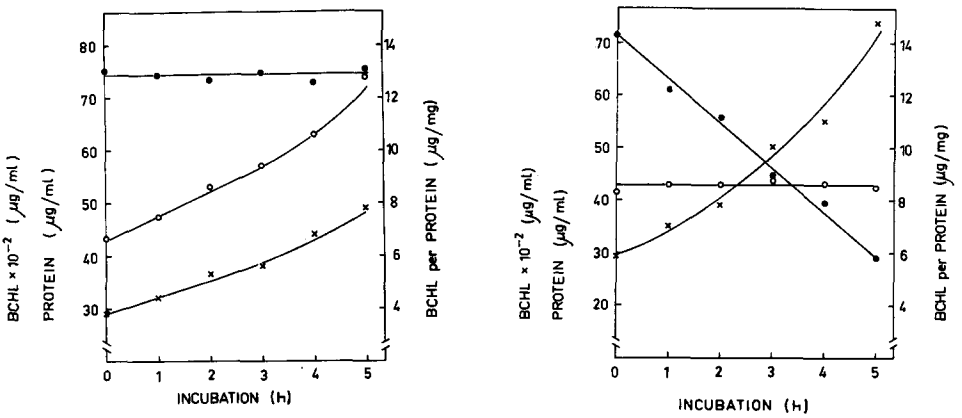

Fig. 1. Growth and bacteriochlorophyll synthesis of *R. rubrum* under anaerobic conditions in the light (4000 lux). For inoculation, cells were cultured 14–18 h anaerobically in the light. ×—×, protein (mg/ml); ○—○, bacteriochlorophyll (µg/ml); ●—●, bacteriochlorophyll per protein (µg/mg).

Fig. 2. Growth of *R. rubrum* under aerobic conditions in the light (4000 lux). For precultivation and symbols see Fig. 1.

For radioactive labeling, cells were cultivated throughout the experiments with 50 µC uniformly-^{14}C-labeled protein hydrolyzate (Radiochemical Centre, Amersham, England) per liter of culture medium. For labelings of the bottled cultures it was necessary to transfer them to 1-l erlenmeyer flasks. To maintain anaerobic conditions the flasks were bubbled with pure nitrogen. After 30 min of nitrogen the radioactive material was added. Thereafter the bottles were filled under nitrogen with the labeled culture suspension and incubated at 400 lux and 30°. After harvesting, the cells were washed in ice-cold phosphate buffer (0.05 M, pH 7.2) and homogenized with a prechilled French pressure cell as described elsewhere[19]. For enzyme measurements all subsequent steps were carried out below 5°.

The methods for measuring NADH oxidase activities were described in a previous paper[14]. Methods for preparation, separation[20] and identification of the protein pattern as well as for tracing of the radioactivity within the different membrane proteins have been published elsewhere[16,17]. Bacteriochlorophyll was determined by using the specific extinction coefficient of SMITH AND BENITEZ[21]. Protein was estimated by the Folin phenol procedure[22].

RESULTS

The NADH oxidase system of thylakoids

Activities of NADH oxidase were tested in purified thylakoids isolated from cells of *R. rubrum*. The cultures were grown under the conditions given in Table I. The results are means of 3–4 independent cultures. Standard deviations (σ) are included[23].

Biochim. Biophys. Acta, 219 (1970) 131–140

TABLE I

NADH OXIDASE ACTIVITY IN THYLAKOIDS OF *R. rubrum*

Thylakoids were isolated[19] from cells cultivated under the conditions indicated. For incubation, organisms were used that had been precultured 14–18 h anaerobically in the light.

Cultural conditions	*NADH oxidized*				*Bacterio-chlorophyll per thylakoid (μg/mg protein)*	*Bacterio-chlorophyll per cell (μg/mg protein)*
	μmole NADH per mg protein per min	± σ	*μmole NADH per μg bacterio-chlorophyll per min*	± σ		
1. Anaerobic, 4000 lux, 14–18 h	0.026	0.004	0.24	0.03	107	17.5
2. Aerobic, 4000 lux, in bottles, 5 h	0.055	0.003	0.97	0.02	61	8.0
↓						
3. + Anaerobic, 400 lux, in bottles, 4 h	0.064	0.004	0.73	0.03	89	12.0
4. Anaerobic, 4000 lux, fermenter, 5 h	0.037	0.007	0.36	0.05	100	14.0
5. Aerobic, dark, fermenter, 5 h	0.052	0.006	0.72	0.11	73	8.5
6. Aerobic, 4000 lux, fermenter, 5 h	0.051	0.016	0.93	0.15	54	5.8

The results in the first line of Table I are representative of thylakoids of organisms used for inoculation in all the subsequent experiments. They were obtained from cells cultured anaerobically in the light for 14–18 h. The thylakoids had bacteriochlorophyll contents of 107 μg/mg of protein. They were isolated from cells with 17.5 μg bacteriochlorophyll per mg of cell protein. The NADH oxidase system showed a relatively low activity of 0.026 μmole NADH per mg of protein per minute. On a bacteriochlorophyll basis this is 0.24. Such cells were diluted with fresh culture medium and then incubated at 4000 lux. This dilution decreases the effect of self shadowing, thus increasing the effective light intensity for each cell. As shown previously, the bacteriochlorophyll contents of the thylakoids and the whole cells are lowered[17]. In contrast, within the purified thylakoids the activity of NADH oxidase increased on a protein basis and even more on a bacteriochlorophyll basis. It should be stressed that at the beginning of these conditions there was a very high oxygen partial pressure in the culture medium. During the 5 h of incubation all the oxygen is consumed[17]. In the dark the same organisms diluted in the same way needed only between 1 and 2 h to use up all the oxygen. When cells grown in bottles at 4000 lux were transferred anaerobically to a lower light intensity (400 lux) the amounts of bacteriochlorophyll per thylakoids and whole cells increased. These conditions resulted in a decrease of NADH oxidase activity on a bacteriochlorophyll basis whereas on a protein basis there was still a small increase.

In order to study the effect of oxygen and light on bottled cultures the following experiments were performed. Cultures were kept for 5 h anaerobically at 4000 lux. This results in a small increase in NADH oxidase activity. The differences in the respiration rate at the beginning and at the end of the incubation were significant, as shown by a *t*-test[23]. The bacteriochlorophyll contents of thylakoids and whole cells were nearly constant (Table I, line 4 and Fig. 1). Oxygen during darkness was even more effective in enhancing NADH oxidase activity (Table I, line 5). After 5 h of growth under these conditions the bacteriochlorophyll contents of thylakoids and cells were diminished. The standard deviations of the respiratory activities are relatively high so that there are no significant differences between the values of the bottled cultures (Table I, Line 2). Differences in growth rate present an additional complication. Under constant aerobic conditions in the light the bacteriochlorophyll contents of whole cells and of thylakoids decreased (Table I, line 6 and Fig. 2). The activity of the NADH oxidase system increased in thylakoids of these cells both on a protein and bacteriochlorophyll basis. The same values were obtained with bottled cultures at 4000 lux and decreasing amounts of oxygen (Table I, line 2).

Changes in protein patterns

When cells precultured anaerobically in the light were maintained under the same conditions after dilution, all thylakoid proteins were synthesized. The upper curve in Fig. 3 illustrates the protein pattern of thylakoids whereas the lower one shows the amount of newly-synthesized proteins as indicated by the incorporation of radioactive amino acids. There are no great differences between the curves.

All the thylakoid proteins (Zones B–G) were synthesized under anaerobic light conditions in growing cultures, and the relation between the rates of synthesis of the different proteins does not seem to be much altered. Protein synthesis in Zone B was perhaps slightly higher, whereas protein synthesis in Zone G may have been smaller.

In such cultures the presence of oxygen instead of nitrogen changed the protein pattern of thylakoid membranes considerably (Fig. 4). Proteins of Zone B were predominantly synthesized. In Zones D, E and F there was less synthesis. A very small portion of Zone G proteins may have been synthesized. Some slower moving proteins

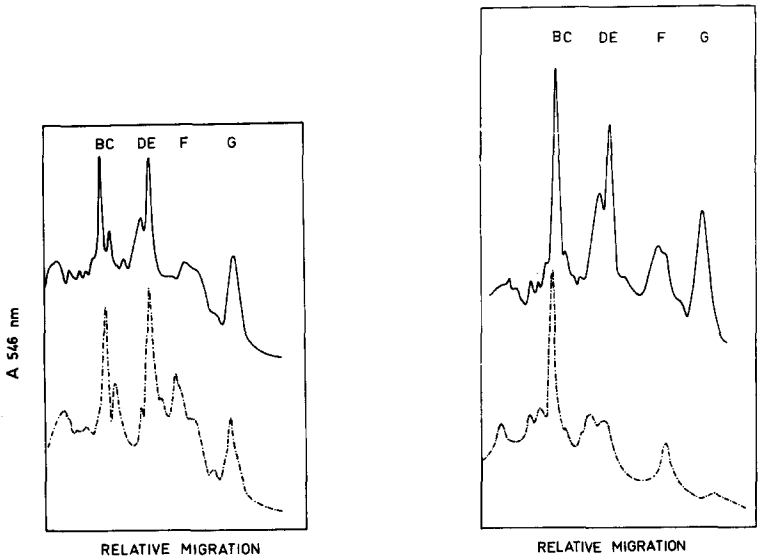

Fig. 3. Synthesis of membrane proteins in thylakoids of *R. rubrum* growing anaerobically in the light (4000 lux). For precultivation see Fig. 1. Thylakoids were isolated from the cells after 5 h of growth in the presence of [^{14}C]protein hydrolyzate (50 μC/l of culture medium). Purification of the thylakoids was achieved by centrifugation in a Ficoll gradient[19]. Preparation and gel-electrophoretic separation of the thylakoid proteins were done according to TAKAYAMA *et al.*[16,17,20]. The radioactivity of the protein zones was detected by autoradiography. The gels as well as the autoradiographs were scanned densitometrically. B–G, protein zones. ———, thylakoid proteins; –·—·–, radioactive thylakoid proteins.

Fig. 4. Synthesis of membrane proteins in thylakoids of *R. rubrum* growing aerobically in the light (4000 lux). For methods and symbols see Fig. 3.

situated in front of Zone B were also visible. Nearly the same change in the protein pattern occurred with thylakoids obtained from cultures grown under decreasing oxygen partial pressure in the light. The protein pattern of thylakoids and newly-synthesized proteins from cultures grown aerobically in the dark are shown in Fig. 5. Synthesis of Zone B proteins predominated. This influenced the protein pattern of the thylakoids drastically. Radioactivity was incorporated in the proteins of the Zones D, E and F. A shoulder at Peak B indicates radioactive proteins in Zone C. Here, too, a slower migrating protein near Zone B was labeled.

The thylakoids of Fig. 6 were prepared in the following manner. Cultures were grown in 50-ml bottles for 5 h at 4000 lux and decreasing oxygen content. At the end of this incubation oxygen had been completely consumed. During this time the specific bacteriochlorophyll content of thylakoids and cells was lowered, and proteins of Zone B predominated over the other thylakoid proteins. When such cells were labeled with protein hydrolyzate for 4 h at 400 lux, thylakoid proteins E and G were synthe-

sized preferentially (Fig. 6). Moreover, Zones D and even F incorporated the label. No synthesis of B or C proteins was observed.

The results indicate that the presence of oxygen always established a predominance of Zone B proteins of thylakoids. This, too, is true for membranes of (not photosynthetically) dark-grown cells[16]. In Fig. 7 we compare the protein pattern of

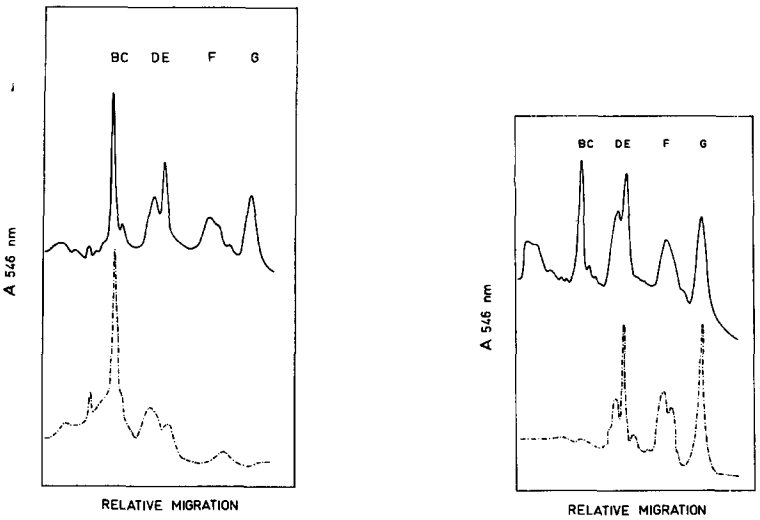

Fig. 5. Synthesis of membrane proteins in thylakoids of *R. rubrum* growing aerobically in darkness. For methods and symbols see Fig. 3.

Fig. 6. Synthesis of membrane proteins in thylakoids of *R. rubrum* growing anaerobically at 400 lux. Cells were precultured as described in Fig. 1. They were then cultivated 5 h aerobically in screw cap bottles at 4000 lux. Thereafter they were incubated for 4 h in the presence of [^{14}C]-protein hydrolyzate (50 μC/l of culture medium). For other methods and symbols see Fig. 3.

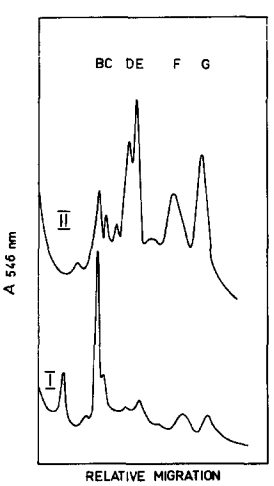

Fig. 7. Protein patterns of thylakoids and a cytoplasmic membrane fraction from 16-h-old anaerobic light-grown cells of *R. rubrum*. The fractions were isolated by centrifugation in a Ficoll gradient[19]. For the other methods see Fig. 3. II, thylakoid proteins; I, proteins of the cytoplasmic membrane fraction.

particles of the upper band of the Ficoll gradient obtained from 16-h-old anaerobic light-grown cells with the protein pattern of thylakoids from the same culture. The features of such particles resembled those of the cytoplasmic membrane[19,24]. Even in this fraction proteins of Zone B were present in the highest amount. All the other proteins of the Zones C–G can also be identified.

DISCUSSION

In 1957 COHEN-BAZIRE *et al.*[15] reported experiments on the influence of oxygen on pigment synthesis in phototrophically-grown cultures of several non-sulfur purple bacteria. They found that with aeration the synthesis of pigments was interrupted immediately. According to KEISTER AND MINTON[9], the composition of the photosynthetic apparatus is not changed under these conditions. This is also true for the amount of bacteriochlorophyll per reaction center (P 870). However, oxygen and darkness result in a significant enhancement of NADH oxidase activity.

Our experiments on the NADH oxidase system in the presence of oxygen show (Table I, Lines 2, 5, 6) that not only in darkness but even in light the activity of the respiratory chain increased in thylakoids of *R. rubrum*. Especially on the basis of bacteriochlorophyll, this increase is convincing. The chosen experimental conditions, taking bacteriochlorophyll as a reference, are most informative. Bacteriochlorophyll is representative of the unchanged photochemical electron-transport system, and the thylakoid differentiation is therefore demonstrated best by relating NADH oxidase activity to bacteriochlorophyll content. In bottled cultures oxygen is consumed very slowly[17]. The presence of oxygen was accompanied by a rise in NADH oxidase activity in thylakoids (Table I, line 2). On a protein basis this activity doubled, whereas on a bacteriochlorophyll basis there was a fourfold increase in comparison with the activities in the inoculum (Table I, line 1).

Similar activities were found in the thylakoids obtained from cells grown for 5 h aerobically either in darkness or in light (Table I, lines 5 and 6). When bottled organisms, after aerobic incubation at 4000 lux, were cultured anaerobically at 400 lux a drop of NADH oxidase activity on a bacteriochlorophyll basis was found (Table I, line 3). Anaerobic conditions together with increased light intensity caused a small but significant increase of activity in thylakoids. This might be the reason for the decreased amount of bacteriochlorophyll per thylakoid protein depending on very high light intensities[25].

The question arises whether changes of NADH oxidase activity are due to physiological changes within the cell. Variations of enzyme activity may be caused by allosteric effects or by synthesis of enzymes. If the proteins of the membranes represent entities with catalytic functions, a new formation of these proteins must occur when membrane-bound enzymes are synthesized. In the following part we discuss the synthesis of thylakoid proteins depending on different culture conditions. Thereafter we intend to correlate changes in membrane function and variations in their protein patterns.

Thylakoids analyzed in Fig. 3 were derived from cells grown anaerobically in the light (4000 lux). Dilution increases the effective light intensity for the organisms of the inoculum. In these cultures growth continued and the amount of bacteriochlorophyll per cell remained constant during the time of investigation (Fig. 1). In

these thylakoids all proteins incorporated radioactive amino acids (Fig. 3). The ratio of synthesis of the different proteins seems to be correlated in such a way that a protein pattern typical for thylakoids resulted. This means that in growing cultures all the membrane proteins are needed for enlargement of the thylakoid reticulum. This includes those proteins typical of thylakoids as well as those typical of membranes of aerobically dark-grown cells (cytoplasmic membrane)[16,17]. A different ratio of synthesis was found among the thylakoid proteins from cells grown highly aerated in the light (Fig. 4). In this condition the synthesis of proteins in Zone B predominates over the small formation of Zone D, E and F proteins. We have demonstrated in an earlier publication[17], by labeling membrane lipids, that in such air-grown cultures the thylakoid infolding may be reversed, thus generating cytoplasmic membrane. In the presence of oxygen the incorporation of newly-synthesized proteins differentiated the thylakoid membrane so that it generated a pattern of membrane proteins typical of cytoplasmic membranes (Figs. 4, 7). This was also true for thylakoids of aerobically dark-grown organisms (Fig. 5). Therefore we believe that in the presence of high oxygen tension light has no direct effect on membrane differentiation. The growth of cultures grown aerobically in the light (4000 lux) stops immediately on transition to anaerobic low light (400 lux) conditions, whereas bacteriochlorophyll is synthesized[17,19]. Newly-synthesized proteins of Zones E and G were incorporated into thylakoids of such cells. No Zone B proteins were formed. Obviously such thylakoids resemble cytoplasmic membranes, which have to be differentiated during thylakoid morphogenesis by incorporation of specific proteins. In all cases discussed below proteins of the heterogeneous Zone F and of Zones C and D were also synthesized. These proteins must have functions common to all modifications of membranes in *R. rubrum*. The amounts of most of the membrane-bound components of the electron transport systems are independent of culture conditions. However, bacteriochlorophyll and the oxidase systems depend very much on such conditions[1,7,9,11]. For this reason the differentiation of membranes may be looked at from a functional point of view.

Results obtained from investigations on different mutant strains of *R. rubrum* have already shown, that formation of proteins in Zone G is correlated with bacteriochlorophyll production[24]. The recent results on thylakoid formation and bacteriochlorophyll production support this conclusion[17] (Fig. 6). Possibly there is a direct connection between Zone G proteins and bacteriochlorophyll. Proteins of Zone E were synthesized in all cases. The rate of synthesis, however, depended on factors inducing thylakoid morphogenesis. The Zone E proteins may reflect compounds present in low amounts in the cytoplasmic membrane and in high amounts in thylakoids.

Proteins of Zone B were synthesized whenever the activity of the NADH oxidase system was enhanced. Zone B proteins predominated in the cytoplasmic membrane fraction of anaerobically light-grown cells, and were only present in small amounts in thylakoids derived from the same cells (Fig. 7). In the preceding paper[14] we showed that there is an unequal distribution of the NADH oxidase system within the total membrane system of one single cell. A high oxidase activity was found in a crude cytoplasmic membrane fraction whereas thylakoids showed low activities. All these results suggest a correlation between Zone B proteins and the NADH oxidase system, which infers that the increase of NADH oxidase activity was due to induction of enzyme synthesis.

In the bottled cultures light partially inhibited the respiratory activity which

caused a slow decrease of the oxygen content. This resulted in a competition between the two energy-generating systems, photosynthesis and respiration. Oxygen caused the total blockage of pigment synthesis, resulting in an inhibition of any further production of the photosynthetic apparatus, and induced the oxidase system. The existing photosynthetic apparatus was thereby diluted. It is apparent that the influence of light is not so important on membrane differentiation as is the presence of oxygen.

ACKNOWLEDGMENTS

This investigation was supported by the Deutsche Forschungsgemeinschaft. We thank Miss H. Heiting for expert technical assistance.

REFERENCES

1 J. LASCELLES, *J. Gen. Microbiol.*, 29 (1962) 47.
2 G. COHEN-BAZIRE AND W. R. SISTROM, in L. P. VERNON AND G. R. SEELEY, *The Chlorophylls*, Academic Press, New York, 1966, p. 313.
3 J. LASCELLES, in A. H. ROSE AND J. F. WILKINSON, *Advances in Microbial Physiology*, Academic Press, New York, 1968, p. 1.
4 G. DREWS, H. H. LAMPE AND R. LADWIG, *Arch. Mikrobiol.*, 65 (1969) 12.
5 T. HORIO AND M. D. KAMEN, *Biochim. Biophys. Acta*, 43 (1960) 382.
6 T. HORIO AND M. D. KAMEN, *Biochemistry*, 1 (1962) 1141.
7 D. M. GELLER, *J. Biol. Chem.*, 237 (1962) 2947.
8 S. TANIGUCHI AND M. D. KAMEN, *Biochim. Biophys. Acta*, 96 (1965) 395.
9 D. L. KEISTER AND N. J. MINTON, in H. METZNER, *Progress in Photosynthesis Research*, Vol. 3, Tübingen, 1969, p. 1299.
10 D. L. KEISTER AND N. J. MINTON, *Biochemistry*, 8 (1969) 167.
11 J. H. KLEMME AND H. G. SCHLEGEL, *Arch. Mikrobiol.*, 68 (1969) 326.
12 A. THORE, D. L. KEISTER AND A. SAN PIETRO, *Arch. Mikrobiol.*, 67 (1969) 378.
13 J. YAMASHITA AND M. D. KAMEN, *Biochem. Biophys. Res. Commun.*, 34 (1969) 418.
14 E. THROM, J. OELZE AND G. DREWS, *Arch. Mikrobiol.*, 72 (1970) 361.
15 G. COHEN-BAZIRE, W. R. SISTROM AND R. Y. STANIER, *J. Cell. Comp. Physiol.*, 49 (1957) 25.
16 J. OELZE AND G. DREWS, *Arch. Mikrobiol.*, 69 (1969) 12.
17 J. OELZE AND G. DREWS, *Biochim. Biophys. Acta*, 203 (1970) 189.
18 G. DREWS, *Arch. Mikrobiol.*, 51 (1965) 186.
19 J. OELZE AND G. DREWS, *Biochim. Biophys. Acta*, 173 (1969) 448.
20 K. TAKAYAMA, D. H. MACLENNAN, A. TZAGOLOFF AND C. D. STONER, *Arch. Biochem. Biophys.*, 114 (1966) 223.
21 J. H. C. SMITH AND A. BENITEZ, in K. PAECH AND M. V. TRACEY, *Moderne Methoden der Pflanzenanalyse*, Vol. IV, Springer, Berlin, 1955, p. 179.
22 O. H. LOWRY, M. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
23 L. CAVALLI-SFORZA, *Biometrie, Grundzüge biologisch-medizinischer Statistik*, Fischer, Stuttgart, 1969.
24 J. OELZE, J. SCHRÖDER AND G. DREWS, *J. Bacteriol.*, 101 (1970) 669.
25 S. C. HOLT AND A. G. MARR, *J. Bacteriol.*, 89 (1965) 1421.