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Light-Activated, -Inhibited and -Independent Denitrification by a Denitrifying Phototrophic Bacterium

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Abstract. Effects of illumination on denitrification by a freshly isolated denitrifying phototrophic bacterium were investigated. Denitrification activity was induced when cells were grown in either light or darkness in the presence of nitrate without oxygen. Denitrification of nitrate with malate as the electron donor by cells at a phase of exponential growth occurred independently of illumination while that by cells in a stationary phase was activated. Effects of illumination on denitrification varied with electron donors. Using malate or succinate, denitrification by cells in a stationary phase was accelerated by illumination, inhibited when glucose or lactate was used, and independent of illumination when pyruvate was used. Denitrification by cells in an exponential phase was independent of illumination when succinate, malate or pyruvate was used and inhibited by it when glucose or lactate was used. Effects of illumination on the denitrification of nitrite were similar to those involving nitrate. Effects of various inhibitors on denitrification were examined in light-succinate and dark-lactate systems. Differences between the two systems are discussed.

Key words: A denitrifying phototrophic bacterium – Effects of illumination on denitrification – *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*.

Previous investigation has developed three new strains of phototrophic bacteria exhibiting denitrifying activity. These were named *Rhodopseudomonas sphaeroides* forma sp. *denitrificans* (Satoh et al., 1976). These denitrifying phototrophic bacteria exhibit nitrate respiration as a biological energy-transforming system in addition to oxygen respiration and photosynthesis common in other phototrophic bacteria. The new phototrophic bacteria raise the following problems: How is the nitrate respiration affected by light? What are the relationships among photosynthesis, nitrate and oxygen respiration as energy-transforming systems? What is the position in which the bacteria are situated in the evolution of energy-transforming systems?

The denitrifying phototrophic bacteria grow anaerobically in the presence of nitrate in either light of darkness. The growth rate of bacteria cultured anaerobically in the presence of nitrate in light was about twice that of those cultured in darkness. Denitrification in light was more intense than that in darkness (Satoh et al., 1976). These phenomena seem to be due to links between denitrification and photosynthesis. In a similar line of thinking, there are relations between oxygen-respiration and photosynthesis, where oxygen uptake is inhibited by illumination (Morita, 1955; Katoh, 1961; Horio and Kamen, 1962; Horio and Yamashita, 1963; Kikuchi et al., 1964; Oelze and Weaver, 1971).

This denitrifying phototrophic bacterium is the first photoorganism showing nitrate-respiration although many denitrifying bacteria are already known (Payne, 1973). In the present paper the effects of illumination on denitrification are described.

MATERIALS AND METHODS

Organism and Growth Conditions. A denitrifying phototrophic bacterium, *Rhodopseudomonas sphaeroides* forma sp. *denitrificans* IL 106, was used, which was newly isolated in our laboratory (Satoh et al., 1976). Cells were cultured in a basal medium supplemented with 0.2% sodium malate as a carbon source, growth factors and 0.01% yeast extract. The composition of the basal medium and growth factors are described in a previous paper (Satoh et al., 1976). For the denitrification cultures, 0.2% KNO_3 was added to the basal medium. The pH of the medium was adjusted to 7.0. Photosyn-

Abbreviations. KCN = potassium cyanide; HQNO = 2-n-heptyl-4-hydroxyquinoline-N-oxide; TTFA = 2-thenoyltrifluoroacetone; CCCP = carbonyl cyanide-m-chlorophenylhydrazone; DCCD = dicyclohexylcarbodiimide

Table 1. Denitrification activities of cells grown under various conditions

Conditions	Aerobic-dark		Semianaerobic-light		Anaerobic-light		Anaerobic-dark
	$-NO_3^-$	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$	$+NO_3^-$
Activity	0	0	0	10	0	12	7

Cells were cultured aerobically in darkness for 15 h with or without nitrate, semianaerobically for 2 days with or without nitrate, anaerobically in light for 3 days without nitrate or 2 days with nitrate, or anaerobically in darkness for 7 days with nitrate, harvested and suspended to give a concentration of 40 mg dry wt. per ml. Denitrification activity was measured with lactate as an electron donor in darkness. The activities are shown as μl N_2 per h per mg dry wt

thetic growth was carried out in light in rubber-plugged 2-l Erlenmeyer flasks filled with the medium. Denitrifying growth was carried out in 1-l Erlenmeyer flasks equipped with gas traps. Semianaerobic cultures were carried out without shaking in light in cotton-plugged 1-l Erlenmeyer flasks filled slightly less than full with the medium. Aerobic cultures were carried out with agitation in darkness in Sakaguchi flasks. In each case the incubation temperature was 30° C and the light intensity was 3000 lux from fluorescent lamps. The intensity was light-limited for growth. However, cells grown under light-saturation (4000 lux from Tungsten lamps) showed the same manner in the effect of illumination on denitrification as those grown under light-limitation.

Manometric Measurements of Denitrification. Manometric measurements were made at 30° C using conventional Warburg vessels with a single side arm. Suspensions of cells for manometric experiments were prepared by centrifuging cultures for 20 min at 5000 × g, washing the cells with saline 2 times, and resuspending the cells in saline to a concentration of 40 mg dry weight of cells per ml. The cells were used for assays within 2 days after harvesting. Each vessel contained 0.2 ml of cell suspension, 0.4 ml of 0.5 M sodium phosphate buffer (pH 7.0), 0.2 ml of 0.5 M each carbon source as electron donors and 1.0 ml of water in the main compartment, 0.2 ml of 0.1 M KNO_3 or 0.1 M $NaNO_2$ in the side arm and 0.2 ml of 20% KOH containing a bit of sodium dithionite in a folded filter paper in the center well. At zero time, 0.2 ml of NO_3^- or NO_2^- was added from the side arm. Production of nitrogen gas was measured under a gas phase of 100% He.

Assay of NO_3^- Quantity with Nitrate Electrode. One ml of the reaction mixture in the Warburg vessel was pipetted into a 50 ml flask after measuring the denitrification activity and diluted tenfold with distilled water. Then, nitrate quantity was assayed using a Corning ion exchange nitrate electrode by direct potentiometric measurements. A calibration plot of millivolts (linear) versus nitrate concentration (logarithmic) was prepared for each assay.

Chemical. The inhibitors, rotenon (Nakarai Chemicals Co.), antimycin A (Kyowa Hakko Co.), HQNO (2-n-heptyl-4-hydroxyquinoline-N-oxide, Sigma Chemical Co.), TTFA (2-thenoyltrifluoroacetone, Tokyo Kasei Co.), gramicidin D (Sigma Chemical Co.), CCCP (carbonyl cyanide-m-chlorophenylhydrazone, Sigma Chemical Co.) and DCCD (dicyclohexylcarbodiimide, Sigma Chemical Co.), were added in ethanol solution. KCN (potassium cyanide), o-phenanthrolin and amytal (Nippon Shinyaku Co.) were dissolved in distilled water and dicoumarol (Tokyo Kasei Co.) in 0.1 M $NaHCO_3$.

RESULTS

Growth Conditions and Denitrifying Activity

It is known that synthesis of denitrifying enzymes is induced when denitrifying bacteria are cultured in the presence of nitrate or nitrite under anaerobic conditions (Nason, 1962). The denitrifying activity of the IL 106 strain cultured under various conditions was examined (Table 1). The denitrifying activity was assayed with lactate as the electron donor in darkness since the activity was inhibited by illumination as shown in Figure 5. Aerobic growth conditions produced denitrifying activity neither in the presence nor absence of nitrate. Under semianaerobic or anaerobic conditions in either light or darkness, cells exhibited denitrifying activity only in the presence of nitrate. The results show that as seen for other denitrifying bacteria, denitrifying activity is induced in the presence of nitrate under anaerobic conditions.

Effect of Illumination on Denitrification of Cells of Different Age

In view of the denitrification process in phototrophic bacteria, it is of great interest how illumination might affect denitrification. The conditions of experiments on the effects of illumination on denitrification were varied. Then, relations between growth age in which cells were harvested and effects of light were studied. A culture (20 ml) of IL 106 grown semianaerobically in light for 3 days was inoculated into each of four flasks (1 l each) of the nitrate containing medium with gas traps. Each culture was allowed to grow anaerobically in light for either 1, 2, 3 or 4 days. Growth (optical density at 660 nm) and volume of nitrogen evolved for each culture were measured and cells harvested for denitrification activity assay. The activity was assayed using malate as the electron donor. When illuminated, the light intensity was adjusted to about 4000 lux from Tungsten lamps because it was sufficient for maximum inhibition of denitrification by 2-day cells with lactate as shown in Figure 1. The growth curve and the gas volume of nitrogen evolved is shown in Figure 2 and the effects of illumination on denitrification in Figure 3. Little effect of illumination was observed in the 1- or 2-day-cultured cells which were in a exponential or early stationary growth phase respectively, showing denitrification independ-

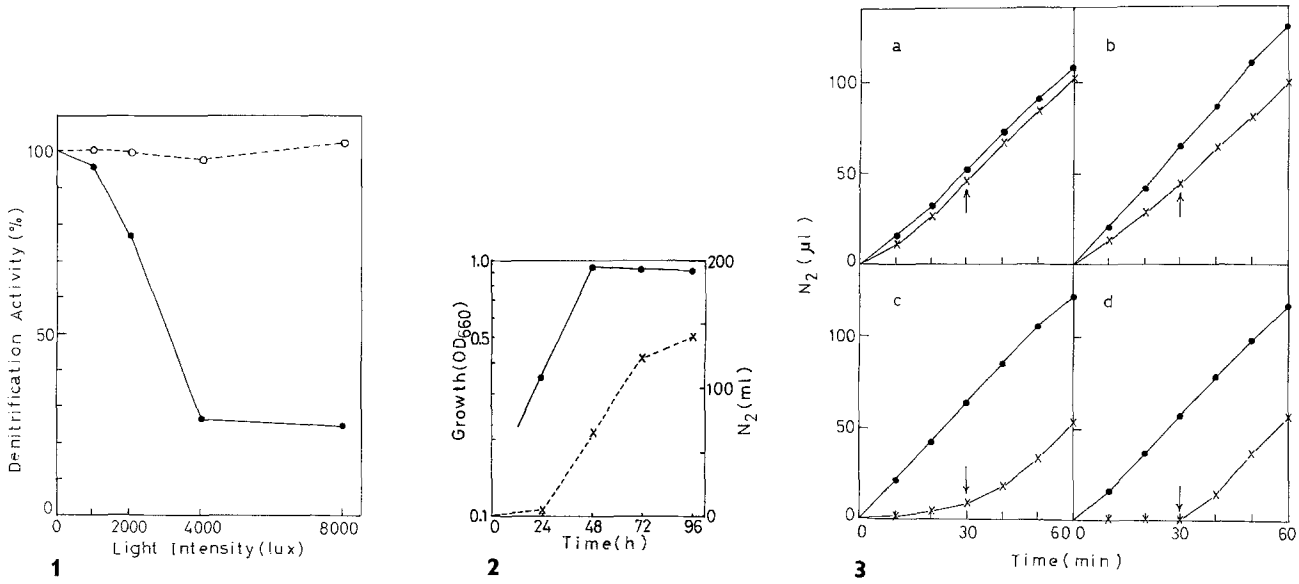

Fig. 1. Inhibition of denitrification of 2-day cells with lactate or succinate by different light intensities. Illumination was given from Tungsten lamps. Denitrification activities are shown as percent of dark denitrification. ●——● lactate; O-----O succinate

Fig. 2. Growth of IL 106 grown anaerobically in light and quantity of N_2 evolved. Cells were grown for each length in each 1-l Erlenmeyer flask equipped with gas traps anaerobically in light in the presence of nitrate. Optical density at 660 nm and the quantity of N_2 evolved were measured for each culture. ●——● growth; ×-----× N_2

Fig. 3a–d. Effects of illumination on the denitrification of nitrate with malate by cells of each culture length. Cells were harvested after culturing for 1 (a), 2 (b), 3 (c) or 4 (d) days and assayed for denitrification activity of nitrate using malate as the electron donor. ●——● illumination lasted throughout the assay period; ×——× illumination began at the arrow after a 30 min dark period

ent of illumination. On the other hand the onset of evolution of nitrogen in the 3- and 4-day cultures, which were in a late stationary growth phase, did not occur in darkness and showed a distinct activation by illumination.

Similar phenomena were observed when nitrite was used as a substrate for denitrification and malate as the electron donor (Fig. 4). Though gas-evolution gave non-linear plots versus time, light-activated denitrification in the 3- or 4-day cultures and light-independent denitrification by the 1- or 2-day cultures were also observed. The gas evolved with nitrite was proved to be nitrogen since no absorption of the gas in the manometric assays was observed with acidified $KMNO_4$ in the center well to absorb nitric oxide (Miyata and Mori, 1968). Nor were nitric oxide and nitrous oxide identified in the gas chromatographic analysis.

Effects of Illumination on Denitrification with Various Electron Donors

Effects of illumination were investigated using various organic compounds as electron donors. Figure 5 shows the denitrification curve of 4-day cultured cells under alternating dark and light phases using various substrates as electron donors. Little denitrification during the first dark period occurred in cultures using succinate or malate. In the next phase, illumination accelerated the denitrification. This phenomenon is the same as that shown in Figure 3. However, resumption of dark period after illumination did not hinder denitrification. Here the effect of illumination is evidently irreversible. When lactate or glucose was used, denitrification occurred in the dark and was inhibited severely by the illumination. In the second dark period denitrification was restored at a rate equal to that in the first, showing the effects of illumination to be reversible. Denitrification using pyruvate was not appreciably affected by light.

Figure 6 shows the denitrification curve of 2-day cells. It shows that the denitrification using glucose or lactate is also inhibited by illumination and restored in darkness. On the other hand, denitrification with succinate, malate or pyruvate was independent of illumination. As shown in Figure 1, denitrification of 2-day cells with succinate was not affected by illumination when saturating or about 2 times saturating light intensity were used.

The results, using nitrite as the substrate for denitrification, of 4-day cells and 2-day cells are shown in Figures 7 and 8, respectively. Since the denitrification of nitrite was not linear against time, the figures are shown separately for each electron donor. The

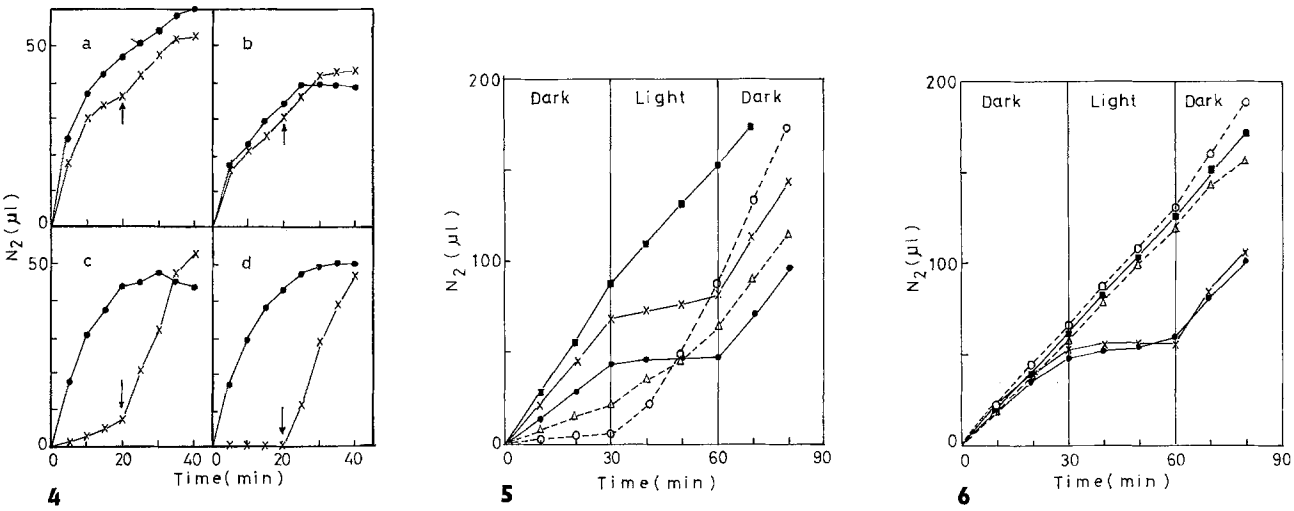

Fig. 4a–d. Effects of illumination on the denitrification of nitrite with malate by cells of each culture length. Cells were harvested after culturing for 1 (a), 2 (b), 3 (c) or 4 (d) days and assayed for denitrification activity of nitrite using malate as the electron donor. ●——● illumination lasted throughout the assay period; ×——× illumination began at the arrow after a 20 min dark period

Fig. 5. Effects of illumination on the denitrification of nitrate of 4-day cells with various electron donors. The denitrification of nitrate of 4-day cells was measured using various electron donors under alternating dark and light phases. ■——■ pyruvate; ●——● lactate; ×——× glucose; O-----O succinate; △-----△ malate

Fig. 6. Effects of illumination on the denitrification of nitrate of 2-day cells with various electron donors. The denitrification of nitrate of the 2-day cells was measured using various electron donors under alternating dark and light phases. Symbols are identical to those in Figure 5

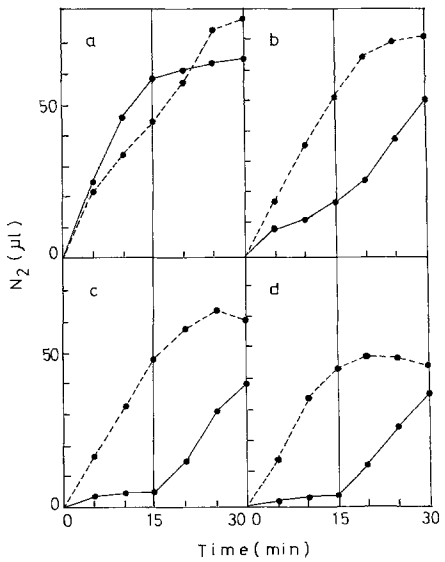

Fig. 7a–d. Effects of illumination on the denitrification of nitrite of 4-day cells with various electron donors. The denitrification of nitrite of the 4-day cells was measured using lactate (a), glucose (b), succinate (c) or malate (d) as the electron donor. Figures are shown separately for each electron donor. ●——● dark for the first 15 min and light in the second 15 min; ●-----● light for the first 15 min and dark in the second 15 min

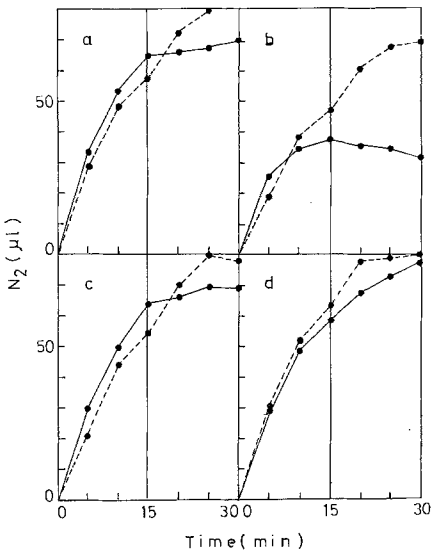

Fig. 8a–d. Effects of illumination on the denitrification of nitrite of 2-day cells with various electron donors. The denitrification of nitrite of the 2-day cells was measured using lactate (a), glucose (b), succinate (c) or malate (d) as the electron donor. Figures are shown separately for each electron donor. Symbols are identical to those in Figure 7

denitrification with succinate or malate by 4-day cells was also severely activated by illumination while that of 2-day cells was independent of illumination similar to the results with nitrate. However, the denitrification with lactate in 4-day cells occurred even during the first 15 min of illumination and then accelerated during the next 15 min of dark period. However, it was also inhibited by illumination after the 15 min of dark period. The denitrification of glucose in 4-day cells was rather activated by light. Though the denitrifica-

Table 2. Effects of inhibitors on denitrification and utilization of nitrate

Inhibitor	Concentration	Denitrification		Nitrate used	
		Light-succinate	Dark-lactate	Light-succinate	Dark-lactate
KCN	0.2 mM	21	38	18	27
o-Phenanthrolin	0.2 mM	0	0	0	0
Rotenon	0.5 mM	100	65	100	61
Amytal	2.5 mM	85	24	67	19
Antimycin A	50 μg/ml	39	74	97	78
HQNO	1.0 μM	32	32	37	33
TTFA	0.5 mM	19	30	15	33
Dicoumarol	0.2 mM	76	90	68	90
Gramicidin D	0.2 mM	84	134	84	138
CCCP	2.0 μM	79	71	75	78
DCCD	0.1 mM	54	74	50	72

Resting cell suspensions of 4-day cells were used to examine the effects of inhibitors. Light-succinate refers to the denitrification of nitrate with succinate in light, while dark-lactate refers to that of nitrate with lactate in darkness. The utilization of nitrate was estimated by measuring with a nitrate electrode. The activities are expressed in percent of that without inhibitor. The data are the average of three experiments

tion with glucose or lactate in 2-day cells occurred in the first 15 min regardless of illumination it was also inhibited under illumination and accelerated in darkness in the following 15 min. The denitrification with succinate or malate occurred in the first 15 min in either darkness or light, but that with succinate was noticeably inhibited by illumination after the first dark period.

As mentioned above, three different phenomena were observed as to the effects of illumination on denitrification. These are: 1) dark denitrification with lactate or glucose; 2) light-reactivated denitrification of 4-day cells with succinate or malate; and 3) light-independent denitrification of 2-day cells with succinate or malate.

Effects of Inhibitors on the Denitrification and Utilization of Nitrate

A cell-free system of nitrate reduction was unsuccessfully attempted in order to elucidate differences of the three distinct effects of illumination. Differences especially between light-reactivated denitrification with succinate and dark denitrification with lactate were to be elucidated by means of employing several inhibitors using the resting 4-day cell suspensions. The denitrification activity was assayed using a Warburg-manometer and the consumption of nitrate was estimated employing nitrate electrode. The results are shown in Table 2. Cyanide and o-phenanthrolin severely inhibited the denitrification and utilization of nitrate in both the light-succinate and the dark-lactate systems. Rotenon and amytal, which are known to inhibit NADH dehydrogenase systems (Izawa and Good, 1972), inhibited more severely the denitrification and utilization of nitrate in the dark-lactate system than that in the light-succinate system. As for antimycin A and HQNO, which both block electron transfer between cytochromes *b* and c_2 in *Rhodopseudomonas* (Nishimura, 1963), antimycin A inhibited more severely the denitrification in the light-succinate system than that in the dark-lactate system although the utilization of nitrate was only slightly affected by it. HQNO inhibited the denitrification and utilization of nitrate in both systems. TTFA, which specifically inhibits the succinate oxidase system in mitochondrial membranes and the particulate fraction from *Escherichia coli* (Kashket and Brodie, 1963), the light-succinate system severely inhibited as well. Uncouplers, such as dicoumarol, gramicidin D and CCCP (Izawa and Good, 1972), inhibited more strongly the light-succinate systems except that of CCCP. Gramicidine D was of special interest in that it activated the denitrification and utilization of nitrate in the dark-lactate system. The light-succinate system was more sensitive to DCCD, an inhibitor of energy transfer (Izawa and Good, 1972).

DISCUSSION

Complicated phenomena were observed concerning the effects of illumination on the denitrification by a denitrifying phototrophic bacterium. The denitrification with malate or succinate as an electron donor by the young cells in an exponential growth phase occurred independently of illumination and that by the cells in a late stationary phase was activated by illumination. The latter phenomenon may show that illumination is necessary for activation of the uptake of succinate or malate by old cells. The more effective

inhibition of denitrification in the light-succinate system by uncouplers and irreversible activation of denitrification by illumination, where light-activated denitrification was not hindered by the 2nd dark period after illumination, may support above-mentioned argument. In this respect, it is of interest in the sense of incorporation and metabolism of pyruvate that little effect of illumination on the denitrification with pyruvate was observed.

The denitrification with lactate or glucose was inhibited by illumination while that with succinate or malate was not affected by it with 2 times saturating light intensity. Similar results are obtained concerning the effects of illumination on oxygen uptake using this bacterium in a exponential phase (data are not shown). The photochemical reduction of NAD by phototrophic bacteria coupled to the oxidation of succinate or H_2 has been demonstrated and the mechanism of photoreduction has been concluded to be an energy-linked reversed electron flow (Keister and Yike, 1967; Klemme and Schlegel, 1967; Lippert and Klemme, 1968; Klemme, 1969; Gest, 1972). In this experiment, rotenone and amytal inhibited the denitrification in the dark-lactate system while gramicidin D accelerated it. It seems likely that NADH is, for the most part, a direct electron donor for electron transport from lactate or glucose to nitrate, and that inhibition by illumination results from energy-linked reversed reduction of NAD as discussed by Oeltze and Weaver (1971) about the light inhibition of oxygen uptake.

Cyanide and o-phenanthrolin strongly inhibited the denitrification using either succinate or lactate. The nitrite reductase of this bacterium is a Cu-enzyme (unpublished data) and cyanide is one of the strongest inhibitors of the enzyme. Iron-sulfur center complements very similar to that of animal mitochondria, where they have been identified as parts of the succinic or NADH dehydrogenases, have been resolved in chromatophores of many phototrophic bacteria (Ingledew and Prince, 1977). For the above-mentioned reasons, the denitrification is assumed to be inhibited by these two inhibitors. However, since the utilization of nitrate was also inhibited by cyanide, it is presumed to act on the other sites of electron transport.

Somewhat similar results were obtained with nitrite as a substrate. The denitrification of 4-day cells with glucose, however, was activated rather than inhibited by illumination. The mechanisms of the complicated phenomena remain to be disclosed.

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