

Regulation of Nitrate Assimilation and Nitrate Respiration in *Aerobacter aerogenes*

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The influence of growth conditions on assimilatory and respiratory nitrate reduction in *Aerobacter aerogenes* was studied. The level of nitrate reductase activity in cells, growing in minimal medium with nitrate as the sole nitrogen source, was much lower under aerobic than anaerobic conditions. Further, the enzyme of the aerobic cultures was very sensitive to sonic disintegration, as distinct from the enzyme of anaerobic cultures. When a culture of *A. aerogenes* was shifted from anaerobic growth in minimal medium with nitrate and NH_4^+ to aerobiosis in the same medium, but without NH_4^+ , the production of nitrite stopped instantaneously and the total activity of nitrate reductase decreased sharply. Moreover, there was a lag in growth of about 3 hr after such a shift. After resumption of growth, the total enzymatic activity increased again slowly and simultaneously became gradually sensitive to sonic disintegration. These findings show that oxygen inactivates the anaerobic nitrate reductase and represses its further formation; only after a de novo synthesis of nitrate reductase with an assimilatory function will growth be resumed. The enzyme in aerobic cultures was not significantly inactivated by air, only by pure oxygen. The formation of the assimilatory enzyme complex was repressed, however, by NH_4^+ , under both aerobic and anaerobic conditions. The results indicate that the formation of the assimilatory enzyme complex and that of the respiratory enzyme complex are regulated differently. We suggest that both complexes have a different composition, but that the nitrate reductase in both cases is the same protein.

Aerobacter aerogenes can utilize nitrate as the sole source of nitrogen in a minimal medium under both aerobic and anaerobic conditions (15). Under anaerobic conditions, nitrate also functions as a terminal hydrogen acceptor (4, 6). Thus, in *A. aerogenes* nitrate assimilation as well as nitrate respiration may occur. In both cases, nitrate is reduced to nitrite, but in the assimilatory process nitrite is reduced eventually to NH_4^+ by nitrite reductase. According to Pichinoty (16), only one nitrate reductase is present in *A. aerogenes*; therefore, this enzyme must have both an assimilatory and a respiratory function.

Oxygen appears to inhibit the formation of enzymes involved in anaerobic respiration (16). The examination of the effect of oxygen on the nitrate reductase in *A. aerogenes* is of interest in view of the enzyme's supposed dual biological function. One might expect that oxygen should not completely abolish nitrate reductase activity, because this would deprive the organism of the assimilatory function of the enzyme under aerobic conditions. Furthermore, it was reported that

NH_4^+ represses the formation of the assimilatory nitrate reductase in some organisms, e.g., fungi (1, 8) and yeast (17), whereas in bacteria, nitrate respiration apparently still occurs in the presence of NH_4^+ (6, 7, 13, 20). We show that respiratory nitrate reductase is inactivated and that its synthesis is blocked by oxygen. Assimilatory nitrate reductase is repressed by NH_4^+ , under both aerobic and anaerobic conditions.

MATERIALS AND METHODS

Organism and growth conditions. *A. aerogenes* strain S 45, which was described previously (5), was used in this study. The minimal nitrate medium contained 7.5 g of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 4.5 g of KH_2PO_4 , 0.05 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005 g of FeSO_4 , 2.8 g of KCl, 4 g of glucose, and 3.5 g of KNO_3 per liter of desalted water (pH 6.8). The minimal nitrate plus NH_4^+ medium contained 7.5 g of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 4.5 g of KH_2PO_4 , 0.05 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005 g of FeSO_4 , 4 g of glucose, 2.0 g of NH_4Cl , and 3.5 g of KNO_3 per liter of desalted water. The minimal NH_4^+ medium was the same as the nitrate plus NH_4^+ medium, except for the omission of KNO_3 .

Bacteria were grown aerobically at 30 C in a Microferm Laboratory Fermentor (New Brunswick Scientific Co., New Brunswick, N.Y.) with vigorous aeration (2.5 liters of air/min per liter medium; agitation at 600 rev/min). Anaerobic growth took place at 30 C either in closed bottles completely filled with medium, or in the Microferm Laboratory Fermentor. In the latter case, nitrogen gas, freed from oxygen by passing through several flasks with alkaline pyrogallol solution, was used instead of air.

A change from aerobic to anaerobic conditions was achieved by switching from aeration to gassing with nitrogen or by putting an appropriate volume of the aerobic culture into bottles which were closed after gassing with nitrogen for about 5 min.

To change from anaerobic to aerobic conditions, the cultures were collected by centrifugation, resuspended in fresh medium, and grown with aeration. In this way, essentially all nitrite that had been produced during anaerobic growth was removed. This was necessary because nitrite is also a good nitrogen source for *A. aerogenes*. Growth was monitored by measuring turbidity at 660 nm in an Engel colorimeter (Kipp en Zn., Delft, Netherlands). Dry weight determinations were performed as described by Hadjipetrou et al. (5).

Preparation of cell-free extracts. After harvesting by centrifugation at 2 C, the bacteria were washed at least three times with cold 0.065 M phosphate buffer (pH 7.0). The pellets of cells grown anaerobically can be stored at -20 C for several weeks without significant loss of activity, but bacteria grown aerobically lose about 80% of their activity by even one cycle of freezing and thawing.

The washed pellets were resuspended in phosphate buffer by means of a Potter-Elvehjem homogenizer with a Teflon pestle. The bacteria were then disrupted by sonic disintegration with a Branson Sonifier for 2 min, with cooling in an ice-ethyl alcohol bath, and centrifuged at 6,000 × *g* in an International centrifuge, model PR 2, for 20 min. The supernatant fluid is designated the cell-free extract.

Assays. For nitrite determination, a modification of the method described by Nicholas et al. (12) was used. To 0.5-ml samples, 0.5 ml of 1% sulfanilamide in 2.5 N HCl was added. After the samples stood for 15 min at 0 C, 0.5 ml of 0.02% N-(1-naphthyl)-ethylenediamine solution was added. After incubation for 30 min at room temperature, 2 ml of water was added, and the absorbancy at 540 nm was measured in a Zeiss spectrophotometer, model PMQ II.

For nitrate determination, a modification of the method of Middleton (11) was used. To 0.5 ml of nitrate solution were added 5 ml of 0.55% Ca(CH₃COO)₂·H₂O in 4% ammonia, 0.1 ml of 1% MnSO₄·4H₂O in 5% acetic acid, and about 0.1 g of finely powdered zinc. This mixture was shaken vigorously for 1 min and filtered; 2 ml of the filtrate was placed in ice, 0.5 ml of 1% sulfanilamide in 5 N HCl was added, and the nitrite content was estimated as described above.

Enzyme activity of the nitrate reductase was determined by the method of Lowe and Evans (9), which uses reduced benzyl viologen as an electron

donor. Unless otherwise stated, the assay mixtures were incubated for 5 min. A unit of enzyme activity was defined as the amount producing 1 μmole of nitrite per min under the conditions employed; the specific activity was expressed as enzyme units per milligram of protein.

Protein was determined according to the method of Lowry et al. (10). This procedure could also be applied to whole cell suspensions with high reproducibility.

RESULTS

Activity of the enzyme under aerobic and anaerobic conditions. The specific activities of nitrate reductase in whole cell suspensions and in extracts of *A. aerogenes* grown under aerobic and anaerobic conditions, respectively, are given in Table 1. Sonic treatment strongly decreases the activity of the aerobic nitrate reductase, whereas the activity of the anaerobic nitrate reductase shows a slight increase. Homogenization in a French pressure cell and the freezing and thawing of bacteria grown aerobically both lead to a considerable loss of nitrate reductase activity, whereas the enzyme of bacteria grown under anaerobic conditions is not sensitive to these procedures. It can be concluded that the aerobic enzyme is very unstable as compared to the anaerobic enzyme. Table 1 shows also that the specific activity of the nitrate reductase is much lower under aerobic than under anaerobic conditions.

The specific nitrate reductase activity, the total activity, and nitrite production are recorded as a function of growth under different conditions (Fig. 1-3). Bacteria were grown aerobically for 16 to 18 hr on NH₄⁺ medium. After centrifugation, the bacteria were either resuspended in nitrate medium and incubated anaerobically (Fig. 1) or aerobically (Fig. 2), or resuspended in nitrate plus NH₄⁺ medium and incubated anaerobically (Fig. 3). Profound differences are

TABLE 1. Specific activities of nitrate reductase in aerobic and anaerobic cultures of *A. aerogenes*^a

Growth conditions	Specific activity ^b	
	Whole cells	Extracts ^c
Anaerobic	0.2 to 0.6	0.35 to 0.8
Aerobic	0.01 to 0.04	0.002 to 0.01

^a Cultures grown in a standard medium with nitrate as the sole nitrogen source were harvested in the middle of the log phase.

^b Expressed as units/mg of protein.

^c Obtained by sonic disintegration of whole cell suspensions.

observed between anaerobic and aerobic cultures grown on nitrate medium (Fig. 1 and 2, respectively). Under anaerobic conditions (Fig. 1), a rapid synthesis of nitrate reductase occurs;

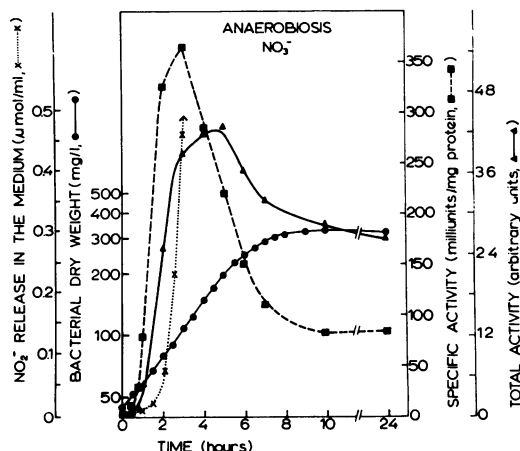


FIG. 1-3. Relation between nitrate reduction and growth of *A. aerogenes*. A preculture grown aerobically for 16 to 18 hr in minimal medium with NH_4^+ as the sole nitrogen source was centrifuged, and the sediment was resuspended (i) in minimal medium with NO_3^- as the sole nitrogen source and reincubated anaerobically (Fig. 1), or (ii) aerobically (Fig. 2), or (iii) in a minimal medium with NH_4^+ and NO_3^- and reincubated anaerobically (Fig. 3). At appropriate intervals, samples were taken for the determination of growth, nitrite release in the medium, and specific nitrate reductase activity. Total nitrate reductase activity was calculated (product of the specific activity and the bacterial dry weight, corresponding to a given absorbance at 660 nm). Note the difference in scale for specific and total activity in Fig. 2 as compared with Fig. 1 and 3.

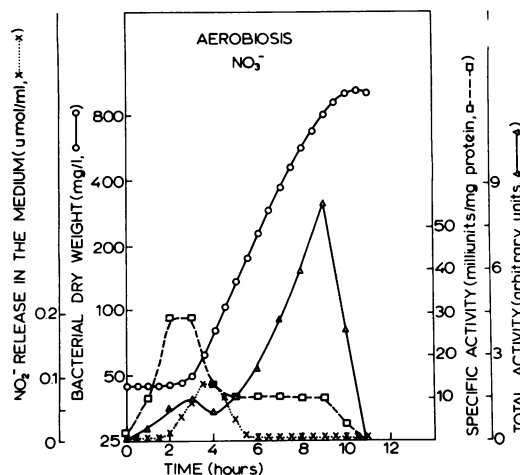


FIG. 2

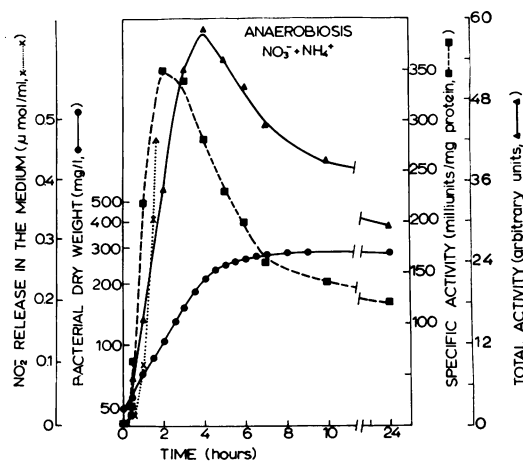


FIG. 3

the specific activity reaches a maximum in the mid-log phase and then decreases rapidly. The total activity attains its maximum in the late log phase. This indicates that net synthesis is stopped at this point, although growth continues for about another 3 hr, suggesting an inactivation or breakdown at a rate which then even exceeds the rate of de novo synthesis.

In aerobiosis (Fig. 2), the specific activity reaches a maximum at the end of the lag phase and then decreases to a constant level during the log phase. The total activity attains a relatively low maximum at the end of the lag phase and a substantially higher one at the end of the log phase. When growth has finished, the activity drops to zero. At the end of the lag phase, some nitrite accumulates in the medium and completely disappears later on. The absence of nitrite accumulation during the log phase can be considered a criterion for true nitrate assimilation. The nitrate reductase present in the aerobic culture at the end of the lag phase has, in contrast with the enzyme present during the log phase, the same behavior towards sonic treatment as the anaerobic enzyme.

In anaerobiosis in nitrate medium (Fig. 1), growth starts at a somewhat slower rate than in nitrate plus NH_4^+ medium (Fig. 3), although no lag is observed. Also, some retardation can be observed in nitrite accumulation in nitrate medium, presumably because in this case nitrite has to be reduced for assimilative purposes. However, the specific activity of nitrate reductase and the total activity show the same growth-dependence in both media.

Shifts from nitrate respiration to nitrate assimilation. If the anaerobically synthesized respiratory enzyme functions also in the aerobic assimilatory

process, shifting the bacteria from anaerobiosis to aerobiosis should cause no lag in growth, even if nitrite and ammonia are not present (Fig. 4). Bacteria growing anaerobically on nitrate medium and on nitrate plus NH_4^+ medium were centrifuged after 3 and 2 hr of growth, respectively (compare Fig. 1 and 3), and then resuspended in nitrate medium. Part of the suspension was then aerated and another part was kept anaerobic. Aerobic bacteria grown on nitrate plus NH_4^+ medium resume growth only after a lag of about 3 hr, whereas the anaerobic culture does so at once. This lag is not due to a dilution effect, because suspensions of different cell densities showed exactly the same lag. The lag is not observed when nitrite is present in the aerobic culture in a nontoxic concentration (5×10^{-4} M; Fig. 4), indicating the presence of nitrite reductase in the cells of the preculture. This suggests that under aerobic conditions growth is resumed only after the induction of the assimilatory nitrate reductase system and that the enzyme synthesized in the anaerobic preculture (nitrate plus NH_4^+ medium) does not function as such under aerobic conditions. This is further illustrated by Table 2, which summarizes the lags that occur in growth after transferring various cultures to aerobic conditions in nitrate medium. Bacteria, precultured aerobically or anaerobically in media containing NH_4^+ show in all cases a lag in subsequent aerobic growth with nitrate as the sole nitrogen source. This suggests that, during growth in the presence of NH_4^+ , the nitrate assimilatory system is not present at all and that its formation under aerobic conditions in nitrate medium takes place rather slowly, causing a lag in growth. The first part of the assumption is confirmed by the obser-

vation that bacteria growing aerobically in the presence of NH_4^+ , with or without nitrate, and bacteria growing anaerobically with NH_4^+ as the sole nitrogen source do not possess any nitrate reductase activity. Moreover, bacteria precultured anaerobically in the presence of NH_4^+ and nitrate (thus displaying nitrate respiration) give rise to the same lag in subsequent aerobic growth in nitrate medium as bacteria precultured aerobically with NH_4^+ as the sole nitrogen source (Fig. 4; Table 2). Obviously, it does not make any difference whether high levels of anaerobic nitrate reductase are present, thus supporting the assumption made above. In addition, no lag in growth was found when bacteria growing anaerobically with nitrate as the sole nitrogen source (thus displaying both nitrate respiration and nitrate assimilation) were shifted to aerobic conditions. A significant lag in subsequent anaerobic growth was never observed, irrespective of the manner of preculturing.

TABLE 2. Lag time for subsequent aerobic growth in minimal nitrate medium of *A. aerogenes* from different precultures

Preculture ^a		Hours of lag time
Medium	Conditions	
NO_3^-	Aerobic	0
NO_3^-	Anaerobic	0
NH_4^+	Aerobic	3 ^b
NH_4^+	Anaerobic	3
$\text{NH}_4^+ + \text{NO}_3^-$	Aerobic	1.5 to 2
$\text{NH}_4^+ + \text{NO}_3^-$	Anaerobic	3 ^c

^a Bacteria grown aerobically for 16 to 18 hr on NH_4^+ medium were centrifuged and resuspended in the preculture medium. After the total bacterial mass was doubled, these bacteria were transferred to nitrate medium.

^b Compare Fig. 2.

^c Compare Fig. 4 and 5.

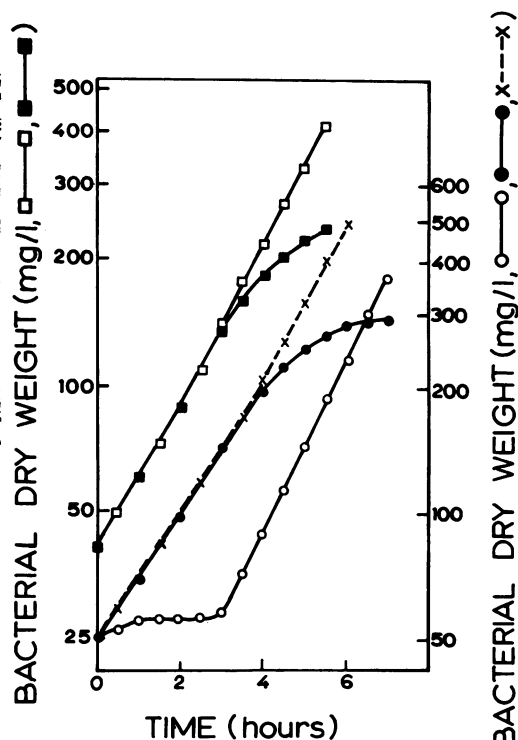


FIG. 4. Influence of a shift from anaerobic to aerobic conditions on growth. Bacteria grown anaerobically for 3 and 2 hr, respectively, in nitrate medium (squares) and nitrate plus NH_4^+ medium (circles), respectively, were centrifuged and resuspended in a twofold volume of nitrate medium. Part of the new suspensions were kept aerobic (open symbols), another part was kept anaerobic (closed symbols). (X) As (O), but with NO_2^- added (final concentration 5×10^{-4} M).

Influence of oxygen on nitrate reductase. If the nitrate reductase in both processes is the same enzyme, oxygen must be concerned with its different behavior under aerobic and anaerobic conditions. In order to investigate a possible influence of oxygen on the amount of active nitrate reductase, we studied in more detail the effects of a change from anaerobic to aerobic conditions (Fig. 5). (For convenience, growth rate is also presented.) Nitrite production during anaerobic

incubation is very large, whereas under aerobic conditions there is no net nitrite production. When a part of the aerobic culture is returned to anaerobic conditions, nitrite production is resumed instantly (Fig. 5b). Similarly, the specific activity of the enzyme decreases rapidly after the switch to aerobic conditions; it regains its original high value after returning to anaerobic conditions (Fig. 5c). Although the specific activity remains low and even keeps decreasing during prolonged aerobic incubation, the total enzymatic activity of the culture increases nearly linearly with time after the first sharp decline (Fig. 5d). This indicates that, in aerobic cultures on nitrate medium, there is still net synthesis of nitrate reductase. The start of this synthesis coincides with the beginning of the aerobic growth after 3 hr (compare Fig. 2), suggesting that the assimilatory enzyme is synthesized thenceforth.

Decline in total activity immediately after the change to aerobic conditions demonstrates that oxygen inactivates the enzyme formed during the anaerobic phase. Rapid increase in specific activity after returning part of the aerobic culture to anaerobic conditions may be explained by a rapid de novo synthesis of the enzyme.

The lack of nitrite production during aerobiosis, although nitrate reductase is present (Fig. 5b, c), could also be demonstrated with resting cells, which were obtained from an anaerobic culture on nitrate plus NH_4^+ medium and which had a high level of nitrate reductase. These cells neither produced nitrite nor reduced nitrate when incubated under aerobic conditions at pH 7.0, with glucose as the hydrogen donor. During anaerobic incubation in Thunberg tubes, however, these cells did produce considerable amounts of nitrite with a concomitant consumption of nitrate if the other conditions were the same.

The inactivation *in vivo* of the anaerobically induced enzyme can also be demonstrated *in vitro* with cell-free extracts from anaerobic cultures (Fig. 6). The degree of inactivation was dependent on the temperature at which the enzyme was exposed to oxygen (Fig. 6a) and on the partial pressure of oxygen (Fig. 6b). The inactivation is never complete. Even after 24 hr of incubation with oxygen, a residual activity of about 10% of the initial activity is found. The inactivation process showed first order kinetics during the first hours, after which the inactivation rate became slower and attained about the same value as the slow inactivation in an extract treated with pure nitrogen gas under the same conditions. The initial inactivation rate was directly proportional to the enzyme concentration. On the whole, the inactivation was found to be irreversible.

Because of high lability towards homogeniza-

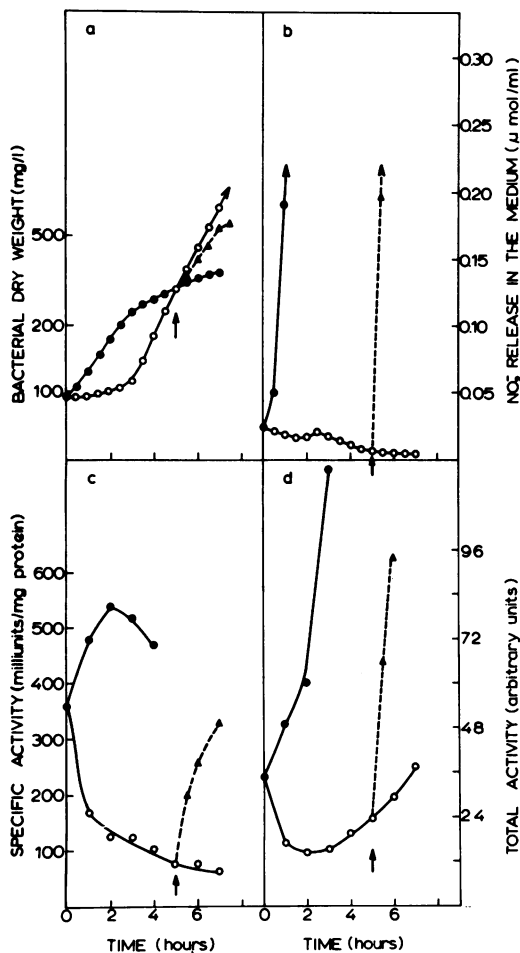


FIG. 5. Influence of a shift from anaerobic to aerobic conditions on nitrate reduction. Bacteria grown anaerobically for 2 hr in nitrate plus NH_4^+ medium were shifted to aerobic conditions in nitrate medium, as in Fig. 4. For comparison, another part of the culture was kept anaerobic. After incubation for 5 hr, part of the aerobic culture was returned to anaerobic conditions (\uparrow). Growth (a), nitrite release in the medium (b), specific nitrate reductase activity in whole cells (c), and total activity (d) were determined at appropriate intervals. Symbols: (●) anaerobiosis; (○), aerobiosis.

tion procedures of the aerobic nitrate reductase (Table 1), the inactivation of this enzyme by oxygen was studied in whole cell suspensions in 0.065 M phosphate buffer (pH 7.0); for the rest, in the same manner as described in the legend to Fig. 6. With pure oxygen, we obtained in three experiments an average inactivation of 65% in 1 hr. By treatment with air, we obtained an average inactivation of only 10%. Therefore, it seems likely that the aerobically induced enzyme is not significantly inactivated by air.

We showed that there is a difference in stability between the aerobic and anaerobic nitrate reductase (Table 1). Furthermore, we assumed that after a shift from anaerobiosis in nitrate plus NH_4^+ medium to aerobiosis in nitrate medium (Fig. 5), growth is resumed only after the induction of assimilatory (and labile) nitrate reductase. Whether this assumption is correct might be determined from the behavior towards sonic treatment of the enzyme present after the shift. The ratio of the specific activities of nitrate reductase in extracts and whole cells remains nearly constant (about 2.5) for the first 3 hr after the shift (Fig. 7). During this period, without growth, the present nitrate reductase is indeed inactivated by oxygen, but the remaining enzyme still shows the same behavior towards sonic treatment as the anaerobic enzyme present before the shift. With the resumption of growth (after 3 hr), the ratio of the specific activities in extracts and whole cells drops gradually, reflecting a decreasing quantity of

anaerobic and stable nitrate reductase (by inactivation and by repressed formation) and an increasing quantity of aerobic and labile nitrate reductase by de novo synthesis. After 6 hr, the major part of the present enzyme is sensitive to sonic treatment, the ratio of the specific activities in extracts and whole cells being then 0.7.

Shifts from aerobiosis to anaerobiosis. When bacteria growing aerobically on nitrate medium and coming from an aerobic preculture on the same medium are shifted to anaerobic conditions, growth is not retarded (Fig. 8a). This does not mean that the aerobic nitrate reductase can function under anaerobic conditions, but simply reflects the rapid synthesis of nitrate reductase under anaerobic conditions. This interpretation is supported by the observation that, in the presence of chloramphenicol, no net synthesis of nitrate reductase takes place and no nitrite accumulates in the medium; also, growth is stopped instantaneously. This result excludes the possibility that nitrate reductase in the aerobic culture is present in an inactivated form, which can be reactivated during anaerobiosis.

The increase in specific activity of the enzyme (Fig. 8c) is dependent on the density of the aerobic culture at the moment of the shift. The rate of nitrite accumulation in the medium (Fig. 8b), which can be taken as a measure of nitrate respiration, shows a nearly linear relationship to the increase in total enzymatic activity (Fig. 8d). Therefore, after a switch from aerobic to anaero-

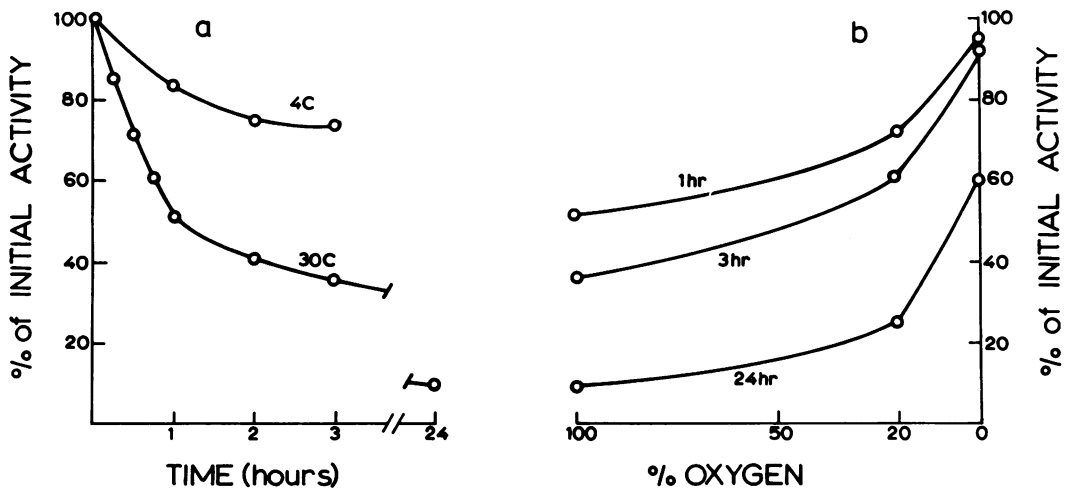


FIG. 6. Inactivation of anaerobic nitrate reductase by oxygen in vitro. Samples of fresh cell-free extracts (10-ml) obtained from anaerobic cultures were gassed for several minutes with pure oxygen, air, or with oxygen-free nitrogen in 500-ml Erlenmeyer flasks. Then the flasks were stoppered and gently shaken for some hours at the indicated temperatures. At appropriate intervals, samples were taken for the determination of nitrate reductase activity. (a) Remaining activity of extracts treated with pure oxygen gas versus time of exposure. (b) Remaining activity of extracts exposed at 30 C to different partial oxygen pressures.

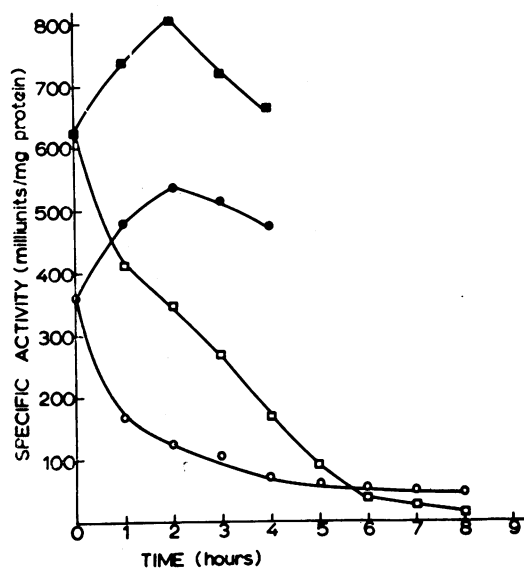


FIG. 7. Effect of sonic disintegration on the specific activity of nitrate reductase in *A. aerogenes*. A shift from anaerobiosis to aerobiosis was performed, as given in Fig. 5. At appropriate intervals, samples were taken from both the anaerobic and the aerobic culture for the determination of the specific activity before and after sonic disintegration. Results obtained are designated as follows: whole cell suspensions (●) and extracts (■) of the anaerobic culture; whole cell suspensions (○) and extracts (□) of the aerobic culture.

bic conditions, nitrate respiration appears to depend completely on de novo synthesis of nitrate reductase and is obviously not carried out by the assimilatory enzyme already present.

DISCUSSION

The experimental data presented show that the nitrate reductase in *A. aerogenes* present under aerobic conditions differs in some respects from the enzyme present under anaerobic conditions. The enzyme in cells grown aerobically is very sensitive to sonic disintegration and to other homogenization procedures, whereas the enzyme in cells grown anaerobically is stable in this respect. The increase in activity after sonic disintegration, often found in the latter case, may be ascribed to an enhanced accessibility of the enzyme during the assay. When a culture of *A. aerogenes* is shifted from anaerobic growth in minimal medium with nitrate and NH_4^+ to aerobiosis in minimal medium with nitrate as the sole nitrogen source, a lag of about 3 hr occurs before logarithmic growth is resumed (Fig. 4). This indicates that the anaerobic respiratory nitrate reductase does not function in the

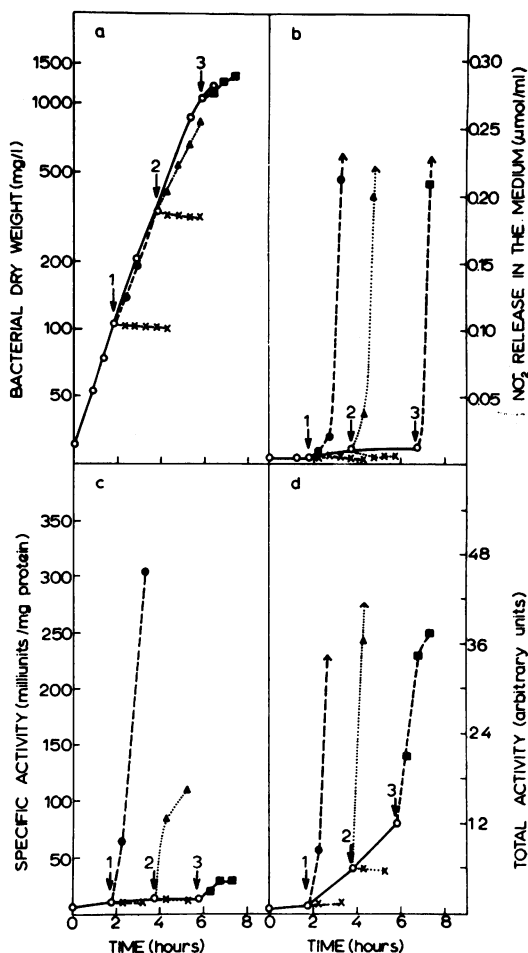


FIG. 8. Influence of a shift from aerobic to anaerobic conditions on growth and nitrate reduction. A preculture of *A. aerogenes*, grown aerobically for 16 to 18 hr in nitrate medium, was diluted in a 25-fold volume of the same fresh medium. Parts of the aerobic culture were shifted to anaerobic conditions at the times indicated by arrows. In two cases (crosslets), chloramphenicol (50 mg/liter) was added 5 min prior to the change from aerobiosis to anaerobiosis. At appropriate intervals samples were taken from all cultures for the determination of: (a) growth; (b) nitrite production; (c) specific nitrate reductase activity (in whole cells); (d) total enzymatic activity. Closed symbols, anaerobic cultures; open symbols, aerobic cultures.

assimilatory process under aerobic conditions. After resumption of growth, the nitrate reductase activity appears to gradually become sensitive to sonic disintegration (Fig. 7), suggesting a de novo synthesis of labile nitrate reductase with an assimilatory function.

The decline in total enzymatic activity immediately after the switch from anaerobic to aerobic

conditions (Fig. 5c) and the results from the experiments with cell-free preparations show that air-oxygen inactivates anaerobic nitrate reductase both in vivo and in vitro. The aerobic assimilatory enzyme is not significantly inactivated by air, however; inactivation could be obtained only by treating whole cell suspensions with pure oxygen gas at atmospheric pressure.

The differences mentioned may be interpreted by assuming the existence of two different nitrate reductases in *A. aerogenes*. One has an assimilatory function, is not inactivated by air, and is sensitive to sonic disintegration. The other one, synthesized under anaerobic conditions, has a respiratory function, is strongly inactivated by air, and is not sensitive to sonic disintegration. However, we have been unable to demonstrate further differences in enzymatic properties (e.g., kinetic parameters, pH -optimum) of the nitrate reductase in the two cases. Moreover, preliminary experiments with partially purified enzyme preparations from anaerobic cultures reveal that in this state (less particulate) the enzyme also becomes sensitive to sonic disintegration. Therefore, the differences described might disappear after extensive purification of the enzyme(s). The experimental data can be explained also by the assumption that in *A. aerogenes* only one nitrate reductase exists, but that the localization of the enzyme is different under aerobic and anaerobic conditions. Immediately after the shift from anaerobic to aerobic conditions, the production of nitrite stops instantly (Fig. 5b), probably because of a preferential flow of the electrons to oxygen. It seems likely that the electron transport chain to nitrate is different in both cases, giving rise to different enzyme complexes. After the transfer of anaerobically growing cells to aerobic conditions, another enzyme complex has to be formed for the reduction of nitrate before growth can be resumed, causing a lag in growth. The suggestion of different enzyme complexes, under aerobic and anaerobic conditions, is supported by an analysis of mutants which are blocked in aerobic growth but not in anaerobic growth with nitrate as the sole nitrogen source (19). The idea of one nitrate reductase, but in different enzyme complexes, has also been proposed by Nicholas and Wilson (14) for *Neurospora crassa*.

If this idea holds true, the regulation of formation might be different for both complexes. In fact, the formation of the assimilatory complex is repressed by NH_4^+ both under aerobic and under anaerobic conditions, whereas that of the respiratory complex is not (Fig. 4; Table 2). It seems likely that the repression by NH_4^+ of the assimilatory complex is not based on the re-

pression of the synthesis of nitrate reductase itself, but rather on the blocked synthesis of one of the other factors in the enzyme complex. This factor is possibly also involved in the formate hydrogenlyase system because the mutants mentioned above showed an altered formate metabolism (19). Furthermore, it was found that the formate dehydrogenizing enzymes in *A. aerogenes* are also very susceptible towards homogenization procedures. If the nitrate reductase in both the assimilatory and the respiratory complex is indeed the same protein, the divergent effect of oxygen and homogenization procedures must be caused by the different composition of both complexes.

From the experimental data, we can deduce that oxygen has some effect on the biosynthesis of nitrate reductase. There is a continuous net production of the enzyme under aerobic conditions, but the measured activity is considerably lower than that found in anaerobic cultures (Fig. 2, 5). The decreased rate of production of active enzyme after a shift from anaerobic to aerobic conditions can result not only from repressed formation, but also from enzyme inactivation. By comparing the increase in total activity for both the anaerobic and the aerobic culture at the same stage of logarithmic growth (i.e., identical cell density), we can calculate roughly that the rate of production of active enzyme in the anaerobic cultures is about seven times as high as in the aerobic cultures (Fig. 5). From the decline in enzymatic activity immediately after the shift to aerobic conditions, the degree of inactivation in vivo can be estimated to be about 60%. Even if this percentage holds true during the entire aerobic incubation (and this is not very likely, as discussed before), inactivation alone cannot explain the lower production of active enzyme in aerobiosis. Therefore, repression of enzyme synthesis seems to be involved in the oxygen effect. This conclusion was confirmed by experiments (Fig. 8), because immediately after shifting a logarithmically growing aerobic culture to anaerobic conditions the rate of production of active enzyme became about 10 times higher than that under aerobic conditions. Similar results were reported very recently by Showe and DeMoss (18), who found that the synthesis of the nitrate reductase in *Escherichia coli* was repressed by aeration even in the presence of nitrate. These authors interpreted their results as evidence for the existence of a redox-sensitive repressor which mediates nitrate reductase regulation.

It is tempting to speculate that in the case of a culture shifted from anaerobic to aerobic conditions (Fig. 5), the oxygen-inactivated form of

the enzyme will bring about a repression of enzyme synthesis one way or another. A similar regulation process, called "inactivation-repression," has recently been suggested by Ferguson and co-workers (3) for the repression of acetate-inducible malate dehydrogenase in yeast by a metabolite of glucose. In a different way also, Cove (2) suggested an active part of nitrate reductase in its own synthesis; in the induction of nitrate reductase in *Aspergillus nidulans*, further synthesis of this enzyme appeared to be blocked when it was not in a substrate-activated form. This kind of regulation may be concerned also in the low nitrate reductase activity in cultures which have been kept aerobic all the time (Fig. 2). Aerobic cultures (in minimal nitrate medium) start growing after a certain lag time, during which nitrate reductase is found to be present already. This early induced enzyme shows the same behavior towards sonic treatment as the nitrate reductase present in anaerobic cultures, in contrast with the enzyme synthesized in exponentially growing aerobic cultures. Therefore, it seems likely that the early induced enzyme is not present in the assimilatory complex, which also can explain the lag in growth. Possibly this early enzyme is similarly inactivated by oxygen and in this way represses its synthesis, eventually causing the low level of nitrate reductase activity always found in aerobic cultures.

Although the results presented do not rule out the possibility of two different nitrate reductases in *A. aerogenes*, they make it most probable that only one nitrate reductase exists in this organism, which has an assimilatory, and a respiratory, function under the appropriate conditions. The assimilatory enzyme complex, the formation of which is repressed by NH_4^+ , and the respiratory enzyme complex, on which oxygen exerts a multiple effect, appear to have a different composition.

Further studies now in progress on the electron transport chain to nitrate might provide, in both cases, additional information on this subject.

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