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Anaerobic Nitrite Production by Plant Cells and Tissues: Evidence for Two Nitrate Pools[1]

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ABSTRACT

Tobacco (*Nicotiana tabacum* L. cv. Xanthi) XD cells containing nitrate and nitrate reductase stopped producing nitrite after approximately 1 hour when incubated under anaerobic conditions. The cessation of nitrite production was not due to an inactivation of the nitrate reducing system. This was shown by the ability of the cells to resume anaerobic nitrite production at a rate similar to the initial rate of nitrite production upon exposure to nitrate, monohydroxy alcohols or pyrazole. Cessation of nitrite production also could not be attributed to leakage of nitrate from the cells. Although some nitrate did leak from the cells, most of the nitrate was still in the cells by the time anaerobic nitrite production ceased. We infer the existence of a small metabolic pool and a large storage pool of nitrate, such that nitrite production ceases when the metabolic pool is depleted of nitrate. The metabolic pool of nitrate in tobacco cells decreased 170-fold as the culture aged from 3 to 5 days. However, total cellular nitrate during this period remained relatively constant.

Anaerobic nitrite production by barley (*Hordeum vulgare*) aleurone layers and corn (*Zea mays*) leaf sections also ceased after only a small fraction of endogenous nitrate was reduced and resumed again upon addition of exogenous nitrate. In contrast to that found with tobacco cells, the metabolic pool of nitrate in corn leaf sections remained constant with age, while total endogenous nitrate increased. These results were interpreted to mean that higher plants in general contain metabolic and storage pools of nitrate, the properties of which vary with species and physiological variables.

Nitrite reduction is inhibited by anaerobiosis in barley aleurone layers (5) and wheat roots (18), whereas the reduction of nitrate to nitrite by nitrate reductase is enhanced (4). Nitrite accumulates under such conditions and the anaerobic production of nitrite has been used to assay nitrate reductase *in situ* in a variety of plant tissues (4, 11–13, 17, 22, 24, 25). The reaction was found to be linear for more than 1 hr and dependent upon the addition of exogenous nitrate (4, 13, 24). However, with barley aleurone layers, a small amount of nitrite accumulated even when exogenous nitrate was omitted from the assay medium (4). When the nitrate of the tissue was measured in later experiments, it was shown that the nitrite produced accounted for only a small fraction of the endogenous nitrate present initially. This observation suggested that not all of the endogenous nitrate was accessible to nitrate reductase. These conditions led us to the idea that under anaerobic conditions nitrate reductase, which is located intracellularly (1), might be used to detect the existence of a distinct nitrate pool *in situ* and might serve as the basis for measuring its size. Plants often contain more than one pool of a compound (20, 21), and there is evidence for more than one pool of nitrate in cultured tobacco cells (10).

Suspensions of tobacco XD cells were used to test the idea. The nitrate assimilation pathway of these cells has been characterized (8–10). In contrast to tissues, organs, or whole plants, every cell in a suspension is exposed to the same exogenous nitrate concentration and this concentration can be easily manipulated. Difficulties of interpretation stemming from cell heterogeneity, intercellular or intertissue transport, and intercellular spaces in organized cell masses do not exist for cell suspensions. Nitrate in plant cell cultures has only one origin—the external medium—and only three fates—uptake by the cell, return to the external medium or reduction to nitrite. Therefore, it should be possible to account for all the nitrate in a plant cell solely in terms of nitrate transport and nitrate reduction.

MATERIALS AND METHODS

MEASUREMENT OF ANAEROBIC NITRITE PRODUCTION

Tobacco Cells. Stationary phase tobacco cells (*Nicotiana tabacum* L. cv. Xanthi) were subcultured by 20-fold dilution into 1-liter Erlenmeyer flasks containing 0.5 liter of M-1D nutrient medium (6). The nitrate concentration in this medium is 2.5 mM. Cells were grown at 28 C and after 3 to 6 days harvested by vacuum filtration on Whatman No. 1 filter paper (two layers for faster filtration) in a Buchner funnel. In the early experiments, the cells were washed three times with a total of approximately 100 ml of 0.85 mM $CaCl_2$ + 0.79 mM KCl solution per wash, weighed and unless otherwise indicated, resuspended for incubation in 20 ml of the same solution per g fresh weight of cells. In the experiments where the effects of washing were studied, cells were first harvested without washing, weighed, resuspended in the $CaCl_2$ + KCl solution at 20 ml/g cells, and reharvested. The resuspending and harvesting was repeated for the desired number of washes. Prior to use, the resuspension medium was flushed with N_2. Between 15 and 25 ml of cell suspension were placed in 30-ml

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tubes and flushed with N_2 an additional 1 to 2 min, after which they were stoppered. At zero time and after incubation at room temperature (23 C) for increasing time periods, nitrite was determined in 0.5-ml aliquots of the cell suspension. Nitrogen was bubbled through the suspension during sample removal to mix the cells, which otherwise settle to the bottom of the vessel, and to prevent oxygen from entering the medium. To the 0.5-ml aliquot, 0.25 ml (0.5 ml in some experiments) each of 1% (w/v) sulfanilamide in 3 N HCl and 0.02% N-(1-napthyl)ethylenediamine dihydrochloride was added (23). After centrifugation at 2000*g* for 10 min, absorbancy at 540 nm of the supernatant was determined and nitrite concentration then determined from a standard curve.

Nitrite production usually ceased after 1 to 2 hr of anaerobiosis; however, cells from 3-day-old cultures whose yields approached 2 g fresh weight/l could reduce so much of their nitrate to nitrite, that even after incubation for 2 or 3 hr a plateau was only being approached. Therefore, the maximum amount of nitrite that could be produced by such cells was estimated after presenting nitrite production against time in a double reciprocal plot, and extrapolating the resultant line to infinite time.

Aleurone Layers. The procedure used to prepare aleurone layers was the same as described earlier (2). Barley seeds (*Hordeum vulgare* L. cv. Himalaya, 1965 harvest) were cut in half, and after 4 to 5 days of imbibition on moist sand, the aleurone layer was aseptically removed from the embryo-less half of the seed. Nitrate reductase was then induced in the aleurone layer by incubation with 50 mM KNO_3 (50 layers/10 ml) at 23 C under aseptic conditions according to method 2 as described earlier (4). After 5.5 hr of induction, aleurone layers were washed three times with approximately 50 ml of deionized distilled water and placed in 4-ml glass vials, 5 layers/vial, with 0.5 ml of 0.1 M potassium phosphate buffer, pH 7.5. The suspension in the vials was flushed with N_2 for 1 min and stoppered. At intervals the assay was stopped by placing the vials on a hot plate until the medium boiled (approximately 30 sec). Nitrite was determined colorimetrically as above after adding to the cooled medium 0.25 ml each of 1% sulfanilamide in 3 N HCl and 0.02% N-(1-napthyl)ethylenediamine dihydrochloride (23).

Corn Leaves. Hybrid corn (*Zea mays,* K61XPr) seeds were planted embryo side down approximately 3 cm deep in vermiculite in a perforated pan. The vermiculite was saturated with 0.1 mM $CaCl_2$, and the pan was placed in darkness for 4 days. Plants were then placed under Gro-lux lamps and watered with White's inorganic nutrient medium (26) supplemented with 10 mM KNO_3, Na_2MoO_4 at 0.01 mg/l and Fe at 5 mg/l in the form of iron EDTA chelate. The same watering solution was used for plants grown in the absence of nitrate, except that KNO_3 and Na_2SO_4 were replaced by equimolar KCl and NH_4Cl, respectively. For measurements of nitrite production, 3 to 4 mm square sections of tertiary leaves were placed in 50-ml Erlenmeyer flasks, 100 mg of leaf tissue per flask. Two ml of 0.1 M potassium phosphate buffer, pH 7.5, was also added to each flask. The solution in the flask was flushed with N_2 for 1 min, after which the flasks were stoppered and incubated at 30 C in the dark (to minimize photosynthetic production of oxygen). During the approach to the plateau of nitrite production, 2 ml of 0.1 M phosphate buffer was added after various times, and the contents of the flasks were boiled to terminate the nitrite production reaction. At the beginning of the plateau, 2 ml 0.1 M phosphate buffer plus 0.1 M potassium nitrate was added to one set of flasks, and phosphate buffer alone was added to a second set of flasks. These flasks were aerated for 1 min and incubated anaerobically in darkness for various times, after which the contents were boiled to terminate nitrite production. Nitrite was determined colorimetrically after adding to 1 ml of the boiled medium 1 ml each of 1% sulfanilamide in 3 N HCl and 0.02% N-(1-napthyl)-ethylenediamine dihydrochloride (23).

Nitrate Measurements. Total nitrate in barley aleurone layers and corn leaves was determined using 0.05 to 0.2 ml boiled aliquots of the medium sample at zero time in the nitrite production assays. In the case of the tobacco cell data in Table I, 0.5 to 1.0 ml of cell suspension (0.05–0.1 g fresh weight/ml) was quickly boiled (approximately 20 sec) to release accumulated nitrate, and the cell debris was sedimented by centrifugation at 2000*g* for 10 min. Rapid boiling minimized volume loss due to evaporation. Nitrate in 0.01 ml of the supernatant was determined according to a modification of the method of Lowe and Hamilton (15). The quantity of nitrate released by boiling tobacco cells was in good agreement with that released by mechanical disruption of the cells (10).

It is difficult to sample dense cell suspensions volumetrically; therefore, in some experiments, aliquots of the cell suspension were harvested and samples (0.5 g) were boiled after resuspension in 5 ml of H_2O. An aliquot was then diluted 20-fold with H_2O and nitrate in 0.3 ml was determined as above.

When measurements of nitrate in washes were first made, it was noticed that nitrate leaches from Whatman No. 1 filters, thereby contaminating the filtrate but not the cells. Therefore, filters were washed with the $CaCl_2$ + KCl solution before use. Nitrate in filtrates of washed cells was assayed in 0.3-ml aliquots of the filtrate or from a suitable dilution.

Nitrate Reductase Assay. Nitrate reductase activity of tobacco cells was determined *in situ* with the anaerobic intact tissue assay method which was described for barley (4), except that 1% propanol was substituted for ethanol in the assay mixture. The assay mixture consisted of the following in a total volume of 1 ml: 0.2 or 0.4 ml of tobacco cell suspension, 0.05 to 0.1 g fresh weight/ml; 0.01 M KNO_3; 0.1 M potassium phosphate buffer, pH 7.5; 1% (v/v) propanol. The assay was started by adding an aliquot of the cell suspension to a mixture of the other components of the assay. The mixture was then flushed with nitrogen gas for 1 min and stoppered. After 30 min at 23 C, the assay was stopped by adding to the medium 0.5 ml of 1% (w/v) sulfanilamide in 3 N HCl and 0.5 ml of 0.02% (w/v) N-(1-napthyl)-ethylenediamine dihydrochloride (23). After centrifugation at 1000*g* for 10 min, absorbance of the supernatant at 540 nm was determined and converted to nitrite by means of a standard curve. Nitrite production was linear with time up to 1 hr, and rates of nitrate reduction were two to three times higher than has been found in previous studies (7, 9, 10) by *in vitro* assays of extractable nitrate reductase activity of tobacco cells.

RESULTS

Kinetics of Anaerobic Nitrite Production. Tobacco XD cells were grown in a medium containing nitrate to allow both accumulation of nitrate and induction of nitrate reductase. Cells were then harvested and placed in a nitrate-less solution containing potassium and calcium chloride at the same concentration as nitrate in the normal culture medium. Such cells produced nitrite when incubated anaerobically (Fig. 1). No nitrite was produced by heat-killed cells. The rate of nitrite production decreased with time until a plateau was reached. After the plateau had been reached, the nitrite level remained constant for at least 1 hr, or in some experiments declined slightly.

Reduction of nitrate to nitrite was the only significant metabolism of nitrate during the anaerobic incubation, and the plateau was due to a cessation of the reduction of nitrate to

nitrite rather than to attainment of a steady state between nitrite production and nitrite consumption. This was shown by comparing the amount of nitrite produced with the amount of nitrate consumed. This experiment was only feasible with cells from young cultures, because with older cultures the decrease in total nitrate due to nitrite production was too small to be measured with sufficient accuracy. According to the averaged results of two separate experiments with 3-day-old cultures, the amount of nitrite produced (7.2 μmoles/g) could account for the amount of nitrate which disappeared (6.8 μmoles/g).

The plateau level of nitrite was found to be dependent on culture age (Table I). From the 3rd to 5th day after subculture, fresh weight increased 3-fold, whereas nitrate reductase activity and total endogenous nitrate (after three washes) varied only slightly. In contrast, anaerobic nitrite production decreased 170-fold: from 10 μmoles/g by cells from 3-day cultures, to 0.06 μmole/g by cells from 5-day cultures (Table I). Cells from still older cultures occasionally produced even smaller amounts of nitrite. Anaerobic nitrite production was also found to increase greatly when a cell culture was diluted 1:1 with fresh medium 24 hr before determining the nitrite plateau level (Fig. 2).

Stability of the Nitrate Reducing System. Nitrite production resumed if exogenous potassium nitrate was added (indicated by arrow in Fig. 1) after the plateau had been reached. Potassium chloride could not replace potassium nitrate (Fig. 1). Nitrite production resumed when potassium nitrate was added after a brief aerobic period or under continuous anaerobic conditions (Fig. 3). Thus, the resumption of nitrite production was due specifically to nitrate and not to the potassium or air which were introduced into the medium when nitrate was added.

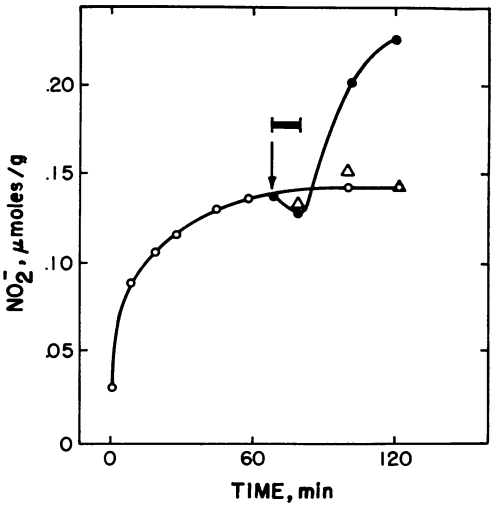

FIG. 1. Kinetics of nitrite formation by tobacco cells. Cells from 5-day-old cultures (8 g/l fresh wt yield) were harvested, washed three times with approximately 100 ml of KCl + $CaCl_2$ solution and then resuspended (15 ml medium/g fresh wt) in KCl + $CaCl_2$ wash medium which had been flushed with N_2. The suspension was immediately gassed with nitrogen an additional 1 to 2 min prior to the start of the assay to remove oxygen which was transferred to the medium during addition of the harvested tobacco cells. Nitrite production was measured by allowing nitrate reductase to convert cellular nitrate to nitrite. At time intervals, 0.5- to 1.0-ml aliquots were removed and analyzed for nitrite as described in "Materials and Methods." At 70 min (indicated by an arrow in this and subsequent figures) a concentrated solution—so as not to change appreciably the volume of the assay medium—of either KNO_3 or KCl was added (10 mM final concentration). Following a 10-min aeration period (indicated by horizontal bar), the cell suspension was deaerated again by bubbling nitrogen gas through the medium for 1 to 2 min. Total nitrate (13.6 μmoles/g fresh wt) of the cells was determined from 10 μl of a boiled aliquot removed from the cell suspension at zero time. Continuous N_2 (○); + KNO_3 (●); + KCl (△).

Table I. *Effect of Culture Age on Yield, Nitrate Reductase, Total Nitrate, and Nitrite Production in Tobacco Cells*

Tobacco cells were harvested after 3, 4, or 5 days growth on M1-D medium containing nitrate. Cells were washed with approximately 100 ml of nitrateless medium containing KCl 0.79 mM and $CaCl_2$ 0.85 mM and were then resuspended in the same medium (10 ml per g fresh wt cells) which had been deaerated by bubbling nitrogen gas through it. Total nitrate and nitrite accumulation were determined as described in Figure 1 and "Materials and Methods." Nitrate reductase was determined in the intact tissue as described in "Materials and Methods."

	Culture Age (days)		
	3	4	5
Culture yield (g fresh wt/l)	2.2	3.2	6.5
Total nitrate (μmoles/g)	31.9	31.0	17.2
Nitrite accumulation (μmoles/g)	10	1.0	0.06
Total/nitrite accumulation	3.2	31	290
Nitrate reductase activity (μmoles/g·hr)	6.5	7.3	4.7

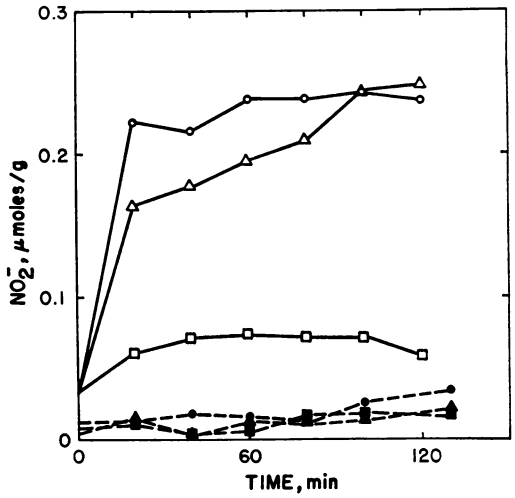

FIG. 2. Effect of fresh culture medium and washing on anaerobic nitrite production. After 3 days growth, one of two sets of cultures was diluted 1:1 with fresh medium. After an additional 24 hr, both sets (diluted and undiluted) of cells were harvested and washed as in "Materials and Methods." After various members of washes nitrite production was assayed during an anaerobic incubation. Open symbols: diluted culture; filled symbols: undiluted culture. Data for one (circles), three (triangles), and five (squares) washed are presented. The nitrate content of the cells after each wash can be found in Table IV.

During anaerobic incubation of cells from 5- to 6-day-old cultures (fresh weight yield, 10.0–12.6 g/l) approximately 1/300 of the endogenous nitrate was converted to nitrite. After nitrite production ceased, various chemicals were added to the incubation mixture, and the amount of nitrite produced in the subsequent hour was determined. Of several chemicals tested, primary and secondary monohydroxy alcohols (Table II) were effective in causing a resumption of nitrite production. In separate experiments this property was also shown for pyrazole (1,2-diazole), propanol (Fig. 4A), and 2,4-dinitrophenol

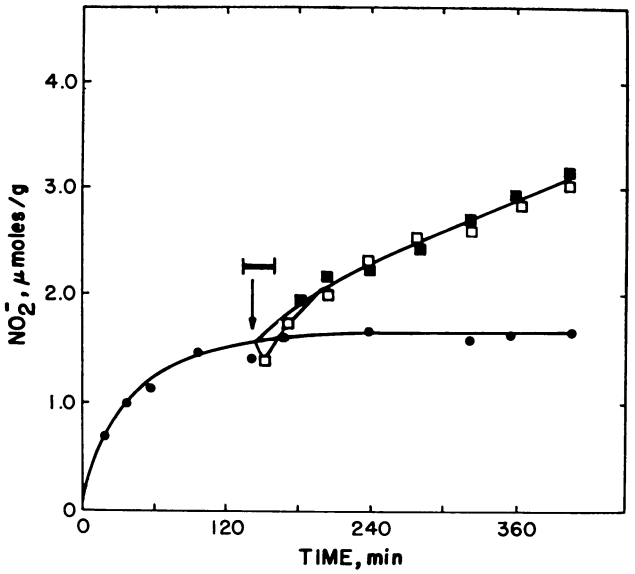

FIG. 3. Effect of air on the resumption of nitrite production. Cells were from 4-day cultures (yield: 3.5 g/l). Nitrite production was followed as in "Materials and Methods." After cessation of nitrite production, 10 mM KNO_3 was added with or without a 10-min aeration period (indicated by bar). Then the suspension was flushed with N_2 and nitrite production was again followed. Continuous N_2 (●); KNO_3 with aeration (□); KNO_3 without aeration (■).

Table II. *Effect of Various Chemicals on Nitrite Formation*

Cells were grown 5 to 6 days on M1-D medium, harvested, washed, and resuspended (10 ml per g fresh wt) in deaerated KCl + $CaCl_2$ medium. Following the cessation of nitrite production (approximately 40 min), 1-ml aliquots of the cell suspension were added under anaerobic conditions to aliquots of the test chemicals. Values represent the amount of nitrite produced during a 60-min period following the cessation of nitrite production. The amount of nitrite produced was considered proportional to the ability of the test chemical to allow unavailable nitrate to become available for reduction. Values represent an average of three determinations from experiments utilizing 5- to 6-day-old cultures yielding 10.0, 11.2, and 12.6 g/l.

	Nitrite Accumulation[1]		
	0.1% (v/v)	1.0% (v/v)	10.0% (v/v)
	nmoles NO_2^- produced/g		
Methanol	15	19	
Ethanol	15	40	
n-Propanol	20	63	
n-Butanol	19	149	
Isopentanol	13	139	
Isopropanol	19	18	
tert-Butanol		15	43
Glycerol	11	16	
DMSO		11	14
Acetone	13	17	
CCl_4	16	15	

[1] Control rate = 13 nmoles produced/g.

(DNP;[4] Fig. 4B). Though a response to DNP alone was consistently observed, the magnitude of the response was variable (*cf.* Figs. 4B and 5). When DNP was added at the start of the incubation, considerably more nitrite was produced and a plateau was not reached (Fig. 5). When nitrate and DNP were added together following the cessation of nitrite production, nitrite production resumed, did not reach a second plateau, and occurred at a rate 45 to 75% faster than the sum of the rates due to either agent alone (range of three experiments). Polyhydroxy alcohols, organic solvents, and *DMSO*, a chemical believed to alter the properties of membranes (10A), were without effect (Table II).

Thus nitrate, monohydroxy alcohols, pyrazole, and DNP,

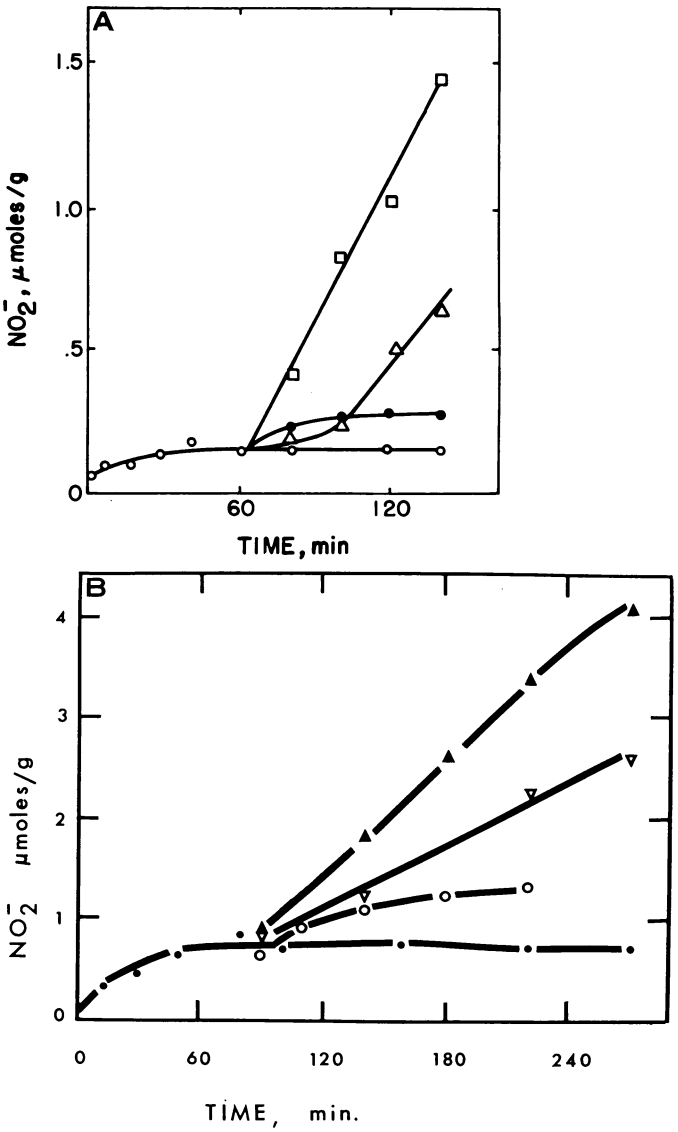

FIG. 4. Effect of propanol pyrazole and DNP on resumption of nitrite production. A: Cells from 5-day cultures (yield: 6.5 g/l) were harvested, washed, and incubated anaerobically as described in "Materials and Methods." Following cessation of nitrite production, 10 mM KNO_3, 1% (v:v) n-propanol, or 10 mM pyrazole were added to separate aliquots of the cell suspension. Nitrate addition was followed by a 5-min aeration period, while continuous anaerobic conditions were used with n-propanol and pyrazole. Continuous N_2 (○); NO_3^- (●); n-propanol (□); pyrazole (△). B: Methods were the same as in A except cells were from a 4-day culture (yield 4 g/l) and DNP (10^{-4} M) was added at the indicated times. Continuous N_2 (●); KNO_3 (○); DNP (△); DNP + KNO (▲).

[4] Abbreviations: DNP: 2,4-dinitrophenol; DMSO: dimethylsulfoxide.

the latter particularly in combination with nitrate, caused a resumption of nitrite production after the plateau had been reached. It therefore appears that all these agents share the common property of being able to release whatever factor is limiting nitrite production. However, they differed qualitatively in that only after the addition of nitrate was a second plateau consistently observed.

Nitrate Leakage and Nitrite Production. The following experiments were done to determine if the cessation of nitrite production was correlated with a loss of cellular nitrate due to leakage.

Kinetics of nitrate leakage from tobacco cells incubated under anaerobic conditions are shown in Table III. Values for leakage (nitrate lost) were derived from amounts of endogenous nitrate remaining in the tissue at various time intervals. (Loss of nitrate due to conversion to nitrite was only a small fraction [less than 2% in the first 15 min] of nitrate lost from the cells and was therefore neglected.) The loss of endogenous nitrate by leakage occurred largely within the first 15 min after resuspension. This was at least three times faster than the time observed for the cessation of nitrite production (*e.g.*, 40–60 min; Fig. 1); consequently, it seems unlikely that the slowing down of nitrite production could be attributed to a leakage of nitrate during anaerobic incubation.

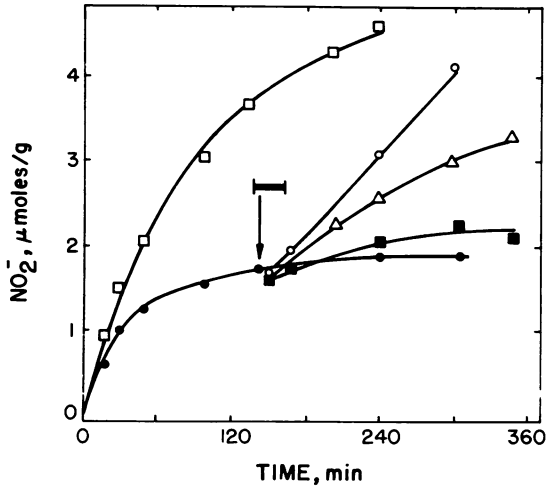

FIG. 5. Effect of 2,4-dinitrophenol on initial and resumed nitrite production. Cells were from 4-day cultures (yield: 3.2 g/l). Cells were harvested, washed, incubated, and assayed for nitrite as in "Materials and Methods." At the indicated times 0.1 mM 2,4-dinitrophenol was added in the presence or absence of 10 mM KNO_3; continuous N_2 (●); DNP at zero time (□). At 140 minutes: DNP (■); KNO_3 (△); KNO_3 + DNP (○).

Table III. *Kinetics of Nitrate Leakage*

Cells were from a 5-day-old culture which had been diluted 1:1 at day 4 with fresh medium. The cells, harvested without washing, were resuspended in $CaCl_2$ + KCl solution (20 ml/g) and incubated under N_2. At the indicated times, aliquots were withdrawn and weighed 0.5-g samples were then boiled and assayed for nitrate.

Incubation Time	Endogenous Nitrate	Nitrate Lost
min	*μmole/g cells*	
0	32.2	—
15	18.4	13.8
30	16.9	15.3
45	16.1	16.1
60	16.3	15.9

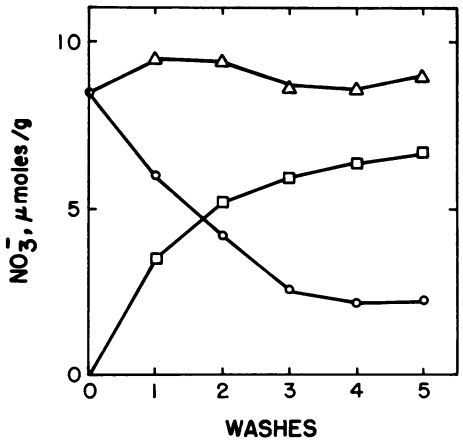

FIG. 6. Dependence of nitrate content on number of washes. Cells were from a 5-day culture (yield: 4.88 g/l). The cells were harvested after each wash by vacuum filtration on prewashed Whatman No. 1 filter paper discs. Each wash consisted of 5-min incubation under N_2 in $CaCl_2$ + KCl solution, 20 ml/g. After each wash, a 0.5-g sample of cells was weighed out and boiled in 5 ml H_2O for nitrate determination. A small portion of the filtrate from each wash was also saved for nitrate determination. Total KNO_3 (△); nitrate washed out (□); nitrate remaining in cells (○).

Table IV. *Washout of Nitrate from Undiluted Cells and Cells Diluted with Fresh Medium*

Same experiment as Figure 2. Cells were grown for 3 days, then one set of cultures was diluted 1:1 with fresh medium and another set was not. After an additional 24 hr, the cells were harvested and washed the indicated number of times. Nitrate was determined in weighed cell samples. Undiluted cells contained 20.5 μmoles/g, and diluted cells contained 30.6 μmoles/g nitrate prior to washing.

Washes	Endogenous Nitrate Remaining	
	Undiluted cells	Diluted cells
No.	*%*	
0	100	100
1	92	75
3	55	54
5	33	28

Nitrate could be released from the cells by washing (Fig. 6). The removal of nitrate from cells by washing—in contrast to nitrite production (Fig. 2)—was only slightly affected by 1:1 dilution of cells with fresh medium 24 hr before the anaerobic incubation (Table IV). This too is inconsistent with the idea that the cessation of anaerobic nitrite production was a consequence of nitrate leakage.

Cold and anaerobiosis are known to cause solute leakage in plant tissues (3, 16). If nitrite production slowed down and eventually ceased as a result of a gradual leakage of nitrate from the cells into the surrounding medium, then preincubation at 0 C would probably allow nitrate to leak out without concomitant enzymatic nitrite production. Then, upon warming the cells, they would not have sufficient endogenous nitrate to reduce to nitrite. However, this effect was not observed (Fig. 7). Cells preincubated at 0 C for 2 hr produced nitrite at a rate comparable to the rate of nitrite production by cells incubated continuously at 23 C. Furthermore, the amount of nitrite produced after transfer from 0 to 23 C was also the same as that produced by cells at 23 C continuously.

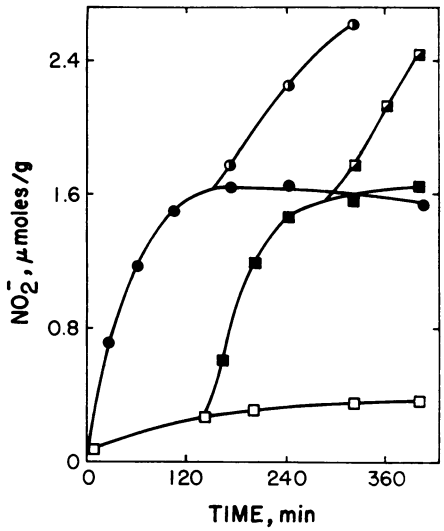

FIG. 7. Effect of cold preincubation on anaerobic nitrite production. Cells from a 4-day culture (yield: 3.5 g/l) were harvested, washed, and assayed as in "Materials and Methods," except that the cells were divided into two portions, one was resuspended in $CaCl_2$ + KCl solution at 0 C (□), the other at 23 C (●). Following cessation of nitrite production at 120 min by the control suspension at 23 C, a portion of the suspension at 0 C was warmed to 23 C (■). Subsequently, 10 mM KNO_3 was added to each suspension (◑, ◪).

Table V. *Independence of Nitrite Plateau and Endogenous Nitrate Concentration*

Cells were from a 4-day-old culture. After the indicated number of washes, the cells were incubated anaerobically. Nitrite was assayed at 20-min intervals with the plateau occurring at about 80 min. The initial rates were computed from the nitrite produced in the first 20 min. At 100 min, 10 mM KNO_3 was added, and the rate for resumption of nitrite production was computed from nitrite produced between 100 and 160 min. Nitrate was determined in boiled, weighed cell samples.

Washes	Nitrate of Cells	Nitrate of Wash Medium	Nitrite Production (Initial Rate)	Nitrite Plateau	Nitrite Production (Resumption Rate)
No.	*μmole/g*	*mM*	*μmole/h·g*	*μmole/g*	*μmole/h·g*
0	16.3	0.094	8.7	6.5	1.7
1	13.2	0.189	6.6	5.6	3.9
2	11.8	0.096	3.0	4.4	3.2
3	9.8	0.046	4.2	6.2	4.1

Effect of Endogenous Nitrate Concentration on Nitrite Production. As already shown, repeated washing lowered extractable nitrate (Fig. 6). Thus, it was possible to examine the influence of different levels of nitrate on nitrite production. Up to three washes had little or no effect on the nitrite plateau, regardless of whether the plateau level was low (0.25 μmole/g; Fig. 2) or high (4.4–6.2 μmoles/g; Table V). In the experiment presented in Table V, 40% of the nitrate was washed from the cells after three washes without affecting a plateau equal to about 40% of the original endogenous nitrate. It should be noted that the plateau level was independent of the rate of nitrite formation (Table V).

Nitrite Production and Exogenous Nitrate Concentration. Anaerobic production of nitrite dependent on an exogenous supply of nitrate, has frequently been used to assay nitrate reductase *in situ* (4, 11–13, 17, 22, 24, 25). This raised the possibility that the plateau level of nitrite production might be determined by the exogenous concentration of nitrate resulting from leakage. This explanation seemed unlikely because widely differening plateau levels were observed with essentially the same exogenous nitrate concentration (about 0.05 mM after three washes) due to leakage.

To examine more critically the possibility that exogenous nitrate governed nitrite production, cells were washed five times to eliminate essentially anaerobic nitrite production (Fig. 2). These exhaustively washed cells were then incubated with various external concentrations of nitrate (Fig. 8A). The initial rate of nitrite production and the plateau level of nitrite were indeed dependent on the concentration of exogenous nitrate, with half-maximal values at 1.32 mM and 1.47 mM nitrate, respectively (Fig. 8B). At 10 mM KNO_3, the maximum plateau height (0.65 μmole/g) and initial rate of nitrite production (3 μmoles/h·g) were measured. It is clear from these results that nitrite production *can* be made dependent on the exogenous nitrate concentration; nevertheless, this dependence *cannot* account for the plateaus observed with cells washed three times and exposed to an external nitrate concentration resulting solely from leakage. For example, after three washes, there existed an external nitrate concentration of 0.046 mM (Table V), a concentration far below that apparently required even for the half-maximal nitrite production rate and plateau height

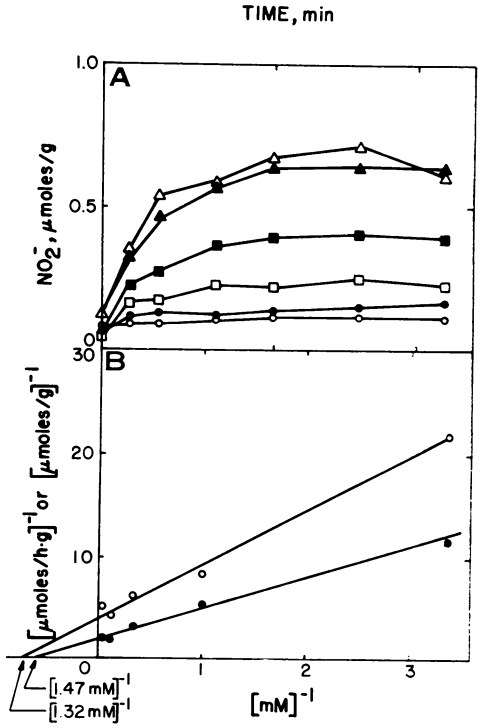

FIG. 8. Dependence of anaerobic nitrite production on exogenous nitrate concentration. Cells were harvested at 2.6 g/l. The nitrate content (30.7 μmoles/g) was determined in a 0.5-g sample which had been boiled. The remainder of the cells were washed five times and another 0.5-g sample was taken for nitrate determination (15.2 μmoles/g). The remainder was divided into 1-g portions which were incubated anaerobically in $CaCl_2$ + KCl solution (20 ml/g) containing 0 (○), 0.3 (●), 1 (□), 3 (■), 10 (▲), or 30 mM KNO_3 (△). Nitrite production was followed as described in "Materials and Methods." A: Nitrite produced versus incubation time. B: double reciprocal plots of initial rate of nitrite production (○) and plateau level of nitrite (●), both derived from data in Figure 8A.

measured (Fig. 8B); yet, in the presence of 0.046 mM external nitrate, there was a high initial rate of nitrite production (4.2 μmoles/h·g), and a high plateau (6.2 μmoles/g; Table V). Clearly, the nitrite produced must be coming from some nitrate source other than the external medium.

Resumption and Cessation of Nitrite Production. Cells from 5-day cultures produced approximately 0.23 μmole nitrite/g during anaerobic incubation (Fig. 9). Following the cessation of nitrite production, nitrate was added to the incubation mixture to final concentrations of 1, 10, or 50 mM. In each case, nitrite production resumed, approached a second plateau and even resumed a third time upon the addition of more nitrate (Fig. 9). The amount of nitrite produced in response to 1 mM nitrate was one-half to one-third that produced in response to 10 or 50 mM nitrate, the latter levels being saturating. In this same experiment, DNP added after establishment of the second plateau caused a burst of nitrite production. The rate of nitrite production in the presence of DNP was dependent on the amount of nitrate added. In the 50 min after establishment of the *second* plateau and addition of DNP, 135, 360, and 305 nmoles nitrite/g were produced by cells which had received 1, 10, and 50 mM nitrate, respectively. This is the same dependence on exogenous nitrate concentration which was observed for establishment of the second plateau (Fig. 9), and for nitrite production by exhaustively washed cells (Fig. 8A). It therefore appears that after the second plateau was established and nitrite production had ceased, DNP made the remaining exogenous nitrate available for reduction.

Data indicate that the height of the first and second plateaus can be related to relatively high levels of externally added nitrate (Figs. 8 and 9). In both of these situations, the question arose: why did nitrite production stop when only a small fraction of the nitrate available was reduced? One possibility which we considered was that the cells deplete the medium and sequester the majority of the added exogenous nitrate. Considering that at 10 mM KNO_3 there are 200 μmoles exogenous nitrate per g cells, and that nitrite production ceases within an hour (accounting for only a tiny fraction of the 200 μmoles), the cells would be required to take up nitrate at 200 μmoles/h·g—about 40 times the measured uptake rates (10). They would also have to achieve a nitrate concentration of 200 μmoles/g—about six times the highest measured concentrations (Table I). These considerations were rejected when it was found that exogenous nitrate was undiminished at the time of cessation of nitrite production.

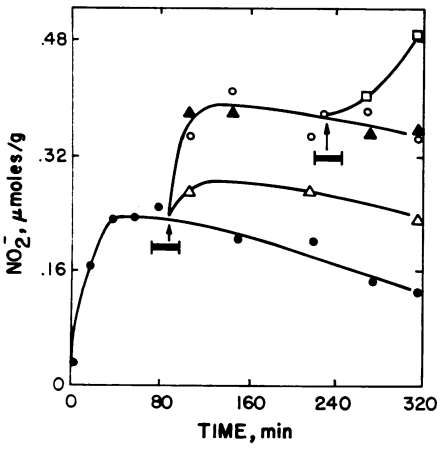

FIG. 9. Dependence of resumption of nitrite production on exogenous nitrate concentration. Cells were from a 5-day culture (yield: 6.1 g/l). They were resuspended in 100 ml of anaerobic $CaCl_2$ + KCl solution and at 80 min, KNO_3 was added to give a final concentration of 1, 10, or 50 mM. At 220 min, 10 mM potassium nitrate was added to each of the suspensions which had previously received KNO_3. Following all nitrate additions, the suspensions were aerated for 10 min, then flushed with N_2, and incubated anaerobically again. Continuous N_2, (●). KNO_3 added at 80 min: 1 mM (△); 10 mM (○); 50 mM (▲). At 220 min, 10 mM KNO_3 added to all but continuous N_2 treatments (data shown for 10 mM treatment only (□).

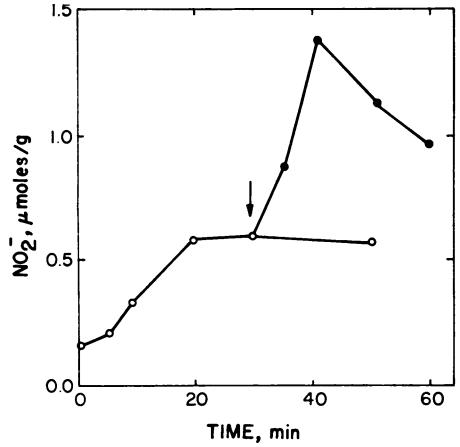

FIG. 10. Anaerobic nitrite production by barley aleurone layers. See "Materials and Methods" for experimental details. At 30 min, 10 mM KNO_3 was added under continuous anaerobic conditions. Continuous N_2 (○); KNO_3 at 30 min (●).

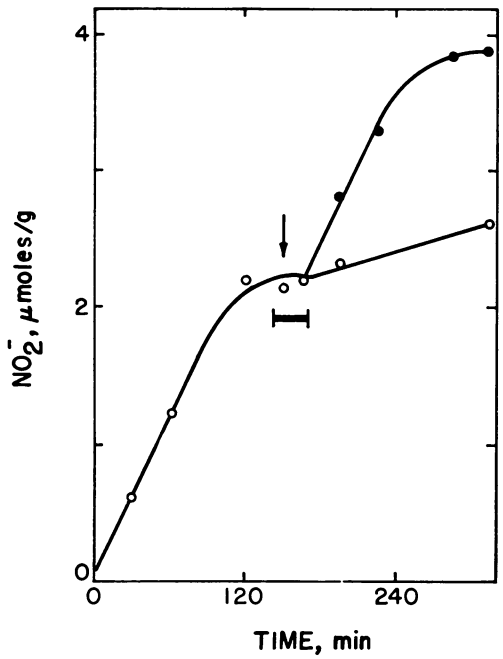

FIG. 11. Anaerobic nitrite production by corn leaf sections. See "Materials and Methods" for experimental details. Continuous N_2 (○); KNO_3 at 160 min (●).

Nitrite Production by Aleurone Layers and Corn Leaf sections. Aleurone layers (Fig. 10) and corn leaf sections (Fig. 11) showed nitrite production kinetics similar to those observed with tobacco cells. In the case of aleurone layers, the second plateau was not level. Instead, the nitrite level decreased with time. This observation is consistent with the finding that this tissue is capable of reducing low levels of nitrite under anaerobic conditions (5). The ratio of nitrite-accumulated to initial

Table VI. *Effect of Age on Total Nitrate and Anaerobic Nitrite Production in Corn Leaves*

Nitrate was supplied at day 15 to the growth medium. Numbers represent nmoles nitrite produced per 100 mg fresh weight leaf tissue.

	Time after Nitrate Addition	Total Nitrate	Nitrite Accumulation	Accumulation Total
	days	*nmoles*		*ratio*
Experiment 1	1	615	190	0.30
	3	1325	210	0.16
	5	2420	210	0.09
Experiment 2	0.3	222	59	0.27
	1	900	190	0.21
	3	2300	235	0.10
	7	4160	280	0.07
	11[1]	3460	145	0.04
	15[1]	2754	200	0.07

[1] Plants in state of decline.

total nitrate in aleurone layers (a mature tissue previously exposed to nitrate for 5.5 hr) or corn leaves (tertiary leaves grown in the presence of nitrate for 3 days) was 0.01, and 0.10 to 0.6, respectively.

The amount of nitrite accumulated in corn leaves during anaerobic incubation approached a maximum 1 to 3 days after administering nitrate to 15-day-old plants while total leaf nitrate continued to increase up to 7 days (Table VI). Little change was evident in the ratio of nitrite accumulated to total nitrate of tertiary leaves when corn plants were germinated and grown in the presence of a continuous supply of nitrate. Under such conditions, the accumulation of nitrite during anaerobiosis represented less than 4% of total extractable nitrate.

DISCUSSION

Suspensions of tobacco cells, isolated aleurone layers, corn leaf sections, and *Angrostemma githago* embryos (12), containing nitrate and nitrate reductase, all produce nitrite when incubated under anaerobic conditions in the absence of exogenous nitrate. Nitrite production decreased with time and eventually ceased, despite the presence of relatively large amounts of nitrate remaining within the tissues and cells.

With tobacco cells, the nitrite plateau level was almost never observed to exceed 50% of the endogenous nitrate present at the beginning of the anaerobic incubation—usually the percentage was far lower. Therefore, cessation of nitrite production was not due to reduction of all nitrate in the incubation mixture. Cessation may have been a result of (*a*) an inactivation of the nitrate reducing system due to decay of one of its components (nitrate reductase and cofactors), (*b*) a loss of endogenous nitrate by leakage or transport out from the cells in an amount sufficient to depress the rate of nitrate reduction, or (*c*) depletion of nitrate in a pool (accessible to nitrate reductase) by reduction to nitrite.

If nitrite production ceased because of the decay of nitrate reductase or a cofactor in the nitrate reducing system, then, because of their ability to cause a resumption in nitrite production, nitrate (Fig. 1), monohydroxy alcohols (Table II), pyrazole and DNP (Fig. 4) would all have to share the common property of virtually being able to restore almost instantaneously the level of the deficient component. A more probable interpretation of these results is that the components of the nitrate reducing system remain fully active after the cessation of nitrite production, and that nitrite production ceased because the concentration of nitrate in the vicinity of the nitrate reducing system drops to a level which at best can only support a very low rate of nitrate reduction.

It seems unlikely that nitrite production ceased because of a gradual leakage of nitrate during the anaerobic incubation. Several observations were inconsistent with such an interpretation. For example, (*a*) nitrite production was found to continue even after nitrate leakage had ceased. (*b*) Nitrite production (Fig. 2), but not nitrate leakage (Table IV), was affected by 1:1 dilution of cells 24 hr prior to determination of the plateau level. (*c*) The plateau level was independent (up to three washes or after removal of 40% of the endogenous nitrate) on the amount of nitrate washed from the cells (Table V). (*d*) The level of nitrate in the external medium was insufficient to cause a plateau of the magnitude observed with thrice-washed cells. (*e*) Finally, incubation of cells under conditions which should have allowed leakage to occur (incubation at 0 C) had no measurable effect on the rate or amount of nitrite produced (Fig. 7).

We concluded that the cessation of anaerobic nitrite production was not determined by either leakage of nitrate from the cells, or by an inactivation of the nitrate reducing system. We were left with the hypothesis that intracellular nitrate exists in two pools: (*a*) a small one, accessible to the nitrate-reducing system in which the nitrate can be converted to nitrite under anaerobic conditions, and (*b*) a large one which is inaccessible and whose nitrate cannot be reduced to nitrate. We propose to call these the metabolic pool and the storage pool of nitrate, respectively. What about the physical reality of the two pools? The nitrate washout experiments (Figs. 2 and 6, Table V) deserve special comment in view of the ability to distinguish wall, cytoplasmic and vacuolar compartments by this technique (21). Based on washout kinetics (Fig. 6), most of the nitrate removed during the first three washes was extracellular or wall bound. Therefore, its removal would not be expected to affect the level of nitrite produced since nitrate reductase is located intracellularly (1). As expected, the level of nitrite produced was unaffected (Fig. 2) or only slightly affected (Table V) up to three washes. However, after five washes, the amount of nitrite produced was reduced nearly 90% from that of unwashed or thrice-washed cells (Fig. 2). This dramatic reduction occurred even though the extractable nitrate was decreased only slightly (approximately 10–20%) from three to five washes (Fig. 6). This indicates that the reducible (metabolic pool nitrate) nitrate remaining after three washes was cytoplasmic (21). The nitrate which was difficult to remove by washing and unavailable for reduction (storage pool nitrate), probably represents vacuolar nitrate (21).

Our data indicated that with tobacco cells the size of the metabolic pool decreased with age, whereas the storage pool remained relatively constant (Table I). This assumes that the metabolic and storage pools of nitrate are strictly separated from the beginning of anaerobic incubation, and that cessation of nitrite production occurs when all of the nitrate in the metabolic pool has been reduced. One possibility which at this time cannot be rejected, is that there was transport from one pool to the other. If this were the case, nitrite production would have ceased when the combination of reduction and transport out had depleted the metabolic pool, or after transport in had ceased and reduction had depleted the metabolic pool. Because of this alternative possibility, the size of the metabolic nitrate pool may not be simply a measure of the plateau level of nitrite produced. However, as discussed, the occurrence of the nitrite plateau indicates the existence of the metabolic pool.

Why nitrite production resumed and then reached a second plateau after the addition of excess nitrate, is not known.

Following the cessation of nitrite production, DNP, pyrazole, and propanol were each able to cause a resumption in nitrite production without establishment of a second plateau. Thus, whatever had limited nitrite production was alleviated by these agents. We have interpreted the data to indicate that these chemicals caused the excess nitrate accumulated in storage pools to leak into the metabolic pool where it became available for reduction. A mixing of two separate pools by DNP was also reported for the malate pools in corn roots (14). Such an effect would initially expose the nitrate-reducing system to an internal nitrate concentration greater than that measured in the external medium. The resulting molarity of internal nitrate (15–20 mM) would then be more than sufficient to cause the observed high rates of nitrite production. *In vivo* nitrate reductase activity in soybean leaves was also found to be enhanced by alcohols of increasing chain length (11).

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