Synthesis and Turnover of Nitrate Reductase Induced by Nitrate in Cultured Tobacco Cells*

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SUMMARY

Synthesis and turnover of nitrate reductase molecules and soluble proteins of tobacco XD cells were studied by labeling pre-existing proteins with 14C-arginine to make them radioactive and with 99 atom % 15N to increase their buoyant density. The cells were then transferred to medium in which new proteins were synthesized from 14N and were radioactively labeled with 3H-arginine. The breakdown of 15N-labeled proteins was followed after transfer of cells to 14N medium by means of the 3H label. The influence of the pre-existing pool of 15N-amino acids on the density of newly synthesized proteins was followed by means of the 3H label. At various times protein was extracted and subjected to isopycnic equilibrium centrifugation in CsCl. Buoyant densities, relative to an added standard, catalase, were determined for nitrate reductase activity, 14C-labeled protein, and 3H-labeled protein. The studies were carried out (a) during induction of nitrate reductase activity, (b) after full induction, and (c) after a shift from inducing to noninducing conditions, when nitrate reductase activity decays.

The buoyant density data show that nitrate reductase activity induced by nitrate is an activity of a protein synthesized de novo after induction of inducer. However, even when the enzyme level was constant, the buoyant density of nitrate reductase decreased from that of 15N-nitrate reductase towards that of 14N-nitrate reductase, indicating that the enzyme turned over. Furthermore, nitrate reductase molecules continued to be synthesized as nitrate reductase activity decayed under noninducing conditions. Turnover of both pre-existing proteins labeled with 14C and newly synthesized proteins labeled with 3H was also observed.

Nitrate reductase (NADH: nitrate oxidoreductase, EC 1.6.6.1) is an ubiquitous enzyme activity of higher plants (1). Although many enzyme activities of higher plants can be induced to increase by a variety of environmental perturbations (2), nitrate reductase is uncommon in that development of nitrate reductase activity is induced by its substrate, nitrate. The phenomenon of substrate-induced synthesis of enzymes is well documented in microorganisms (3). The occurrence of a comparable phenomenon in higher plants has not been rigorously proven, although it is considered highly probable, largely because of the fact that nitrate induces development of nitrate reductase activity. The substrate-induced development of nitrate reductase activity has been shown, with the aid of inhibitors, to depend on nucleic acid synthesis and protein synthesis (4–9), but attempts to demonstrate that the nitrate reductase molecule itself is synthesized during induction have been unsuccessful (7) or have not been sufficiently rigorous (9).

In cultured tobacco cells, nitrate induces, whereas casein hydrolysate or certain amino acids inhibit, development of the three activities of the nitrate assimilation pathway: the nitrate uptake system (10), nitrate reductase (11), and nitrite reductase (12). The nitrate reductase activity of XD cells decays as the cells deplete the medium of nitrate or if the cells are transferred to medium lacking nitrate. Addition of casein hydrolysate also brings about decay of nitrate reductase activity after it has been induced by nitrate.

Any increase in an enzyme level in higher plants and animals has to be viewed against a background of continual synthesis and degradation of cell proteins (13–15). Merely showing that an enzyme activity increases is not sufficient to prove that synthesis of enzyme has occurred, and, if it has occurred, that it was induced. A decrease in the rate of degradation could also elevate an enzyme level. Schimke, Sweeney, and Berlin (16) have shown that tryptophan increases the level of tryptophan pyrrolase in vivo by stabilizing pre-existing enzyme rather than through increasing the rate of synthesis. The rate of degradation also varies from enzyme to enzyme in a single tissue, e.g. a half-life of 70 min has been determined for δ-aminolevulinate synthetase (17) compared to a half-life of 4 to 5 days for arginase (18) in rat liver.

With these considerations in mind, the contributions of both synthesis and degradation of nitrate reductase molecules to the observed changes in nitrate reductase activity levels were investigated. A density difference between pre-existing macromolecules and newly synthesized macromolecules can be introduced by labeling with stable, heavy isotopes (19–22). Then the old and new molecules can be resolved from each other ac-
cording to density by isopycnic equilibrium centrifugation. This technique has been applied successfully in determining the origins of increases of a number of enzyme activities, including β-galactosidase (40) and aspartate transcarbamylase (39) in cell-free systems from Escherichia coli, arginyl- and histidyl-tRNA synthetase in E. coli (34), valyl-tRNA synthetase in phage-infected E. coli (25), α-amylase (29) and protease (28) induced by gibberellic acid in the aleurone cells of barley seed, and the glyoxylate cycle enzymes, malate synthetase (27) and isocitrate (27, 28), which develop in the cotyledons of germinating peanut seed. In the present study, the technique has been used to determine the origin of nitrate reductase activity induced by nitrate in the XD strain of cultured tobacco cells.

EXPERIMENTAL PROCEDURE

Materials—Optically pure CsCl was purchased from the Harshaw Chemical Company. Ninety-nine atom % 15N-enriched KNO3 was obtained from Bio-Rad, Richmond, California and the radioactive amino acids were purchased from New England Nuclear. Miracloth is a product of Chiopee Mill, Inc., New York. Crystalline bovine liver catalase was purchased from Worthington.

Preparation of Nitrate Reductase from 14N, 15N, 3H- and 14C, 15N, 14C-Labeled Cells—Stationary cells growing on 15N-nitrate were subcultured into 1 liter of 14N nitrate medium with 20 μCi of U-14C-arginine and 0.1 mM unlabeled arginine. After 5 days of growth, cells were harvested by filtration on Miracloth and resuspended in cold 0.1 M Tris-HCl, 1 mM cysteine-HCl, 1 mM EDTA, and 1 mM FAD, 5.0 ml per g fresh weight. The cell homogenate was centrifuged at 30,000 × g for 10 min. The supernatant was stirred with an equal volume of saturated ammonium sulfate, pH 7.5, for 30 min at 3°C. The suspension was centrifuged at 27,000 × g for 20 min. The precipitate was dissolved in a small volume of 0.1 M Buffer A (variable concentration of potassium phosphate, pH 7.5, 1 mM cysteine-HCl, 1 mM EDTA, 1 mM FAD) and used directly for isopycnic equilibrium centrifugation in CsCl. Occasionally the enzyme was further purified before centrifugation. The dissolved precipitate was dialyzed against 0.01 M Buffer A and adsorbed to DEAE-cellulose equilibrated with the same buffer, washed with 0.05 M Buffer A, and eluted with 0.12 M Buffer A. Nitrate reductase from 15N, 14C-labeled cells was obtained by subculturing stationary cells that had been grown over 10 generations in medium with 15N-nitrate as the sole nitrogen source into 1 liter of 14N nitrate medium with 20 μCi of U-14C-arginine and 0.1 mM unlabeled arginine. After 5 days of growth, cells were harvested and extracted as above.

Induction of Nitrate Reductase with 14N, 15N, 3H-Labeled Stationary Phase Cells—Stationary phase cells that had been grown over 10 generations on 15N-nitrate were subcultured into 1 liter of 14N nitrate medium with 20 μCi of U-14C-arginine and 0.1 mM unlabeled arginine. After 14 days of growth, the cells were in the stationary phase, the proteins were labeled with 14C amino acids and 15N, and the cells contained essentially no nitrate reductase activity. The cells were then steriley harvested on Miracloth, washed with nitrate-less medium, and resuspended in 2 liters of 14N nitrate medium supplemented with 40 μCi of U-14H-arginine, and a mixture of 17 amino acids, each at 0.1 mM. The 17 amino acids were: L-alanine, L-arginine, L-aspartate, L-cysteine, L-glutamate, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, and L-valine. This mixture has little effect on nitrate reductase induction and was added in an effort to dilute any pre-existing 15N-amino acid pools. Preparations of nitrate reductase were made from 250-ml aliquots of the culture taken at time of transfer (zero time) and at subsequent times up to 72 hours.

Turnover and Decay of Nitrate Reductase in Exponentially Growing 15N, 14C-Labeled Cells—Cells which had grown continuously on 15N-nitrate were subcultured into 1 liter of 14N nitrate medium with 10 μCi of U-14C-arginine and 0.1 mM unlabeled arginine. After 5 days the exponentially growing cells with nitrate reductase activity were steriley harvested, washed as above, and divided into two portions. One portion was transferred to 1 liter of 14N nitrate medium with 40 μCi of U-14H-arginine and the above mixture of 17 amino acids. The second portion was transferred to 1 liter of nitrate-less medium with casein hydrolysate, 3 g per liter, as a nitrogen source and 30 μCi of U-14H-arginine. Enzyme preparations were made at the times indicated in the tables and figures. The breakdown of 15N-labeled proteins was followed after transfer of cells to 14N medium by means of the 14C label. The influence of the pre-existing pool of 15N-amino acids on the density of newly synthesized proteins was followed by means of the 14H label.

Centrifugation in CsCl—The isopycnic equilibrium centrifugation was performed in a Beckman L2-65I ultracentrifuge using a SW56L rotor at 37,500 rpm or a SW65L rotor at 40,000 rpm for 65 hours at 3°C. Two milliliters of enzyme (500 to 3,000 nmoles of nitrate reduced per hour) containing 0.5 to 1.5 mg of protein were added to each tube, followed by 700 units of catalase (specific activity was 25,000 units per mg) and 1.0 ml of a saturated solution (3°C) of CsCl in water adjusted to pH 7.5. The contents were mixed and 2.0 ml of paraffin oil were layered over the aqueous phase in each tube. After centrifugation the tubes were punctured at the bottom and 1- to 3-drop fractions were collected. One-drop fractions were used for the determination of catalase activity (31) and refractive index with a Bausch and Lomb Abbe-32 refractometer. Refractive index values were converted to density units by means of a standard curve. One- or two-drop fractions were used for determination of radioactivity and were collected directly in scintillation vials. Two- or three-drop fractions were collected in 5-ml vials containing 0.9

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Fig. 1. Resolution of $^{15}$N-nitrate reductase and $^{14}$N,$^{14}$C-proteins from $^{15}$N-nitrate reductase and $^{14}$N,$^{3}$H-proteins by CsCl isopycnic equilibrium centrifugation. Centrifugation and enzyme preparations were carried out according to the methods under “Experimental Procedure.” Catalase was used as an internal marker in all tubes. The short vertical lines intersecting the bands indicate the band centers. The short vertical arrows in the bottom panel indicate the position of $^{14}$N-nitrate reductase and $^{15}$N-nitrate reductase. All peak heights are normalized. The density in the CsCl gradient increases toward the left. $\bigcirc$, nitrate reductase activity; $\triangle$, catalase activity; $\bigtriangleup$, $^{3}$H-labeled proteins; $\square$, $^{14}$C-labeled proteins; $\blacktriangle$, buoyant density. Panels in a given figure are aligned on the center of the catalase bands. In subsequent figures the center of the catalase band is only indicated by a broken vertical line. NR, nitrate reductase. $W_{b}$, band width in drops as measured at half the activity found at band center.

ml of assay mix for the NADH nitrate reductase assay (11) minus the NADH, but supplemented with bovine serum albumin, 1 mg per ml. After the vials had warmed to room temperature, 0.1 ml of 1 mm NADH was added to initiate the 45-min assay. Recovery of nitrate reductase activity from the gradient was always greater than 80%. Catalase and nitrate reductase activities were proportional to enzyme concentration under the conditions employed. One unit of nitrate reductase activity is defined as the amount of enzyme required to reduce 1 nmole of nitrate per hour.

Determination of Radioactivity—Ten milliliters of Bray’s scintillation fluid (32) were added to each scintillation vial. The radioactivity was determined with an error of less than 5% in the vicinity of the peak of each band in a Beckman LS-133 liquid scintillation system with the gain set so that there was less than 0.1% $^{3}$H spillover into the $^{14}$C channel. The $^{3}$H counts were calculated by subtracting the appropriate percentage of counts due to spillover of $^{14}$C counts into the $^{3}$H channel, as determined with a standard. An average of 6,800 cpm of $^{3}$H and 12,000 cpm of $^{14}$C, after correction for background and overlap, was put on each gradient.

RESULTS

Resolution of $^{15}$N-Nitrate Reductase, $^{14}$N-Nitrate Reductase, $^{15}$N,$^{14}$C-Protein, and $^{15}$N,$^{3}$H-Protein—The density increase due to substitution of $^{15}$N for $^{14}$N is insufficient for complete resolution of high density enzyme from low density enzyme by isopycnic equilibrium centrifugation in CsCl. However, the overlapping distribution of $^{15}$N-nitrate reductase and $^{14}$N-nitrate reductase can be resolved by banding them in separate tubes along with catalase to provide a common reference point in the two gradients (Fig. 1). The nitrate reductase band centers were separated by 16 ± 0.5 drops. Band centers were determined from a plot on Gaussian paper (33) because in defining the band center such a plot gives weight to points throughout the distribu-

![Gaussian Plot](image-url)
and 3H-arginine, the former to induce nitrate reductase activity, phase XD cells containing 15N-protein, which was labeled with two curves. This observation allows one to distinguish between greater than either 15N-nitrate reductase or 14N-nitrate reductase alone. This is precisely the band width found if r5N-nitrate resolved in the mixture. An increase in the band width, as nitrate reductase and 14N-nitrate reductase peaks were not alone and 14N-nitrate reductase alone (Fig. 1). Discrete lsN-half-way between the positions expected for '5N-nitrate reductase.

The band center is the intersection at 100% (Fig. 2). By this procedure 15N-nitrate reductase was found to be 0.92% more dense than 14N-nitrate reductase. The same density difference was found between bulk soluble protein labeled by synthesis from 15N-nitrate plus 14C-arginine and bulk protein labeled by synthesis from 14N-nitrate plus 15N-arginine, in good agreement with an earlier determination (22). The average soluble 15N-protein is 0.51% less dense than 15N-nitrate reductase, and the same is true for 14N-protein compared to 14N-nitrate reductase.

When 16N-nitrate reductase and 14N-nitrate reductase were mixed in equal proportions, and the mixture was subjected to equilibrium centrifugation in CsCl, the center of the distribution of nitrate reductase activity was located approximately half-way between the positions expected for 15N-nitrate reductase alone and 15N nitrate reductase alone (Fig. 1). Discrete 15N-nitrate reductase and 15N-nitrate reductase peaks were not resolved in the mixture. An increase in the band width, as measured at half the activity found at band center, was observed (Inset, Fig. 1). The band widths were also determined from plots on Gaussian paper. The mixture had a band width 20% greater than either 15N-nitrate reductase or 15N-nitrate reductase alone. This is precisely the band width found if 15N-nitrate reductase and 15N-nitrate reductase curves are plotted independently and the band width is determined from the sum of the two curves. This observation allows one to distinguish between a mixture of all 15N- and all 15N-nitrate reductase molecules and a sample of nitrate reductase which may have the same buoyant density but which consists of one protein species synthesized from a mixture of 15N- and 14N-amino acids.

**Synthesis and Turnover of Old and New Proteins—**Stationary phase XD cells containing 15N-protein, which was labeled with 14C-arginine, were transferred to medium containing 15N-nitrate and 14C-arginine, the former to induce nitrate reductase activity, the latter to label new protein. During the first 12 hours there was little growth as measured by fresh weight or net synthesis of protein. Nevertheless, protein synthesis occurred since 3H-arginine was incorporated into protein (Table I). There was no lag before 14H-arginine incorporation into protein began and the rate of incorporation varied little during most of the next 72 hours. This indicates that exogenously supplied 14H-arginine readily entered the protein precursor pool.

### Table I

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Fresh weight (g/liter)</th>
<th>Protein mg/g cells</th>
<th>mg/liter medium</th>
<th>Nitrate reductase activity</th>
<th>14C-arginine incorporated</th>
<th>3H-arginine incorporated</th>
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</table>

The curves for 0 and 72 hours are not shown. NR, nitrate reductase.

### Fig. 3

Change in buoyant density with time of nitrate reductase and protein after induction of nitrate reductase in 15N,14C-labeled stationary phase cells by 15N nitrate in the presence of 14C-arginine. See "Experimental Procedure" for details of centrifugation and enzyme preparations. 15N-Grown cells were radioactively labeled by 14-day growth in 15N-nitrate medium supplemented with 20 μCi of U-14C-arginine per liter. At the end of this period the cells were sterilely transferred to 2 liters of 14N nitrate medium with 40 μCi of U-14H-arginine and a mixture of 17 amino acids, each at 0.1 mM. Percentage of band height is plotted versus hours after transfer to inducing conditions. The scales on the ordinate are displaced by 20%. The broken vertical lines represent the centers of the catalase bands (see Fig. 1). O, nitrate reductase activity; △, 1H-labeled proteins; □, 14C-labeled proteins. The curves for 0 and 72 hours are not shown. NR, nitrate reductase.
The changes in buoyant density with time of the various fractions were small, so that it was not possible to read band centers with the necessary precision directly from conventional plots of the data (Fig. 3). However, the curves are smooth and closely approximate Gaussian distributions. The band centers were therefore determined from Gaussian plots (see Fig. 2). The fractional change in buoyant density from that of \(^{15}N\)-protein toward that of all \(^{14}N\)-protein was calculated from the buoyant densities at band centers for nitrate reductase, \(^3H\)-labeled protein, and \(^14C\)-labeled protein. This normalization procedure facilitates direct comparisons of the kinetics of the density shift for the induced nitrate reductase, \(^3H\)-labeled proteins, and \(^14C\)-labeled proteins (Fig. 4).

The percentage by which the mean buoyant density of newly synthesized protein shifts from the density of the \(^{15}N\)-protein towards that of the \(^{14}N\)-protein can be considered a measure of the percentage of \(^{14}N\)-amino acids in the protein at any given time. If the protein precursor pool was immediately saturated with the added \(^{15}N\)-amino acids, the proteins synthesized after the transfer should contain only \(^{14}N\)-amino acids. However, during the first 4 hours \(^1H\)-arginine was incorporated into proteins that had approximately the buoyant density of \(^{15}N\)-proteins. This indicates that during the first few hours after transfer from \(^{15}N\) medium to \(^{14}N\) medium, the protein precursor pool contained predominantly \(^{15}N\)-amino acids. At times later than 4 hours, the density of \(^3H\)-labeled protein progressively shifted towards that of exclusively \(^{14}N\)-labeled protein. This must reflect both an increasing percentage of \(^{14}N\)-amino acids in the protein precursor pool and dilution of \(^{15}N\)-labeled protein. However, the decrease in buoyant density of the \(^3H\)-labeled protein from 24 to 48 hours is greater than can be explained by dilution because of net protein synthesis. During this 1-day period, both total protein and total \(^1H\)-arginine incorporated into protein doubled. Assuming that the protein precursor pool is completely saturated with \(^{15}N\)-amino acids at 24 hours and that no degradation of \(^3H\)-labeled protein occurred during the next 24 hours, the mean buoyant density of the \(^3H\)-labeled protein at 48 hours would be expected to be halfway between the buoyant density of the \(^3H\)-labeled protein at 24 hours and exclusively \(^{14}N\)-labeled protein. At 24 hours the buoyant density for \(^3H\)-labeled protein corresponded to a 50:50 mixture of \(^{15}N\)- and \(^{14}N\)-amino acids. By the above reasoning, the mean buoyant density for \(^{14}N\)-labeled protein at 48 hours should correspond to about 75\% \(^{14}N\). However, the observed buoyant density was close to that expected for protein containing 90\% \(^{14}N\). This result indicates degradation of some \(^3H\)-labeled protein containing 80\% or less \(^{14}N\)-amino acids, and its replacement by \(^3H\)-labeled protein containing a greater proportion of \(^{14}N\) amino acids.

The \(^14C\)-labeled proteins, mainly representing protein synthesized before the transfer to inducing conditions, initially have the mean buoyant density of exclusively \(^{15}N\)-labeled protein. However, the buoyant density of the \(^14C\)-labeled protein gradually decreased towards that of exclusively \(^{14}N\)-labeled protein, reaching the density of 50\% \(^{14}N\)-protein after about 48 hours. This indicates that the older cellular proteins, i.e. those which existed before the density shift, also undergo turnover. Since there is little or no net loss of \(^14C\) from proteins, degradation of \(^{12}N\), \(^{14}C\)-protein must occur, and the \(^14C\) must be reincorporated into the newly synthesized protein which probably contains a mixture of \(^{12}N\)- and \(^{14}N\)-amino acids as well as \(^1H\)-arginine. There must be very efficient recycling of \(^14C\) from \(^1H\)-arginine since the total \(^14C\) radioactivity in the soluble protein fraction remained relatively constant over 72 hours (Table I).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time after transfer (hrs)</th>
<th>Nitrate reductase</th>
<th>Catalase</th>
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<tr>
<td>Control</td>
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</tr>
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<td>(^{15}N)-Nitrate reductase</td>
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<td>31</td>
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<tr>
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<td>41</td>
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<td>41</td>
<td>34</td>
<td></td>
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<td>Induced nitrate reductase</td>
<td>31</td>
<td>31</td>
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The table above shows the results of the experiments, with the width at half-height for nitrate reductase and catalase bands after isopycnic equilibrium centrifugation in CsCl.
density of nitrate reductase continued to decrease more rapidly than that of the H-labeled protein. This quite unexpected result can be interpreted in at least two ways. Assuming that nitrate reductase and the average H-labeled protein are synthesized from the same pool of amino acids, the more rapid decrease in nitrate reductase density may simply reflect a more rapid turnover rate for nitrate reductase and resynthesis from a pool richer in 14N-amino acids. Consequently, the density of nitrate reductase reflects the higher proportion of 14N-amino acids in the precursor pool at later times than in the case of longer lived H-labeled proteins. Alternatively, nitrate reductase and H-labeled proteins may be synthesized from different pools of amino acids, with different rates of replacement of 15N-amino acids by 14N-amino acids.

Indeed, turnover of nitrate reductase is quite apparent from the fact that, although the enzyme level is relatively constant after 8 hours, the buoyant density of nitrate reductase continues to decrease. Also, the width of the nitrate reductase activity band was constant at all times (Table II) and corresponded to that of a population of nitrate reductase molecules which was homogeneous with respect to buoyant density, although the buoyant density changed with time. Thus, at no time did the nitrate reductase activity behave as a mixture of exclusively 15N-nitrate reductase molecules and exclusively 14N-nitrate reductase molecules. This is further evidence of the rapid rate of turnover of nitrate reductase molecules.

Turnover of Nitrate Reductase under Inducing and Noninducing Conditions—In the previous experiment, turnover of nitrate reductase was observed in cells during the course of induction of nitrate reductase. The turnover of the enzyme was also studied in cells which were fully induced at the time of transfer. Cells were grown on 15N nitrate medium with 14C-arginine and then transferred to the exponential phase, when nitrate reductase activity was fully induced, to 14N nitrate medium with H-arginine. Unexpectedly, the cell weight and protein content remained relatively constant with time after the transfer (Table III). Ideally the cells should have continued to grow exponentially. Nevertheless, the nitrate reductase level also remained relatively constant, so that the behavior of enzyme molecules at constant activity could be examined. There was continuous turnover of nitrate reductase and proteins, both

![Table III](https://example.com/table_iii.png)

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Fresh weight (g/liter)</th>
<th>Protein (mg/g cells)</th>
<th>Nitrate reductase activity</th>
<th>$^{14}$C-arginine incorporated (cpm/mg protein)</th>
<th>$^{3}$H-arginine incorporated (cpm/mg protein)</th>
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![Figure 5](https://example.com/fig_5.png)

**FIG. 5.** Decrease in buoyant density of nitrate reductase and protein in exponential phase $^{14}$N, $^{14}$C-cells transferred to inducing and noninducing conditions. A, exponential phase $^{14}$N, $^{14}$C-cells were transferred to $^{15}$N nitrate medium, plus 40 µCi of H-arginine per liter. B, $^{15}$N, $^{14}$C-cells were transferred to nitrate-free medium supplemented with casein hydrolysate, 3 g per liter, and 30 µCi of U-$^{14}$-arginine. See "Experimental Procedure" for details. O, nitrate reductase activity; △, $^{14}$H-labeled proteins; □, $^{3}$H-labeled proteins.

![Table IV](https://example.com/table_iv.png)

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<th>Time (hrs)</th>
<th>Fresh weight (g/liter)</th>
<th>Protein (mg/g cells)</th>
<th>Nitrate reductase activity</th>
<th>$^{14}$C-arginine incorporated (cpm/mg protein)</th>
<th>$^{3}$H-arginine incorporated (cpm/mg protein)</th>
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</table>

$^{14}$C- and $^{3}$H-labeled (Fig. 5), indicating that synthesis of both nitrate reductase and soluble protein occurs in cells with a constant level of enzyme.

Nitrate reductase activity is known to decay under noninducing conditions. Heimer and Filner (10) observed that nitrate reductase activity decays exponentially if cultured tobacco cells with nitrate reductase activity are transferred to medium lacking nitrate or with nitrate and casein hydrolysate present. To study the nitrate reductase molecules during the decay of enzyme activity, exponential phase cells grown on 15N nitrate medium with 14C-arginine were transferred to medium with casein hydrolysate as the sole nitrogen source, plus $^{3}$H-arginine. These conditions allow the cells to utilize 14N-amino acids for synthesis of 14N, $^{3}$H-labeled proteins which can be detected by their decreased buoyant density. After the cells were transferred, the level of nitrate reductase per liter of medium decreased expon-
nentially (Table IV). If synthesis of nitrate reductase ceased when decay of activity began, the density of the remaining nitrate reductase would remain the same as that of \(^{15}\)N-nitrate reductase. However, the buoyant density of nitrate reductase decreased with time, indicating that even while the enzyme activity was decaying, nitrate reductase was being synthesized (Fig. 5).

**DISCUSSION**

The criterion for synthesis de novo of an induced enzyme is that the protein chains composing the enzyme be synthesized from the amino acid pool available at the time of enzyme appearance. It was shown in this study that the nitrate reductase activity induced by nitrate in cultured tobacco cells satisfies this criterion.

If a density marker is included at the time of enzyme induction, all of the newly synthesized proteins, including the enzyme in question, should contain the density marker. The buoyant density difference between all heavy \((^{15}\)N) and all light \((^{14}\)N) nitrate reductase is 0.92\%. This density difference can be anticipated only if no pre-existing amino acid pool is available for new protein synthesis, or if the pre-existing pool is rapidly depleted or diluted. Furthermore, if proteins synthesized before the induction phase are degraded into free amino acids and then are reincorporated into newly synthesized proteins, the newly synthesized proteins will have density characteristics intermediate between those of the pre-existing proteins and proteins synthesized solely from the density marker added at the time of induction.

Both a substantial pre-existing \(^{15}\)N amino acid pool and degradation of pre-existing \(^{15}\)N-proteins were encountered in this study. The \(^{14}\)H-labeled proteins, synthesized de novo after transfer of cells to \(^{14}\)N medium, were initially as dense as proteins exclusively labeled with \(^{14}\)N (Figs. 4 and 5). This clearly indicated that the protein precursor pool was largely composed of \(^{15}\)N-amino acids at early times and that even the inclusion of 17 \(^{15}\)N-amino acids was not sufficient to completely dilute out the \(^{15}\)N-amino acids. A further source of \(^{15}\)N-amino acids was the turnover of \(^{15}\)N-labeled proteins which was continually evident throughout the time courses of the experiments (Figs. 4 and 5). It is of interest that turnover of \(^{15}\)N, \(^{14}\)C-labeled proteins could be detected by the density shift method (Fig. 4), although the total amount of \(^{14}\)C label remained constant in the soluble protein pool (Table I).

Continuous turnover of nitrate reductase and protein was evident in all three experiments in which cells grown on \(^{15}\)N-nitrate were transferred to \(^{14}\)N medium. In all cases the changes in buoyant density of nitrate reductase and of both the \(^{14}\)C-labeled and \(^{14}\)H-labeled proteins were greater than could be accounted for by dilution because of net protein synthesis. The continuous turnover of proteins in cultured tobacco cells is in complete agreement with similar observations in mammalian tissue (13). However, it should be noted that the tobacco cells are a proliferating cell population, not a mature tissue as in the mammalian systems. Thus, rapidly growing tobacco cells are unlike rapidly growing bacteria in which protein turnover is at a minimum (13).

The apparent rates of decrease in buoyant density of nitrate reductase, and of \(^{14}\)C-labeled and \(^{14}\)H-labeled proteins differ when stationary cells are transferred to inducing conditions. However, when cells in exponential phase are transferred to inducing or noninducing conditions, the decrease in buoyant density with time was the same within experimental limits for all three fractions (Fig. 5). The factors responsible for these results have not yet been established, although the different physiological states of the cells may be one possible explanation. For instance, if the rate of replacement of \(^{15}\)N-amino acids in the protein precursor pool was much slower in exponential phase cells than in stationary phase cells, then pool turnover rate rather than protein turnover rate would determine the kinetics of the decrease in buoyant density. If all proteins are made from the same precursor pool, then they would all decrease in density with the same kinetics, even if turnover rates were different.

We had initially hoped to be able to derive some quantitative estimates of the turnover rates for nitrate reductase from these experiments. This would have been possible if the average protein had a long life time with respect to the time required to replace the \(^{15}\)N-amino acids with \(^{14}\)N-amino acids in the protein precursor pool. Then \(^{15}\)N-amino acids derived from breakdown of nitrate reductase and other proteins would have been a minor source of amino acids and would have been efficiently diluted out by \(^{14}\)N-amino acids. Under these conditions an estimate of the turnover rate of nitrate reductase could have been derived from the rate of shift in buoyant density. Unfortunately, the turnover rates of the proteins are sufficiently high to supply a high proportion of \(^{15}\)N-amino acids to the protein precursor pool. This conclusion is based on the fact that \(^{14}\)C-labeled protein, which initially is identical with exclusively \(^{15}\)N-labeled protein, decreases in density with time. This can occur only if a high percentage of the pre-existing proteins turns over. A further, and yet unexplained, complication is that the amount of \(^{14}\)C-arginine incorporated into protein after transfer of cells with induced nitrate reductase activity to inducing or noninducing conditions does not continue to increase with time (Table III and IV). However, this does not negate the conclusion that protein in these experiments is turning over, since the buoyant density of both \(^{14}\)C- and \(^{14}\)H-labeled proteins decreases with time.

Although the data are not amenable to calculations of exact turnover rates, estimates of the lower limit of rates can be made. In Table I the ratio of the increment of net protein synthesized to the increment of \(^{14}\)C-arginine incorporated between any two time points after 8 hours varies only by a factor of approximately two. Similarly, if the net increase of protein per liter from the beginning of the experiment is divided into the total cpm of \(^{14}\)C-arginine incorporated into soluble proteins, a constant value of about 20,000 cpm per mg of protein synthesized (net) is obtained from about 24 hours on. Although the results from later periods are complicated by extensive turnover of \(^{15}\)N-labeled proteins, in the first 4 hours there is very little \(^{14}\)H-labeled protein available for turnover. Consequently, the amount of \(^{14}\)C-arginine incorporated into the soluble protein is an approximate measure of the amount of new protein synthesized. Since about 104,000 cpm of \(^{14}\)C-arginine was incorporated in the first 4 hours, and its maximum specific activity may be estimated to be 20,000 cpm per mg, at least 5 mg of protein or approximately 13% of the original amount of soluble protein per liter of cells were synthesized. This value is a minimum one since immediate equilibration of the pre-existing amino acid pool with \(^{14}\)C-arginine is assumed. No net increase of protein per liter was observed up to 12 hours during the early part of the experiment, although an easily detectable increase of at least 28% would be expected from the incorporation at 12 hours. Apparently, the
soluble cell proteins were turning over fast enough to eliminate an expected 28% increase in 12 hours. However, it is not known whether this rate is maintained.

The half-life of nitrate reductase activity when cells are transferred to noninducing conditions is 4.3 hours (Table IV). This agrees well with the half-life of 4 hours estimated for nitrate reductase when corn seedlings first grown in presence of nitrate were transferred to nitrate-free medium (34). Heimer and Filner (10) observed a half-life of about 6.5 hours for nitrate reductase activity when nitrate-grown cells were transferred to nitrate-free medium, compared to a half-life of 3.7 hours when casein hydrolysate, 1.5 g per liter, was added to cells growing on nitrate.

This study has clearly shown that the observed nitrate reductase activity is an activity of a protein synthesized de novo after addition of inducer. The mechanism of induction of nitrate reductase still remains to be determined. One possibility is that synthesis of nitrate reductase is constant but the rate of degradation is so rapid in noninduced cells that nitrate reductase activity is not observed. During induction the rate of degradation decreases and allows accumulation of nitrate reductase molecules in the cell. A more probable mechanism is an increase in the rate of enzyme synthesis during induction. A third possibility is that the enzyme level may be regulated by changes in both the rate of synthesis and degradation.

Nevertheless, degradation of nitrate reductase is an important part of the control of the nitrate assimilation pathway in cultured tobacco cells. Not only does the nitrate reductase activity decay rapidly under noninducing conditions, but the enzyme is also rapidly degraded and resynthesized during the rise in activity, during the period of constant activity after induction is complete, and during decay of enzymatic activity after removal of nitrate and addition of casein hydrolysate. It has yet to be determined what contributions are made to the decrease in enzyme level by changes in the rate of synthesis or the rate of degradation (or both). The results from this study are in agreement with Schimke's suggestion that continual turnover of proteins in the slowly dividing cells of higher organisms may allow adjustment of their enzyme levels to their environment (35).

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