

# Nitrogen Metabolism in Plant Cell Suspension Cultures

## II. ROLE OF ORGANIC ACIDS DURING GROWTH ON AMMONIA<sup>1</sup>

Received for publication March 8, 1976 and in revised form June 19, 1976

JOSEF BEHREND AND RICHARD I. MATELES

Laboratory of Applied Microbiology, Institute of Microbiology, Hebrew University-Hadassah Medical School, Jerusalem, Israel

### ABSTRACT

Tobacco cells (*Nicotiana tabacum*) are capable of growth on ammonia as a sole nitrogen source only when succinate, malate, fumarate, citrate,  $\alpha$ -ketoglutarate, glutamate, or pyruvate is added to the growth medium. A ratio between the molar concentrations of ammonia to succinate (as a complementary organic acid) in the growth medium of 1.5 was optimal. Succinate had no effect on the rate of uptake of ammonia from the medium into the cells although it did affect the intracellular concentration of ammonia. However, the changes were not sufficient to explain inhibition of growth as being due to ammonia toxicity. The radioactivity from <sup>14</sup>C-succinate was incorporated into malate, glutamate, and aspartate within 2 minutes.

It appears that the role of organic acids is neither connected to ammonium transport nor to relief of ammonia toxicity, but may be related to the need for additional carbon skeletons for synthesis of amino acids.

Plant cell cultures do not usually grow on ammonia as a sole nitrogen source unless organic acids are added to the growth medium. Gomborg (6) described the growth of soybean cultures on ammonia in a medium containing intermediates of the tricarboxylic acid cycle. Butenko (4) described the growth of sunflower and tobacco tumor tissues on ammonium succinate as the source of nitrogen. The use of ammonium succinate or ammonium citrate as nitrogen sources was also described recently by Dougall (5), Oaks (10), and Behrend and Mateles (2), and can be considered to be a general technique for obtaining growth on ammonia as sole source of nitrogen.

This work was undertaken to try to clarify the role of organic acids in the utilization of ammonia by plant cell cultures.

### MATERIALS AND METHODS

**Growth.** Tobacco cell culture (*Nicotiana tabacum*) was obtained from Dr. P. Filner (Michigan State University). The cultures and growth media have been described (2). T (NO<sub>3</sub>) is a growth medium containing nitrate as sole nitrogen source. Medium T (NH<sub>4</sub>) contains ammonium succinate as the nitrogen source, and T-N is the same growth medium without any nitrogen source.

Organic acids were sterilized by filtration through a 0.22- $\mu$ m Millipore filter.

**Ammonia Determination.** Ammonia was assayed according to Weatherburn (11) with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> as standard. For determina-

tion of ammonia in the cells, the cells were collected by vacuum filtration on Whatman No. 1 filter paper. The cells were washed with T-N medium and resuspended in 2 volumes of distilled H<sub>2</sub>O. The suspension was heated for 5 min in boiling water, centrifuged, and the supernatant liquid was assayed for ammonia.

**Determination of Succinic Acid in the Growth Medium.** Ten  $\mu$ Ci succinic acid (2-<sup>3</sup><sup>14</sup>C) were added to cells growing in 50 ml T (NH<sub>4</sub>) medium to give a final specific radioactivity of 0.02  $\mu$ Ci/ $\mu$ mol succinic acid. Samples of 10 ml were taken every 2 days. Growth (settled cell volume) was determined, and the cells were removed by centrifugation. The supernatant liquid was acidified by 1 N HCl to pH 3 and nitrogen was bubbled through the liquid for 1 min to strip out <sup>14</sup>CO<sub>2</sub>. Aliquots were taken from each sample for paper chromatography to examine whether radioactive metabolites other than succinate were present in the growth medium. The chromatogram was assayed in a Packard model 720 radiochromatograph scanner. No radioactive spots other than succinate could be detected.

Aliquots of 0.5 ml of each sample were counted in Bray's solution (2) and the amount of succinate in the growth medium was calculated according to the radioactivity found.

**Incorporation of <sup>14</sup>C-Succinic Acid into Cell Constituents.** Cells grown on T (NH<sub>4</sub>) medium were harvested by vacuum filtration, washed with the same medium, and resuspended in T (NH<sub>4</sub>) medium containing 1 mM ammonium succinate. The incorporation of <sup>14</sup>C-succinic acid was carried out in a flask containing 28 ml of a reaction mixture. The reaction mixture consisted of 8 g (fresh weight) of cells, 20 ml T (NH<sub>4</sub>) medium containing <sup>14</sup>C-succinic acid (30  $\mu$ Ci, 20  $\mu$ mol).

Incubation was undertaken with stirring at 30 C. At suitable time intervals, 2.5 ml of the mixture were removed, vacuum-filtered through GF/C (Whatman) filter paper, and the cells were rinsed with cold T-N medium and transferred immediately into 5 ml boiling ethanol.

**Extraction of <sup>14</sup>C-labeled Compounds.** The cells transferred to the hot ethanol were boiled for 5 min and centrifuged. The solids were washed with 5 ml of 20% ethanol and centrifuged. The combined supernatant was evaporated to dryness under reduced pressure at 45 C. The residue was dissolved in 200  $\mu$ l of 20% ethanol. An aliquot of 20  $\mu$ l was applied to filter paper (Whatman No. 1, 40  $\times$  30 cm) for ascending paper chromatography and to cellulose plates for two-dimensional TLC.

**Chromatography.** The paper was developed using the following solvent: 100 ml 1-butanol, 100 ml water, 16.2 ml formic acid, and 100 bromophenol blue. The two phases were separated with a separating funnel, and the butanol phase was used for the chromatography. For the separation of amino acids, two-dimensional TLC according to Heimer and Filner (8) was carried out.

**Identification of Labeled Compounds.** Radioactive compounds on paper chromatograms were detected and measured

<sup>1</sup> This work will be submitted in partial fulfillment of the requirements for the degree of doctor of philosophy in microbiology of the Hebrew University.

using a Packard model 720 radiochromatogram scanner. Radioactive compounds were eluted from the paper chromatogram with 20% ethanol and identified by co-chromatography with authentic compounds, on Whatman No. 1 paper, using amyl alcohol-formic acid (1:1, v/v) and 0.01% bromophenol blue.

For detection of amino acids, the plates were sprayed with 0.02% ninhydrin in 1-butanol and heated to 100 C for 2 min. Spots were scraped off and placed in a vial containing 10 ml of Bray's (3) scintillation mixture. Radioactivity was measured by a liquid scintillation spectrometer (Packard Tri-Carb, model 3320).

**RESULTS**

**Effect of Organic Acids on the Growth of Tobacco Cells on Ammonia.** Several organic acids were tested for their ability to promote growth of tobacco cells on ammonia as a sole nitrogen source. The results are summarized in Table I.

No growth was obtained in NH<sub>4</sub>Cl medium without any organic acid added. The addition of succinate, malate, fumarate, citrate, α-ketoglutarate, glutamate, or pyruvate promotes growth on ammonia. Succinate is seen to be one of the most effective organic acids for supporting growth of tobacco cells on ammonia, and therefore, it was used as a complementary organic acid throughout the following experiments.

In 10 mM NH<sub>4</sub>Cl medium, the addition of succinate increased the yield of the cells proportionately up to 6 mM (Fig. 1). By changing the NH<sub>4</sub>Cl concentration in the growth medium (Table II), it was found that a ratio of the molar concentrations of ammonia to succinate of 1.5 is needed for optimal growth. At this optimal ratio, the intracellular concentration of ammonia is 12.3 μmol/g cells. Lowering the relative concentration of succinate in the growth medium brought about reduction of growth and an increase in the intracellular ammonia concentration.

The optimum initial pH for growth on ammonium succinate is between 5 and 6.5. There is very little growth above or below this range of pH. The pH drops to 4.3 to 4.7 during growth. The utilization of succinate is parallel to that of ammonia and proceeds until the end of the logarithmic phase of growth (Fig. 2).

Table I. Effect of Organic Acids on Growth of Tobacco Cells on Ammonia

Organic acid added	Growth
	% settled cell vol
None	5
Succinate	60
Fumarate	30
Malate	32
Citrate	22
Acetate	2
Γ-ketoglutarate	60
Tartrate	6
Pyruvate	40
Lactate	5
Glycolate	4
Glutamic acid	65

T(NH<sub>4</sub>) grown cells were inoculated into T-N medium containing 10 mM NH<sub>4</sub>Cl and 10 mM organic acid. Growth was determined after 14 days with shaking at 27 C.

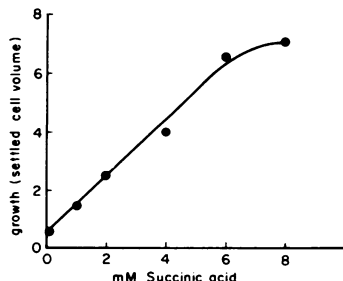


FIG. 1. Effect of succinic acid concentration on growth of plant cells on ammonia. T (NH<sub>4</sub>) grown cells were inoculated into T-N medium containing 10 mM NH<sub>4</sub>Cl and various concentrations of succinic acid. Growth was determined after 14 days.

Table II. Effect of Molar Concentration Ratio of Ammonia to Succinate on Growth and Intracellular Concentration of Ammonia

Medium		Growth	Intracellular ammonia
Succinate + NH <sub>4</sub> Cl		% settled cell vol	μmol/gr fresh wt
mM	mM		
5	0	13	0.9
5	5	31	1.6
5	10	58	13
5	15	30	37
5	20	20	55
10	0	9	0.9
10	5	33	0.9
10	10	56	0.9
10	15	67	12.3
10	20	50	33.6
10	25	38	36
10	30	26	52

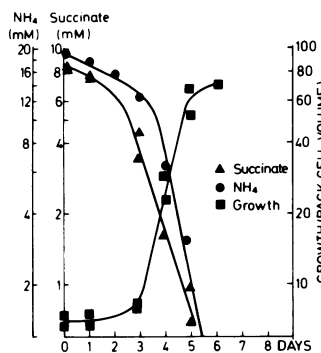


FIG. 2. Kinetics of ammonia and succinate consumption during growth. T (NH<sub>4</sub>) grown cells were inoculated into fresh medium. Aliquots of 5 ml were taken every 2 days for ammonia, succinate, and growth determination.

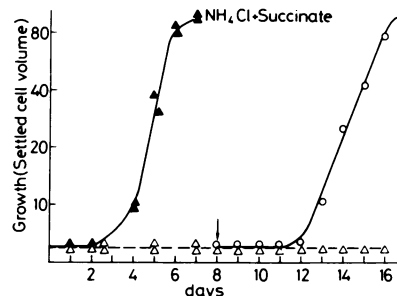


FIG. 3. Effect of stepwise addition of NH<sub>4</sub>Cl during growth. T (NH<sub>4</sub>)-grown cells were inoculated into T-N medium. Ammonium chloride was added daily in an amount sufficient to raise its concentration equivalent to 0.5 mM. ▲: NH<sub>4</sub>Cl + succinate; △: NH<sub>4</sub>Cl only; ○: addition of succinate to NH<sub>4</sub>Cl medium after 8 days.

**Effect of Stepwise Addition of Ammonia during Growth.**

Little growth of tobacco cells took place in the absence of an organic acid in the growth medium (Table I). There is a possibility that the lack of growth might have been due to the accumulation of ammonia inside the cells. In that case, the role of organic acids might be to bind the free intracellular ammonia and prevent a toxic effect. If this was so, then one might expect to obtain growth by adding NH<sub>4</sub>Cl stepwise to the growth medium striving for very low ammonium ion concentrations. No growth was observed during 8 days (Fig. 3). However, the addition of 10 mM succinate to the growth medium after this period brought about the growth of the cells after a further lag period of 3 days.

**Uptake of Ammonium Ion by Tobacco Cells.** The rate of uptake of ammonium ion by nitrogen-starved tobacco cells is similar when the cultures were presented with 1 mM ammonium chloride or ammonium lactate which do not support growth, or ammonium succinate which does support growth (Fig. 4). The uptake of ammonium ion began immediately after the transfer of

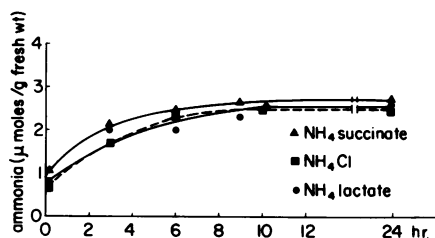


FIG. 4. Uptake of ammonium ion in different media. Nitrogen-starved cells were inoculated into different media to give 50 g cells (fresh weight)/liter. Aliquots of 5 ml were taken for intracellular ammonia determination.

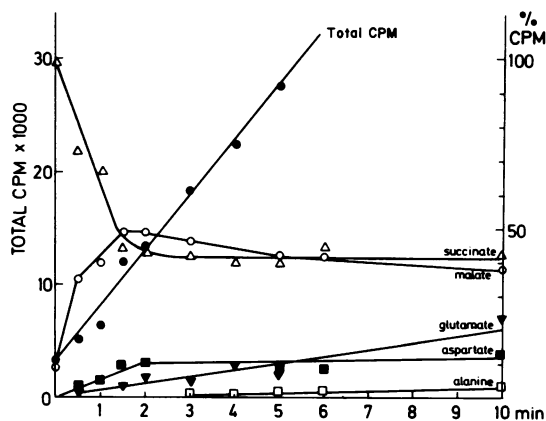


FIG. 5. Incorporation of  $^{14}\text{C}$ -succinate into cell constituents.

cells to ammonia medium, and reached a maximal level after 10 hr. Thus, succinate does not appear to affect the rate of uptake of ammonium ion from the medium into the cells.

**Incorporation of  $^{14}\text{C}$ -Succinic Acid into Cell Constituents.** The incorporation of radioactivity of  $^{14}\text{C}$ -succinic acid into cell constituents of tobacco cells grown on ammonium succinate was examined. The radioactivity of  $^{14}\text{C}$ -succinic acid appeared rapidly in malic acid, aspartic acid, and glutamic acid (Fig. 5). After 1 min, almost 50% of total radioactivity was found in malic acid. After 2 min, about 15% of radioactivity was found in aspartate and glutamate. No other significantly labeled compounds could be detected.

## DISCUSSION

That organic acids permit growth of plant cells on ammonia as a nitrogen source has been shown in several cultures (2, 4-6, 10) and seems to be a general phenomenon. In most of the experiments described in this report, succinate was used as a complementary organic acid for growth since it was one of the most effective organic acids tested (Table I). Glutamate, which is at least as effective, was not used because it can serve as a nitrogen source by itself.

We found (Fig. 4) that the rate of ammonium ion uptake is not influenced by the presence of succinate in the growth medium. Ammonia is accumulated by resting cells in 1 mM ammonium chloride medium or ammonium lactate medium, as well as in ammonium succinate. It seems, therefore, that the role of or-

ganic acids is not connected to transport of ammonium ions.

The intracellular concentration of ammonia decreases following addition of succinate to the growth medium (Table II). This intracellular concentration changes from 0.9  $\mu\text{mol/g}$  in cells growing well, but in the presence of higher than optimal succinate concentration, to more than 30  $\mu\text{mol/g}$  in cells growing in the presence of suboptimal concentrations of succinate. Thus, growth can occur at a wide range of intracellular ammonium concentrations, and it would appear that the intracellular concentration of ammonium is not a primary cause of growth inhibition.

Furthermore, we found (Fig. 3) that the cells cannot grow on  $\text{NH}_4\text{Cl}$  even when it was added slowly in an attempt to minimize the possible accumulation of ammonia inside the cells. Ammonia was added at a rate equivalent to an increase of 0.5 mM/day. At these conditions, the intracellular concentration of ammonia does not exceed 30  $\mu\text{mol/g}$  (Fig. 4)—an intracellular concentration which still permitted good growth (Table II) when succinate was also present.

It appears that organic acids are connected directly to ammonia metabolism, since a relatively fixed molar ratio between ammonia and organic acids is needed (Table II). This concentration ratio is preserved throughout growth, since ammonia and succinate are consumed in parallel to the growth (Fig. 2). Moreover, the radioactivity of  $^{14}\text{C}$ -succinate is incorporated via malate into glutamate and aspartate. Glutamic acid is generally considered to be the first step of ammonia assimilation into the  $\alpha$ -amino group of amino acids, either by glutamic dehydrogenase (1, 7), or by glutamate synthase (5, 9). We suggest that the role of organic acids is to supply  $\alpha$ -ketoglutarate, which is a substrate for these enzymes. Withdrawal of  $\alpha$ -ketoglutarate from the Krebs' cycle would stop further operation of this cycle unless another intermediate is replaced. This replacement can be done via the glyoxylic acid cycle or by exogenous supply of Krebs' cycle intermediates.

One may hypothesize that in cells growing on ammonia, the replacement of this intermediate is inhibited, and an exogenous supply of intermediate is required. Possible mechanisms are under consideration.

## LITERATURE CITED

- BARASH, I., T. SADON, AND H. MOR. 1973. Induction of specific isoenzyme of glutamate dehydrogenase by ammonia in oat leaves. *Nature New Biol.* 244: 150-152.
- BEHREND, J. AND R. I. MATELES. 1975. Nitrogen metabolism in plant cell suspension cultures. I. Effect of amino acids on growth. *Plant Physiol.* 56: 584-589.
- BRAY, C. A. 1960. A simple efficient liquid scintillator for counting aqueous solution in liquid scintillation counter. *Anal. Biochem.* 1: 279-288.
- BUTENKO, R. G. 1968. *Plant Tissue Culture and Plant Morphogenesis*. Israel Program for Scientific Translations, Jerusalem. p. 27.
- DOUGALL, D. K. 1974. Evidence for the presence of glutamate synthase in extracts of carrot cell culture. *Biochem. Biophys. Res. Commun.* 58: 639-646.
- GAMBORG, O. L. AND J. P. SHYLUK. 1970. The culture of plant cells with ammonium salts as the sole nitrogen source. *Plant Physiol.* 45: 598-600.
- GIVAN, C. V. AND R. M. LEECH. 1971. Biochemical anatomy of higher plant chloroplasts and their synthesis of small molecules. *Biol. Rev.* 46: 409-428.
- HEIMER, Y. M. AND P. FILNER. 1970. Regulation of nitrate assimilation pathway of cultural tobacco cells. II. Properties of a variant cell line. *Biochim. Biophys. Acta* 215: 152-165.
- LEA, P. J. AND B. J. MIFLIN. 1974. Alternative route for nitrogen assimilation in higher plants. *Nature* 251: 614-616.
- OAKS, A. 1974. The regulation of nitrate reductase in suspension cultures of soybean cells. *Biochim. Biophys. Acta* 372: 122-126.
- WEATHERBURN, M. W. 1967. Phenol hypochlorite reaction for determination of ammonia. *Anal. Chem.* 39: 971-974.