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# Sulfate Transport in Cultured Tobacco Cells

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## ABSTRACT

**Sulfate transport by tobacco (*Nicotiana tabacum* L. var. Xanthi) cells cultured on either L-cysteine or sulfate as a sole sulfur source was measured. The transport rate on either sulfur source was low during pre-exponential growth, increased during exponential growth, and was maximal in late exponential cells. The initial increase in transport rate was correlated with a decline in the intracellular sulfate, but was not correlated with the amino acid content of the cells which remained relatively constant before the depletion of the endogenous sulfate pool. The previously reported inhibition of sulfate transport by L-cysteine was shown to be caused by an elevation in intracellular sulfate resulting from the degradation of cysteine to sulfate. It is proposed that the intracellular sulfate pool is the major factor regulating the entry of sulfate into tobacco cells.**

In 1964 Dreyfuss (4) demonstrated that sulfate and thiosulfate were actively transported by *Salmonella typhimurium*, and that the transport was under genetic control. The sulfate-transporting system was repressed by cysteine and derepression occurred when growth occurred in the absence of sulfate. Pardee and coworkers isolated (18) and crystallized (19) a sulfate-binding protein, and presented evidence for its location on or outside of the cell membrane (5, 21). The properties of the sulfate-binding system suggested that it might be part of the active transport system (20). This was not unequivocally established because the binder gene does not map in the *cys*-A region (sulfate-transport gene) (17) and the binding protein is present in sulfate negative mutants (20). Recently, Kaback (10) has suggested that binding proteins may be involved in other surface phenomena, such as chemotaxis, and only indirectly in transport.

In *Neurospora crassa*, evidence has been presented for two distinct sulfate permeases coded for by two unlinked genes (12–14, 26). One of the permeases predominates in the conidial stage and the other predominates during mycelial growth (13). The transport system is energy-dependent, highly temperature-dependent, repressed by high methionine, and subject to positive control by the product of the *cys*-3 locus (15). Other filamentous fungi also contain a metabolically controlled sulfate permease system that is repressed by L-methionine, and feedback inhibited by intracellular sulfate and possibly cysteine or a cysteine metabolite (1, 32).

Sulfate transport in higher plants has been investigated using either plant roots (6, 11, 16, 23, 24) or plant cells cultured in liquid media (9). In cultured tobacco cells, there seems to be a negative feedback control of sulfate assimilation by methionine and cysteine (9), similar to that previously reported in microorganisms.

Although Pardee and coworkers (17–21) presented evidence to support protein-mediated active transport of sulfate in bacteria, most of the work with eukaryotes is based on indirect evidence. Studies were initiated to obtain direct evidence for the presence of a sulfate permease system in cultured tobacco cells. Preliminary investigations indicated that the size of the intracellular sulfate pool might be a major factor in controlling the rate of sulfate transport in these cells.

## MATERIALS AND METHODS

Tobacco XD-cell line (*Nicotiana tabacum* L. var. Xanthi) was obtained from P. Filner and cultured as previously described (9). Sulfateless M-1D medium was prepared, and sulfate (50 $\mu$M, final concentration) was added before sterilization, or L-cysteine (50 $\mu$M, final concentration) was added through a Millipore G.S. filter following medium sterilization.

**Transport Experiments.** Cells were harvested by vacuum filtration and washed with 100 ml of medium M-1D (minus sulfate). The washed cells (0.25–0.5 g fresh weight) were placed in a 50-ml Erlenmeyer flask containing 20 ml of M-1D medium and 0.2 ml of 5 mM $Na_2$ $^{35}SO_4$ (1 $\mu$Ci). The flasks were stoppered with cotton plugs and placed on a rotary shaker at 25 C. At the termination of the transport experiment, cells were harvested by vacuum filtration and washed with 0.5 mM sodium sulfate (100 ml) to remove adsorbed radioactive sulfate. Transported sulfate was extracted by placing cells in 20 ml of boiling $H_2O$ for 1 min. A 5-ml aliquot of this extract was added to 10 ml of Aquasol liquid scintillation fluid and was counted in a Packard 3310 Tri-Carb scintillation spectrometer with external standardization.

In preincubation experiments, cells were washed as decribed above, suspended in 20 ml of preincubation medium in an Erlenmeyer flask, and shaken on a rotary shaker at 25 C. Following preincubation, cells were harvested and washed with 100 ml of M-ID medium (minus sulfate) before the measurement of transport.

All operations before the addition of radioactive sulfate were conducted in a sterile room using sterilized media and equipment.

**Measurement of Sulfate, Cysteine, and Methionine Content of Tobacco Cells.** Tobacco cells were grown on agar containing M-1D medium supplemented with $Na_2{}^{35}SO_4$ (final concentration 50 $\mu$M, with a specific radioactivity of 1 Ci/mole). The callus (0.2–0.4 g fresh weight) was transferred to 100 ml of liquid medium, and after a 15-day growth period, a 10-ml aliquot was used to reinoculate a further 100 ml of medium. All media used had the same specific radioactivity as above, and it was assumed that after the second transfer the specific radioactivity of the sulfur-containing compounds would be essentially the same as that of the added sulfate.

Cells were harvested by vacuum filtration and washed with 100 ml 0.5 mM sodium sulfate to remove adsorbed radioactive sulfate. Cells (0.25–0.6 g fresh weight) were extracted for 1 min in 25 ml of boiling $H_2O$, containing 5 $\mu$moles of L-methionine, 5 $\mu$moles of L-cysteine, 5 $\mu$moles of glutathione, and 400 $\mu$moles of sodium sulfate. Cellular debris was sedimented by centrifugation and discarded. The supernatant solution was passed through a Dowex 50 $H^+$-form column (7 × 0.9 cm), and the column was washed with 10 ml of $H_2O$. This fraction is designated noncationic fraction. Cationic material was eluted from the column with 20 ml of 3N $NH_4OH$.

Sulfate present in the noncationic fraction was quantified by the method of Segel and Johnson (28).

Cysteine, glutathione, and methionine present in the Dowex 50 eluate were determined following oxidation by the method of Giovanelli *et al.* (8). Individual amino acids were identified by decending paper chromatography on Whatman No. 3MM paper, using methanol-pyridine-1.25 N HCl (37:4:8) as solvent (29).

## RESULTS AND DISCUSSION

**Effects of Culture Age on Sulfate Transport.** Sulfate transport by cells grown on sulfate was linear with time (for at least 2 hr) and with the amount of tissue in the range of 0.25 to 0.75 g fresh weight. There was no efflux of transported sulfate when cells were suspended in sulfate-free medium for 6 hr. These observations are in agreement with previous work (9).

The rate of transport was dependent upon the age of the culture (Fig. 1); it was low in pre-exponential cells, increased during exponential growth, and was maximal in stationary cultures. This pattern was also observed in cells grown on L-cysteine as a source of sulfur (Fig. 1). The source of sulfur did not significantly affect either the growth curves (Fig. 2) or the sulfate transport rates (Fig. 1). During the first 3 days of exponential growth, 90% of the added sulfur source is removed from the medium. Thus, the increase in transport rate coincided with the depletion of external medium of the sulfur source. The effect of medium depletion on the endogenous sulfur-containing compounds was determined by direct measurement.

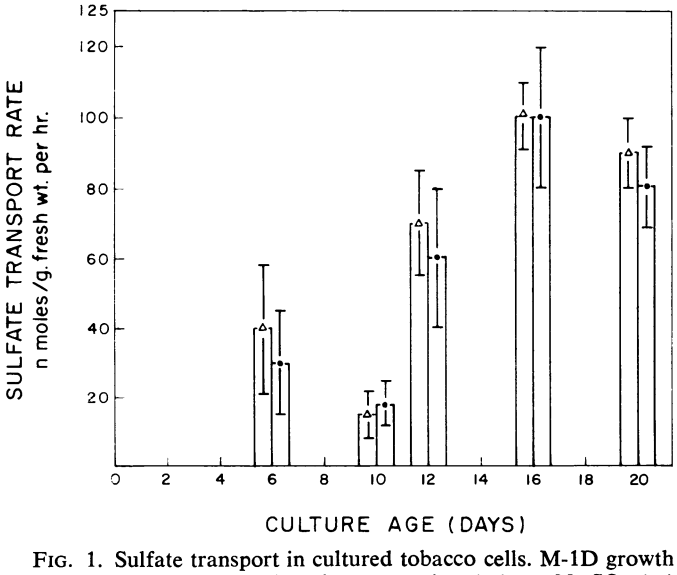


FIG. 1. Sulfate transport in cultured tobacco cells. M-1D growth medium supplemented with either L-cysteine (△) or $Na_2SO_4$ (●) as the sole sulfur source.

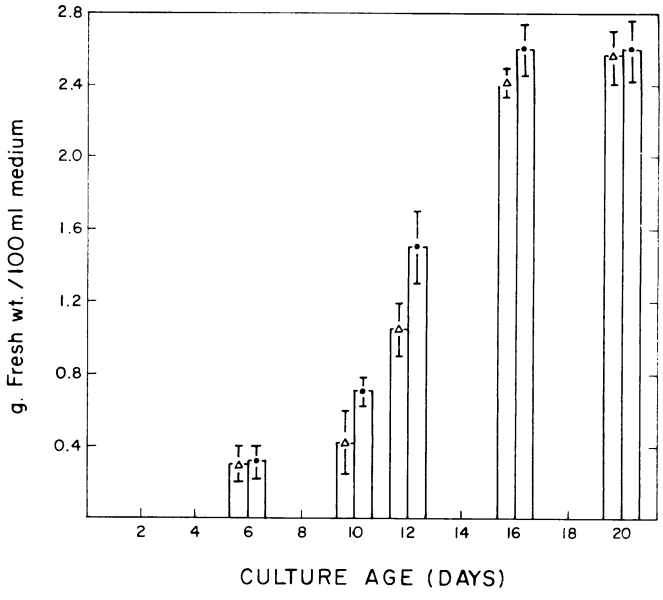


FIG. 2. Growth of cultured tobacco cells. M-1D medium supplemented with either L-cysteine (△) or $Na_2SO_4$ (●) as the sole sulfur source.

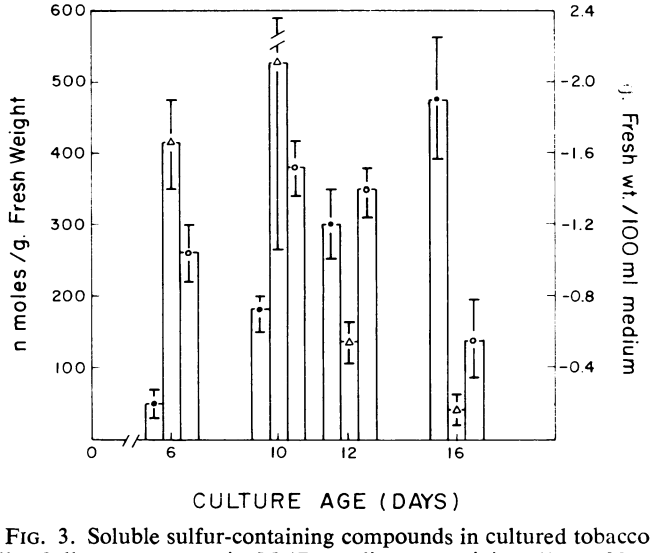


FIG. 3. Soluble sulfur-containing compounds in cultured tobacco cells. Cells were grown in M-1D medium containing 50 $\mu$M $Na_2{}^{35}SO_4$ (1 Ci/mole). Fresh weight (●), sulfate (△), Dowex 50 eluate (○).

**Soluble Sulfur-containing Compounds in Cultured Tobacco Cells.** Cells used as inoculum were 15 days old, and initially had small pools of soluble constituents. Before exponential growth (less than 8 days), the transport of sulfate caused an increase in the endogenous sulfate and amino acid pools (Fig. 3). Maximal pools were observed in 10-day-old cultures. As exponential growth proceeded, the medium was depleted of sulfate, and the utilization of sulfur metabolites for growth reduced the sulfate content of the tissue (12 days). This decline initially had no effect on the amino acid content of the cells. However, when the medium and tissue were depleted of sulfate, continued growth caused a reduction in the amino acid pool (16 days).

The individual amino acids present in the Dowex 50 eluate were measured. Using the methods outlined, authentic [$^{35}$S]-cysteine and [$^{14}$C]-methionine were recovered in 75 ± 5%

yield. In cell extracts, 65 ± 5% of the radioactivity in the Dowex 50 eluate was recovered in cysteine, glutathione, and methionine; these amino acids therefore represent greater than 75% of the material present in this fraction. All amino acid quantities quoted are actual amounts determined and are not corrected for the 75 ± 5% yield reported above. The relative stability in the total organic pool through 12 days was observed in the individual amino acids (Fig. 4). The major decline thereafter was in the glutathione content of the tissue.

The above experiments were repeated using cells grown on [$^{35}$S]-cysteine as a sulfur source. The pools of sulfate, cysteine, glutathione, and methionine were within the limits determined for sulfate grown cells (Figs. 3 and 4). More specifically, the cysteine pool was not elevated in cysteine-grown cells above the level observed in sulfate-grown cells; 10-day-old cells utilizing cysteine as a sulfur source contained 600 ± 200 nmoles of sulfate/g fresh weight.

To demonstrate that L-cysteine may be degraded to sulfate in tobacco cells, cysteine ([$^{32}$S] or [$^{35}$S] 50 μM, final concentration; specific radioactivity, 1 Ci/mole) was added to cells growing on $Na_2{}^{35}SO_4$ (50 μM; specific radioactivity, 1 Ci/mole). The sulfur-containing compounds were assayed 16 hr later. The use of nonradioacive cysteine allowed determination of the effect of this amino acid on existing sulfur metabolites, whereas differences in pool sizes beween nonradioactive cysteine and [$^{35}$S]-cysteine represented the direct contribution from [$^{35}$S]-cysteine. Nonradioactive cysteine had no significant effect on any of the existing pools during the 16-hr period of the experiment (Table 1). In contrast, a significant amount of the radioactive cysteine was either converted to sulfate or incorporated into the cysteine, glutathione, and methionine pools. This conclusion was supported by the addition of [$^{35}$S]-cysteine to cells growing on nonradioactive sulfate. After a 16-hr growth period the cells contained, in nmoles/g fresh weight: [$^{35}$S]-labeled sulfate, 180; cysteine, 30; glutathione, 70; and methionine, 8; whereas 95% of the radioactivity in the medium was present as cysteine. The latter observation supports intracellular degradation of cysteine to sulfate rather than extracellular degradation followed by sulfate transport.

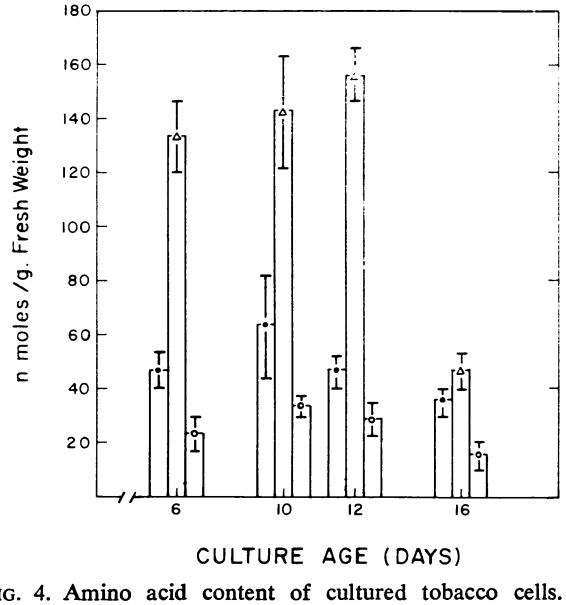


FIG. 4. Amino acid content of cultured tobacco cells. Cells were grown in M-1D medium containing 50 μM $Na_2{}^{35}SO_4$ (1 Ci/mole). Cysteine (●), glutathione (△), methionine (○).

The individual amino acid pools, after [$^{35}$S] cysteine addition, were approximately the same in young and old cells (Table I). The elevation of the endogenous sulfate pool was greatest in young cells, which initially had the highest amino acid content. It is concluded that initially, cysteine enters the cells and raises the amino acid pools to maximal levels; subsequently, any additional cysteine is degraded to sulfate.

**Preincubation Experiments.** It was shown (Figs. 1 and 3) that the variation in sulfate transport rate by cultures of differing ages was correlated with the endogenous sulfate pool. However, this may not be a causal relationship, because cultures of different ages may contain different populations of cells. Therefore, cultures of different ages were preincubated in media expected to perturb endogenous sulfur constituents, for time periods insufficient for any marked change in cell distribution to occur (preincubation period 1–24 hr; generation time of cells 48–60 hr).

The rate of sulfate transport by cells which initially had low transport rates was increased by preincubation in sulfate-free media (Table II). Conversely, the rate of sulfate transport by cells which initially had high transport rates was decreased by preincubation with sulfate (Table III).

Cysteine inhibited sulfate transport. Because cysteine was not present during measurement of transport in the present study, the conclusion of Hart and Filner (9), that cysteine does not act extracellularly, is confirmed. L-Cysteine (0.1 mM) was more effective than sulfate (0.1 mM) as an inhibitor of transport by young cultures (Table II). These cultures have low transport rates and therefore, relatively small quantities of sulfate enter to increase the total sulfate pool. However, these cultures have large amino acid pools initially, and relatively large quantities of cysteine are degraded to sulfate (Table I). Conversely, in older cultures, sulfate (0.1 mM) is more effective than cysteine (0.1 mM) as a sulfate transport inhibitor (Table III). These cultures have high transport rates and the transported sulfate may cause an immediate and relatively large

Table I. *Effect of Cysteine Addition on Sulfur-containing Metabolites in Cultured Tobacco Cells*

Cells were grown in M-1D medium containing $Na_2{}^{35}SO_4$ (50 μM, final concentration; specific radioactivity, 1 Ci/mole) for 10 and 17 days; nonradioactive L-cysteine (50 μM, final concentration) or [$^{35}$S]-cysteine (50 μM; specific radioactivity, 1 Ci/mole) was added 16 hr before measurement of the pools. Parentheses in table indicate that these figures are not the total pool sizes.

| Addition | Fresh Wt/100 ml Medium | Sulfate | Dowex 50 Eluate | Cysteine | Glutathione | Methionine |
|---|---|---|---|---|---|---|
| | | *nmoles/g fresh wt* | | | | |
| None | 0.40 ± 0.31 | 427 ± 73 | 284 ± 55 | 68 ± 7 | 117 ± 20 | 36 ± 6 |
| L-Cysteine | 0.49 ± 0.16 | (578 ± 76) | (270 ± 116) | (84 ± 16) | (146 ± 20) | (38 ± 7) |
| [$^{35}$S]-cysteine | 0.39 ± 0.23 | 913 ± 167 | 515 ± 92 | 131 ± 20 | 253 ± 40 | 52 ± 10 |
| None | 1.90 ± .20 | 43 ± 16 | 140 ± 58 | 35 ± 7 | 47 ± 10 | 15 ± 5 |
| L-Cysteine | 1.80 ± .37 | (81 ± 23) | (169 ± 52) | (55 ± 9) | (54 ± 6) | (20 ± 1) |
| [$^{35}$S]-cysteine | 1.80 ± .10 | 227 ± 50 | 475 ± 75 | 167 ± 21 | 214 ± 52 | 33 ± 1 |

Table II. *Sulfate Transport by Tobacco Cells Preincubated in the Presence of Sulfate, L-Cysteine, or Sulfur-free Medium*

The 10-day-old cultures yielded 0.86 ± 0.28 g fresh wt/100 ml of medium.

| Addition to M-1D Medium | Preincubation Period | Sulfate Transport Rate |
|---|---|---|
| | *hr* | *nmoles/g fresh wt·hr* |
| None | 0 | 8.6 ± 2.0 |
| Sulfur-free | 8 | 26.5 ± 0.8 |
| Sulfur-free | 16 | 63.9 ± 9.1 |
| Sulfur-free | 24 | 65.1 ± 5.0 |
| Sulfate (0.1 mM, final concn) | 3 | 5.0 ± 0.8 |
| L-Cysteine (0.1 mM) | 3 | 1.0 ± 0.1 |
| L-Cysteine (50 μM) | 16 | 3.7 ± 2.1 |

Table III. *Sulfate Transport by Tobacco Cells Preincubated in the Presence of Sulfate or Cysteine*

The 17-day-old cultures yielded 2.68 ± 0.04 g fresh weight/100 ml of medium.

| Additions to M-1D Medium | Preincubation Period | Sulfate Transport Rate |
|---|---|---|
| | *hr* | *nmoles/g fresh wt·hr* |
| None | 0 | 78.6 ± 21.6 |
| Sulfate (0.1 mM, final concn) | 1 | 70.3 ± 5.0 |
| Sulfate (0.1 mM, final concn) | 3 | 19.9 ± 3.9 |
| Sulfate (0.1 mM, final concn) | 6 | 10.3 ± 1.5 |
| L-Cysteine (0.1 mM) | 3 | 36.0 ± 3.6 |
| L-Cysteine (50 μM) | 16 | 45.7 ± 9.6 |

elevation in the total sulfate pool. In contrast, older cells have small amino acid pools initially, and much less cysteine is degraded to sulfate (Table I). In summary, comparing L-cysteine and sulfate as potential contributors to the sulfate pool, it would be predicted that cysteine would contribute more sulfate in young cultures and sulfate would be the major contributor in older cultures.

Preincubation with cysteine (50 μM) for 16 hr inhibited sulfate transport (Tables II and III); the inhibited rate, however, was dependent on the uninhibited rate. Because preincubation with cysteine increased the amino acid pools in either young or old cultures to approximately the same level (Table I), it was concluded that the size of the endogenous sulfate pool is the major determinant of the sulfate transport rate.

## CONCLUSION

The conclusion that the size of the intracellular sulfate pool is the major factor regulating the transport of sulfate into tobacco cells is supported by three lines of evidence. First, 10-day-old cells have low transport rates (Fig. 1) and high sulfate pools (Fig. 3). As exponential growth proceeds (12 days), the transport rate increases (Fig. 1) and the sulfate pool decreases (Fig. 3). These changes precede any decrease in the amino acids constituents of the cells (Figs. 3, 4). The fact that the decline in the intracellular sulfate initially has no effect on the organic constituents is probably attributable to the unfavorable equilibrium ($K = 10^{-5}$) (25) of the sulfate activation reaction. Second, when 10-day-old cells with large sulfate pools (Fig. 3) were starved of sulfate, their ability to transport sulfate increased (Table II). Conversely, when 17-day-old cells which had small sulfate pools (Fig. 3) were preincubated with sulfate, their ability to transport sulfate decreased (Table III). Third, the relative inhibition of sulfate transport by either sulfate or cysteine (Tables II and III) depended upon their relative contribution to the sulfate pool (Table I). No evidence was obtained in the present study for regulation of sulfate transport by the sulfur-containing amino acids.

The role of intracellular sulfate pool as a regulator of sulfate transport has been reported in lower organisms. Scott and Spencer (27) reported that, in contrast to wild type, the sulfate transport activity of ATP-sulfurylase negative mutants of *Aspergillus nidulans* was not increased by sulfate starvation. They concluded that the level of the intracellular sulfate pool controlled sulfate transport. In contrast, Bradfield and co-workers (1) were able to derepress (or deinhibit) sulfate transport by sulfur starvation in three mutants of *A. nidulans* that had negligible ATP-sulfurylase activity. Correlations between the size of the internal sulfate pool and the rate of sulfate transport have been reported in *Chlorella* (30).

An additional conclusion is that the size of the cysteine pool in tobacco cells is regulated in part by degradative enzymes. Cells utilizing cysteine as a sulfur source had an amino acid content similar to cells utilizing sulfate as a sulfur source (*cf. Escherichia coli*, ref. 31). Cysteine transported in excess of that required to maintain the metabolic pool was apparently degraded to sulfate. Similarly, when cysteine was added to cells growing on sulfate, the amino acid pools were increased to a maximum and excess cysteine was degraded to sulfate (Table I). Recently, Collins and co-workers (2, 3) reported that cysteine levels in *Salmonella typhimurium* may be reduced by the degradative action of cysteine desulfhydrase. Depletion of the cysteine pool is prevented by a substrate-velocity relationship which results in the enzyme being relatively inactive below 0.1 mM cysteine. Mechanisms for the oxidation of sulfide (the product of the above reaction) or cysteine to sulfate have been described in various organisms (7, 22). Experiments are in progress to elucidate the pathway of cysteine degradation to sulfate.

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