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Characterization of Sulfate Transport in Cultured Tobacco Cells

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ABSTRACT

Sulfate transport by tobacco cells (*Nicotiana tabacum* L. var. Xanthi) cultured in liquid medium was investigated.

Transport was linear with time, had a sharp pH optimum between 6.5 and 7.5, and obeyed Michaelis-Menten kinetics. The *Km* varied within the range 2×10^{-5} M and 4×10^{-5} M and the maximum velocity was in the range 100 to 400 nanomoles per gram fresh weight·hour.

Transport was inhibited more than 90% by 10^{-4} M sulfite, thiosulfate, metabisulfite, sulfide, selenate, and chromate, but was inhibited less than 40% by 10^{-3} M chloride, nitrate, or phosphate. Selenate was a competitive and sulfide a noncompetitive inhibitor of sulfate transport.

The oxidative respiration inhibitors, azide and cyanide, uncoupling reagents, carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and dinitrophenol, and the ATPase inhibitor N,N'-dicyclohexylcarbodiimide (DCCD) were all potent inhibitors of transport. Inhibition by CCCP was not prevented by preincubation of cells with dithiothreitol. Removal of CCCP from the transporting medium resulted in a partial resumption of transport, in contrast removal of DCCD had no effect.

Sulfate transport was inhibited more than 90% by 10^{-4} M mercaptoethanol, dithiothreitol, or D-cysteine and was abolished by either 10^{-5} M N-ethylmaleimide or 10^{-4} M iodoacetamide. Removal of mercaptoethanol from the transporting medium resulted in a return to maximal rates of transport whereas when either N-ethylmaleimide or iodoacetamide were removed transport remained inhibited.

N-ethylmaleimide (10^{-5} M) and iodoacetamide (10^{-4} M), which inhibited transport completely, induced the efflux of between 70 and 90% of the transported sulfate in 5 hours. Metabolite efflux was induced by the following compounds, which are listed according to their effectiveness, DCCD, CCCP, mercaptoethanol, and selenate. Increasing the concentration of an inhibitor, in excess of that required to inhibit transport 100%, increased the rate of nonspecific metabolite efflux from the cells.

The concept that ion uptake in plants is an active, carrier-mediated process has been supported by numerous investigations (3, 9, 21). Sulfate transport has been studied in intact plants and plant parts (8, 10, 15, 19, 20, 23, 26) and cultured plant cells (6, 24, 25). Sulfate transport into tobacco cells cultured in liquid medium obeys Michaelis-Menten kinetics (6), is regulated by the intracellular sulfate pool (24), and is inhibited by dithiothreitol and CCCP[1] (25). The present communication reports a further characterization of this sulfate transport system.

MATERIALS AND METHODS

Tobacco XD-cell line (*Nicotiana tabacum* L. var. Xanthi) was obtained originally from P. Filner and cultured in B-5 medium (5). Inhibitors were purchased from Sigma Chemical Co., $Na_2{}^{35}SO_4$ from Amersham/Searle Corp., and Aquasol liquid scintillation fluid from New England Nuclear. Analytical grade chemicals were purchased from commercial suppliers.

Transport Experiments. Sulfate transport was measured as reported previously (24) with one modification; washed cells were placed in a 125-ml Erlenmeyer flask containing 40 ml of M-1D medium, 0.4 ml of 5 mM $Na_2{}^{35}SO_4$ (2 μCi), and 10 mM HEPES, pH 7.

CCCP and DCCD were dissolved in 95% ethanol and 0.2 ml added to 40 ml of transport medium prior to the addition of the cells. For comparison purposes 0.2 ml of 95% ethanol was added to all transport media used as controls in experiments with these compounds. This concentration of ethanol depressed transport rates between 25 and 35%, but had no effect upon the linearity of transport. The nature of this inhibition was not investigated.

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

Metabolite Efflux Experiments. Tobacco cells were grown in 100 ml of M-1D medium (6) supplemented with $Na_2{}^{35}SO_4$ (final concentration 50 μM with a specific radioactivity of 1 Ci/mol). After a 15-day growth period a 10-ml aliquot was used to reinoculate an additional 100 ml of radioactive medium.

Cells (0.25-0.6 g fresh weight) were harvested by vacuum filtration, washed with 70 ml of sulfate-free M-ID medium, and placed in 40 ml of the latter medium in 125-ml Erlenmeyer flasks containing the transport inhibitor. The flasks were placed on a rotary shaker at 25 C and 2-ml aliquots of the medium were removed at 2-hr intervals. At the end of the experiment the cells were harvested, washed with 100 ml of 0.5 mM sodium sulfate and sulfur-containing metabolites extracted for 1 min in 25 ml of boiling water.

The sulfate and sulfur-containing amino acids present in the effluxed material and in the cells were separated and quantified as reported previously (24).

RESULTS AND DISCUSSION

General Characteristics. Cells transferred from B-5 growth medium to M-1D transport medium initially had low rates of sulfate transport, within 2 hr the transport rate reached a maximum and remained linear for at least 6 hr. Unless otherwise stated all rates reported are maximal rates measured after the 2-hr lag.

The pH optimum for sulfate transport into cultured tobacco cells was between 6.5 and 7.5, using either 0.01 M bis-tris-propane or HEPES as buffer (Fig. 1). Outside of the pH optimum range the transport rate declined rapidly, so that at pH values lower than 5 or higher than 8.5 no significant transport occurred. Fisher and Albersheim (4) showed that the pH optimum for active H^+ efflux from sycamore cells is 7. To determine whether cell membrane integrity was maintained in the pH range 5 to 9 cells grown in the presence of $Na_2{}^{35}SO_4$ were placed in medium buffered at either pH 2, 4 (maleate-buffer), 6 (HEPES), 8, or 10 (Bicine) and the efflux of sulfur-containing

[1] Abbreviations: CCCP: carbonylcyanide *m*-chlorophenylhydrazone; DNP: dinitrophenol; DCCD: N,N'-dicyclohexylcarbodiimide.

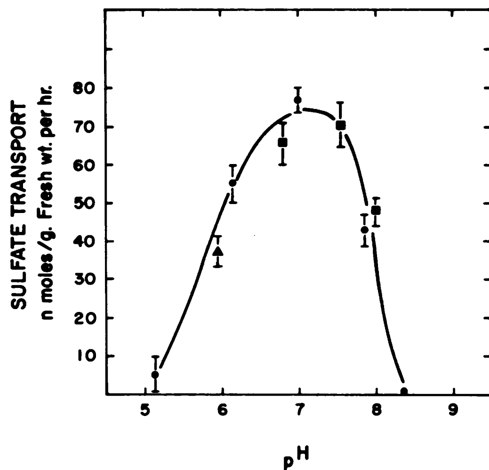

FIG. 1. Effect of pH on sulfate transport. Transport was measured in M-1D medium containing 5×10^{-5} M sodium sulfate and 0.01 M buffer. ●——●: bis-tris-propane; ▲: sodium phosphate; ■——■: HEPES.

metabolites measured. At pH 2 and pH 4, 80% and 20% of the sulfur-containing metabolites effluxed in a 6-hr period, the remaining pH values did not induce any significant metabolite efflux. Since no metabolite efflux occurred in the pH range 6 to 10 I concluded that the differences in transport rate in this range are due to the specific effect of pH on the transporting system and not due to a general effect of OH^- or H^+ upon membrane integrity. This conclusion is supported by the observation that the pH optimum for cysteine transport into these cells is 4.5 (Harrington and Smith, unpublished results).

Variation of the sucrose concentration in the transport medium, in the range 10 to 50 g/l, had no significant effect on sulfate transport (Fig. 2A). The sucrose used in all experiments was passed through Dowex 1 OH^--form resin to remove sulfate impurities.

Variation of the total inorganic salt content of the transport medium, from no salts to five times the concentration of inorganic salts present in M-1D medium, caused a decrease in sulfate transport rate (Fig. 2B). Whether this effect was due to the specific effect of a particular inorganic ion or due to the general effect of ionic strength was not investigated.

Sulfate transport obeyed Michaelis-Menten kinetics (Fig. 3), the *Km* varied in the range 2×10^{-5} M to 4×10^{-5} M which is in agreement with previous studies of sulfate transport in cultured tobacco cells, *Km* 1.5×10^{-5} M (6) and barley roots, *Km* 1×10^{-5} M (15). The maximum velocity of transport was usually within the range 100 to 400 nmol/g fresh weight·hr, although rates as high as 1000 nmol/g fresh weight·hr were recorded.

A previous investigation of sulfate transport into tobacco cells indicated that cells with large endogenous sulfate pools transported sulfate slowly relative to cells with small endogenous sulfate pools (24). Cells were preincubated for 6 hr in the presence of 10^{-3} M sodium sulfate prior to measurement of the kinetics of transport. As shown (Fig. 3) preincubation with nonradioactive sulfate lowered the maximum velocity of transport but had no effect on the *Km*; in other experiments cells with a maximum transport rate as low as 10 nmol/g fresh weight·hr had a *Km* of 2×10^{-5} M. Apparently the endogenous sulfate pool is a noncompetitive inhibitor of sulfate transport.

Nissen and his coworkers (10, 19–21, 26) reported that the uptake of sulfate by roots and leaf slices of barley is mediated by a single multiphasic mechanism. In rape two separate sulfate transporting systems have been proposed (23). No evidence for multiphasic uptake was obtained in the present study or in a previous investigation of sulfate transport into cultured tobacco cells (6). However, in this study a very limited range of concentrations was used and complex kinetics were not discovered in other systems until wide concentration ranges were investigated.

Anion Inhibitors. Sulfate transport was inhibited more than 90% by low concentration (10^{-4} M) of the sulfur-containing anions sulfite, thiosulfate, metabisulfite, and by structurally similar ions selenate and chromate (Table I). In the case of selenate the inhibition was shown to be competitive (Fig. 4); the *Ki* was 1.3×10^{-5} M. In contrast, the anions chloride, nitrate, and phosphate were less effective and inhibited transport less than 40% at relatively high concentrations (10^{-3} M). These observations support the conclusions of other workers (15, 26) that sulfate transport systems are highly selective.

Sulfide was a noncompetitive inhibitor of sulfate transport (Fig. 5). Sulfide may be regarded as an end-product of sulfate transport and metabolism and potentially an allosteric inhibitor of the transporting system.

Respiratory Metabolism Inhibitors. Sulfate transport was inhibited by the oxidative respiration inhibitors cyanide and azide, by the uncoupling reagents CCCP and DNP and by the ATPase inhibitor DCCD (Table II). The requirement for a relatively high concentration of DNP for inhibition may be due to the slow entry of this compound into the cells. In *Euglena gracilis* DNP is only transported rapidly when the medium pH is below its pK of 3.96 (14).

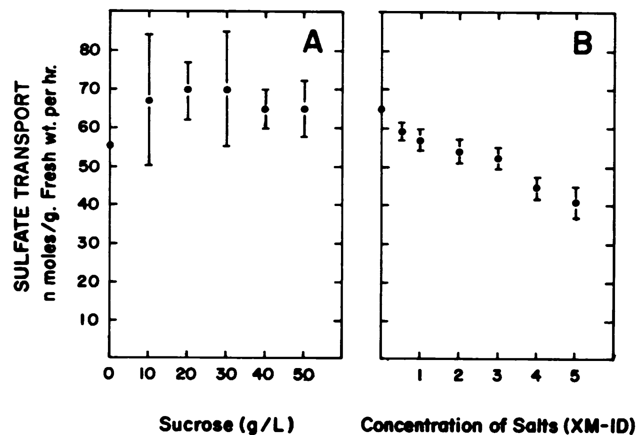

FIG. 2. A: Effect of sucrose concentration on sulfate transport. Transport medium contained M-1D salts, 5×10^{-5} M sodium sulfate, and 0.01 M HEPES, pH 7. B: Effect of ionic strength on sulfate transport. Transport medium contained sucrose (20 g/l), 5×10^{-5} M sodium sulfate, and 0.01 M HEPES, pH 7. 1 indicates salts present in M-1D medium, 5 indicates five times the concentration of salts in M-1D medium.

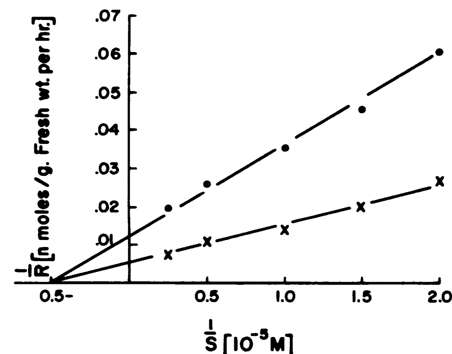

FIG. 3. Effect of sodium sulfate concentration on the rate of sulfate transport. Transport was measured as described in text; range of sulfate concentration was from 5×10^{-6} M to 4×10^{-5} M. ●——●: transport in first 2 hr after transfer from high sulfate medium; ×——×: transport in the next 2 hr.

Table I. *Effect of Various Anions on Transport of Sulfate by Cultured Tobacco Cells*

Transport was measured as described in the text, sulfate concentration 10^{-5} M. Figures indicate percentage of inhibition of transport at indicated concentrations of inhibitor.

Inhibitor	Inhibitor Concentration (M)			
	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Sodium sulfite	98	91 ± 0	40 ± 10	29 ± 4
Sodium thiosulfate	99	91 ± 1	59 ± 4	27 ± 4
Sodium metabisulfite	99	90 ± 1	57 ± 3	16 ± 2
Sodium sulfide	100	98 ± 1	60 ± 4	20 ± 1
Sodium selenate	99	92 ± 2	58 ± 2	26 ± 2
Potassium chromate	-	95 ± 1	59 ± 2	27 ± 1
Sodium chloride	39 ± 6	21 ± 4	0	
Potassium chloride	0	14 ± 1	0	
Sodium phosphate	34 ± 10	28 ± 8	0	
Potassium nitrate	13 ± 1	13 ± 8	0	

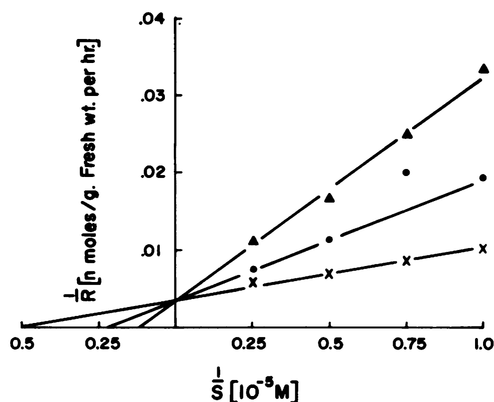

FIG. 4. Effect of sodium selenate on sulfate transport. Transport was measured as described in text; range of sulfate concentration was from 10^{-5} M to 4×10^{-5} M. ×——×: no sodium selenate; ●——●: 2.5×10^{-5} M sodium selenate; ▲——▲: 5×10^{-5} M sodium selenate. The *Km* for sulfate was 2×10^{-5} M and the *Ki* for selenate was 1.3×10^{-5} M.

CCCP was the most potent inhibitor and the effective concentration, namely 100% inhibition with 10^{-6} M CCCP, was the same as reported for the uncoupling of plant mitochondria (7) and is an order of magnitude lower than the concentration used to inhibit efflux of protons from cultured sycamore cells (4). The sensitivity of cells to CCCP is independent of the initial transport rate. Cells in which the rate of sulfate metabolism limits transport have low transport rates, whereas high transport rates are observed in cells in which sulfate metabolism is not a limiting factor (24). The observation that both types of cells are equally sensitive to CCCP supports the conclusion that CCCP affects a process prior to the activation of sulfate by ATP-sulfurylase.

Previous investigators showed that thiol reagents protect mitochondria from the uncoupling effects of CCCP (7) and that dithiothreitol reverses CCCP-induced inhibition of transport into bacterial vesicles (13). In contrast, inhibition of sulfate transport into tobacco cells by 10^{-6} M CCCP was not affected by preincubation of cells for either 0, 2, or 4 hr with 10^{-5} M dithiothreitol (Table II) (25). Higher concentrations of dithiothreitol could not be used in the present experiments because transport is also inhibited by sulfhydryl reducing agents (Table III). This is not the case in either mitochondria, where 10^{-3} M cysteine has no effect on electron transport (7), or bacterial vesicles, where 10^{-2} M mercaptoethanol or dithiothreitol has no effect on transport (13).

Removal of CCCP resulted in partial restoration of transport (Table IV).

Inhibition of transport by respiratory inhibitors and uncoupling reagents indicate that oxidative respiration is required for transport (1-3, 8-10, 21-23), although the nature of this requirement has not been determined. In this regard Cram (2), investigating the influx of chloride into carrot root cells, concluded that the active influx of chloride at the plasmalemma was linked to respiratory redox reactions, whereas active influx of chloride at the tonoplast required a high energy respiratory intermediate other than ATP.

Sulfhydryl Reagent Inhibitors. Sulfate transport was inhibited

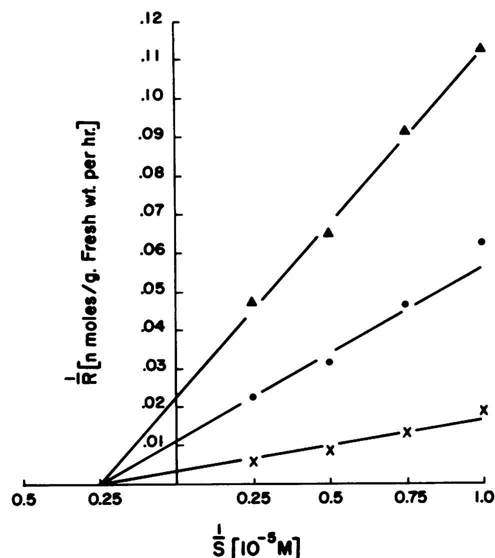

FIG. 5. Effect of sodium sulfide on sulfate transport. Transport measured as described in text; range of sulfate concentration was from 10^{-5} M to × 10^{-5} M. ×——×: no sodium sulfide; ●——●: 10^{-5} M sodium sulfide; ▲——▲: 2×10^{-5} M sodium sulfide.

Table II. *Effect of Metabolic Inhibitors on Transport of Sulfate by Cultured Tobacco Cells*

Transport was measured as described in the text, sulfate concentration 10^{-5} M. Figures indicate percentage of inhibition of transport at indicated concentration of the inhibitor.

Inhibitor	Inhibitor Concentration			
	10^{-4}	10^{-5}	10^{-6}	10^{-7}
		M		
Dinitrophenol	98	54 ± 6	32 ± 2	16
DCCD		100	64 ± 10	1
CCCP			100	24 ± 0
Potassium cyanide	98 ± 1	58 ± 10	43 ± 8	
Sodium azide	97 ± 1	42 ± 3	27 ± 6	
Dithiothreitol + CCCP			100[1]	

[1] Cells incubated with 10^{-5} M dithiothreitol for either 0, 2, or 4 hr prior to addition of CCCP and $Na_2{}^{35}SO_4$. Sulfate concentration was 5×10^{-5} M; at this concentration of sulfate dithiothreitol inhibits transport less than 10%.

Table III. *Effect of Sulfhydryl Reagents on Transport of Sulfate by Cultured Tobacco Cells*

Transport was measured as described in the text; sulfate concentration 10^{-5} M. Figures indicate percentage of inhibition of transport at indicated concentration of inhibitor.

Inhibitor	Inhibitor Concentration (M)			
	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Mercaptoethanol	98	96 ± 1	42 ± 12	11 ± 1
Dithiothreitol	99	98	50 ± 16	68
D-Cysteine	99	81 ± 7	50 ± 8	44
N-Ethylmaleimide		100	99	0
Iodoacetamide		99 ± 1	[70 ± 30][a]	27 ± 7
N-Ethylmaleimide + mercaptoethanol		-	100[b]	-

[a] Indicates stimulation of transport.

[b] Cells were incubated with 10^{-5} M N-ethylmaleimide for 2 hr and washed with medium minus inhibitor prior to the addition of 10^{-6} M mercaptoethanol and $Na_2{}^{35}SO_4$.

Table IV. *Effect of Inhibitors on Sulfate Transport and Efflux*

Cells were allowed to transport sulfate for 5 hr in the presence of various inhibitors (column 1). These cells were washed with sulfate-free medium and allowed to transport in inhibitor-free medium for a further 5 hr (column 2). Cells which had transported sulfate for 5 hr in the absence of any inhibitors were incubated in sulfate-free medium containing inhibitors for 5 hr to determine efflux (column 3).

		Inhibition of Transport		Total Sulfate to Efflux
	CONC	In Presence of Inhibitor	After Removal of Inhibitor	
Inhibitor	M	%	%	
Mercaptoethanol	10^{-4}	93	0	5
	10^{-3}	-	-	8
N-Ethylmaleimide	10^{-5}	97	96	86
	10^{-4}	-	-	91
Iodoacetamide	10^{-4}	100	100	76
	10^{-3}	-	-	70
CCCP	10^{-6}	100	63	7
	10^{-5}	-	-	21
DCCD	10^{-5}	100	100	13
	10^{-4}	-	-	54

more than 90% by low concentrations (10^{-4} M) of thiol reducing agents mercaptoethanol, dithiothreitol, and D-cysteine (Table III). These observations are consistent with the transport model proposed by Kaback and co-workers (12, 16) in which the carriers possess high affinity for their substrates only in the oxidized (disulfide) form, whereas the reduced (sulfhydryl) form has low substrate affinity. Removal of mercaptoethanol resulted in a complete recovery of transport (Table IV).

The sulfhydryl reagents N-ethylmaleimide (10^{-5} M) and iodoacetamide (10^{-4} M) completely abolish transport (Table III), which is in agreement with the observations of other investigators using a variety of organisms (10, 11, 17, 18). In the case of iodoacetamide a noninhibitory concentration (10^{-5} M) stimulated transport (Table III); this stimulation was always observed, but the amount of stimulation was extremely variable, ranging from 40 to 150%. The reason for this stimulation is unknown and is the subject of further investigation.

There are two major differences between tobacco cells and other systems with regard to the effects of sulfhydryl reagents. First, the inhibition of transport by N-ethylmaleimide was not reversed by thiol-reducing agents (Table III) (*cf.* refs. 17 and 18), because at inhibitory concentrations the former compound caused irreversible membrane disruption as indicated by massive nonspecific efflux of sulfur-containing metabolites (Table IV). Second, in tobacco cells sulfate transport is inhibited by relatively low concentrations of thiol-reducing agents, so that concentrations of thiol reagents which in other systems reverse the effects of N-ethylmaleimide, *p*-chloromercuribenzoate (12, 16–18), and CCCP (13) are themselves inhibitory to transport.

Effect of Inhibitors on Sulfur-Metabolite Efflux. The possibility that the effect of inhibitors of sulfate transport are due to an effect on membrane integrity was tested by measuring efflux of sulfur-containing metabolites from cells preloaded with $^{35}SO_4$.

In one series of experiments, cells which had transported sulfate for 5 hr were washed with sulfate-free medium and placed in new medium containing inhibitors (Table IV). Two inhibitor concentrations were used, a concentration known to inhibit transport 100% and 10 times this concentration. N-Ethylmaleimide (10^{-5} M) and iodacetamide (10^{-4} M) induced the efflux of between 70 and 90% of the accumulated sulfate in a 5-hr period. In this period the cell fresh weight decreased 50% and the cells were sticky when filtered. I concluded that these compounds react covalently with —SH groups in the cell membrane which are required for the maintenance of cell membrane integrity. This conclusion is supported by the observation that transport was not restored when these inhibitors were removed (Table IV).

The remaining results presented in Table IV indicate a correlation between inhibitor-induced metabolite efflux and the inability to restore transport when that inhibitor is removed. Substantial efflux was induced by DCCD (10^{-4} M) and removal of this inhibitor did not restore transport, CCCP induced lower rates of efflux and transport was partially restored, mercaptoethanol and selenate (not shown) induced low rates of efflux and transport was completely restored when these inhibitors were removed.

In the above experiments most of the material lost by the cells was sulfate because of the short duration of the experiment. A second series of experiments was performed to determine the composition of material leaving cells grown on sulfate for several generations, in which all of the sulfur-containing metabolites were labeled.

In the presence of 10^{-6} M CCCP, which inhibited transport 100%, 6% of the total soluble pool left the cells in an 8-hr period (Fig. 6). The efflux occurred at a linear rate and when the CCCP concentration was increased the rate of efflux increased. The material lost by the cells was representative of the total soluble sulfur pool (Table V). Sulfate was lost most rapidly followed by the neutral and anionic sulfur-containing compounds (nonsulfate) and finally the cationic material. These differences in efflux rate do not imply any selectivity but may be due to the relative size of the molecules; the smallest component of the cationic material is the amino acid cysteine and the major

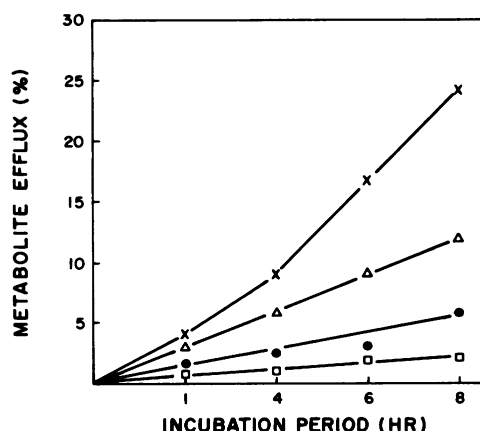

FIG. 6. Effect of CCCP on efflux of sulfur-containing metabolites from tobacco cells. CCCP concentrations: □: none, ●: 10^{-6} M; △: 10^{-5} M; ×: 10^{-4} M.

Table V. *Effect of CCCP on Sulfur-containing Metabolite Efflux from Cultured Tobacco Cells*

Cells were incubated in the presence of CCCP in sulfate-free M-1D medium for the time periods indicated. Nonsulfate fraction refers to material which was not retained by Dowex 50 H⁺-form resin and was not precipitated by barium salts. Amino acid fraction refers to material which is retained by Dowex 50 H⁺-form resin; this material has been shown to consist of cysteine, glutathione, and methionine (24). The total cellular content of sulfur containing metabolites in nmol/g fresh weight were: nonsulfate: 114 ± 9; sulfate: 365 ± 173; and amino acid: 536 ± 147.

CCCP	TIME	NON-SO_4	SO_4	AMINO ACID
M	HR	% TOTAL TO EFFLUX		
0	2	2.6 ± 0.2	2.6 ± 0.6	1.1 ± 0.1
0	4	4.1 ± 0.1	2.6 ± 0.6	1.9 ± 0.1
0	6	4.1 ± 0.3	3.2 ± 0.7	2.7 ± 0.1
10^{-6}	2	4.2 ± 0.4	5.4 ± 0.6	1.7 ± 0.1
10^{-6}	4	4.6 ± 0.6	7.7 ± 0.9	2.8 ± 0.2
10^{-6}	6	6.0 ± 0.5	9.2 ± 0.5	5.0 ± 0.6
10^{-5}	2	3.7 ± 0.2	5.5 ± 1.0	2.7 ± 0.3
10^{-5}	4	7.8 ± 0.3	12.1 ± 2.1	6.6 ± 0.4
10^{-5}	6	10.2 ± 0.1	18.9 ± 3.0	8.4 ± 1.0
10^{-4}	2	6.8 ± 0.4	9.6 ± 0.2	5.7 ± 0.9
10^{-4}	4	12.2 ± 1.7	21.4 ± 4.8	10.2 ± 0.4
10^{-4}	6	20.2 ± 3.5	32.8 ± 9.8	17.6 ± 4.4

component of this fraction is the tripeptide glutathione (24). I concluded that if 10^{-6} M CCCP which abolishes transport also inhibits ATP synthesis 100% the increased rates of metabolite efflux at concentrations of CCCP in excess of 10^{-6} M must be due to effects on the membrane which are not associated with ATP. Kaback *et al.* proposed that the effects of CCCP on bacterial membranes are due to effects not associated with ATP generation (13).

Analysis of the data presented in Table V indicates that the inhibition of sulfate transport by CCCP is not due exclusively to stimulation of sulfate efflux. In the absence of CCCP 2.6% of the sulfate associated with the cells was released in 2 hr, this material probably represented loss from the free space. Subsequently, relatively small amounts of sulfate were released (Table V); so that influx rate and transport rate are similar, *i.e.* greater than 50 nmol/g fresh weight·hr, 10^{-6} M CCCP induced the loss of an additional 1%/hr or 3.65 nmol/g fresh weight·hr (Table V). Transport was inhibited 100% at this concentration of inhibitor, therefore the influx rate could not have exceeded 3.65 nmol/g fresh weight·hr. The effect of CCCP is 2-fold, inhibition of influx and stimulation of efflux.

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