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Transinhibition Kinetics of the Sulfate Transport System of *Penicillium notatum*: Analysis Based on an Iso Uni Uni Velocity Equation

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Summary. Inorganic, intracellular sulfate, selenate, and molybdate strongly transinhibit the activity of the sulfate transport system in a mutant of *Penicillium notatum* lacking ATP sulfurylase. All three Group VI anions are substrates of the sulfate transport system. No effect was observed on the activity of the choline-O-sulfate transport system. Choline-O-sulfate transport was transinhibited only by intracellular choline-O-sulfate. The results confirm the earlier conclusions of Bradfield *et al.* (*Plant Physiol.* **46**:720, 1970) and Bellenger *et al.* (*J. Bacteriol.* **96**:1574, 1968) that transinhibition results from the action of the unchanged, internal substrate, and not a metabolite. The sulfate transport system is quite sensitive to L-cysteine. L-cysteine may be a feedback inhibitor of the sulfate transport system, or, alternately, some component of the sulfate transport system may be chemically (and nonspecifically) inactivated by reducing agents. Internal unlabeled sulfate, selenate, and molybdate acted as mixed-type inhibitors with respect to $^{35}SO_4^{2-}$ transport, in agreement with the simple Iso Uni Uni model. The kinetic constants for sulfate are: $K_{m_S}=1.25\times10^{-5}$ M, $V_{\max}=2.5$ μmoles $\times g^{-1}\times min^{-1}$, $K_{m_P}=2.5\times10^{-3}$ M, $K_{ip}=5\times10^{-3}$ M. The apparent equilibrium ratio of internal-to-external sulfate was about 10^4 at low initial substrate concentrations. External unlabeled 10^{-2} M sulfate promotes exchange at a rate of 0.15 μmoles $\times g^{-1}\times min^{-1}$. DNP ($10^{-4}$ M) and anaerobiosis promote a small efflux. DNP inhibits the exchange reaction.

The sulfate transport system of filamentous fungi is subject to transhibition [20–22, 31]. That is, preloading the mycelium with unlabeled inorganic sulfate (or a number of other sulfur compounds) causes a marked decrease in the activity of the transport system when measured with $^{35}SO_4^{2-}$. Recently, Marzluf [20] reported that preloading with unlabeled sulfate had no effect on the sulfate transport activity of an ATP sulfurylase negative mutant of *Neurospora crassa*. He concluded that the true intracellular

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effector was not sulfate, but rather, APS[1] or PAPS. Earlier, Bradfield *et al.* [2] performed the identical experiment but came to the opposite conclusion: The sulfate transport system of ATP sulfurylase negative mutants of *Aspergillus nidulans* and *Penicillium notatum* was strongly inhibited by internal sulfate. Bradfield *et al.* also reported that the sulfate transport activity of mycelium grown on L-methionine was significantly lower than that of sulfate-grown mycelium (wild type) or mycelium grown on a low level of L-cysteic acid and then preloaded with sulfate. These workers concluded that internal sulfate was a transinhibitor of sulfate transport but L-methionine was a repressor of the transport system [2]. Marzluf [19] also suggested that L-methionine is a repressor. It is possible, of course, that the mutants used by Bradfield *et al.* contain a very low level of ATP sulfurylase, undetectable by the usual assay, and insufficient to support growth on sulfate, but enough to yield an inhibiting level of APS. It occurred to us that selenate and molybdate might be used to advantage. These Group VI analogues of sulfate are substrates of the sulfate transport system and are transported as rapidly as, or faster than, sulfate [26]. Selenate and molybdate are also substrates of ATP sulfurylase [27, 28]. However, the products of the reaction, APSe and APMo, are exceedingly unstable and spontaneously decompose to AMP and SeO_4^{2-} or MoO_4^{2-}. APSe does have a finite life [30] but it is doubtful that free APMo exists. If SeO_4^{2-} and MoO_4^{2-} transinhibit the sulfate transport system as strongly as SO_4^{2-}, then there would be no doubt that the effector is the internal substrate itself, and not a metabolite. This paper also discusses the kinetic consequences of the Iso Uni Uni model of transport and transinhibition.

Materials and Methods

Organisms and Cultivation Methods

Most of the experiments were performed with *Penicillium notatum* strain 38632R, a white-spored, ATP sulfurylase-negative mutant. Strain 38632R is a sulfate transport-positive spontaneous revertant which was isolated from a sulfate transport-negative, ATP sulfurylase-negative parent, 38632M (listed as strain 38632 by the Commonwealth Mycological Institute). Some experiments were performed with *Penicillium chrysogenum*, wild type strain PS-75 (ATCC 24791) and *Aspergillus nidulans*, strain eta (PAPS reductase-negative).

The organisms were grown in 500-ml Erlenmeyer flasks containing 100 ml of synthetic medium. The medium has the following composition (per liter): $(NH_4)_2HPO_4$, 6 g; $(NH_4)_2H$ citrate, 10 g; K_2HPO_4, 16 g; trace metals [31], 10 ml; glucose (sterilized

1 Abbreviations: APS, adenosine-5′-phosphosulfate; APSe, adenosine-5′-phosphoselenate; APMo, adenosine-5′-phosphomolybdate; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PPO, 2,5-diphenyloxazole; DNP, 2,4-dinitrophenol.

separately), 40 g; and the desired sulfur source (usually 0.10 g/liter L-cysteic acid). The cultures were incubated at 25 °C *(Penicillia)* or 28 °C *(A. nidulans)* on a rotary shaker (250 rpm–1 inch circle).

Permease Assays

Transport was measured at room temperature (ca. 25 °C) in the following way: Mycelium from 2-day cultures was filtered, washed several times with 0.057 M K^+-NH_4^+-phosphate buffer, pH 6.0 (0.05 M KH_2PO_4, 0.007 M $(NH_4)_2HPO_4$), and then resuspended at a density of 0.25 or 0.5 g wet weight per 25 ml of the same buffer containing 0.8% (w/v) glucose. Transport rates were measured after 15 min of aeration or after preincubation with a desired compound. In all preincubation experiments the added compound was washed away before measuring transport. Rates were calculated from four 5-ml samples taken at 15- or 30-sec intervals after adding the labeled substrate (specific activity ca. 10^6 cpm/μmole). Each aliquot was filtered rapidly with suction through Whatman No. 1 filter paper discs (1.6 cm) or Whatman GF/B glass fiber paper discs. The resulting mycelial pad was washed once with 15 ml of ice-cold water, then peeled off the filter and counted in a scintillation vial containing 0.5 ml of water (used to disperse the mycelium) and 5 ml of scintillation fluid (6 g PPO plus 100 g naphthalene in 1 liter of dioxane). Each aliquot contained 7 or 14 mg (dry weight) of the mycelium. Since four time samples are used to calculate transport rates, the problem of different amounts of extracellular substrate sticking to the mycelium of different strains was eliminated.

Chemicals

Carrier-free $^{35}SO_4^{2-}$ was obtained from Nuclear Chicago and mixed with solutions of unlabeled Na_2SO_4 to obtain the desired specific activity. Carrier-free ^{35}S-labeled choline-O-sulfate was synthesized by the method of Segel and Johnson [24] and purified as described by Bellenger *et al.* [1]. Unlabeled choline-O-sulfate was synthesized as described by Bellenger *et al.* [1]. The sodium salt of L-cysteine-S-sulfate was prepared as described by Segel and Johnson [23].

Results

Effect of Selenate and Molybdate

When the mycelium of *P. notatum*, 38632R, is preincubated with unlabeled sulfate, selenate, or molybdate, the specific activity of the sulfate transport system rapidly decreases (Fig. 1). In all cases, the inhibition must be exerted from the cytoplasmic side of the membrane since the mycelium is thoroughly washed before activity is measured with $^{35}SO_4^{2-}$. After 2 hr, when the transinhibition is near maximal, the mycelium contained approximately 55 μmoles of SO_4^{2-} per g dry weight (determined in a separate experiment in which the mycelium was preloaded with $^{35}SO_4^{2-}$). A more extensive study is shown in Table 1. It can be seen that sulfate, selenate, molybdate, and thiosulfate strongly transinhibit the sulfate transport system, but have no effect at all on choline-O-sulfate transport. This result eliminates the possibility that selenate and molybdate acted in some nonspecific, toxic manner. When 0.05 M MES buffer, pH 6.4, without glucose was used for

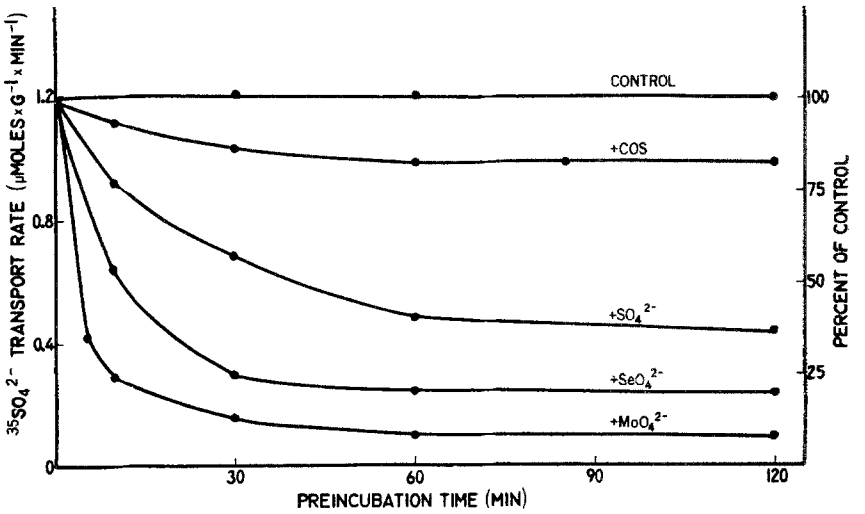

Fig. 1. *P. notatum*, strain 38632R, was grown in synthetic medium containing 100 mg/liter L-cysteic acid as sole sulfur source. After two days, the mycelium was filtered, washed several times with 0.057 M K^+-NH_4^+ phosphate buffer, pH 6.0, and then resuspended at a density of 0.50 g wet weight per 25 ml of the same buffer containing 1% glucose and the indicated addition (10^{-3} M). The suspensions were incubated at room temperature on a rotary shaker. Periodically, the mycelium was filtered, washed several times with the usual buffer (minus the additions) and then resuspended at the original density in fresh buffer plus glucose. The $^{35}SO_4^{2-}$ transport rate was measured at 5×10^{-5} M initial substrate concentration as described in Materials and Methods

the preloading and the subsequent assay, the results were essentially identical to those shown in Table 1. Choline-O-sulfate caused some decrease in sulfate transport, but this is not surprising since the organism possesses a choline sulfatase [1]. Of the first nine compounds shown in Table 1, only choline-O-sulfate strongly transinhibited the choline-O-sulfate transport system. The only sulfur-containing metabolite of choline-O-sulfate in *P. notatum*, 38632R, is inorganic sulfate, which was inactive. The results confirm our original conclusions [1, 2] that the transinhibitors are the unchanged, internal substrates of the respective transport systems. Chromate at 10^{-3} M and 10^{-4} M abolished the activity of both transport systems. Chromate is believed to be a substrate of the sulfate transport system [18] but its general inhibitory effect very likely results from its being a strong oxidizing agent. Chromate was equally effective in destroying choline-O-sulfate transport by *P. notatum*, 38632M, the sulfate transport-negative parent of strain 38632R. Sulfite had no effect on choline-O-sulfate transport but reduced sulfate transport by 51%. Sulfite in dilute solutions is quite unstable, oxidizing rapidly to sulfate. The reduction in sulfate transport activity very likely results from sulfate produced during the preincubation period. No

Table 1. Effect of preincubating with various compounds on the activity of the sulfate and choline-O-sulfate transport systems [a]

Preincubation conditions [b]	$^{35}SO_4^{2-}$ Transport Rate [c]		Choline-O-$^{35}SO_4^{2-}$ Transport Rate [c]	
	μmoles × g^{-1} × min^{-1}	% of control	μmoles × g^{-1} × min^{-1}	% of control
Control	1.35	100	0.69	100
+ Na_2SO_4	0.50	37	0.67	98
+ K_2SeO_4	0.24	18	0.67	98
+ Na_2MoO_4	0.11	8	0.67	98
+ $Na_2S_2O_3$	0.47	35	0.68	99
+ Choline-O-sulfate	0.71	53	0.18	27
+ Choline-O-phosphate	1.79	133	0.80	117
+ Choline chloride	1.25	93	0.75	109
+ L-Methionine	0.86	64	0.66	97
+ L-Cysteic acid	1.36	100	0.64	93
+ Na_2CrO_4	0.03	2	0.02	3
+ Na_2SO_3	0.67	49	0.69	100
+ L-Cysteine	0.10	7	0.48	70
+ L-Cysteine-S-sulfate	0.09	7	0.44	65

[a] The mycelium (*P. notatum*, 38632R) was grown and treated as described in the legend of Fig. 1.

[b] All additions were at 10^{-3} M.

[c] Transport rates (reported on a dry weight basis) were measured after 2 hr of preincubation with the indicated additions. The initial labeled substrate concentration was 5×10^{-5} M. In all cases, transport was measured after the mycelium was thoroughly washed. Thus, an effect cannot be ascribed to external competition for a common carrier or dilution of the specific activity of the labeled substrate.

definite conclusions can be drawn concerning the effects of cysteine and cysteine-S-sulfate. Cysteine which is transported [25], is a strong reducing agent. Cysteine-S-sulfate decomposes to cysteine plus sulfite in the presence of thiols. Thus, both compounds may act in a general toxic manner. It is noteworthy though, that sulfate transport is severely reduced after preloading the mycelium with cysteine or cysteine-S-sulfate. This may indicate that (a) cysteine is a specific feedback inhibitor of the sulfate transport system [2, 8], or (b) some component of the sulfate transport system contains linkages that can be reduced by cysteine (in a purely chemical reaction). The results with other fungal strains were qualitatively similar to those shown in Table 1 except that selenate strongly suppressed sulfate and choline-O-sulfate transport in strains that could reduce the selenate (as evidenced by a strong, disaggreeable odor of what was probably dimethylselenide [9]).

Kinetics of Transinhibition

Hunter and Segel [12] suggested that the simplest model of a carrier-mediated transport process could be described by the velocity Eq. (1) shown below:

$$\frac{v}{V_{\max_f}} = \frac{[S]}{K_{m_s}\left(1+\frac{[P]}{K_{m_P}}\right)+[S]\left(1+\frac{[P]}{K_{iip}}\right)} \tag{1}$$

where v is the initial velocity of labeled substrate transport, $[S]$ is the external concentration of labeled substrate, $[P]$ is the internal concentration of unlabeled substrate, K_{m_s} is the Michaelis constant for labeled substrate transport, $V_{\max_f}$ is the maximal velocity of labeled substrate influx, K_{m_P} is the Michaelis constant for unlabeled substrate efflux (and also an inhibition constant affecting the slope of the $1/v$ versus $1/[S]$ plot), and K_{iip} is an isoinhibition constant affecting the $1/v$-axis intercept of the $1/v$ versus $1/[S]$ plot. Each kinetic constant is composed of a number of forward and reverse rate constants (*see* Discussion). The equation predicts that if there are no unusual rate constants, unlabeled internal substrate will act as a linear mixed-type inhibitor with respect to the transport of labeled substrate. If, coincidentally, $K_{m_P} = K_{iip}$, the inhibition will be noncompetitive. As noted in the Discussion, Eq. (1) is actually a special case of a more complete equation derived by Schachter [21]. Fig. 2 shows the results of an experiment designed to determine the nature of the transinhibition of $^{35}SO_4^{2-}$ transport by internal unlabeled SO_4^{2-}. As predicted, the inhibition is mixed type. The inserts show the slope and $1/v$-axis intercept replots from which all the kinetic constants can be obtained. Intracellular selenate and molybdate were also mixed-type inhibitors with respect to $^{35}SO_4^{2-}$ transport. The data are summarized in Table 2.

Equilibrium Ratio of Internal-to-External Sulfate

An attempt was made to determine the equilibrium ratio of internal-to-external sulfate. The mycelium (0.50 g wet weight) was incubated over a 3-hr period in 50 ml of the usual buffer containing carrier-free $^{35}SO_4^{2-}$ (71,000 cpm/0.5 ml) or 10^{-6} M $^{35}SO_4^{2-}$ (89,000 cpm/0.5 ml) at zero time. Periodically a 5-ml aliquot was taken and filtered. The mycelial pad (0.05 g wet weight) and 0.5 ml of the filtrate were counted. K_{eq} was calculated as (11.6 × cpm of 0.05 g wet weight mycelium)/(cpm of 0.5 ml of filtrate) from samples taken between 30 and 120 min (when no further change was noted). The factor 11.6 takes into account the internal/external volume ratios and the 86% water content of the mycelium. In another experiment, 0.05 g wet

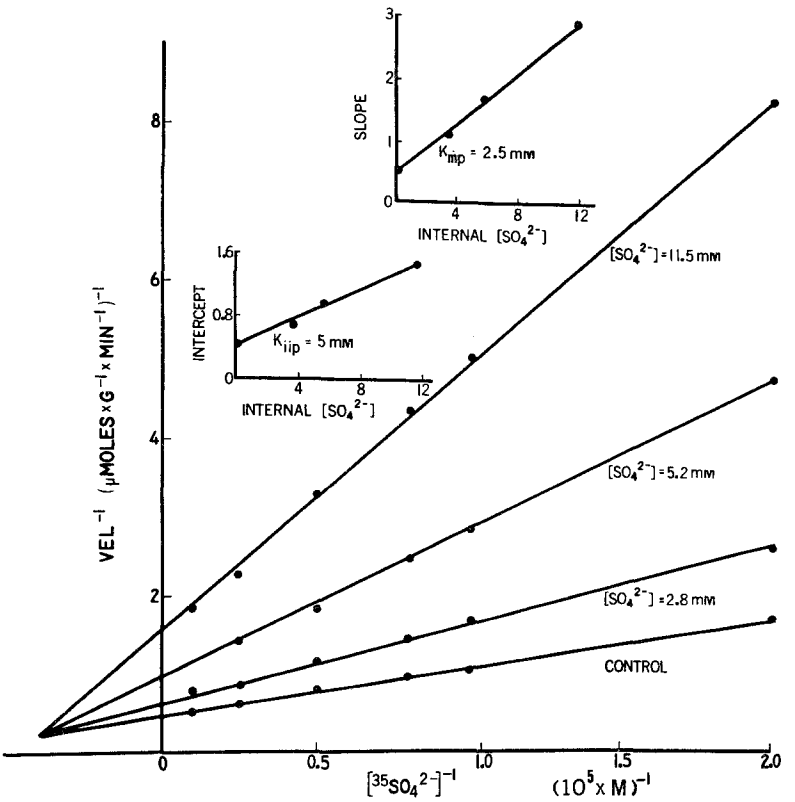

Fig. 2. Effect of unlabeled intracellular sulfate on the kinetics of $^{35}SO_4^{2-}$ transport. The mycelium was preincubated at 0.25 g wet weight per 25 ml of 0.057 M K^+-NH_4^+ phosphate buffer, pH 6.0, containing 1% glucose and 0 (control), 3×10^{-5} M, 5×10^{-5} M, and 10^{-4} M unlabeled sulfate. After 2 hr, the mycelium was filtered, washed, and resuspended at the same density in fresh buffer plus glucose. Sulfate transport by each batch of mycelium was measured at six different initial concentrations of $^{35}SO_4^{2-}$ (5×10^{-6} to 10^{-4} M). In a separate experiment, the mycelium was preincubated with 3×10^{-5} M, 5×10^{-5} M, and 10^{-4} M $^{35}SO_4^{2-}$ to establish the intracellular (preloaded) concentrations. Essentially all the $^{35}SO_4^{2-}$ added was taken up by the mycelium in 2 hr. The intracellular concentrations indicated for each curve and used for the slope and intercept replots assume that all the cell water is intracellular. (The fresh, blotted mycelium is 86% water.) The primary plots intersect at $1/[S] = -K_{m_P}/K_{m_s}K_{iip}$ and $1/v = (1/V_{max})(1 - K_{m_P}/K_{iip})$. The units of the vertical axis of the intercept replot are the same as those of the vertical axis of the primary plot. Slopes were read directly off the primary plot (ignoring the 10^5 factor). The constants of interest (K_{m_P} and K_{iip}) were obtained by extrapolating the slope and intercept replots, respectively, to the horizontal axes

weight mycelium was suspended in 50 ml of the usual buffer containing 10^{-7} M $^{35}SO_4^{2-}$ (76,500 cpm/0.5 ml) at zero time. The $^{35}SO_4^{2-}$ remaining in the medium was measured after 2 and 3 hr of incubation. (At this low mycelial density, it was impossible to obtain uniform mycelial samples.) K_{eq} was calculated from the decrease in the $^{35}SO_4^{2-}$ content of the filtrate.

Table 2. Kinetic constants for transinhibition of the sulfate transport system of *Penicillium notatum*, 38632R [a]

Constant	Transinhibiting compound [b]		
	Sulfate	Selenate	Molybdate
K_{m_s}	1.25×10^{-5} M	—	—
$V_{\max_f}$ [c]	2.5 μmoles × g^{-1} × min^{-1}	2.0 μmoles × g^{-1} × min^{-1}	1.0 μmole × g^{-1} × min^{-1}
K_{m_P} or $K_{i_{slope}}$ [d]	2.5×10^{-3} M	2.2×10^{-3} M	7.5×10^{-4} M
$K_{i_{ip}}$ or $K_{i_{int}}$ [d]	5.0×10^{-3} M	2.0×10^{-2} M	1.5×10^{-3} M
$K_{i_{is}}$ [e]	2.5×10^{-5} M	—	—
$V_{\max_r}$ [f]	0.05 μmoles × g^{-1} × min^{-1}	—	—

[a] The assays were performed as described in the legend of Fig. 2.

[b] The mycelium was preloaded for 2 hr with various concentrations of the indicated unlabeled compound. The intracellular sulfate levels were determined directly in a separate experiment in which the mycelium was preloaded with $^{35}SO_4^{2-}$. It was assumed that the low-cysteic acid-grown mycelium did not contain a significant pre-existing internal sulfate pool. The linear $slope_{1/S}$ and intercept replots support this assumption. The intracellular selenate and molybdate levels were calculated assuming that all the added compound was taken up by the mycelium.

[c] $V_{\max_f}$ represents the maximal transport velocity of the indicated compound (dry weight basis). $V_{\max_f}$ for sulfate was obtained from control plots (Fig. 2). $V_{\max_f}$ for selenate and molybdate are those reported by Tweedie and Segel (26).

[d] When sulfate is the transinhibitor, $K_{i_{slope}} = K_{m_P}$, $K_{i_{int}} = K_{i_{ip}}$. Since selenate and molybdate are alternate products, $K_{i_{slope}}$ and $K_{i_{int}}$ may not be identical to K_{m_P} and $K_{i_{ip}}$ because $^{35}SO_4^{2-}$ was used as the substrate (as opposed to $^{75}SeO_4^{2-}$ and $^{99}MoO_4^{2-}$).

[e] Calculated from the relationship $K_{i_{is}} = K_{i_{ip}} K_{m_s} / K_{m_P}$.

[f] Calculated from K_{m_s}, K_{m_P}, $V_{\max_f}$, and $K_{eq} = 10^4$.

The low initial $^{35}SO_4^{2-}$ concentrations were chosen to minimize transinhibition which would slow the rate of equilibration and give erroneous "equilibrium" $[P]/[S]$ ratios. However, sulfur contamination of the phosphate (listed as 0.005% maximum) could have contributed up to 2.5×10^{-6} M SO_4^{2-}. K_{eq} was calculated to be 7,000 to 16,000. Essentially the same values were obtained when a 1:5 dilution of the growth medium was used in place of the usual buffer. When mycelium preloaded with $^{35}SO_4^{2-}$ is filtered, washed, and resuspended in fresh buffer, a small efflux occurs until the internal/external concentration ratio stabilizes at about 10^4. The finite value of K_{eq} shows that none of the reverse rate constants equal zero (*see* Discussion). The experimental K_{eq} is an apparent value in the same way that the K_{eq} for a H^+ ion-dependent reaction varies with pH. In the present case, K_{eq} will vary with the activity of the energy coupling system (presumably a proton motive force [10, 13]). Furthermore, the *apparent*

$[P]/[S]$ equilibrium ratio will decrease with decreasing mycelial density and increasing initial concentration of S (once $[S]$ exceeds a certain value). For example, at a 100:1 medium/mycelium volume ratio and an initial $[S] = 10^{-2}$ M, the mycelium would have to accumulate almost 1 M $[P]$ to deplete the medium sufficiently to yield a K_{eq} of 10^4. The mycelium may be unable to accumulate 1 M $[P]$ for reasons unrelated to the operation of the transport system.

The high value for K_{eq} made it impractical to study the kinetics of $^{35}SO_4^{2-}$ efflux from preloaded mycelium into sulfate-free medium. The system would come to equilibrium after only a small increase in external $^{35}SO_4^{2-}$. Initial efflux velocities would be very difficult to determine. The possibility of employing an external sulfate trapping agent is under consideration.

Efflux and Exchange

When mycelium preloaded with $^{35}SO_4^{2-}$ is washed and resuspended in fresh buffer, pH 6.0, no sustained efflux is observed. Similarly, the loaded mycelium can be incubated for several hours in the original medium (minus

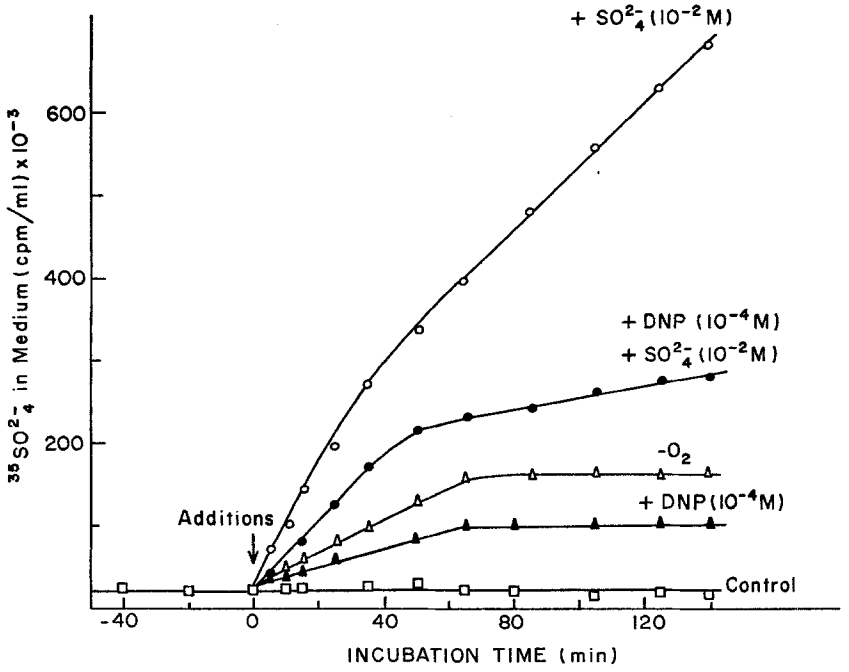

Fig. 3. The mycelium was preloaded at a density of 5 g wet weight per 500 ml of 0.057 M K^+-NH_4^+-phosphate buffer, pH 6.0, containing 10^{-4} M $^{35}SO_4^{2-}$ (2×10^7 cpm/mole). After 3 hr when the $[P]/[S]$ ratio was about 10^4, the suspension was divided into five parts and the indicated additions made. Periodically, 0.25 ml of filtered incubation medium was counted. The minus O_2 flask was stoppered and left unshaken

glucose) without any loss of accumulated label (Fig. 3, control). The addition of 10^{-4} M DNP or the cessation of aeration causes a small amount of efflux. In the presence of 10^{-2} M unlabeled sulfate efflux is observed. DNP (10^{-4} M) inhibited the efflux stimulated by external sulfate.

Glucose was omitted from the medium in order to avoid a decrease in pH during the incubation period. A small decrease in pH would have no effect on the activity of the sulfate transport system [2, 31] but would increase the concentration of unionized DNP markedly. (The pK_a of DNP is about 4.) In a separate experiment, it was established that the mycelium could be incubated in buffer for several hours without any decrease in transport activity.

Discussion

Our results show conclusively that internal sulfate, selenate, and molybdate are transinhibitors of the sulfate transport system of *P. notatum*. This is not at all surprising since even the simplest model for carrier-mediated transport predicts such inhibition as long as (a) the carrier in its "internal" conformation retains some affinity for the substrate, and (b) the internal level of the substrate is in the region of the inhibition constants. The model shown below is identical to an Iso Uni Uni enzyme mechanism [5] or the mobile carrier model of Cirillo [4]. The latter has been treated mathematically by Jacquez [14], Kalsow and Doyle [15], Kotyk [16] and by Hoare [11].

$$\begin{array}{ccccc} S+C & \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} & C'+P & & \\ \Big\uparrow\Big\downarrow{\scriptstyle k_1}\ {\scriptstyle k_{-1}} & & \Big\uparrow\Big\downarrow{\scriptstyle k_{-3}}\ {\scriptstyle k_3} & & \\ \text{(external)} & & \text{(membrane)} & & \text{(internal)} \\ CS & \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} & C'P & & \end{array}$$

C represents the membrane carrier in a conformation accessible to external substrate, S. C' represents the membrane carrier in a conformation accessible to internal substrate, P.

A steady-state treatment yields the velocity equation shown below:

$$\frac{v}{[E]_t}=\frac{k_1k_2k_3k_4[S]-k_{-1}k_{-2}k_{-3}k_{-4}[P]}{(k_4+k_{-4})(k_{-1}k_{-2}+k_{-1}k_3+k_2k_3)+k_1(k_2k_3+k_2k_4+k_{-2}k_4+k_3k_4)[S]+k_{-3}(k_{-1}k_{-2}+k_{-1}k_{-4}+k_2k_{-4}+k_{-2}k_{-4})[P]+k_1k_{-3}(k_2+k_{-2})[S][P]}.$$

The equation is not very useful unless the resulting groups of rate constants are defined in terms of experimentally obtainable kinetic constants. The

definitions of Cleland [5–7] lead to Eq. (2):

$$\frac{v}{V_{\max_f}}=\frac{[S]-\frac{[P]}{K_{eq}}}{K_{m_s}\left(1+\frac{[P]}{K_{m_P}}\right)+[S]\left(1+\frac{[P]}{K_{iip}}\right)} \tag{2}$$

where:

$$K_{m_s}=\frac{(k_4+k_{-4})(k_{-1}k_{-2}+k_{-1}k_3+k_2k_3)}{k_1(k_2k_3+k_2k_4+k_{-2}k_4+k_3k_4)}$$

$$V_{\max_f}=\frac{k_2k_3k_4[C]_t}{k_2k_3+k_2k_4+k_{-2}k_4+k_3k_4}$$

$$K_{m_P}=\frac{(k_4+k_{-4})(k_{-1}k_{-2}+k_{-1}k_3+k_2k_3)}{k_{-3}(k_{-1}k_{-2}+k_{-1}k_{-4}+k_2k_{-4}+k_{-2}k_{-4})}$$

$$K_{iip}=\frac{k_2k_3+k_2k_4+k_{-2}k_4+k_3k_4}{k_{-3}(k_2+k_{-2})}.$$

The additional kinetic constants for the symmetrical efflux reaction are:

$$V_{\max_r}=\frac{k_{-1}k_{-2}k_{-4}[C]_t}{k_{-1}k_{-2}+k_{-1}k_{-4}+k_2k_{-4}+k_{-2}k_{-4}}$$

$$K_{iis}=\frac{k_{-1}k_{-2}+k_{-1}k_{-4}+k_2k_{-4}+k_{-2}k_{-4}}{k_1(k_2+k_{-2})}.$$

The $C \rightleftharpoons C'$ isomerization does not change the Haldane equation for the system. Therefore:

$$K_{eq}=\frac{K_{m_P}V_{\max_f}}{K_{ms}V_{\max_r}}=\frac{k_1k_2k_3k_4}{k_{-1}k_{-2}k_{-3}k_{-4}}=\frac{[P]_{eq}}{[S]_{eq}}.$$

It can also be shown that $K_{m_P}/K_{ms}=K_{iip}/K_{iis}$. Eq. (2) gives the *net* $S \rightarrow P$ velocity. If S is radioactive (S^*) and P is not, and it is further assumed that all free carrier available for combination with S results from the catalytic cycle $C \xrightarrow{k_1} CS^* \xrightarrow{k_2} C'P^* \xrightarrow{k_3} C' \xrightarrow{k_4} C$, then Eq. (1) will describe the observed influx of labeled S. Schachter [21] has presented a more rigorous treatment in which labeled S and unlabeled S are considered separately. This treatment takes into account the efflux sequence $C' \xrightarrow{k_{-3}} C'P \xrightarrow{k_{-2}} CS \xrightarrow{k_{-1}} C$ which provides an additional path for C regeneration. The same final equation is obtained using the somewhat simpler procedure of Cleland [7] for deriving isotope exchange equations. The complete velocity

equation for labeled S influx is then given by:

$$\frac{v}{V_{\max_f}} = \frac{[S]\left(1+\frac{[P]}{K_I}\right)}{K_{m_s}\left(1+\frac{[P]}{K_{m_P}}\right)+[S]\left(1+\frac{[P]}{K_{iip}}\right)} = \frac{[S]}{K_{m_s}\frac{\left(1+\frac{[P]}{K_{m_P}}\right)}{\left(1+\frac{[P]}{K_I}\right)}+[S]\frac{\left(1+\frac{[P]}{K_{iip}}\right)}{\left(1+\frac{[P]}{K_I}\right)}}. \quad (3)$$

Eq. (3) was derived as Eq. (33) in reference [21]. The identities are: $K_{m_s} = K_1$, $K_{m_P} = K_2$, $K_I = K_4$, $K_{iip} = K_3 K_4/K_1$, and $V_{\max_f} = [K_1 k_4 k_{-4}/K_4'(k_4+k_{-4})][C]_t$. Also, $K_4 = k_4(k_{-1}k_{-2}+k_{-1}k_3+k_2k_3)/k_{-1}k_{-2}k_{-3}$, $K_4' = k_{-4}(k_{-1}k_{-2}+k_{-1}k_3+k_2k_3)/k_1k_2k_3$, and $K_{eq} = K_4/K_4'$. (*See also* Eq. (1) of reference [3].) Eq. (3) predicts either hyperbolic slope and intercept replots (activation or inhibition) if $K_{m_P} \neq K_I$, and $K_{iip} \neq K_I$ or no effect of P if $K_{m_P} = K_{iip} = K_I$. Eq. (1) predicts only linear transinhibition. Eq. (3) reduces to Eq. (1) if $[P]/K_I \ll [P]/K_{m_P}$ and $[P]/K_I \ll [P]/K_{iip}$; i.e., if $K_I \gg K_{m_P}$ and $K_I \gg K_{iip}$. This condition appears to be met for S^* influx into *P. notatum* (at least for the $[P]$ range investigated) since the replots are linear. (Actually, it would not take more than a 10 to 15% error in the values of the slopes and intercepts to make the replots appear linear even if $K_I = 5K_{iip} = 10K_{m_P}$). The efflux of labeled P stimulated by unlabeled, external S represents transstimulation, as predicted by Eq. (34) of reference [21]. The Iso Uni Uni equation for efflux [symmetrical to Eq. (1)] predicts only transinhibition. Thus, a significant fraction of the C' available for combination with P^* arises from the sequence $C \xrightarrow{k_1} CS \xrightarrow{k_2} C'P \xrightarrow{k_3} C'$ as well as from the $C \xrightarrow{k_{-4}} C'$ step. In this case, the reduction of the full velocity equation to an Iso Uni Uni equation is not valid.

The fact that the observed transinhibition of sulfate transport is mixed type shows that the translocation of the uncharged carrier is partially rate limiting. If the rate of isomerization of the uncharged carrier (the k_4/k_{-4} step) is extremely rapid compared to the other steps, P would behave as a competitive inhibitor. Recently, Britton [3] showed that the intercept $[P]$ term would also disappear (i.e., K_{iip} becomes very large) if the $C \rightleftharpoons C'$ isomerization were extremely slow compared to the other steps. The model of Britton omits the $CS \rightleftharpoons C'P$ step. In terms of the model shown above, the $[P]/K_{iip}$ term disappears when k_4 is very small and k_3 is much larger than k_{-3} and k_{-2}. If k_{-2} or $k_{-4} = 0$, the kinetics of influx and transinhibition would be unchanged but $V_{\max_r}$ would be zero (no efflux). A "revolving door" model in which both k_{-2} and k_{-4} are zero would yield uncompetitive inhibition by $P(K_{m_P} = \infty)$. This is essentially the model of Vallee [29] if it is assumed that P and M of that model represent different conformational

states of the same protein. Kotyk and Rihova [17] also assumed that k_{-2} and k_{-4} were zero. However, they observed that internal α-aminoisobutyric acid acted as a pure noncompetitive inhibitor with respect to the transport of labeled external α-aminoisobutyric acid. To account for the discrepancy, they proposed a distinct feedback site on the hypothetical catalyst of the $CS \leftarrow C'P$ translocation reaction. It is unnecessary to postulate an additional site. We can see from Eq. (1) that P will act as a pure noncompetitive inhibitor when $K_{m_P} = K_{i_P}$. If we consider the expressions for K_{m_P} and K_{i_P}, we can probably find numerous combinations of relative values of the rate constants that result in $K_{m_P} \simeq K_{i_P}$. Also, the exchange (trans-stimulation) confirms that $k_{-2} \neq 0$. The efflux stimulated by DNP alone or by anaerobiosis confirms that $k_{-4} \neq 0$.

Our results do not exclude the possibility that APS or PAPS also affect the activity of the transport system, as suggested by Marzluf [20]. It is difficult, however, to evaluate the report of Marzluf [20] that the cys-11 mutant of *Neurospora crassa* is not transinhibited by internal sulfate because a complete kinetic analysis was not performed. The K_{m_s} for the mycelial sulfate transport system of *Neurospora crassa* is reported to be about 8×10^{-6} M [19]. Five-minute transport assays were conducted at an extracellular $^{35}SO_4^{2-}$ concentration between 10^{-4} M and 2×10^{-4} M [20]. Thus, if the major effect of internal sulfate in the cys-11 system is on K_{m_s} rather than V_{max}, very little transinhibition would be observed.

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