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UPTAKE AND EFFLUX OF SULFATE IN *NEUROSPORA CRASSA*

GEORGE A. MARZLUF

Department of Biochemistry, Ohio State University, Columbus, Ohio 43210 (U.S.A.)

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SUMMARY

Sulfate efflux from an intracellular pool was observed with both wild-type and *cys-11* cells of *Neurospora* and apparently occurs by way of the sulfate transport system. Efflux requires the presence of external sulfate or the related ions, chromate, selenate, or thiosulfate, and probably occurs by an exchange reaction. The sulfur amino acids, cysteine or methionine, do not promote sulfate efflux. The K_m for efflux is much greater than the K_m for sulfate uptake, which permits the accumulation of a considerable intracellular pool before efflux becomes significant. Substantial transmembrane movement of sulfate, both influx and exit, was found to occur in azide-treated cells, although the net uptake of sulfate was abolished by this inhibitor. Both sulfate uptake and efflux are inhibited by *p*-chloromercuribenzoate which suggests that the sulfate permease possesses an essential sulfhydryl group.

INTRODUCTION

Sulfate transport in *Neurospora crassa* has been demonstrated to be a highly regulated process. This organism possesses two distinct sulfate permeases, one of which predominates in the mycelial stage while a second form is the only one present in conidiospores [1, 2]. The synthesis of both of these sulfate permeases is repressed by a metabolite derived from methionine [3]. The permeases, as well as a number of related enzymes of sulfur metabolism, are also controlled by at least two regulatory genes, *cys-3* and *scon* [4, 5]. Recent studies have demonstrated that the sulfate transport systems are also regulated by feedback inhibition, which is not due to an intracellular pool of inorganic sulfate, but is instead exerted by an early intermediate of the assimilatory pathway [6]. Finally, the sulfate permeases are subject to turnover, such that once new synthesis is terminated, the remaining activity is inactivated and declines to a low level [7].

In *Salmonella*, an "overshoot" phenomenon was observed [8] in which a rapid entry of sulfate into the cells was followed by an immediate efflux of a fraction of the

Abbreviation: PCMB, *p*-chloromercuribenzoate.

internal sulfate. Earlier studies, however, suggested that sulfate uptake was unidirectional in *Neurospora* and that no exchange or exit from an intracellular sulfate pool occurred [1, 9, 10]. Furthermore, sulfate uptake was found to be completely inhibited in azide-treated cells. After cells had accumulated an intracellular pool of sulfate, the addition of azide immediately prevented further uptake but did not result in any loss from the internal pool [1, 10]. This result also suggested that sulfate transport was unidirectional, moving only into the cell, and that azide inhibited any transmembrane movement of the ion. The objective of the present study was to determine whether sulfate transport was indeed unidirectional or whether efflux could be detected under appropriate conditions. The results indicate that efflux does indeed occur and may help to regulate the size of the intracellular pool of sulfate.

MATERIALS AND METHODS

Neurospora strains and growth conditions

The wild type *Neurospora* strain *74-OR23-1A* and the *cys-11* mutant (allele NM86) were obtained from the Fungal Genetics Stock Center, Humboldt State College, Arcata, Calif. Erlenmeyer flasks (250 ml) containing 40 ml of Fries' medium [11] which lacked the usual sulfur source but contained 0.25 mM L-methionine and 1.5% sucrose were used for outgrowth of conidia into mycelia. This concentration of methionine allows derepression of the sulfate permeases and related enzymes of sulfur metabolism [1, 3]. The media was inoculated with a filtered conidial suspension to a final absorbance of 0.1 at 420 nm and shaken at 25 °C for approximately 16 h.

Sulfate transport and efflux

Sulfate uptake assays were conducted as previously described [1]. For study of efflux, mycelial samples were preloaded by incubating with 0.1 mM radioactive sulfate for various times when the cells were collected by gentle filtration and washed free of all external sulfate. The cells were then resuspended in Fries' medium and incubated for 5 min when nonradioactive sulfate was added to initiate efflux. Samples (2 ml) were then taken immediately and after various time intervals; the cells were collected on a glass fiber filter and washed twice with 3-ml aliquots of distilled water. The combined medium and wash solutions were collected, 1 ml of saturated $BaCl_2$ was added, and the resulting precipitate of $BaSO_4$ was collected on a Millipore filter. The radioactivity present in the cells and that in the medium was determined by placing the corresponding filters in vials with a dioxane-based fluid and counting them in a Beckman Model LS133 scintillation counter.

Intracellular sulfate concentration

The intracellular concentration of the sulfate ion was determined by use of the relationship derived by Slayman and Tatum [12]. By knowing the total accumulated sulfate and the dry weight of each mycelial sample, the intracellular concentration was calculated by the expression:

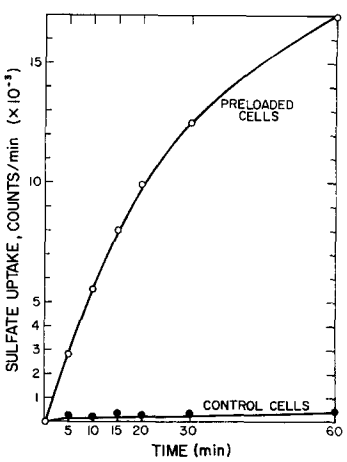
$$\text{Intracellular concentration (mM)} = \frac{\text{total sulfate within cells (nmoles)}}{2.54 \times \text{dry weight of cells (mg)}}.$$

RESULTS

Sulfate influx in the presence of azide

Since azide prevents the uptake of sulfate but allows near complete retention of a pre-existing intracellular sulfate pool, it was suggested [1, 9] that the movement of sulfate was unidirectional, passing only into the cells. It seemed that azide prevented any movement of sulfate across the cell membrane, whether exit or entry, so that azide-treated cells were virtually impermeable to sulfate. The results given below show that this is not at all the case, but rather that considerable transmembrane movement of sulfate can occur in the presence of azide, although net uptake of the ion is indeed abolished.

Fig. 1 shows that when cells were preloaded with unlabeled sulfate before the addition of azide, considerable subsequent uptake of radioactive sulfate occurred. The influx of sulfate into such preloaded cells in the presence of azide was found to be nearly linear for 15 min, after which its uptake leveled off. In contrast, 5 min of incubation with azide completely inhibited any $^{35}SO_4^{2-}$ uptake by cells which had not been first preloaded with inorganic sulfate.



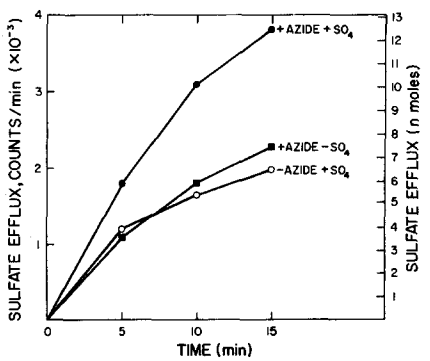


Fig. 1. Time dependence of sulfate uptake in presence of azide. *cys-11* cells were preloaded with non-radioactive sulfate by incubation for 5 min with 0.1 mM sulfate when azide was added (1 mM) and incubation continued for 5 min. Radioactive sulfate (1 mM) was added and its uptake into the cells was followed for 0–60 min (○–○). An identical series of *cys-11* cells were not preloaded with sulfate but were similarly incubated for 5 min before receiving azide and then radioactive sulfate (●–●).

Fig. 2. Sulfate efflux in the presence and absence of azide. *cys-11* cells were preloaded with $^{35}SO_4^{2-}$ for 30 min as described in Materials and Methods. After resuspending in minimal medium lacking sulfate, the cells were incubated for 5 min when NaN_3 was added to a final concentration of 1 mM. After 3 min of additional incubation, non-radioactive sulfate (to 1 mM) was added to one flask (●–●) while another received an equal volume of water (■–■). A similar sample of preincubated cells was incubated without azide for 6 min when unlabeled sulfate (1 mM) was added (○–○). Samples (2 ml) from each flask were taken immediately upon addition of the nonradioactive sulfate and at intervals thereafter and processed to determine the amount of sulfate efflux as described in Materials and Methods.

Efflux of sulfate

Cells of the *cys-11* mutant were preloaded with $^{35}SO_4^{2-}$ and then resuspended and incubated in medium containing nonradioactive sulfate, both in the presence and absence of azide. Considerable loss of radioactive sulfate from the cells occurred, which was accompanied by a parallel appearance of $^{35}SO_4^{2-}$ in the extracellular medium (Fig. 2). Although the efflux of $^{35}SO_4^{2-}$ occurred in both cases, its exit was nearly twice as rapid in the presence of azide. Fig. 2 also shows that sulfate efflux was found to occur in the presence of azide even when external sulfate was lacking, although in this case the rate of exit was significantly lower. These results show that sulfate can move in both directions across the cell membrane, both in the presence and absence of azide. Since the *cys-11* mutant is blocked in the very first reaction of sulfate assimilation, it is obvious that no metabolism of the ion is required, but that such transmembrane movements involve inorganic sulfate itself.

Requirement of external sulfate for efflux

Additional studies of sulfate efflux were all carried out in the absence of azide. Fig. 3 shows that absolutely no $^{35}SO_4^{2-}$ efflux took place from preloaded cells unless non-radioactive sulfate was added to the external medium. In the presence of external

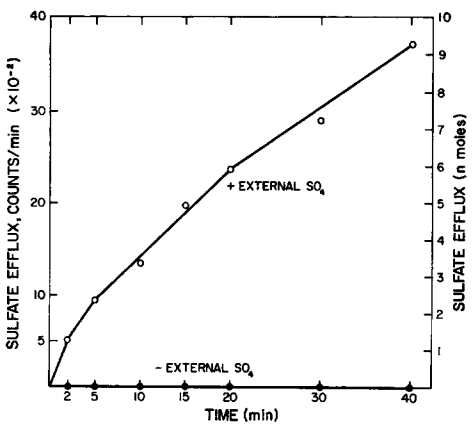


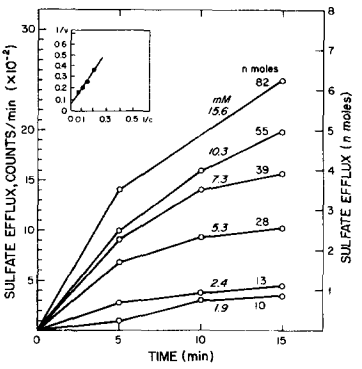
Fig. 3. Time dependence of sulfate efflux. Wild-type cells were preloaded with radioactive sulfate to an intracellular pool of 29.2 nmoles (11 700 cpm) per 2-ml sample. The washed cells were resuspended in minimal medium, when non-radioactive sulfate (2 mM) was added to one series while a second series received no addition. Efflux of the radioactive sulfate was then determined for 0–40 min. All values were corrected for the counts appearing in the medium at zero time.

sulfate, efflux of radioactive sulfate continued throughout the period studied (40 min), although the rate of its exit was not linear for the entire time. After 30 min, approximately 20% of the original intracellular pool of sulfate (20 nmoles) was found to have exited from the cells. The dependence of efflux upon time suggests that true exit from an intracellular sulfate pool is being observed rather than an exchange with externally bound ion. In the absence of external sulfate, either efflux does not take place or else any sulfate lost from the cells is immediately recaptured.

Dependence of efflux upon pool size

It was of interest to determine whether the rate of efflux was dependent upon the intracellular concentration of inorganic sulfate. To create different intracellular pool sizes, *cys-11* mycelia were preloaded for various times with radioactive sulfate. The use of the *cys-11* mutant insures that the entire intracellular pool exists as inorganic sulfate since this strain lacks ATP sulfurylase activity [1]. It is clear that the rate of efflux was strongly dependent upon the intracellular sulfate concentration and that saturation of the efflux system was not achieved even with the greatest pool size (Fig. 4).

These data permitted an estimation of the K_m for efflux; it was determined to be approximately 20 mM from a standard Lineweaver–Burk plot of the initial efflux values. Thus, the K_m for efflux is much greater, approximately 2000 times, than the K_m for sulfate uptake (0.01 mM).



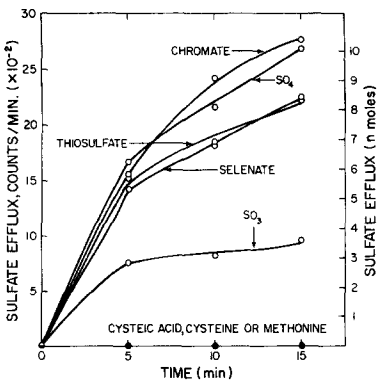


Fig. 4. Efflux of sulfate as a function of intracellular sulfate concentration. *cys-11* mycelia were preloaded to different levels by incubation with 0.1 mM $^{35}SO_4^{2-}$ for various times (0–30 min). The washed cells were resuspended in minimal medium and incubated for 5 min when non-radioactive sulfate (2 mM) was added to initiate efflux. Efflux was then determined by removing 2-ml samples at 0, 5, 10, and 15 min and immediately separating the cells from the medium. Each 2-ml sample contained 2.1 mg dry weight of mycelia. The intracellular sulfate pool in nmoles is indicated by each curve and the corresponding intracellular sulfate concentration (mM) is also given. The insert shows a Lineweaver–Burk plot of sulfate efflux^{-1} versus intracellular sulfate concentration^{-1}.

Fig. 5. Substitution of other ions for external sulfate in $^{35}SO_4^{2-}$ efflux. *cys-11* mycelia were preloaded to an intracellular concentration of 15 mM radioactive sulfate (152 nmoles per 4 mg dry weight of mycelia in 2-ml samples). The washed cells were resuspended in minimal medium and incubated for 5 min when the indicated compounds were added to a final concentration of 1 mM. Sulfate efflux was then determined with 2-ml samples at the indicated times.

Dependence of efflux upon external sulfate concentration

Efflux of sulfate also depended upon the concentration of extracellular sulfate (Table I). A maximum efflux rate was observed with 1 mM extracellular sulfate and about 50% of the optimal efflux was found when the external sulfate concentration was only 0.05 mM. However, no efflux was detected in the absence of external sulfate as noted before. These results suggest that the uptake system for sulfate is involved in the efflux of the ion since external sulfate is required for efflux, but the necessary

TABLE I

DEPENDENCE OF EFFLUX UPON EXTERNAL SULFATE CONCENTRATION

cys-11 mycelia were preloaded with radioactive sulfate to an intracellular concentration of 7.6 mM (50 nmoles of sulfate per sample of 2.6 mg dry weight). The washed cells were resuspended in minimal medium and efflux was initiated by the addition of the indicated amount of non-radioactive sulfate. Efflux was determined as described in Materials and Methods.

External sulfate concn (mM)	5 min sulfate efflux		Percent of optimal efflux
	cpm	nmoles	
0	0	0	0
0.05	600	1.50	51
0.10	732	1.83	62
0.2	914	2.29	77
0.5	1014	2.54	86
1.0	1187	2.97	100
2.0	1156	2.89	97

concentration is very low, in the range of the K_m for sulfate transport. Lower concentrations of external sulfate were not studied since efflux of the labeled ion would be followed by immediate re-entry under such conditions.

Substitution for external sulfate

Several other ions can substitute for external sulfate for the efflux of $^{35}SO_4^{2-}$ from an intracellular pool (Fig. 5). Chromate, selenate, and thiosulfate all promote sulfate efflux and to about the same extent as does sulfate itself. Sulfite, which inhibits sulfate transport [1] and thus must somehow interact with sulfate permease, permits efflux at about 50% of the rate observed with external sulfate. The sulfur amino acids, cysteine, methionine, and cysteic acid, are all completely ineffective in promoting efflux.

TABLE II

REVERSAL OF PCMB INHIBITION OF MYCELIAL SULFATE TRANSPORT BY DITHIOTHREITOL

Samples of *cys-11* mycelia were incubated with PCMB ($2 \cdot 10^{-4}$ M) for 5 min, when duplicate samples were assayed for sulfate transport. After incubating with PCMB for 5 min, a second series was then incubated with dithiothreitol (final concentration 25 mM) for 2 additional min before assaying sulfate transport. The average results of two experiments are given. Preincubation with PCMB for 30 min caused 98 % inhibition of sulfate transport.

Sample	Sulfate transported		Percent inhibition
	cpm	nmoles	
Control	2948	11.1	0
+PCMB	261	1.0	91
+dithiothreitol	2622	9.9	11
+PCMB+dithiothreitol	1650	6.2	44

PCMB inhibition of sulfate uptake and efflux

Incubation of mycelia with 0.2 mM *p*-chloromercuribenzoate (PCMB) for 5–30 min inhibited sulfate transport by 90–98%. This result suggests that the sulfate transport system possesses an essential sulfhydryl group. This possibility is supported by the finding that the inhibition caused by PCMB could be largely reversed by dithiothreitol (Table II). When preloaded cells were similarly treated with PCMB for 10, 20, and 30 min, sulfate efflux was found to be inhibited by 34, 37, and 57%, respectively.

DISCUSSION

The finding that the rate of sulfate efflux depended upon the internal pool size supports the idea that true efflux from an intracellular pool was being detected. The alternative possibility that a release of externally bound sulfate is taking place seems unlikely since the amount of external binding would be expected to be similar after incubation of cells with sulfate regardless of the size of the internal pool created by uptake. Sulfate efflux apparently occurs by way of the sulfate transport system. This possibility is supported by the finding that extracellular sulfate is required for efflux, particularly since only low concentrations, in the range of the K_m for uptake, are needed. The external sulfate required for efflux can be replaced by chromate, selenate, or thiosulfate, all ions which are also probably transported by the uptake system for sulfate. We have previously shown [10] that chromate is transported by the sulfate permease system of *Neurospora* and Tweedie and Segel [13] have demonstrated that thiosulfate, selenate, and molybdate all enter via the sulfate transport system in *Penicillium* and *Aspergillus* species. Furthermore, both the uptake and the efflux of sulfate was found to be inhibited by PCMB. The results imply that efflux actually occurs by way of an exchange reaction, in which the exit of sulfate is accompanied by the entry of either sulfate or a related ion.

Some efflux of sulfate can be detected in azide-treated cells in the absence of external sulfate although a more rapid exit occurs when sulfate is present. It appears that considerable transmembrane movement of sulfate occurs in the presence of azide, both influx and exit, but that its net uptake is abolished. When azide is added to cells in the process of sulfate accumulation, further uptake is immediately inhibited although no loss from the internal pool is observed. Apparently, under these conditions the exit of a sulfate ion is coupled to the entrance of another so that no net change occurs.

Sulfate efflux was not detected in previous experiments [1, 9, 10], presumably because its magnitude is low until the intracellular sulfate concentration is appreciable. The apparent K_m for sulfate for its efflux from cells is approximately 2000 times greater than that for uptake. Such a relationship permits the creation of a considerable intracellular sulfate pool before any efflux becomes significant. When the internal pool reaches a certain size, efflux could then act to decrease and eventually to prevent further accumulation. It thus appears that efflux may constitute still another mechanism to control the size of the intracellular sulfate pool and thereby contribute to the complex regulation of sulfate assimilation in *Neurospora*

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