ACCUMULATION OF INTRACELLULAR INORGANIC SULFATE BY *PENICILLIUM CHRYSOGENUM*[1]

IRWIN H. SEGEL AND MARVIN J. JOHNSON

Department of Biochemistry, University of Wisconsin, Madison, Wisconsin

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The cell membranes of microorganisms appear to be rather complex, metabolically active entities, equipped with many specific active transport mechanisms which mediate the uptake and accumulation of a variety of compounds.

Bacteria have been shown to accumulate amino acids (Cohen and Rickenberg, 1956; Gale, 1947; 1953), carbohydrates (Cohen and Monod, 1957; Rickenberg et al., 1956), and inorganic phosphate (Mitchell and Moyle, 1953; Mitchell, 1954) against apparent concentration gradients. Still more recent reports suggest the presence of specific permease systems for acetylornithine (Vogel, 1960) and citrate (Waterbury and MacDonald, 1960) in bacteria. Except for the recent studies of the galactoside permease system, very little is understood about the mechanism of these active transport systems.

This paper presents evidence for a sulfate-transfer mechanism in strains of *Penicillium chrysogenum*. One high penicillin-yielding mutant, strain 51-20F3, has been shown to be able to accumulate inorganic sulfate within its mycelium to a concentration of 25 to 35 mM.

MATERIALS AND METHODS

Fermentation techniques. The strains of *P. chrysogenum* were maintained in the laboratory as soil stocks. Spore plates were prepared by sprinkling some of the soil onto sterile sporulation medium contained in a narrow-mouth, 6-ounce rectangular bottle and incubating about 2 weeks at 25 C. The sporulation medium contained, per liter: honey, 60 g; peptone, 10 g; and agar, 25 g. Spores from one or two plates were suspended in 5 to 10 ml of distilled water and used to inoculate 100 ml of sterile glucose-lactate-$CaCO_3$ inoculum medium (Tardrew and Johnson, 1958) contained in a 500-ml Erlenmeyer flask. The inoculum was grown for 45 to 48 hr at 25 C on a rotary shaker operating at a speed of 250 rpm and describing a 2-in. circle. After 45 to 48 hr, 10 ml of the resulting dense mycelial suspension were used to inoculate 100 ml of the lactose-glucose-lactate fermentation medium (Tardrew and Johnson, 1958) contained in a 500-ml Erlenmeyer flask. Nonradioactive Na_2SO_4 (usually 100 mg per 110 ml fermentation) and carrier-free $S^{35}O_4^{=}$ (usually about 1 mc) were added to the mineral portion of the fermentation medium prior to sterilization. All fermentations were conducted at 25 C as described above. No penicillin R-group precursor was added to any of the fermentations.

Analytical procedures. The distribution of sulfur in the fermentations was determined in the following way. At various times during the fermentation, 10 to 20 ml of the mycelial suspension were removed aseptically, the pH determined immediately, and the mycelium separated from the medium by suction filtration through coarse filter paper. The filtrate (cell-free medium) was refiltered if necessary, through fine filter paper, to remove any suspended matter, diluted (usually 50- to 100-fold), and the radioactivity determined as described below. The mycelial pad was washed twice while still in the suction funnel by suspending it in approximately 25 ml of distilled water and sucking dry rapidly. The mycelium was then removed, blotted to remove any excess extracellular water, and divided into two approximately equal portions. Each portion was weighed immediately, before any significant loss of intracellular water occurred. One of the portions was then dried by heating overnight at 98 C. The second portion (0.3 to 0.7 g wet weight) was extracted by dropping it into 20 to 40 ml of boiling distilled water and dispersing with a stirring rod for 30 to 45 sec. The water was kept at the boiling point throughout the extraction. After 30 to 45 sec, the suspension was cooled slightly by the addition of room-temperature

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distilled water, filtered, the insoluble residue washed, and the extract and washings made up to volume (usually 100 ml). Total S^{35} was determined in both the medium and extract by diluting a 1.0-ml portion of the diluted sample with 1.0 ml of 2 per cent gelatin (made up in 0.02 N NaOH) and pipetting exactly 1.0 ml of the resulting solution onto a round 5-cm^2 copper planchet. After the planchet had dried, the radioactivity was counted with a Geiger-Mueller thin-window counter. Organic S^{35} in the diluted samples was determined after precipitating the inorganic sulfate. The sulfate was precipitated in the following manner: to a 5.0-ml sample of the diluted samples, 2.5 ml of 0.02 M nonradioactive Na_2SO_4 (pH about 2) as carrier, and 2.5 ml of 0.10 M $BaCl_2$ (pH about 2) were added. The tube was loosely stoppered and placed in a boiling water bath for about 3 to 5 min. After cooling, the contents were filtered through Whatman no. 42 filter paper. A 1-ml portion of the sulfate-free filtrate was diluted with 1.0 ml of gelatin solution and counted as described above. Inorganic $S^{35}O_4^=$ was calculated as the difference between the total and organic S^{35}. It was assumed that the precipitation procedure introduced an additional dilution factor of 2; any volume changes occurring during the precipitation were negligible. Under the above conditions, only heat- and acid-stable, barium-soluble organic sulfur was determined. In all cases the final count was corrected for background, coincidence, and self-absorption. From the amount of S^{35} counted in the extracts and the amount of intracellular water present in the mycelium as determined by drying, the intracellular concentration of inorganic and organic S^{35} could be calculated (counts:min:ml intracellular water). In all fermentations, a zero-time sample of the medium was taken. Since the amount of nonradioactive Na_2SO_4 added to each flask at the beginning of the fermentation was known, the specific activity of the S^{35} could be calculated. Consequently, the actual concentrations (μmoles per ml) of organic and inorganic sulfur in the medium and in the intracellular soluble fraction of the mycelium could be calculated.

Labeled methionine - S^{35}. Radioactive methionine-S^{35} was isolated by column chromatography on cellulose powder from a 6 N HCl hydrolyzate of strain 51-20F3 mycelium grown on $Na_2S^{35}O_4$ (50 mg Na_2SO_4 and 39 $\times$ 10^8 counts/min of $S^{35}O_4^=$ per flask) as the sole sulfur source. The final product, after recrystallization with carrier nonradioactive methionine had a constant specific activity of 3.18 $\times$ 10^5 counts:min:mg and showed a single ninhydrin- and S^{35}-positive spot coinciding with that of pure methionine on paper chromatograms.

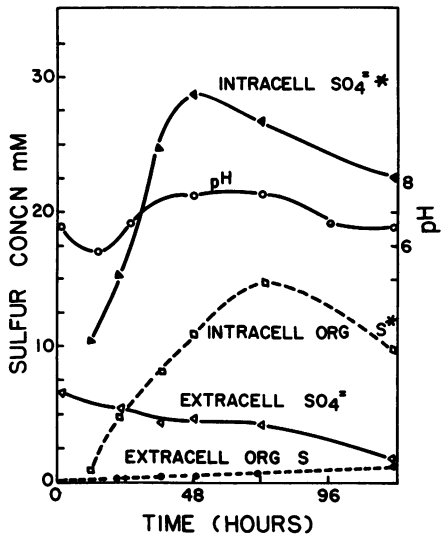

Figure 1. Distribution and concentration of sulfur in fermentations by *Penicillium chrysogenum* strain 51-20F3 (high penicillin-yielding strain). Pooled results of three fermentations. Mycelium grown on defined medium.

* Intracellular sulfur refers to sulfur extracted from mycelium with boiling water.

RESULTS

Sulfur distribution in fermentations. Figure 1 shows the distribution and concentrations of sulfur in a typical strain 51-20F3 fermentation. In all cases, the term "intracellular" sulfur refers to the soluble mycelial sulfur which can be extracted by the boiling water. The values for the intracellular concentrations were based on the assumption that all the water determined by drying was intracellular. This amount consistently ran between 78 and 80 per cent of the blotted mycelium. The assumption is conservative because certainly some extracellular water remained after blotting. However, since all of the mycelium samples were blotted to the same degree of "wetness" before drying ($\pm$ 1 to 2 per cent), a quantitative comparison can be made between strains and samples. It can be seen from

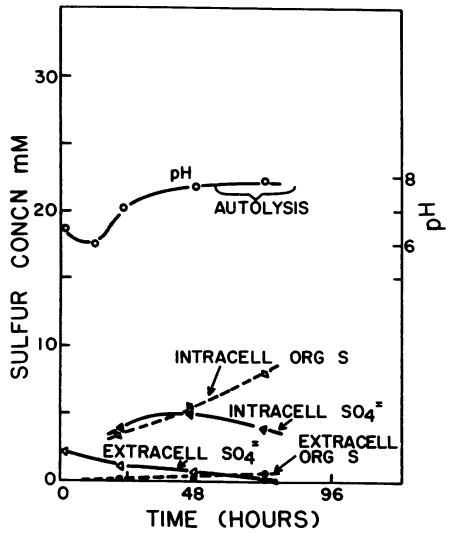

Figure 2. Distribution and concentration of sulfur in fermentations by *Penicillium chrysogenum* strain NRRL 1951-B25 (low penicillin-yielding strain), at low (2.5 mM) initial inorganic sulfate level.

figure 1 that strain 51-20F3 rapidly concentrated inorganic sulfate within the mycelium during the initial growth phase to a level 5 to 6 times that in the extracellular medium. From 48 to 120 hr (during the penicillin-producing phase), the intracellular sulfate level decreased slightly with time but was maintained well above the extracellular inorganic sulfate concentration. Further experiments showed that the intracellular level would drop sharply when the medium became depleted of inorganic sulfate. When the low penicillin-yielding strain NRRL 1951-B25 was cultured under the same conditions in the high sulfate medium (6.9 mM), the intracellular sulfate concentration did not rise above that in the medium, suggesting that in this case the sulfate-transfer mechanism is either absent or not as active as that in strain 51-20F3. The low yielding strain could not be cultured for more than 24 to 48 hr on the usual fermentation medium because of the rise in pH resulting in subsequent autolysis of the mycelium. To determine whether strain NRRL 1951-B25 could concentrate inorganic sulfate against a concentration gradient, a fermentation was conducted in which the initial sulfate concentration in the medium was decreased to 2.5 mM, or about one-half of the previously determined maximal intracellular concentration. Figure 2 summarizes the results. It can be seen that under these conditions, strain NRRL 1951-B25 accumulated inorganic sulfate to about the same extent (about 5 mM) as when the high sulfate medium was employed. It appears then, that both strains of *P. chrysogenum* are able to concentrate inorganic sulfate against a concentration gradient, i.e., some mechanism exists for the transport and retention of sulfate. The level of intracellular sulfate attained however, is much higher in the high penicillin-yielding strain 51-20F3. It was interesting to note that of the total organic sulfur in the mycelium of 51-20F3, about 40 per cent was present as intracellular, soluble, extractable sulfur.

Other strains of P. chrysogenum. Tardrew and Johnson (1958) showed that the high penicillin-yielding strains of *P. chrysogenum* generally (i) utilized inorganic sulfate at a faster rate and (ii) excreted into the medium more organic sulfur compounds (in addition to penicillin) compared to the low penicillin-yielding strains. It was considered worth while to determine whether the ability to accumulate inorganic sulfate was also common to all the high yielding strains. Fermentations were conducted with several strains of *P. chrysogenum.* Inorganic $S^{35}O_4^-$ (initial concentration 6.63 mM) was the sole sulfur source. The intracellular sulfur distribution and concentrations were determined as previously described. The results are shown in table 1. It can be seen that of all the strains tested, only strain 51-20F3 accumulated intracellular sulfate to any great extent. The other high penicillin-yielding strains (Q-176 and 49-133) did not accumulate inorganic sulfate to any higher concentrations than the low (NRRL 1951-B25) and intermediate (X-1612) strains.

Isotopic competition experiments. Because of the unusually high internal concentrations of sulfate observed with strain 51-20F3, it was considered possible that much of the intracellular sulfate may not be truly intracellular at all, but rather, residual sulfate from the medium occluded with or adsorbed on the mycelium, and that the two rapid washes given the mycelium prior to extraction did not remove all of the extracellular sulfate. Mechanical occlusion could be ruled out immediately since the amount of medium that would have to be occluded by the mycelium to yield such high results would be greater than the total amount of water in the

TABLE 1

Intracellular sulfur concentrations in several strains of Penicillium chrysogenum[a]

Strain	Geneological Sequence[b]	Peak Penicillin Production[c]	Intracellular Sulfur Concn			
			Inorganic		Organic	
			24 hr	48 hr	24 hr	48 hr
		units/ml	*mM*	*mM*	*mM*	*mM*
NRRL 1951-B25	Parent strain	45	5.5	—[d]	4.4	—[d]
X-1612	X (1)	90	2.2	0.00	5.3	5.1
Q-176	UV (1)	230	3.3	2.1	5.0	6.5
49-133	UV, S, NM (4)	290	5.4	2.6	5.7	8.2
51-20F3	NM, S (5)	260	24.9	22.0	7.6	8.2

[a] Grown on defined medium without penicillin R-group precursor. Initial sulfate concentration was 6.63 mM for all fermentations.

[b] Backus and Stauffer (1955). Each strain was derived from the preceding by treatment with X-rays (X), Ultraviolet radiation (UV), nitrogen mustards (NM) or by spontaneous mutation and selection (S). Numbers in parentheses refer to number of known successive mutations between strains.

[c] On defined medium without penicillin R-group precursor.

[d] The pH was 8.2—autolysis of mycelium.

sample. Physical adsorption of the extracellular sulfate could not be immediately discounted. To determine if any of the intracellular sulfate was really adsorbed sulfate, the following experiment was performed. A series of eight 110-ml fermentations were conducted as previously described. Each flask contained 6.9 mM $Na_2S^{35}O_4$ of the same specific activity. Seven of the flasks contained in addition to the sulfate, 6.9 mM sulfur-32 in one of the following forms: Na_2SO_3 $Na_2S_2O_3$, L-cysteic acid, taurine, L-cysteine·HCl, cystine, or L-methionine. The fermentations were sampled periodically as described and the apparent concentrations of sulfur in the intracellular extractable inorganic and organic fractions determined. The total yield of mycelium was approximately the same for each flask (about 2.5 g dry weight at 120 hr). The pH of each fermentation differed by no more than a few tenths of a unit at comparable times; those flasks containing the added sulfur amino acids generally ran at a slightly lower pH. The results obtained for the apparent intracellular sulfur distribution and concentrations at 48 hr are shown in table 2. The results at 24, 72, and 96 hr were essentially the same as those shown for 48 hr. If the sulfate observed as intracellular was adsorbed extracellular sulfate, then all eight flasks which contained the same amount of $Na_2S^{35}O_4$ and were washed and extracted identically should have yielded the same results,

TABLE 2

Isotopic competition: effect of unlabeled sulfur-containing compounds on amount of intracellular extractable sulfur in mycelium of strain 51-20F3[a]

Flask No.	S^{32} Compound Added	Apparent Intracellular Sulfur Concn[b]	
		Inorganic	Organic
		mM	*mM*
1	None[c]	32.0	10.0
2	Na_2SO_3	13.5	6.3
3	$Na_2S_2O_3$	15.7	8.6
4	L-Cysteic acid	27.0	6.8
5	Taurine	21.9	7.0
6	L-Cystine	11.3	2.3
7	L-Cysteine·HCl	7.5	2.8
8	DL-Methionine	7.4	3.8

[a] Grown on defined medium with the above additions. Each flask contained 6.9 mM $Na_2S^{35}O_4$ of the same specific activity. Flasks 2 to 8 contained in addition, 6.9 mM S^{32} in one of the indicated forms.

[b] Average results of two fermentations. Results based on the assumption that all of the intracellular sulfur had the same specific activity as the $Na_2S^{35}O_4$ added to each flask.

[c] No additions, 6.9 mM $Na_2S^{35}O_4$ alone.

However, it can be seen that either methionine, cysteine, or cystine, when present in equimolar amounts with sulfate, apparently suppressed the intracellular sulfate concentration by about 65

to 80 per cent. Sulfite and thiosulfate caused an apparent suppression of about 50 per cent, suggesting that they are just as available to the mold as a sulfur source as sulfate, or, more likely, in the case of sulfite, that it was oxidized to sulfate during the sterilization and incubation. Cysteic acid and taurine produced only a 15 to 30 per cent apparent suppression. (The values for the intracellular sulfur concentrations were calculated from the specific activity of the $Na_2S^{35}O_4$ added to each flask at zero time.) If the intracellular sulfate was in reality adsorbed extracellular sulfate and the organic sulfur compounds added had any effect on the adsorption, then one would expect cysteic acid and taurine to compete with sulfate adsorption to a greater extent than either cysteine or methionine since the former two compounds resemble sulfate ionically (—SO_3H) more closely than either cysteine (—SH) or methionine (—S—CH_3). (The mycelium would grow equally well on any of the added sulfur compounds alone.) It may be concluded then that the high levels of intracellular sulfate actually exist and are not artifacts of the experimental procedure.

Figure 3 shows the effect of methionine added at various times during the fermentation on the apparent total intracellular sulfur concentration in strain 51-20F3. It was interesting to note that the addition of methionine to a concentration of 6.9 mM at 34 or 53 hr to fermentations, grown initially on $Na_2S^{35}O_4$ as the sole sulfur source, caused a rapid depression of intracellular sulfur, even after the usual maximal level had been reached. The intracellular sulfur level approached that found when methionine was added to the fermentation at zero time. The values for the intracellular concentrations of sulfur were based on the specific activity of the $Na_2S^{35}O_4$ added to the medium at zero time.

Reciprocal labeling experiments. Three possible explanations for the apparent depression of sulfate accumulation by mycelium of strain 51-20F3 growing in the presence of the more reduced forms of organic sulfur are: (i) the organic sulfur partially or completely suppressed the sulfate-transport mechanism, (ii) the organic sulfur was oxidized intracellularly to sulfate and whereas the actual intracellular concentrations of sulfate may or may not have been the same as in the control mycelium (grown on sulfate alone), the specific activity of the intracellular

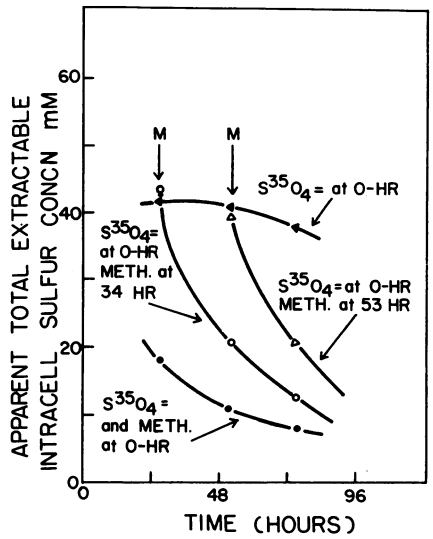

Figure 3. Effect of methionine added at various times during the fermentation on the apparent total intracellular extractable sulfur concentration in mycelium of strain 51-20F3. Concentrations were based on the assumption that all of the intracellular sulfur had the same specific activity as the $Na_2S^{35}O_4$ present at zero time. *M* indicates time at which methionine-S^{32} was added to a concentration of 6.9 mM. Initial $Na_2S^{35}O_4$ concentration was 6.9 mM in all fermentations.

sulfate was decreased by dilution. Consequently, calculations of the intracellular sulfate concentration based on the specific activity of the $S^{35}O_4^-$ originally added to the medium would yield low results, (iii) both of the above phenomena are occurring. To distinguish between these possibilities, four fermentations were conducted. One flask contained initially 6.9 mM $Na_2S^{35}O_4$, a second flask contained 6.9 mM methionine-S^{35}, a third 6.9 mM $Na_2S^{32}O_4$ plus 6.9 mM methionine-S^{35}, and the fourth 6.9 mM $Na_2S^{35}O_4$ plus 6.9 mM methionine-S^{32}. Mycelium samples were taken at 24, 48, and 72 hr and analyzed as previously described. The results are shown in figure 4. Calculations of intracellular sulfate concentrations were based on the specific activity of the radioactive sulfur source. It is apparent that the mold is able to oxidize the methionine to sulfate and accumulate this sulfate against a concentration gradient (curves B and C). However, the concentration of inorganic intracellular sulfate does not rise over about 10 mM. The amount of $S^{35}O_4^-$ produced from methionine-S^{35}

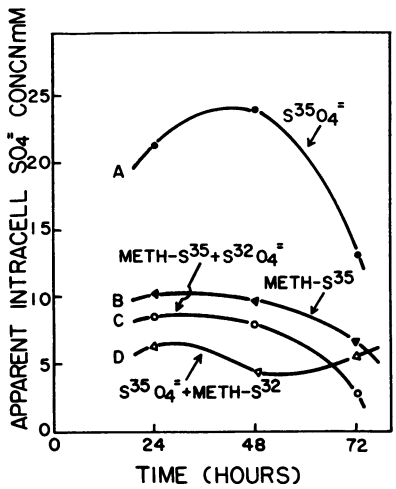

Figure 4. Apparent intracellular sulfate concentration in mycelium of strain 51-20F3 grown in the presence of different sulfur sources. Medium *A* contained 6.9 mM $Na_2S^{35}O_4$ alone. Medium *B* contained 6.9 mM methionine-S^{35} alone. Medium *C* contained 6.9 mM methionine-S^{35} plus 6.9 mM $Na_2S^{32}O_4$. Medium *D* contained 6.9 mM $Na_2S^{35}O_4$ plus 6.9 mM methionine-S^{32}. Concentrations were based on the assumption that all of the intracellular sulfate had the same specific activity as the radioactive sulfur source added at zero time.

never rises over about 1.5 mM in the medium. The total intracellular sulfate derived from both extracellular sulfate and methionine (sum of curves C and D) is less than the concentration of intracellular sulfate achieved when only inorganic sulfate is present in the medium. It appears, that hypothesis (iii) is the more likely, i.e., when both inorganic sulfate and methionine are present in the medium in substrate amounts, the mold will oxidize intracellularly a portion of the methionine, but will not transport and accumulate inorganic sulfate from the medium to any great extent. It was interesting to see to what extent the extracellular methionine-S^{32} concentration could be reduced and still affect the transport of inorganic sulfate. Table 3 summarizes the results. It can be seen that as the concentration of added methionine is reduced while maintaining the high (6.63 mM) extracellular inorganic sulfate concentration, the effect on sulfate transport and utilization is decreased. At the 6.2 mM level of added methionine, very little $S^{35}O_4^-$ from the medium is used over the 48-hr fermentation

TABLE 3

Apparent sulfur distribution during fermentations of strain 51-20F3 on defined media containing different ratios of methionine-S^{32} to $Na_2S^{35}O_4$

Age of Culture	Initial Methionine-S^{32} Concn	Initial $Na_2S^{35}O_4$ Concn	Apparent Sulfur Distribution[a]			
			Medium		Intracellular	
			Inorganic	Organic	Inorganic	Organic
hr	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*
24	6.2	6.6	6.2	0.02	6.7	0.7
	3.0	6.6	5.6	0.05	10.3	2.0
	0.89	6.6	5.1	0.05	15.1	3.1
	0.30	6.6	5.0	0.13	28.1	5.7
	0.00	6.6	5.4	0.20	24.1	8.5
	6.2	1.8			3.0	0.6
48	6.2	6.6	6.2	0.07	5.1	1.4
	3.0	6.6	5.8	0.11	17.1	2.8
	0.89	6.6	4.6	0.27	22.6	4.3
	0.30	6.6	4.3	0.32	23.5	5.3
	0.00	6.6	5.0	0.53	21.7	7.7
	6.2	1.8			4.2	0.9

[a] Concentrations based on specific activity of $Na_2S^{35}O_4$ as described in table 2.

period, and the apparent intracellular $S^{35}O_4^-$ concentration never rises above 6.7 mM. As the concentration of added methionine is reduced or decreased by the metabolism of the mold, the utilization and apparent accumulation of $S^{35}O_4^-$ increases. At the 0.3 mM level of added methionine, no depression of sulfate utilization and accumulation can be noticed. As also shown in table 3, when the mycelium was grown in a medium containing 6.2 mM methionine-S^{32} and an initial $S^{35}O_4^-$ concentration of 1.83 mM, the apparent intracellular $S^{35}O_4^-$ concentration still rose to 3 to 4 mM. Thus it appears that the sulfate transport mechanism is suppressed by the methionine but not completely inhibited.

Chemical nature of the intracellular sulfate. It was considered possible that much, if not all, of the intracellular sulfate determined by the method outlined may not be free ionic sulfate but rather, some labile organic compound which gave rise to sulfate during the extraction and precipitation. To test this hypothesis, the methods of extraction and precipitation were varied. Overnight extractions of mycelium of strain 51-20F3 with cold (4 C) 5 per cent trichloroacetic

acid or with cold (4 C) 70 per cent aqueous ethanol and precipitations with neutral carrier sulfate and neutral $BaCl_2$ at 4 C or at room temperature did not show any significant differences from the boiling water-acid precipitation method. The intracellular sulfate then appears to be free ionic sulfate, although the possibility of a very labile organic covalent sulfate or ionic sulfate bound to some high molecular weight, nondiffusible molecule cannot be disregarded.

DISCUSSION

All of the high penicillin-producing strains tested thus far have in common the ability to metabolize inorganic sulfate and excrete organic sulfur to a much greater extent than their low yielding ancestors. However, of all the strains tested, only strain 51-20F3 possesses the ability to accumulate intracellularly inorganic sulfate to an unusually high level. Since strain 51-20F3 is the latest in the line of mutations descended from strain NRRL 1951-B25, it appears that this characteristic is a recent mutation. It is probable that all of the strains of *P. chrysogenum* tested possess an identical mechanism for the transport and accumulation of inorganic sulfate. The enhanced ability of strain 51-20F3 may be due to the loss, by mutation, of some specific control mechanism that limits the operation of the transport mechanism, rather than a mutation that caused an actual acquisition of a new or enhanced mechanism. The fact that methionine and cysteine appear to suppress sulfate uptake suggests that the compound that controls the sulfate transport mechanism can be easily synthesized by strain 51-20F3 from the sulfur amino acids, but not from sulfate. Since low levels of added methionine cannot suppress sulfate uptake and since 51-20F3 must be able to synthesize methionine from inorganic sulfate (it can use inorganic sulfate as the sole sulfur source), it is likely that the compound that exerts the negative feedback must be present in fairly high concentrations to be effective. In all of the other strains tested, the control compound is probably easily synthesized from sulfate directly. The enhanced ability of strain 51-20F3 to produce penicillin on natural media or on media in which the penicillin R-group precursor is not the limiting factor may be a reflection of the same mutation.

SUMMARY

The intracellular distribution and concentration of sulfur-35 was investigated in the mycelium of several related strains of *Penicillium chrysogenum* grown on $Na_2S^{35}O_4$ as the sole sulfur source. It was found that a high penicillin-producing strain (51-20F3) could concentrate inorganic sulfate within its mycelium to a level of 25 to 35 mM (five to six times that in the extracellular medium). Other high and low penicillin-producing strains could concentrate inorganic sulfate only to a level of about 5 mM. The enhanced ability of strain 51-20F3 to accumulate inorganic sulfate may be a reflection of the same mutation that resulted in the ability of this strain to produce increased yields of penicillin on media containing R-group precursor. The mutation probably resulted in the loss of a specific control mechanism that limits the operation of the sulfate-transfer mechanism. The accumulated sulfate appears to be free ionic sulfate. It was observed that substrate amounts of methionine or cysteine inhibited the sulfate transfer mechanism. A possible explanation for the effects of the sulfur amino acids is presented.

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