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Regulation of Choline Sulphatase Synthesis and Activity in *Aspergillus nidulans*

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1. Choline *O*-sulphate is taken up from the growth medium to the same extent by sulphur-deficient and sulphur-sufficient mycelia of *Aspergillus nidulans*, but hydrolysis of the transported sulphate ester *in vivo* only occurs in the sulphur-deficient mycelia. 2. Choline sulphatase activity could not be detected *in vitro* in sulphur-sufficient mycelia of wild-type and sulphur mutants of *A. nidulans*, but after sulphur starvation all strains showed appreciable activity of this enzyme. 3. Optimum activity of choline sulphatase in an ultrasonically treated preparation of sulphur-deficient mycelia was at pH 7·5. The optimum substrate concentration was in excess of 25mM and K_m was 0·035M. The enzyme was completely inhibited by 10mM-SO_3^{2-}, PO_4^{3-}, CN^- and cysteine. 4. Growth of sulphur-deficient mycelia on various sulphur sources resulted in a decrease of choline sulphatase activity *in vitro*. The decrease appeared to be due to a repression of choline sulphatase synthesis rather than to inhibition of activity. De-repression by growth on a sulphur-deficient medium was prevented by cycloheximide. Unlike the choline sulphatase of bacteria the fungal enzyme did not need to be substrate-induced. 5. By using sulphur mutants the identity of the co-repressor was limited to $S_2O_3^{2-}$, cysteine-*S*-sulphonate, cysteine or compounds derived directly from them. Circumstantial evidence suggests that the co-repressor is cysteine. 6. Inhibition of choline sulphatase activity *in vivo* was demonstrated with cysteine as the sulphur source for growth.

Choline *O*-sulphate is thought to act as a store of sulphur in the higher fungi (Spencer & Harada, 1960), but the mechanism of its utilization has been in some doubt. Hydrolysis by a sulphatase, to give inorganic sulphate, which would then be reduced to cysteine, seemed the most feasible mechanism, but previous authors had failed to detect a choline sulphatase in *Alternaria* (Itahashi, 1961), *Penicillium chrysogenum* (Ballio *et al.* 1960) and disrupted germinating conidiospores of *Aspergillus niger* (Takebe, 1961), all of which fungi can synthesize choline sulphate. An inducible choline sulphatase has been reported in *Pseudomonas nitroreducences* (Takebe, 1961) and *Pseudomonas aeruginosa* (Harada, 1964), but neither of these bacteria can synthesize choline sulphate. With choline [^{35}S]sulphate of very high specific activity, weak choline sulphatase activity was detected in the choline sulphate-producing fungi *Aspergillus sydowi* and *P. chrysogenum*, but the physiological significance of such low activity was uncertain (Segel & Johnson, 1963*a*).

The various attempts to demonstrate choline sulphatases in fungi have been carried out with sulphur-sufficient mycelia, whereas it seemed reasonable to suppose that fungi might only utilize their stored choline sulphate during sulphur deficiency. Preliminary studies on sulphur-starved mycelia of *Aspergillus nidulans* revealed considerable choline sulphatase activity. By using this technique choline sulphatase was demonstrated in a number of mutants of *A. nidulans* and the key role of this enzyme in the utilization of the sulphur of choline sulphate was established (Spencer, Hussey, Orsi & Scott, 1968).

In the present study the factors that regulate the activity and synthesis of choline sulphatase were investigated. A preliminary account of this work has appeared (Scott & Spencer, 1965).

EXPERIMENTAL

Organisms. A. nidulans A69 (wild-type) [C.M.I. 38576 (ii)], and mutants *gamma* (C.M.I. 38581S), blocked at ATP sulphurylase, *zeta* (C.M.I. 38584S), blocked between SO_3^{2-} and $S_2O_3^{2-}$, and *eta* (C.M.I. 38595S), blocked at adenosine 3′-phosphate 5′-sulphatophosphate reductase, were those described in the preceding paper (Spencer *et al.* 1968). *A. nidulans* mutant *bimeth*2, which required both biotin

and methionine for growth, was kindly supplied by Professor J. A. Roper.

Culture conditions. Inoculations (10^7 spores/l. of medium) were carried out with spore suspensions washed from potato–glucose–agar slopes (Difco) with sterile water. Fungi were grown at 28° with aeration in 5l. or 10l. flasks containing 3l. or 8l. respectively of a sulphur-sufficient medium. After growth for 5 days, the fungi were harvested by filtration through sterile muslin and washed thoroughly with sterile distilled water. The mycelium was pressed between muslin and then between filter papers until all excess of fluid had been removed. The pressed mycelium was transferred to the same volume of a sulphur-deficient medium as that used initially and incubated under the same conditions. Samples of the mycelium were removed at intervals for choline sulphatase determination. After 36hr. growth the remaining mycelium was harvested and pressed as before. To study the effects of various sulphur compounds or further sulphur starvation, 1·5g. portions of the pressed sulphur-starved mycelium were reincubated in 125ml. of the appropriate medium contained in 250ml. conical flasks. Incubation was carried out at 28° with shaking at 100 oscillations/min. (Gyrotory incubator shaker; New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). Fungi from these flasks were harvested after different periods of growth, washed, pressed and used for enzyme assay. Contamination during these procedures occurred infrequently.

The basic medium used in this study was a modified Czapek–Dox liquid medium which contained: glucose, 50g.; trisodium citrate, 5g.; $NaNO_3$, 2g.; KH_2PO_4, 0·4g.; KCl, 0·5g.; $MgCl_2,6H_2O$, 0·5g.; $FeCl_3,6H_2O$, 0·01g.; distilled water to 1l. The appropriate sulphur source was added and the pH adjusted to 5·6 before sterilization at 15lb./in.^{2} for 30min. for the bulk amounts, or for 10min. for the 250ml. flasks. Sulphur sources other than Na_2SO_4, choline sulphate and taurine were adjusted to pH5·6, Seitz-filtered and added aseptically to the sterilized medium. For the initial sulphur-sufficient medium the sulphur source was 0·4mM-taurine except for mutants *zeta* and *bimeth*2, when 0·4mM-cysteine and 0·4mM-methionine respectively were used. For the *bimeth*2 mutant 20μg. of biotin/l. was included in the media. Sulphur sources, except 20μg. of biotin/l. for mutant *bimeth*2, were omitted for the sulphur-deficient media. In the reincubation repression studies carried out in 125ml. of medium, a 1·5mM concentration of the appropriate sulphur source was used.

Chemicals and assay methods. $Na_2{}^{35}SO_4$ (code SJS1, specific activity greater than 100mc/mg. of S) was obtained from The Radiochemical Centre, Amersham, Bucks. Choline [^{35}S]sulphate (1mc/m-mole) was prepared as described by Spencer *et al.* (1968). Adjustments of the specific activity were made by adding unlabelled choline sulphate prepared by the method of Schmidt & Wagner (1904).

Protein was determined by the biuret method (Layne, 1957), with crystalline bovine serum albumin as the standard.

For the standard assay of choline sulphatase *in vitro* in growth experiments, 1g. wet wt. of pressed mycelium was suspended in 5ml. of 1mM-tris–HCl buffer, pH7·5, and ground in a glass homogenizer with 0·5g. of glass beads (no. 20; Jencons Ltd., Hemel Hempstead, Herts.) for 1min.

After cooling to 2°, the homogenate was treated with ultrasonics for 2·75min. in 15sec. bursts with a 20kcyc./sec. Soniprobe type 1130A (Dawe Instruments Ltd.) at 100w with cooling to prevent the temperature rising above 5°. The ultrasonically treated preparation was centrifuged at 38000*g* for 20min. at 2° and the supernatant used for choline sulphatase assay and protein determination. In the assay 10μl. of 40mM-choline [^{35}S]sulphate (5000 counts/min./μl. under the conditions of measurement used) in 0·2M-tris–HCl buffer, pH7·5, was added to 10μl. of the supernatant and incubated for 1hr. at 25°. The reaction was stopped by heating in a boiling-water bath for 90sec. and the cooled tubes were then centrifuged at 2000*g* for 10min. A portion (15μl.) of the supernatant was chromatographed for 3hr. on Whatman no. 1 paper with acetone–water (9:1, v/v) as solvent. The $^{35}SO_4{}^{2-}$ split off by choline sulphatase action remained at the origin. The radioactivity of the $^{35}SO_4{}^{2-}$ spot was measured with an Actigraph strip-scanner (Nuclear–Chicago Corp.) and related to a standard of the original choline [^{35}S]sulphate measured under the same conditions. In the control determination, enzyme and substrate were incubated separately and added together immediately before heating at 100°.

A unit of choline sulphatase activity was defined as that causing the hydrolysis of 1μmole of choline sulphate/hr. and the specific activity as units of choline sulphatase/mg. of protein of the extract.

In the assay of choline sulphatase *in vivo* 1g. wet wt. of pressed mycelium was suspended in 5ml. of the 'sulphur-deficient' medium to which was added choline [^{35}S]sulphate (approx. 5μc/m-mole) to give a concentration of 0·4mM. After incubation at 28° for the required period the mycelium was filtered under vacuum through Whatman no. 54 filter paper and washed with distilled water. The mycelium was transferred to 5ml. of water, heated at 100° for 2min. and then cooled. After centrifugation, the supernatant was chromatographed and analysed for choline [^{34}S]sulphate and $^{35}SO_4{}^{2-}$ as described in the preceding paper (Spencer *et al.* 1968). With mutant *gamma*, blocked at ATP sulphurylase, the $^{35}SO_4{}^{2-}$ liberated from the choline [^{35}S]sulphate is not further metabolized. In the wild-type the $^{35}SO_4{}^{2-}$ liberated can be metabolized, but many of the expected metabolites have the same R_F value (0·0) as $^{35}SO_4{}^{2-}$ and are thus counted as $^{35}SO_4{}^{2-}$. Only very faint radioactivity occurred at positions other than those occupied by $^{35}SO_4{}^{2-}$ and choline [^{35}S]sulphate. When significant, these spots were counted and the values added to that of $^{35}SO_4{}^{2-}$. Choline sulphatase activity *in vivo* was expressed as the percentage of the total radioactivity that was no longer present as choline [^{35}S]sulphate.

RESULTS

Hydrolysis of choline sulphate in vivo

Mutant *gamma* was grown on 0·4mM-taurine as the sulphur source for 5 days, harvested and divided into two portions. One half was grown further in a sulphur-deficient medium and the other half grown in a medium containing 1·5mM-cysteine. After 2 days' growth the mycelia were harvested and portions (1g. of pressed mycelium) incubated in 5ml. of 0·4mM-choline [^{35}S]sulphate (5μc/m-mole) at 28°. At different time-intervals the uptake of choline [^{35}S]sulphate and the hydrolysis of the transported sulphate ester *in vivo* were determined.

The sulphur-deficient and sulphur-sufficient my-

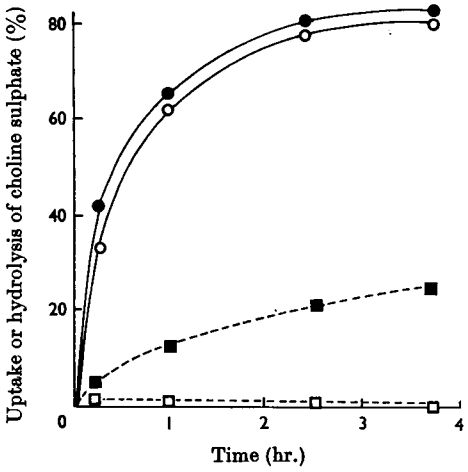


Fig. 1. Uptake and hydrolysis of choline sulphate *in vivo* in *A. nidulans* mutant *gamma*. Mycelia were incubated at 28° in a medium containing 0·4mM-choline [^{35}S]sulphate and the disappearance of choline sulphate from the medium was measured at different times: ○, mycelia grown on cysteine; ●, mycelia starved of sulphur for 2 days. After incubation the mycelia were extracted and the $^{35}SO_4^{2-}$ present was expressed as a percentage of the total mycelial ^{35}S; □, mycelia grown on cysteine; ■, mycelia starved of sulphur for 2 days.

celia took up choline [^{35}S]sulphate at the same rate (Fig. 1), but though the sulphur-deficient mycelium showed considerable hydrolysis of the transported choline [^{35}S]sulphate *in vivo* no such hydrolysis occurred in the sulphur-sufficient mycelium.

Hydrolysis of choline sulphate in vitro

Confirmation of these results was sought *in vitro*. Wild-type *A. nidulans* and two mutants, *gamma*, blocked at ATP sulphurylase, and *eta*, blocked at adenosine 3′-phosphate 5′-sulphatophosphate reductase, were grown on a medium containing 0·4mM-taurine as the sole sulphur source for 5 days and then harvested. Portions of the harvested mycelia were assayed for choline sulphatase activity by using arbitrary conditions of assay involving treatment with ultrasonics for 3min. in 0·1M-tris–hydrochloric acid buffer, pH 8·3, centrifugation and incubation of the supernatant with 0·2mM-choline [^{35}S]sulphate in 0·1M-tris–hydrochloric acid buffer, pH 8·3, for 1hr. at 37°. Other portions of the mycelia were grown for a further 3 days in a sulphur-deficient medium, harvested and the choline sulphatase activity was assayed.

No choline sulphatase could be detected in any of the sulphur-sufficient mycelia, but after sulphur starvation the wild-type, mutant *gamma* and mutant *eta* (specific activities $1·33 \times 10^{-5}$, $1·33 \times 10^{-5}$ and $0·74 \times 10^{-5}$ units/mg. of protein respectively) showed appreciable activity of this enzyme.

Conditions for assay of choline sulphatase

Duration of ultrasonic treatment. The optimum time for extraction of the enzyme was 2·75min. Further treatment with ultrasonics, although extracting more protein, caused inactivation of the enzyme. The supernatant from the preparation treated with ultrasonics for 2·75min. was used in subsequent experiments as a source of choline sulphatase.

Effect of substrate concentration. Unlabelled choline sulphate was added to 0·4mM-choline [^{35}S]sulphate (1mc/μmole) to give the required substrate concentrations and choline sulphatase activity was assayed in 0·1M-tris–hydrochloric acid buffer at pH 8·3 over 1hr. at 37°. The optimum substrate concentration was in excess of 0·25M and a plot of $1/v$ against 1/[S] gave K_m 0·035M for the enzyme in the crude extract under these conditions.

Effect of pH. With 0·1M-tris–hydrochloric acid buffers and incubation for 1hr. at 37°, the effect of pH on the hydrolysis of 20mM-choline [^{35}S]-sulphate by the *A. nidulans* extract was investigated. Optimum activity occurred at about pH 7·5. At pH 6·8 and 8·3 the activity was only about 60% that at the optimum pH.

Effect of temperature. When choline sulphatase was assayed with 20mM-choline [^{35}S]sulphate in 0·1M-tris–hydrochloric acid buffer at pH 7·5 and an incubation period of 1hr. the optimum temperature appeared to be at least 28°. The sharpness of the fall in activity after 28° suggested that assay at a lower temperature might be more reliable and for subsequent assay an incubation temperature of 25° was chosen.

Effect of inhibitors. The effect of various inorganic inhibitors known to affect different sulphatases was tested (Table 1). At 10mM, SO_3^{2-}, PO_4^{3-} and CN^- caused complete or almost complete inhibition, whereas SO_4^{2-} and F^- showed only partial inhibition.

Preliminary attempts to purify the choline sulphatase of sulphur-deficient *A. nidulans* were hampered by the rapid rate of inactivation of the enzyme. The addition of cysteine as a possible stabilizing agent caused immediate loss of activity, and further investigation (Table 1) showed that cysteine at a concentration of 5mM caused complete inhibition of choline sulphatase.

Choline sulphatase synthesis

Repression in sulphur-sufficient media. Choline sulphatase could not be detected in mycelia of *A. nidulans* after 5 days' growth on a sulphur-sufficient medium (0·4mM-taurine), but enzyme activity had

Table 1. *Effect of inorganic ions and cysteine on choline sulphatase activity of* A. nidulans

Choline sulphatase was assayed under standard conditions.

Addition	Concn. (mM)	$10^2 \times$ Sp. activity of choline sulphatase	Inhibition (%)
None	—	8·0	—
Na_2SO_3	0·1	8·0	0
	10	0	100
K_2SO_4	0·1	8·1	0
	10	6·0	25
NaF	0·1	6·5	19
	10	6·0	25
Na_2HPO_4	0·1	5·0	38
	10	0·5	94
KCN	0·1	7·1	11
	10	0	100
None	—	16·0	—
Cysteine	0·001	15·8	1
	0·1	13·5	16
	1·0	4·0	75
	5·0	0	100
	10	0	100

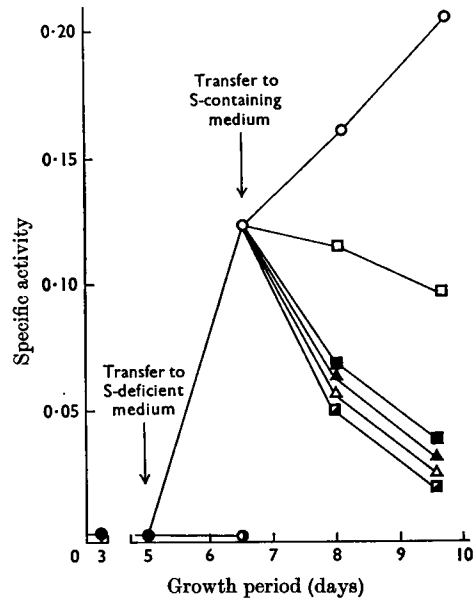
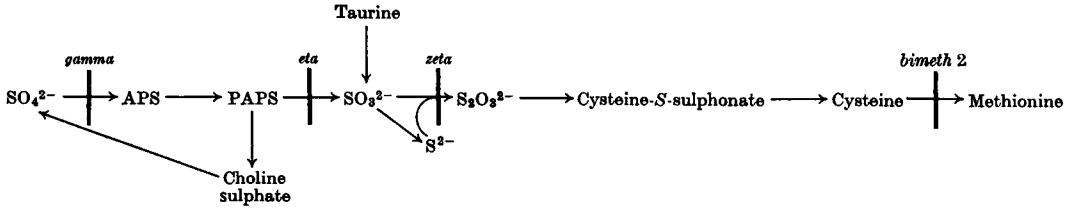


Fig. 2. Effect of sulphur compounds on choline sulphatase activity of wild-type *A. nidulans*. The fungus was grown on 0·4mM-taurine for 5 days (●), and then transferred to a sulphur-deficient medium (○). After 36hr. portions of the sulphur-deficient mycelium were transferred to media containing 1·5mM-taurine (□), SO_3^{2-} (■), methionine (▲), SO_4^{2-} (△), cysteine (◪) or no sulphur (○), and grown for a further 3 days. In another experiment, the mycelium obtained after growth for 5 days on taurine was transferred to a sulphur-deficient medium supplemented with cycloheximide (100μg./ml.) (◑).

developed 36hr. after transfer to the sulphur-deficient medium (Fig. 2). Subsequent addition of various sulphur compounds caused a decrease in choline sulphatase activity that was least marked with taurine. That the decrease in enzyme activity was due to repression of synthesis rather than to inhibition was shown by the failure of preparations from sulphur-sufficient mycelia to decrease choline sulphatase activity of sulphur-deficient mycelia when added together *in vitro*. This was confirmed by the prevention of choline sulphatase synthesis during sulphur deficiency in the presence of cycloheximide (100μg./ml.) (Fig. 2).

Repression of choline sulphatase in A. nidulans *mutants*. As in other cases of end-product repression it seemed likely that the various metabolites were converted into a single co-repressor. To find the identity of the common co-repressor use was made of four mutants that were blocked as shown in Scheme 1 (Spencer *et al.* 1968). The mutants were grown on sulphur sources at a concentration of 0·4mM: mutants *gamma* and *eta* on taurine, mutant *zeta* on cysteine and mutant *bimeth* 2 on methionine as the respective sulphur sources. After 5 days' growth the fungi were harvested and regrown on the sulphur-deficient medium for a further 2 days. At this stage the mycelia were divided, samples of each being further starved of sulphur for 2 days, whereas the other samples were incubated with various sulphur sources. It was expected that the 2 days' growth on the sulphur-deficient medium would relieve any repression and allow the synthesis of choline sulphatase. The reincubation for a further 2 days on various sulphur sources would result in a lowering of choline sulphatase synthesis if the co-repressor was formed in that particular mutant. None of the compounds SO_4^{2-}, SO_3^{2-} and taurine, which are intermediates in the cysteine-synthesis pathway before $S_2O_3^{2-}$ (Scheme 1), caused repression of choline sulphatase in sulphur-deficient mutant *zeta*, which is blocked between SO_3^{2-} and $S_2O_3^{2-}$ (Table 2). The ability of these compounds to act as co-repressors in the wild-type must result from their further metabolism. Confirmation that SO_4^{2-} was not the co-repressor was obtained with mutant *gamma*, blocked at ATP sulphurylase, and mutant *eta*, blocked before SO_3^{2-}. On the other hand, all sulphur compounds except taurine (cf. Fig. 2) acted as co-repressors with mutant *bimeth* 2. Since SO_4^{2-}, SO_3^{2-} and cysteine cannot be used as the precursors of methionine in this mutant the



Scheme 1. APS, adenosine 5′-sulphatophosphate; PAPS, adenosine 3′-phosphate 5′-sulphatophosphate.

Table 2. *Effect of various sulphur compounds on choline sulphatase activity of* A. nidulans

Mutants were grown on a sulphur-sufficient medium for 4 days, and then made sulphur-deficient by growing on a medium devoid of sulphur for a further 2 days. Portions of the sulphur-deficient mycelia were then grown on various sulphur sources for 2 days. Choline sulphatase was assayed under standard conditions.

	$10^2 \times$ Sp. activity of choline sulphatase	
Mutant	Sulphur-deficient	After 2 days' further growth on sulphur source in parentheses
gamma	12·0	17·4 (no sulphur) 22·4 (SO_4^{2-}) 6·0 (SO_3^{2-}) 6·4 (cysteine) 5·0 (methionine)
eta	8·5	12·0 (no sulphur) 9·5 (SO_4^{2-}) 0·6 (SO_3^{2-}) 0·1 (cysteine) 0·2 (methionone)
zeta	12·1	20·7 (no sulphur) 20·1 (SO_4^{2-}) 20·1 (SO_3^{2-}) 0·7 (cysteine-*S*-sulphonate) 0·4 (cysteine) 0·5 (methionine) 13·6 (taurine)
*bimeth*2	10·2	15·0 (no sulphur) 8·0 (SO_4^{2-}) 5·0 (SO_3^{3-}) 2·5 (cysteine) 3·0 (methionine) 12·7 (taurine)

co-repressor of choline sulphatase cannot solely be methionine, and the ability of methionine to act as a co-repressor is probably due to its metabolism to other sulphur compounds. The results limit the co-repressor to $S_2O_3^{2-}$, cysteine-*S*-sulphonate or cysteine or compounds derived directly from them. Circumstantial evidence, discussed below, suggests that the co-repressor is cysteine.

Although primary growth in taurine prevented choline sulphatase synthesis the transfer of sulphur-deficient mycelia to a taurine medium resulted in only partial repression (Fig. 2 and Table 2). This suggests that in the later circumstances the metabolism of taurine is not rapid enough to build up a cysteine pool of sufficient concentration to cause complete repression. Similar partial repression was obtained by transferring sulphur-deficient mycelia to media containing different concentrations of cysteine. After 72hr. growth choline sulphatase activity in mycelia grown on 1·3mM-cysteine was very low, but on 0·013mM-cysteine choline sulphatase synthesis was seen to have continued at a slightly faster rate than in the sulphur-deficient mycelia. Cysteine at a concentration of 0·13mM gave only partial repression (Fig. 3). Whether the internal cysteine pool depended directly on the external cysteine concentration or whether derepression was brought about by using up all the cysteine for growth was not clear from this experiment.

End-product inhibition of choline sulphatase. Both cysteine and SO_3^{2-} inhibit choline sulphatase. Although in the preparation of the mycelial extracts the concentrations of these metabolites were decreased to a non-inhibitory level and did not interfere in the assay of the enzyme *in vitro* it was possible that the endogenous concentrations of cysteine or SO_3^{2-} could cause an inhibition of choline sulphatase *in vivo*.

The experiment illustrated in Fig. 2, which showed the effect of sulphur sufficiency and sulphur deficiency on choline sulphatase activity, was repeated. Mycelium of wild-type *A. nidulans* was grown for 5 days on a sulphur-sufficient medium in which taurine was the sulphur source, transferred to a sulphur-deficient medium for 2 days and then divided into two portions, one of which was grown for a further 12hr. on the sulphur-deficient medium and the other on the medium containing 1·5mM-cysteine. Mycelial samples were removed at intervals as shown in Fig. 4, and choline sulphatase activities *in vitro* and *in vivo* were determined. During the initial growth on the sulphur-sufficient medium no choline sulphatase activity was seen

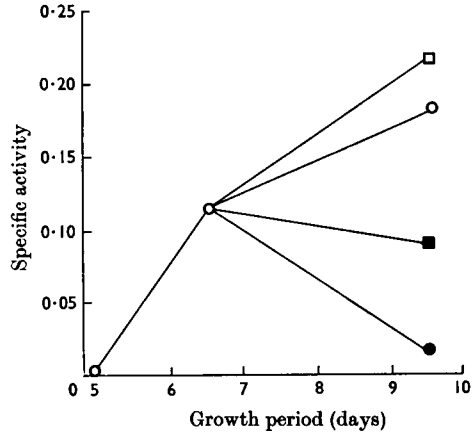


Fig. 3. Repression of choline sulphatase synthesis in wild-type *A. nidulans* grown on different concentrations of cysteine. Portions of sulphur-deficient mycelia obtained by growing for 5 days on 0·4mM-taurine and then for 36hr. on a sulphur-deficient medium (○) were transferred to media containing different concentrations of cysteine and grown for a further 3 days: 0·013 mM-cysteine (□), 0·13mM-cysteine (■), 1·3mM-cysteine (●) or no sulphur (○).

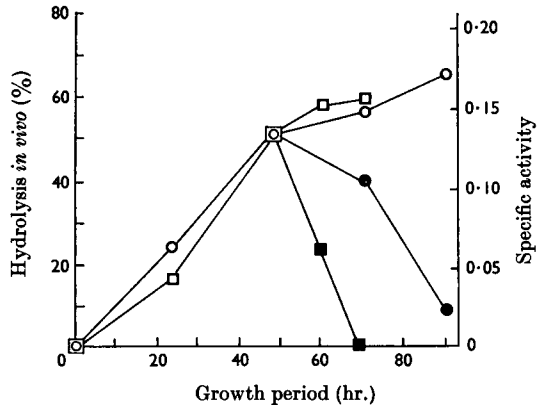


Fig. 4. Repression and inhibition of choline sulphatase in wild-type *A. nidulans*. The fungus was grown initially for 5 days on 0·4mM-taurine and then transferred at zero time to a sulphur-deficient medium. After 48hr. the mycelium was divided into two portions and grown further on the sulphur-deficient medium or on the same medium supplemented with 1·5mM-cysteine. Choline sulphatase activity *in vivo* was measured during sulphur deficiency (□) and after transfer to the cysteine-containing medium (■). Choline sulphatase activity *in vitro* was measured during sulphur deficiency (○) and after transfer to cysteine-containing medium (●).

either *in vivo* or *in vitro*. With increasing sulphur deficiency both the activities *in vitro* and *in vivo* increased at approximately the same rate. After 48hr. on the sulphur-deficient medium the mycelia that were transferred to the cysteine-containing medium showed a decrease in choline sulphatase activity that was much more rapid *in vivo* than *in vitro*. This can be interpreted as showing inhibition of choline sulphatase *in vivo* by the internal cysteine pool.

DISCUSSION

The ability of various sulphur compounds to repress the synthesis of choline sulphatase suggested that they were being converted into a common co-repressor. By using mutants the identity of the co-repressor was restricted to metabolites between SO_3^{2-} and methionine. Though the pathway from SO_3^{2-} to methionine has not been completely described in *A. nidulans*, current knowledge suggests that the main metabolites include $S_2O_3^{2-}$, cysteine-*S*-sulphonate and cysteine (Nakamura & Sato, 1962). The mutants that were used to define these metabolites (Nakamura & Sato, 1962) and that would enable a closer definition of the co-repressor, are unfortunately no longer available (T. Nakamura, personal communication). However, the fact that choline sulphatase synthesis in mutant *zeta* is repressed by cysteine but not by SO_3^{2-} strongly suggests that it is cysteine, or a compound derived directly from cysteine, that is the actual co-repressor rather than $S_2O_3^{2-}$ or cysteine-*S*-sulphonate. In this mutant the synthesis of $S_2O_3^{2-}$ and thus of cysteine-*S*-sulphonate, from SO_3^{2-} formed by oxidation of the exogenous cysteine is blocked (see Scheme 1), and the possibility that cysteine-*S*-sulphonate could be formed directly by reaction between cysteine and SO_3^{2-} has been ruled out (Nakamura & Sato, 1962).

In *A. nidulans* mutants the repression and derepression of the synthesis of arylsulphatase by various sulphur sources runs exactly in parallel with choline sulphatase (cf. Hussey & Spencer, 1967), although fractionation studies show the enzymes to be distinct (H. McDonnell & B. Spencer, unpublished work). Similar parallel behaviour between these two enzymes was observed in *Neurospora crassa* (Metzenberg & Parson, 1966). In this organism arylsulphatase was repressed by SO_3^{2-} but not by cystine, and it was suggested that S^{2-} was the actual co-repressor. It should be noted that the sulphate-reduction pathways in *A. nidulans* and *N. crassa* are different, $S_2O_3^{2-}$ being implicated as an intermediate in *A. nidulans* (Nakamura & Sato, 1962; Segel & Johnson, 1963*b*) but not in *N. crassa* (Leinweber & Monty, 1965).

That a compound, cysteine, at the end of the sulphate-reduction pathway reaches a concentration during sulphur sufficiency that causes repression of choline sulphatase synthesis is advan-

tageous to the organism, since it prevents the use of endogenous reserves of SO_4^{2-} when sufficient readily metabolizable exogenous sulphur is available. When a nutritional sulphur deficiency occurs the co-repressor decreases in concentration, choline sulphatase synthesis is de-repressed and the sulphur store is mobilized. More immediate control, which is not dependent on protein catabolism and synthesis, is given by feedback inhibition of choline sulphatase by cysteine and possibly SO_3^{2-}. In Scheme 1, if choline sulphatase is regarded as the first reaction in a sequence of SO_4^{2-} utilization, the regulation of choline sulphatase by cysteine then conforms with the classical end-product feedback inhibition and repression, such as is shown by histidine, CTP, tryptophan, isoleucine and valine for the first enzymes in their respective synthetic pathways (for review see Cohen, 1966). Unlike the analogous hydrolytic enzyme, alkaline phosphatase (Horiuchi, Horiuchi & Mizuni, 1959), the immediate product, in this case SO_4^{2-}, does not inhibit the enzyme.

There are differences between the choline sulphatase of *A. nidulans*, which produces and stores choline sulphate, and that of bacteria, which can utilize choline sulphate but cannot synthesize it. In the fungus the enzyme appears to be repressible whereas in the bacteria it is substrate-inducible. That choline sulphatase synthesis in *A. nidulans* does not need to be induced by endogenous substrate after de-repression is shown by the rapid formation of the enzyme in mutant *gamma*, which lacks ATP sulphurylase and thus does not produce choline sulphate. The inducible choline sulphatase of *Pseudomonas nitroreducences* (Takebe, 1961) and the repressible enzyme of *A. nidulans* both had high K_m values, 0·04M and 0·035M respectively, and similar pH optima, 8·3 and 7·5, but their behaviour towards inhibitors showed differences. Both enzymes were inhibited by 0·01 mM-SO_3^{2-} but the bacterial choline sulphatase was almost unaffected by 0·01 mM-PO_4^{3-} and CN^-, though the fungal enzyme was completely inhibited by these ions.

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