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Choline-O-sulphate utilization in *Aspergillus nidulans*

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SUMMARY

The role of choline-O-sulphate (COS) as a sulphur storage compound in *Aspergillus nidulans* was examined by comparing a normal strain and one unable to utilize COS in a sulphur-starvation experiment designed to measure the mobilization of sulphur stores. Efforts to isolate the necessary mutants deficient in choline sulphatase activity produced two nutritionally distinct classes of mutants unable to utilize COS. They were found to be allelic on the basis of genetic complementation and fine structures mapping and represent either leaky or tight mutants with respect to choline sulphatase activity. One of these mutants with no detectable choline sulphatase activity was selected for a growth experiment which demonstrated that COS is a major, though not the only source of the endogenous sulphur supply which can be mobilized during growth in sulphur-limiting conditions.

1. INTRODUCTION

Choline-O-sulphate (COS, see Fig. 1 for abbreviations), the sulphate ester of choline, appears to play a role in the storage of sulphur in higher fungi (Spencer & Harada, 1960). It was first isolated from *Aspergillus sidowi* in yields of more than 0·2% of the dry weight of the mycelium (Wooley & Peterson, 1937), and conidia have been found to contain up to 1·5% COS (Takebe & Yanagita, 1959; Takebe, 1960). COS is produced by most higher fungi, though not by the yeasts, Phycomycetes, or bacteria (Harada & Spencer, 1960; Itahashi, 1961). It is synthesized by sulphate transfer from PAPS (Kaji & Gregory, 1959; Orsi & Spencer, 1964), while its utilization occurs by hydrolysis to choline and sulphate (Fig. 1, Spencer & Harada,1960; Spencer *et al.* 1968).

Early investigations of the role of COS in fungi demonstrated that various Ascomycetes assimilate sulphate preferentially to COS but can utilize the latter as the sole source of sulphur (Hockenhull, 1948, 1949; DeFlines, 1955; Stevens & Vohra, 1955). Spencer & Harada (1960) compared the growth of COS producing fungi to that of Phycomycetes which do not synthesize COS. The COS producers more than doubled their weights after transfer from medium containing a high concentration of sulphate to medium devoid of sulphur, while the non-producers

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showed only slight weight increases. The authors concluded that COS was responsible for virtually all of the additional growth in those species which could accumulate COS. They postulated that higher fungi produce large quantities of COS during growth in S-rich medium. Then, as exogenous sulphur becomes limiting, the sulphur of stored COS becomes available for assimilation.

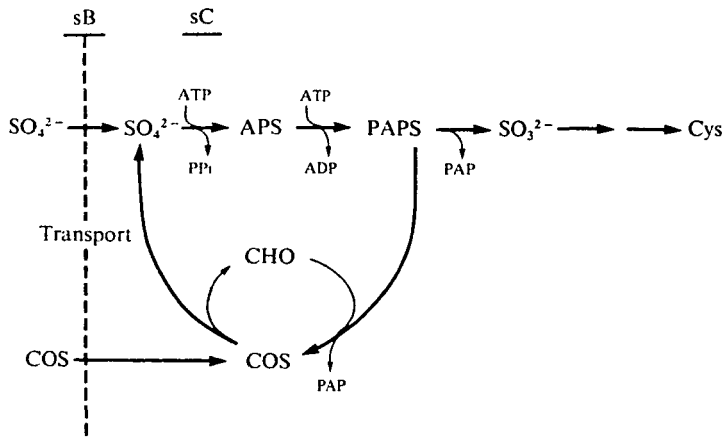

Fig. 1. Pathway showing the synthesis and utilization of COS occurring as a shunt off the main pathway of sulphate assimilation. The synthesis of COS, by sulphate transfer from PAPS, is catalysed by choline sulphotransferase (enzyme 1) and its hydrolysis is catalysed by choline sulphatase (enzyme 2). Mutations at the sB locus produce defective sulphate transport, at the sC locus block ATP sulphurylase. Abbreviations: APS = adenosine-5′-phosphosulphate; PAPS = 3′-phosphoadenosine-5′-phosphosulphate; CHO = choline; COS = choline-O-sulphate.

In support of the sulphur storage hypothesis is the evidence that while COS is extensively accumulated during growth in S-rich medium, the enzyme mediating the hydrolysis of COS, choline sulphatase, is only detectable in extracts from S-starved mycelium and is repressed during growth in the presence of various assimilatory sulphur metabolites, including sulphate (Scott & Spencer, 1968).

The hypothesis that COS is a sulphur reservoir in Aspergillus is examined in this investigation by comparing the growth of a wild-type strain of *A. nidulans* with one lacking choline sulphatase activity in a sulphur starvation experiment similar to that of Spencer & Harada (1960). Efforts to isolate the necessary mutants deficient in choline sulphatase activity produced two nutritionally distinct classes of isolates unable to utilize COS. They were found to be allelic on the basis of complementation tests and genetic fine structure mapping. One of these mutants with no detectable choline sulphatase activity was selected for the growth experiment which demonstrated that COS is a major, though not the only source of the endogenous sulphur supply responsible for the continued growth of *A. nidulans* after shifting mycelium from S-rich to S-free medium.

2. MATERIALS AND METHODS

(i) *Strains.* All strains used in this investigation originate from the same wild-type haploid strain (Pontecorvo *et al.* 1953) and were kindly provided by E. Käfer-Boothroyd or the Fungal Genetics Stock Centre. The strains used for the isolation of mutants were FGSC no. 26 *biA1* and Montreal no. 771 *suA1adE20 adE20 biA1*; *AcrA1*; *choA1*; *chaA1*. The strains used for the construction of diploids were FGSC no. 159 *suA1adE20 pabaA1 adE20*; *AcrA1*; *pheA2*; *pyroA4*; *lysB5*; *sB3*; *nicB8*; *riboB2* and FGSC no. 133 *pabaA1 yA2 adE20*; *wA2*; *pyroA4*; *lysB5*; *sB3*; *choA1*. One of the new mutants, *csuA101*, was outcrossed to Montreal no. *proA1 pabaA1 yA2 adE20*; *lysB5*; *nicB8 choA1 ma1A1 pa1F15*. The genetic markers used and the origins of strains are described by Barratt, Johnson & Ogata (1965), Käfer (1965, and personal communication), and Dorn (1967) following the nomenclature of Roper (1970). Strain 26, employed in the isolation of mutants, has previously been confirmed as translocation-free (Barratt *et al.* 1965).

During the course of this investigation, Arst (1971) reported the isolation of mutants unable to utilize COS and has kindly made available his entire collection. He gave these mutants the gene symbol *csu* and the mutant *csuA6* (complete genotype: *suA1adE20 pabaA1 biA1*; *csuA6*; *choA1*; *chaA1*), derived from a cross to *pabaA1* after nitrosoguanidine treatment of FGSC no. 168 (Arst, 1971, and personal communication), was characterized in this study.

(ii) *Media, chemicals, and growth conditions.* Details of the composition of standard media have been described by Pontecorvo *et al.* (1953), Käfer (1958), and Barratt *et al.* (1965). Sulphur-free minimal medium, MM (S-free), is identical to MM except that equimolar quantities of chloride salts have been substituted for the sulphate salts of the salt solution and trace elements. In addition, agarose (1 % w/v) was sometimes used in place of agar (1·25 % w/v) in solid medium. This was done because agar dissolved in medium provides enough sulphate for optimal growth of Aspergillus. The agarose was purchased from Sigma Chemical Company, St Louis, Mo. A number of other brands of agarose proved impractical for use in S-free medium.

The following sulphur sources were used as supplements: Na_2SO_4 (up to 5 mM), Na_2SO_3 (5 mM), L-cysteine (0·2 mM), L-methionine (0·2 mM), COS (0·2 mM), and djenkolic acid (0·2 mM). COS was synthesized according to the method of Schmidt & Wagner (1904) and ^{35}S-COS was synthesized according to the method of Segel & Johnson (1967). All media and supplements, except the following, were sterilized by autoclaving at 15 psi and 125 °C. Na_2SO_3, cysteine, and COS were filter-sterilized through Millipore filters; Na_2SO_3 and cysteine solutions were prepared immediately prior to each use.

Cultures prepared for assays of choline sulphatase or for the uptake of COS or sulphate were grown in liquid MM (S-free) supplemented with required growth factors and a sulphur source. The cultures, 500 ml in 2000 ml fluted flasks, were incubated at 37 °C in a New Brunswick Gyrotory Shaker at 300–350 rpm. Best

growth yields were obtained when conidia were inoculated at concentrations ranging from 2×10^5 to 4×10^5/ml.

The standard sulphur sources for the liquid cultures were Na_2SO_4 or djenkolic acid. Na_2SO_4 (2 or 5 mM), a normal constituent of MM, produced mycelium repressed for choline sulphatase (Scott & Spencer, 1968). Growth yields of about 15 g wet weight per 500 ml medium were obtained after 24–28 h incubation. Djenkolic acid (0·2 mM) was selected as the best sulphur source for derepression of choline sulphatase after preliminary comparison with 0·2 mM cysteic acid or methionine. The growth of *A. nidulans* in djenkolic acid is reduced, producing at best 10–11 g wet weight per 500 ml medium after 34–38 h incubation. Further growth resulted in darkening of the mycelium in the growth flasks and produced choline sulphatase of highly variable specific activity.

Cultures were harvested by draining the medium by suction through porous filter paper followed by washing with three volumes of distilled water. For the choline sulphatase assay, the wet mycelium was stored at −15 °C, usually no longer than overnight.

(iii) *Isolation of mutants.* At the beginning of this study, mutants unable to utilize COS had yet to be isolated. It was expected that such mutants would behave like wild-type in MM since they should still be able to assimilate sulphate. Nevertheless initial attempts concentrated on the use of sulphur auxotrophy as a primary selection technique in the hope of isolating some pleiotropic sulphur mutants as have been found in Neurospora (Marzluf & Metzenberg, 1968). This was unsuccessful in ten mutant hunts which produced about 500 sulphur auxotrophs after enrichment with the biotin starvation technique of Macdonald & Pontecorvo (1953), with the exception that one cysteine-requiring (cys^-) isolate simultaneously defective in the utilization of COS and in sulphate assimilation was recovered. It was outcrossed to strain 174 to put in a *choA1* background. Arst (1968, 1971) had found that *choA1* strains will grow on COS as a choline source and that mutants unable to utilize COS are auxotrophic for choline on COS medium. Out of 108 cho^- progeny, 27 were csu^+cys^-, 32 were csu^-cys^+, and the remainder were of the parental genotypes (χ^2 for independent assortment = 0·93, not significant at the 5% level). Therefore, at least two mutations were induced in the original isolate, a mutation producing a deficiency in COS utilization, designated *csuA101*, and a second mutation identified by complementation tests as an *sB* allele (sulphate uptake deficiency). One of the *csuA101* progeny, complete genotype: *pabaA1 yA1 adE20*; *csuA101*; *choA1*, was investigated further in this study and is described as strain *csuA101*.

The second selection procedure depended on isolating mutants in a *choA1* background. Arst (1971) selected his *csu* mutants in a *sB3*; *choA1* background which made COS both the sole choline and sole sulphur source. For these mutant hunts strain 771, which has normal sulphate uptake but requires choline for growth, was used as the strain of origin.

Mutants were obtained following u.v. irradiation (Giles, 1951) or after treatment of conidia with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG), Aldrich Chemical

Company, by a modification of the technique of Clutterbuck & Sinha (1966). Without selection, *csu* auxotrophs were produced in a yield of one per 10^5 isolates tested (determined by calculation). The desired classes of mutants were greatly enriched to one per 440 isolates tested, by the application of the biotin starvation technique of Macdonald & Pontecorvo (1953). By using COS as the sole sulphur source in the starvation medium, *csu* mutants were enriched as 'sulphur' auxotrophs. Following up to 95 h in the starvation medium, conidia were plated and *csu* mutants were then recovered by replica-plating as 'choline' auxotrophs.

(iv) *Complementation tests.* Complementation tests were done between *csu* mutants in heterokaryons or diploids according to the procedures of Roper (1952) and Pontecorvo *et al.* (1953). The tests were done on appropriately supplemented medium containing COS as the choline source versus control medium containing choline. It was essential that all tests be done in a *choA1/choA1* genetic background to ensure an absolute growth requirement for COS.

(v) *Genetic mapping.* Linkage group assignments were made by the selection of haploid mitotic recombinants from heterozygous diploids (Käfer, 1958; McCully & Forbes, 1965). Diploids were constructed homozygous for *choA1* or *sB3* in order that all haploids recovered could be tested on COS as a choline or sulphur source, respectively, in order to follow the segregation of the *csu* marker. Similarly for the fine structure mapping all crosses were constructed in a genetic background homozygous for *choA1* and isolates were tested for their ability to utilize COS as a choline source.

(vi) *Choline sulphatase determination.* Frozen mycelium was lyophilized to dryness and ground with a Wiley mill or mortar and pestle with glass beads in 20 ml of 0·05 M Tris-HCl, pH 7·5, per gram dry weight. All procedures were performed at 4 °C unless otherwise indicated. The mycelial suspension was stirred at 1 h followed by centrifugation at 15000 rpm for 45 min in a Sorvall RC-2 centrifuge with a Type SS-34 head to remove cell debris. The supernatant was recovered and dialysed against two changes of 0·05 M Tris-HCl, pH 7·5, over a $2\frac{1}{2}$ h period and the extracts were frozen at -15 °C until ready for use, usually no longer than overnight.

Choline sulphatase was assayed using the conditions described by Scott & Spencer (1968) and activity was determined by the barium precipitation method of Metzenberg & Parson (1966). The incubation mix contained, in a total volume of 1 ml: 50 μmol Tris-HCl, pH 7·5; 20 μmol ^{35}S-COS containing 2×10^6 cpm (unless otherwise indicated); and crude extract containing up to 2 mg protein. Incubation was done at 25 °C and the assay was started with the addition of substrate. At intervals of 15 or 30 min, 0·1 ml samples were withdrawn and transferred to a test-tube containing 5 ml of 0·01 M Na_2SO_4 in 0·1 N-HCl to stop the reaction and add carrier sulphate. Two drops of 1 M-$BaCl_2$ were added to precipitate the sulphate. When single time points were obtained, determined after 1 h of incubation, the assay tubes were acidified directly with the addition of 0·1 ml of 0·1 M-Na_2SO_4 in N-HCl followed by two drops of 1 M-$BaCl_2$. Activity was expressed as micromoles of COS hydrolysed per milligram protein per hour.

(vii) *Sulphate and COS uptake.* The sulphate and COS uptake systems were assayed based on procedures used for *A. nidulans* by Bradfield *et al.* (1970) for sulphate uptake and Bellenger *et al.* (1968) for COS uptake. These authors had shown that both uptake systems are unidirectional, confirmed in preliminary experiments for these studies. Bradfield *et al.* (1970) also observed similar sulphate uptake rates in strains of *A. nidulans* whether defective or normal for the first enzyme of the sulphur pathway, ATP sulphurylase. Similar results were obtained for COS uptake in the presence or absence or choline sulphatase deficiency in this investigation. These results indicate that the observed uptake in both systems is a measure of transport into the sulphate or COS pool.

Sulphate and COS uptake were determined as follows: 0·1 g wet weight of fresh, washed mycelium, grown in 0·2 mM djenkolic acid as the sole source of sulphur, was suspended in 4 ml of 0·1 M Tris-citrate buffer, pH 6·0, in a round bottom flask and agitated with a Vortex mixer until it was evenly distributed. The suspension was preincubated at 37 °C with shaking in a reciprocating water bath for 15 min. At zero time, 1·0 ml of 5×10^{-4} M [^{35}S]Na_2SO_4 containing $1{\cdot}8 \times 10^7$ cpm or [^{35}S]COS containing 3×10^7 cpm was added to begin transport. After 1 h of incubation, the samples were filtered with suction through glass fibre filters followed by washing with three volumes of distilled water. The samples were dried and dispersed in scintillation fluid for counting. Tests were done in duplicate and amount of uptake was expressed as micromoles of compound taken up per gram dry weight in 1 h. Dry weight estimates were made by drying samples of mycelium equivalent to those used in the experiments.

(viii) *Growth yields in sulphur-limiting media.* Strain *csuA101* and strain 165 (*csuA*$^+$) were compared for their abilities to mobilize COS under conditions of sulphur starvation. For a valid interpretation of the results, it was essential to harvest the mycelium after growth was terminated but before general lysis began to affect the growth yield. The optimal time of harvest was determined by plotting a growth curve for strain *csuA101* grown in 20 μM djenkolic acid as the sole source of sulphur. This, coupled with a preliminary run of the main experiment, indicated that cultures, using the procedure described below, reached maximum growth yield before 72 h incubation. They also showed that losses due to cell lysis were minor (less than 10% of the total yield) if growth was prolonged up to 96 h. Therefore, cells were harvested 72 h after inoculation. It was also necessary that the growth yield of each strain grown in a given limiting quantity of sulphur supplement (growth yield per unit sulphur) be similar. This was determined as part of the main experiment.

Strain 165 and the choline sulphatase mutant, *csuA101*, were grown in liquid MM (S-free) containing 2 mM-Na_2SO_4, as the sole source of sulphur, and other essential supplements. The cultures were harvested and washed with four volumes of the same medium without sulphur in aseptic conditions and were transferred in 0·2 g wet weight aliquots to fluted 250 ml flasks containing 50 ml liquid MM (S-free) containing essential growth requirements and 0·8, 16, 24 or 40 μM-Na_2SO_4 as the sole source of sulphur. Each flask was made up in duplicate. They were grown in

shake culture for 72 h at 37 °C and harvested. Dry weights were determined by drying the total harvest from each flask at 95 °C for 24 h and weighing. In addition, duplicate flasks were made up as described above but were harvested without incubation for determination of dry weights at zero time.

3. RESULTS

(i) *Characterization of mutants.*

Seventy-four mutants of *A. nidulans* unable to utilize COS were recovered from over 30000 colonies examined after u.v. or NG treatment of conidia. These mutants are unable to grow on COS as a choline source when tested in a *choA1* genetic background, deficient in the endogenous synthesis of choline. With mutant *csuA6*

Table 1. *Nutritional classification of mutants*

Nutritional designation or genotype	Number of isolates in group	Additions to basal medium*						
		SO_4^{2-}	Cho SO_4^{2-}	COS	SO_4^{2-} COS	Met COS	Cho SO_4^{2-} COS	or Met
I	34	0†	+	0	0	0	+	
*csuA*6(I)‡		0	+	0	0	0	+	
II	40	0	+	+	0	±	+	
csuA$^+$		0	+	+	+	+	+	

* The basal medium is S-free MM containing essential growth factors except choline and a sulphur source.

† Growth symbols: + = optimal growth; 0 = no or reduced growth; ± = reduced growth.

‡ Mutant provided by H. N. Arst.

(Arst, 1971), they were further subdivided into two nutritionally distinct groups according to their responses to the effects of other exogenous sulphur sources in the COS growth medium (Table 1). Group I mutants have a permanent loss of COS utilization in all media tested, while group II mutants retain the ability to respond to COS as a choline source in medium devoid of other sources of sulphur. However, group II mutants are growth inhibited in COS medium if other sulphur sources, including sulphate, sulphite, cysteine, or methionine, are present. The further addition of choline to any of these media restores the growth of both classes of mutants.

One of the group I mutants, *csuA106*, was outcrossed to a *choA*$^+$ strain in order to determine if the *csu* marker is expressed in a *choA*$^+$ background. The *choA*$^-$ progeny segregated 44 csu$^-$:45 csu$^+$ in COS medium containing sulphate, but the 106 cho$^+$ progeny were all phenotypically csu$^+$. On the other hand, if medium containing COS as the sole source of sulphur was used, it was possible to identify csu$^-$cho$^+$ recombinants as weak growers. In this medium, the cho$^+$ progeny segregated 44 csu$^-$:62 csu$^+$ (χ^2 = 3·0, not significantly different from 1:1 at the 5% level). Therefore, although a mutant unable to utilize COS does grow on mini-

mal medium, it can be made to express deficiencies in both the sulphur and choline pathways in appropriate nutritional tests.

Mutants unable to utilize COS as a choline source are expected to be defective in either COS uptake or the hydrolysis of COS to choline and sulphate. Initial screening of these mutants showed that all of them can transport COS as well as wild type. Choline sulphatase activity was determined after growth of mycelium

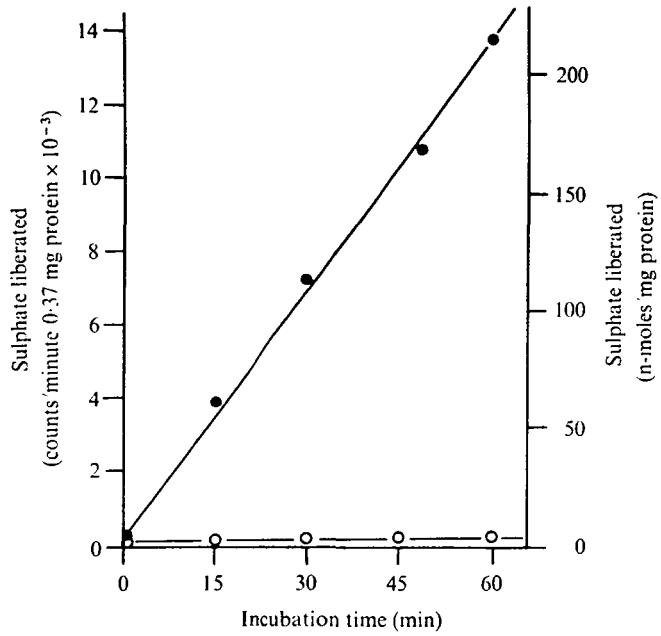

Fig. 2. Hydrolysis of choline-O-sulphate by dialysed extracts of strain 165 prepared from mycelium grown in 5 mM-Na_2SO_4 (○) and 0·2 mM djenkolic acid (●) at 25 °C. Assays were performed at 25 °C. Protein concentrations = 3·7 mg/ml (djenkolate grown), 5·2 mg/ml (sulphate grown). COS concentration = 0·02 M, specific activity = $1·7 \times 10^5$ cpm/μmole.

Table 2. *Sulphur transport and choline sulphatase determinations in representative mutants**

Strain	Nutritional group	COS transported†	[Sulphate transported†	Choline sulphatase‡
csuA6	I	—	—	0
csuA101	I	3·4	5·3	0
csuA106	I	—	—	0
csuA84	II	1·2	5·1	0·02
csuA108	II	2·1	7·0	0·02
csuA$^+$		1·6	5·6	0·72

* Assays were performed on duplicate samples incubated for 1 h as detailed in Materials and Methods.

† Specific activity: μmol/h/g mycelium. —, No assay was performed.

‡ Specific activity: μmol/h/mg protein. The normal range observed for *csuA*$^+$ strains was 0·7–1·2 units.

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on djenkolic acid to ensure maximum derepression of the enzyme (Fig. 2). Mutants tested from group I have no discernible choline sulphatase activity (Table 2). Thus the failure of group I mutants to grow on COS as a choline source correlates with the lack of enzyme activity in crude extracts. Similarly, the trace enzyme activity of group II mutants may be adequate to produce growth in COS medium devoid of other sulphur sources. The growth inhibition produced by adding sulphur supplements to the COS medium might be caused by end-product repression or inhibition of the mutant enzyme (Scott & Spencer, 1968) to levels below which a 'physiologically induced' auxotrophy would result.

Support for this rationale comes from the effect of crossing sulphur pathway mutations into a group II mutant. Mutant *csuA108* was crossed to *sB3*, defective in sulphate transport, and in another cross to *sC12*, defective in the first step of sulphate activation (Fig. 1). In the presence of either *s* mutation as well as *choA1*, the growth response of *csuA180* is restored to normal in COS medium containing sulphate. At the same time, however, the growth inhibiting effect of sulphite, cysteine or methionine is not appreciably corrected. These compounds are found at steps beyond the introduced metabolic defects and presumably lead to a feedback effector of choline sulphatase, suggested by Scott & Spencer (1968) to be at the level of cysteine. Thus it appears that the residual choline sulphatase activity of the group II mutants is adequate to support growth on COS as a choline source, while the addition of sulphur pathway metabolites to the medium may be responsible for the reduction of residual activity to levels too low to promote COS hydrolysis.

(ii) *Genetic analysis*

A genetic analysis, employing the use of complementation tests, linkage group localization, and fine structure mapping, was undertaken to determine the gene relationship between the two groups of choline sulphatase deficient mutants. Complementation tests were done in forced heterokaryons or diploids homozygous for *choA1* in which non-complementing *csu* mutants would express a choline requirement in medium containing COS as the choline source. Diploids heterozygous for *csuA101* (group I) or *csuA108* (group II) gave wild-type growth responses to COS medium containing sulphate and as such behave as recessive mutations. Twenty-three mutants from both nutritional groups were examined for complementation in pairwise combinations in heterokaryons or diploids. None of the tests resulted in a recovery of the ability to utilize COS in the presence of sulphate. Thus the two classes of mutants do not complement each other in spite of the nutritional differences between them.

The possibility that the two classes of mutants map at the same locus was tested by genetic mapping. First, *csuA101* and *csuA108* were both mapped by mitotic haploidization to linkage group V, although crosses involving various *csuA* alleles revealed no linkage to *lysB5* which had previously been mapped to linkage group V (Käfer, 1958). Secondly, crosses were made between mutants, within or between nutritional groups, from which a fine structure map was constructed on the basis of

recombination frequencies, shown in Fig. 3 (Pritchard, 1955; Case & Giles, 1958). Twenty crosses were done which yielded fertile cleistothecia involving ten mutants, six from group I and four from group II. Generally, the cleistothecia contained at least 10^5 ascospores so that the recombination data were usually based on the analysis of single cleistothecia. This avoided the complications produced by combining large numbers of selfed and hybrid cleistothecia dealt with by Pritchard (1955) and Siddiqi (1962). Reversion rates were considerably lower than even the smallest recombination frequencies and did not interfere with the results. Only one

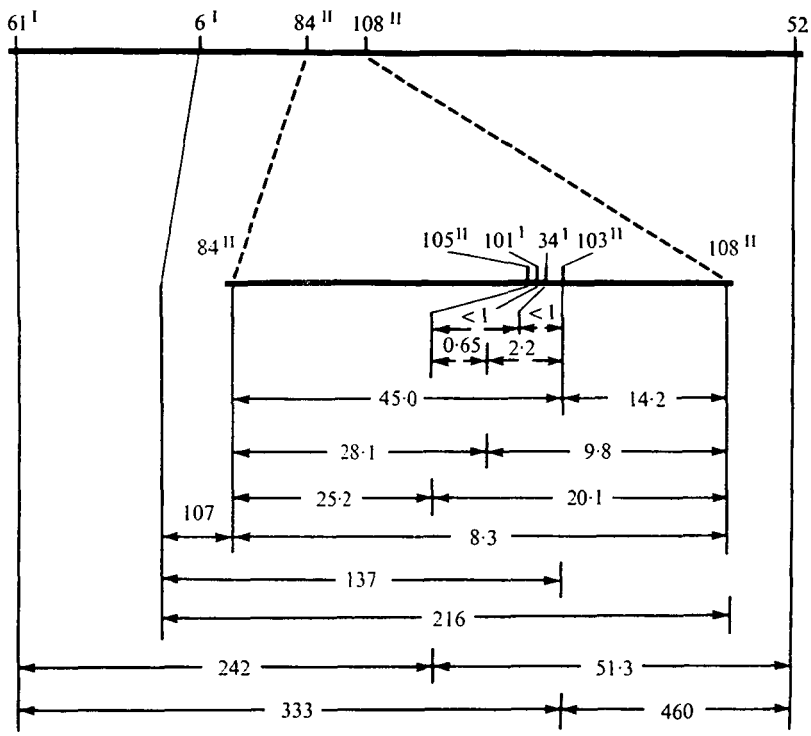

Fig. 3. Fine structure map of the choline sulphatase region. Map distances are the recombination frequencies per 10^5 viable ascospores. Allele number superscripts refer to nutritional groups.

recombination value is significantly inconsistent with the additivity of map distances for surrounding markers. The recombination frequency between *csuA84* and *csuA108* is $8{\cdot}3 \times 10^{-5}$. The relative distance between these markers inferred by their positions on the map is of the order of 40×10^{-5} to 60×10^{-5} based on the results of three crosses involving the markers in question (Fig. 3).

The fine structure map constructed from the recombination data shows that the groups I and II alleles are interspersed along its length. It is not possible to construct the fine structure map so that the two classes of mutants map at adjacent clusters, analogous to the B5 and B3 segments of the *pan*-2 locus in *Neurospora*

crassa (Case & Giles, 1958), without producing a large number of non-additive map distances.

Therefore, it is concluded that the mutants of groups I and II are defective at the same genetic locus on the basis of genetic fine structure mapping and complementation tests; that group II mutants are able to grow in medium containing limiting sulphur in the COS growth test due to the presence of residual choline sulphatase activity, while group I mutants are more tightly blocked; and that the growth inhibition of group II mutants in a rich sulphur source in the COS growth test must be due to the effect of normal feedback controls acting on the residual activity of the leaky mutants.

(iii) *The COS storage hypothesis*

Mutant *csuA101* was selected for comparison with strain 165 (*csuA*$^+$) in a test of the COS storage hypothesis. The mutant has no detectable choline sulphatase in crude extracts and it shows reduced growth on COS as the sole source of sulphur. It has normal sulphate uptake and, by its ability to grow on sulphate as the sole source of sulphur, it presumably has normal sulphate assimilation.

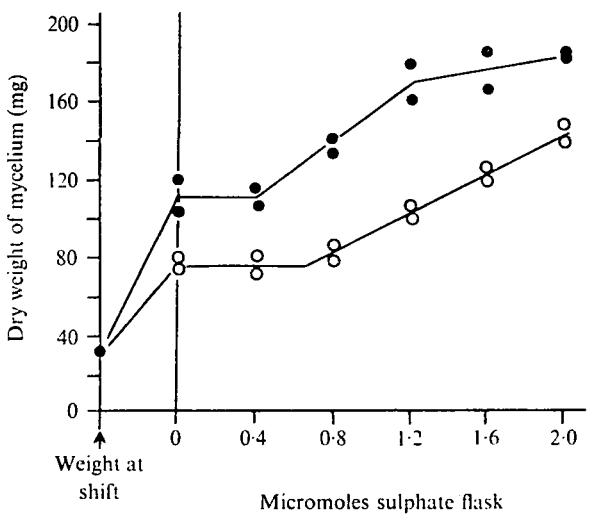

Fig. 4. Storage of choline-O-sulphate. Mycelia were harvested from cultures grown in 2 mM sulphate as sole source of sulphur. Duplicate aliquots (0·2 g wet weight; average dry weights are indicated on the left ordinate) were transferred to flasks of media containing the indicated amounts of sulphate. After 72 h growth, dry weights were obtained and plotted as shown. ●, *csuA*$^+$; ○, *csuA101*.

The two strains were grown in sulphate-rich medium in which they were expected to accumulate COS (Spencer & Harada, 1960) as well as assimilate the sulphate for growth. After thoroughly washing and transferring the mycelium to S-free medium, any further weight increase would have to be attributed to the ability to mobilize endogenous sulphur stores. Fig. 4 shows that the dry weight of the *csu*$^+$ strain

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increased from 28 to 110 mg after transfer to S-free medium, an increase of 290 %, while the dry weight of mutant *csuA101* increased by only 160 % to 74 mg.

The interpretation of the COS storage experiment depends on the ability of the strains being tested to have the same increment of weight increase per unit sulphur taken up. The graph in Fig. 4 presents the dry weight yields of mycelia transferred from S-rich media to media containing different amounts of sulphate. The slopes of the curves represent the final dry weight increments per unit additional sulphur. As can be seen the slopes are roughly equal. This confirms that the difference in growth yield between the *csuA*$^+$ and *csuA101* strains is due to a difference in the available sulphur accumulated in the S-rich medium and not due to a difference in their capacity to assimilate sulphate. Since, as far as can be determined, the sulphur pathways of these strains differ only with respect to choline sulphatase activity, it is concluded that COS is responsible for the additional growth of the *csuA*$^+$ strain above the 160 % increase observed for mutant *csuA101* in the S-free medium. Using a growth yield of 52 mg/μmol sulphate taken from the curve for mutant *csuA101*, the results indicate that the total sulphur pool available for further growth in the S-free medium was 56 mmol/gm dry weight for the *csuA*$^+$ strain and 31 mmol/g dry weight for the mutant. Thus the difference of 25 mmol/gm is apparently due to COS and is equivalent to 0·45 % of the dry weight of the mycelium. This is in good agreement with a yield of more than 0·2 % COS found in *A. sydowi* by Wooley & Peterson (1937).

4. DISCUSSION

Two classes of mutants were recovered which are unable to grow in medium containing COS as the sole source of choline. One group of mutants with no detectable choline sulphatase activity is unable to produce choline from COS in any growth medium. The members of the second group have enough residual enzyme activity to meet the choline requirement in growth medium designed to produce maximal derepression of choline sulphatase. These mutants are unable to grow in COS medium to which other sulphur sources have been added, presumably because these compounds lead to repression or inhibition of residual choline sulphatase activity to levels too low to cope with the choline requirement. Thus the introduction of the *sB* or *sC* mutation blocks the growth-inhibitory effect of sulphate but not of other sulphur compounds which can be metabolized to feedback effectors of choline sulphatase. This view is upheld by the results of complementation tests and fine structure mapping. The lack of complementation between any mutants of the two groups and the random distribution of alleles in the fine structure map demonstrate that the mutants of both groups are defective at the same locus. Whether the *csuA* region encodes the choline sulphatase is not obvious from the data and such a conclusion must await biochemical confirmation of primary modification of the enzyme of group II mutants or revertants of either group.

The results of the S-starvation experiment confirm the ability of *A. nidulans* to mobilize previously accumulated sulphur stores during periods of S-depletion.

Spencer & Harada (1960) postulated that the sulphur reservoir is made up predominantly of COS. Yet the present results show that a significant amount of growth was attained by a mutant lacking any demonstrable choline sulphatase activity in a similar S-starvation experiment, and it was inferred that COS made up less than half of the sulphur made available after shift to S-free medium. The evidence for the regulation of the COS (Spencer & Harada, 1960; Scott & Spencer, 1968) and the demonstration of the accumulation of COS to very high levels (0·43 % by weight in this study) certainly support the concept of sulphur storage, but it may be worthwhile to reconsider the role of COS as a sulphur storage compound in view of its considerably smaller contribution to the total weight increase of *A. nidulans* than anticipated by the experiment of Spencer & Harada.

The ability of *Aspergillus* to accumulate and retain large quantities of sulphate would seem to obviate a need for a unique sulphur storage mechanism. Bradfield *et al.* (1970) found that sulphate uptake is a uni-directional process and that the sulphate pool could be maintained against a concentration gradient or in the presence of a variety of exogenously supplied sulphur metabolites. They measured a maximal sulphate pool size of up to 175 μmol/g dry weight in a mutant of *A. nidulans* with defective sulphate activation and preconditioned in S-limiting medium. This is seven times the COS pool size and three times the size of the total sulphur pool available for later assimilation reported in this investigation. Thus the maximal capacity of the sulphate pool by far exceeds the size of the COS pool measured in this report and elsewhere. In Neurospora, McGuire & Marzluf (1974) have demonstrated an extremely complex sulphur storage system involving COS, cysteine, methionine, and an unidentified sulphur compound of high molecular weight containing cysteine and homocysteine; COS predominates in ascospores but is a minor component in conidia and mycelia. These data argue against a primary role for COS in sulphur storage.

If there has been no selective pressure for the evolution of sulphur storage as COS, then what is the basis for the COS shunt in higher fungi? It is possible to envisage a role for the COS shunt in the regulation of the pool sizes of the active sulphates, APS and PAPS. In bacteria and yeast, which do not synthesize COS, the first enzyme of sulphate activation, ATP sulphurylase, as well as APS kinase in *E. coli*, has been reported to be repressible by later sulphur metabolites (DeVito & Dreyfuss, 1964; Wheldrake & Pasternak, 1965; Wheldrake, 1967). In *Aspergillus* the regulation of the sulphate activating enzymes has not been investigated, although the observation of the accumulation of COS in S-rich medium, with the active sulphates as intermediates, indicates that these enzymes are synthesized constitutively (Spencer *et al.* 1968). Similarly, in *Neurospora crassa* ATP sulphurylase is not repressed by methionine nor is it lost in the mutant, *cys-3*, which has a pleiotropic loss of several repressible enzymes of the sulphur pathway (Marzluf, 1970). Thus in the absence of regulation of sulphate activating enzymes, APS and PAPS might accumulate to levels high enough to interfere with the energy balance of the cell or to inhibit adenylate-dependent reactions.

The synthesis of COS in S-rich medium may reflect a role in the regulation of the

pool sizes of the active sulphates. COS provides a route for the disposal of the sulphate moiety of PAPS such that the sulphate ion does not become available for reactivation by ATP sulphurylase. Any mechanism for the hydrolysis of PAPS which regenerated inorganic sulphate would be lethal in S-rich medium. It would produce a never-ending, energy requiring cycle consisting of sulphate activation to APS and PAPS with hydrolysis of PAPS to regenerate the sulphate for reactivation. With COS acting as an end-product in S-rich medium, the accumulation of the active sulphates is prevented without liberating free sulphate. This leaves to be answered the question of whether excessive accumulation of the active sulphates could be toxic to growth.

An interesting phenomenom which might bear on the toxicity of the active sulphates was reported by Gillespie, Demerec & Itikawa (1968). They observed that some old cultures of *Salmonella typhimurium* defective in the reduction of PAPS tend to accumulate second mutations which, in every case tested, occurred at earlier steps in the sulphur pathway. They concluded that the accumulation of intermediates before the metabolic block is deleterious to cell viability, while double mutants with an additional, earlier defect have a selective advantage. Similar double mutants have also been observed among some original *cys* mutants of Neurospora (Murray, 1965) and this phenomenon has been observed for other pathways as well (discussed by Gillespie *et al*, 1968).

The potential for the sparing effect of COS is best exemplified by an experiment reported by Segel & Johnson (1963). The principal sulphur compounds produced by mycelia of *Penicillium chrysogenum* conditioned in S-rich medium after a 33-sec pulse with [^{35}S]sulphate were [^{35}S]PAPS and [^{35}S]COS. The use of pulses of increasing duration up to 90 sec revealed that while all absolute pool sizes increased, the relative size of the PAPS and sulphite pools as a function of total sulphur decreased and the relative amount of COS increased sharply. All other sulphur pool sizes produced much smaller changes. The data might indicate a responsiveness of COS synthesis to the levels of the active sulphates accumulated by mycelium grown in S-rich medium.

It is apparent that a proper understanding of the role of COS in higher fungi will have to await the recovery of mutants with defective choline sulphotransferase. If the COS shunt does exert control over the pool sizes of the active sulphates then such mutants might be expected to be growth-inhibited in high sulphate medium. Conversely, if the role of COS is limited to the storage of sulphur then the mutants should not be affected by the increased supply of sulphate. Arst (personal communication) has isolated some mutants which are growth-inhibited by high concentrations of exogenous sulphate, although they display normal growth in low sulphate medium. These mutants have not been characterized biochemically. It might be interesting to test these for the *in vivo* accumulation of COS on the possibility that one might prove to lack choline sulphotransferase.

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