Mechanism of Choline O-Sulphate Utilization in Fungi

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1. The position of the enzyme blocks in a number of parathiotrophic mutants of *Aspergillus nidulans* A 69 and mutants *A* and *C* of a biotinless mutant of *Aspergillus nidulans* were examined by nutritional and heterokaryosis experiments and by assay *in vitro* of enzyme systems and specific enzymes. 2. The mutants were in five groups: *A* and *C* blocked at sulphate transport; *gamma* at ATP sulphurylase; *iota* at adenosine 5'-sulphatophosphate kinase; *eta* at the adenosine 3'-phosphate 5'-sulphatophosphate reductase system; *alpha*, *beta* and *zeta* between sulphite and thiosulphate. 3. The ability of the various mutants to synthesize choline O-sulphate *in vivo* and *in vitro* and to utilize choline O-sulphate as a source of sulphur indicated that the utilization of endogenously formed choline O-sulphate involved the splitting off of inorganic sulphate, which was then reduced. 4. Choline O-sulphate acted as a source of choline for cholineless strains of *Neurospora crassa*, suggesting that choline O-sulphate breakdown occurred by simple hydrolysis involving a choline sulphatase. 5. After de-repression of mycelia by growing for a period on a sulphur-free medium the presence of choline sulphatase in physiologically significant amounts was demonstrated in all the *A. nidulans* strains tested. 6. Choline O-sulphate is transported across the mycelial wall by a mechanism different to that responsible for inorganic sulphate transport.

Choline O-sulphate is found in the higher fungi (Harada & Spencer, 1960), lichens of the *Rocella* species (Lindberg, 1956b), the lichen *Dermatiscum thunbergii* (Harper & Letcher, 1966), the red alga *Gelidium cartilagineum* (Lindberg, 1956a) and in plants such as corn, barley and the sunflower (Nissen & Benson, 1961). The synthesis of choline sulphate in *Aspergillus sydowi* (Kaji & McElroy, 1958) and other fungi (Spencer & Harada, 1960) involves the formation of PAPS* and the subsequent transfer of sulphate to choline by the enzyme choline sulphotransferase (EC 2.8.2 group, PAPS-choline sulphotransferase) (Orsi & Spencer, 1964). The failure of the lower fungi to synthesize choline sulphate is due to a lack of choline sulphotransferase rather than of the sulphate-activating system (Spencer & Harada, 1960).

The relatively large amounts of choline sulphate found in fungal mycelia suggest that the ester is an end product of sulphur or choline metabolism rather than an active intermediate. It is more likely that it acts as a store of sulphur rather than a waste product, since it can be used as the sole source of sulphur for the growth of various fungi and endogenously formed choline sulphate will support the growth of *Aspergillus oryzae* and

Penicillium chrysogenum for some time after transfer of the fungi to sulphur-deficient media (Spencer & Harada, 1960). The sulphur of the choline sulphate of conidiospores of *A. niger* is readily incorporated into the sulphur-containing amino acids of the cellular protein during spore germination (Takebe & Yanageta, 1959; Takebe, 1960).

The mechanism of utilization of the sulphur of the stored choline sulphate is not clearly understood. A reversal of the choline sulphotransferase reaction to give PAPS, which could be reduced to SO_3^{2-} and then to cysteine, has been ruled out (Kaji & Gregory, 1959; Orsi & Spencer, 1964). Several workers have failed to observe a choline sulphatase in fungi (Ballio *et al.* 1960; Itahashi, 1961) and, though this enzyme has been detected in *A. sydowi* and *P. chrysogenum* (Segel & Johnson, 1963), the activity of the enzyme was very low and its physiological significance uncertain.

In the present study, the positions of enzyme blocks in a series of parathiotrophic mutants of *Aspergillus nidulans* were elucidated and indicate that hydrolysis of choline sulphate is obligatory for the utilization of this compound. Subsequently, the presence of choline sulphatase in physiologically significant amounts was demonstrated after de-repression of the enzyme by sulphur starvation (cf. Scott & Spencer, 1965a).

* Abbreviations: PAPS, adenosine 3'-phosphate 5'-sulphatophosphate; APS, adenosine 5'-sulphatophosphate.

A preliminary account of this work has been given (Hussey, Orsi, Scott & Spencer, 1965).

EXPERIMENTAL

Organisms and culture conditions

Aspergillus nidulans A69 (wild-type) was obtained from Mr Yuill (late of John and E. Sturge Ltd.). This strain has been accepted by the Commonwealth Mycological Institute as a replacement of C.M.I. 38576 and is listed as C.M.I. 38576(ii). Six parathiotrophic mutants of C.M.I. 38576, namely *alpha* (C.M.I. 38579S), *beta* (C.M.I. 38580S), *gamma* (C.M.I. 38581S), *zeta* (C.M.I. 38584S), *iota* (C.M.I. 38586S), and *eta* (C.M.I. 38595S), described by Hockenhull (1949), were obtained from the Commonwealth Mycological Institute. Two other *A. nidulans* mutants, *A* (*bi*1; W_3 :S10) and *C* (*bi*1; W_3 :S9), were kindly supplied by Professor J. A. Roper. *Neurospora crassa* A.T.C.C. 9279 (wild-type) and two cholineless mutants (A.T.C.C. 14078 and 14714) have been described by Horowitz & Beadle (1943) and Horowitz (1946).

Bulk amounts of mycelia for most enzyme assays were obtained by aerated growth in 10l. of glucose-peptone medium (Orsi & Spencer, 1964) or Czapek-Dox liquid medium (Spencer & Harada, 1960). In nutritional studies the medium was a modified Czapek-Dox medium containing (per l.) 30g. of glucose, 10.5g. of citric acid, 2g. of $NaNO_3$, 0.5g. of KCl, 0.4g. of KH_2PO_4 , 0.4g. of $MgCl_2 \cdot 6H_2O$, 0.005g. of $FeCl_3$ (anhydrous) and the appropriate amount of sulphur-containing compound. The pH was adjusted to 6.0 with NaOH and the medium distributed in 100ml. portions in 500ml. conical flasks. Sulphur sources were added to give 3.2mg. of S/100ml. and sterilized in the medium at 10lb./in.² for 20min. for Na_2SO_4 and choline sulphate. Dimethylaminoethanol sulphate, monomethylaminoethanol sulphate, aminoethanol sulphate, Na_2SO_3 , $Na_2S_2O_3$ and cysteine were sterilized by filtration and added to the autoclaved medium. Biotin (1 μ g./100ml. of medium) was incorporated for the biotin-requiring mutants *A* and *C*. The inoculum was 1 ml. of a spore suspension from a potato-glucose-agar or a maltose-agar slope containing between 5×10^6 and 5×10^7 spores/ml. The flasks were incubated at 28° in static culture for 9 days. Growth was determined by filtering the mycelia on to a dried weighed filter paper and drying at 80° to constant weight. In the absence of any sulphur source slight growth of up to 30mg. dry wt. of mycelium/100ml. of medium was obtained (Table 1).

For the assay of choline sulphatase and for measurement of sulphate and choline sulphate uptake, mycelia were grown at 28° with shaking on the modified Czapek-Dox medium with taurine (0.136g./l.) as the sulphur source. After 4 days the mycelia were transferred to a further batch of the same medium but without any sulphur source and shaken at 28° for a further 4 days. Biotin (1 μ g./100ml. of medium) was incorporated for mutants *A* and *C*.

With *N. crassa* strains the medium used for investigation of choline requirements was that described by Beadle & Tatum (1945). For investigation of the sulphur requirements the sulphate salts in this medium were replaced by chloride salts.

Heterokaryosis tests were carried out as described by Hockenhull (1949).

Substrates and methods

$Na_2^{35}SO_4$ (code SJS1, specific activity greater than 100mc/mg. of S) was obtained from The Radiochemical Centre, Amersham, Bucks.

The synthesis of choline [^{35}S]sulphate *in vivo* was demonstrated by the method of Harada & Spencer (1960) with the difference that the SO_4^{2-} content of the medium was decreased from 0.06% to 0.01% Na_2SO_4 (w/v) and the amount of carrier-free $^{35}SO_4^{2-}$ was raised from 100 μ C to 600 μ C/100ml. of medium.

The synthesis of [^{35}S]PAPS and choline [^{35}S]sulphate *in vitro* was shown by the method of Spencer & Harada (1960). The assay of ATP sulphurylase (ATP-sulphate adenylyltransferase, EC 2.7.7.4), APS kinase (ATP-APS 3'-phosphotransferase, EC 2.7.1.25) and choline sulphotransferase, protein determination and preparation of [^{35}S]PAPS, [^{35}S]APS and choline [^{35}S]sulphate and analogues were as described by Orsi & Spencer (1964).

Choline sulphatase activity was measured in mycelia that were grown for 4 days with shaking in the modified Czapek-Dox medium containing cysteine (1.5mm) as the sulphur source and then transferred to the sulphur-deficient medium for a further 4 days. The assay method was that of Scott & Spencer (1968).

Choline [^{35}S]sulphate. Choline [^{35}S]sulphate of high specific activity (1 mc/m-mole) was prepared by a modification of the method of Segel & Johnson (1963). To 50mg. of choline chloride were added 0.2ml. of 10N-HCl, 1mg. of Na_2SO_4 and 7mc of $Na_2^{35}SO_4$. The mixture was kept at 98° for 10hr. and then dissolved in 0.5ml. of water and distributed as a streak on Whatman 3MM paper. After electrophoresis in 0.1M-ammonium acetate buffer, pH 8.6, for 6hr. at 10v/cm. the radioactivity remaining at the origin was eluted with 10ml. of water. The solution of choline [^{35}S]sulphate so obtained contained 85% of the radioactivity of the original $Na_2^{35}SO_4$. The preparation was free of choline and $^{35}SO_4^{2-}$ but slowly decomposed even at 0° and pH 8.6; decomposition was faster at acid pH. The choline sulphate so prepared had a specific activity of 1 mc/m-mole. This was added to unlabelled choline sulphate to give the specific activities required in the assay methods for choline sulphatase and choline sulphate transport.

Unlabelled and low-specific-activity [^{35}S]sulphate esters of choline and its demethylated analogues were prepared as described by Orsi & Spencer (1964).

Synthesis of choline sulphate in vivo. The synthesis of non-labelled choline sulphate *in vivo*, with inorganic sulphate produced endogenously from organic sulphur sources supplied in the medium, was determined by extracting the choline sulphate from the harvested mycelia, hydrolysing with acid and estimating the liberated SO_4^{2-} . The dried mycelium (1g.) was extracted with 5ml. of water at 100° and the extract centrifuged. A portion of the supernatant (2ml.) was passed through a 10cm. $\times$ 0.5cm. column of Dowex mixed-bed resin [equal quantities of Dowex 1 (OH⁻ form) and Dowex 50 (H⁺ form)] to remove SO_4^{2-} and other interfering ions. A total of 5ml. of effluent was collected and boiled with 2ml. of 3N-HCl for 2hr. The hydrolysate was freeze-dried, redissolved in 1ml. of water and the SO_4^{2-} content determined by the turbidimetric method of Dodgson (1961).

Sulphate and choline sulphate uptake. For measurement of sulphate uptake, 1g. wet wt. of mycelium that had been

sulphur-starved for 2 days (see section on choline sulphatase assay) was suspended in 5 ml. of the modified Czapek-Dox medium in which the only sulphur source was $0.05 \mu\text{M}$ - Na_2SO_4 plus $4.25 \mu\text{C}$ of $\text{Na}_2^{35}\text{SO}_4$. After incubation at room temperature for 10 min. or 1 hr. the suspended mycelium was filtered quickly under vacuum and the radioactivity remaining in the supernatant was measured by spotting $15 \mu\text{l}$. on Whatman no. 1 paper and scanning with the Actigraph strip-scanner (Nuclear-Chicago Corp.). The amount of sulphate taken up by the mycelium was obtained by subtracting the 10 min. and 1 hr. radioactivity values from a zero-time sample.

A similar technique was used for the measurement of choline sulphate, dimethylaminoethanol sulphate, monomethylaminoethanol sulphate and aminoethanol sulphate uptakes by mycelia but $4 \mu\text{M}$ solutions of the ^{35}S sulphate esters (1 mc/m-mole) were substituted for the Na_2SO_4 and $\text{Na}_2^{35}\text{SO}_4$. Controls carried out with boiled mycelia showed an apparent $^{35}\text{SO}_4^{2-}$ or choline ^{35}S sulphate uptake of about 2–5%. This was due to dilution of the uptake medium by water present in the pressed mycelium.

RESULTS

Growth experiments

Nutritional studies. The growth of the wild-type and of the eight mutants of *A. nidulans* on the modified Czapek-Dox medium containing various sulphur compounds as the sole sulphur source is shown in Table 1. The results confirmed Hockenfull's (1949) findings that the wild-type could utilize SO_4^{2-} , SO_3^{2-} , $\text{S}_2\text{O}_3^{2-}$ and choline sulphate equally as well as cysteine. In addition, dimethylaminoethanol sulphate, monomethylaminoethanol sulphate and aminoethanol sulphate, which are not known to occur naturally, also acted as good sulphur sources. The five sulphateless mutants

(*gamma*, *iota*, *eta*, *A* and *C*), which could all utilize SO_3^{2-} and $\text{S}_2\text{O}_3^{2-}$ but not SO_4^{2-} , fell into two groups: group I, mutants *gamma*, *iota* and *eta*, which showed only trace growth on the sulphate esters of choline and its demethylated analogues; group II, mutants *A* and *C*, which grew quite well on these sulphate esters. Both the group II mutants (from Professor Roper) were biotin-requiring, whereas the group I mutants did not require biotin.

Table 2. *Growth of A. nidulans mutant A on various sulphur sources*

The mutant was grown in 100 ml. of modified Czapek-Dox medium containing $1 \mu\text{g}$. of biotin and sulphur sources at the concentrations shown. Growth was for 10 days in static culture at 27° .

Sulphur source	Growth (mg. dry wt. of mycelium)
Na_2SO_4 (0.1 mM)	5
Na_2SO_4 (1.0 mM)	9
Na_2SO_4 (10 mM)	7
Na_2SO_3 (1.0 mM)	192
$\text{Na}_2\text{S}_2\text{O}_3$ (0.5 mM)	124
Choline sulphate (1.0 mM)	265
Choline sulphate (1.0 mM) + Na_2SO_3 (1.0 mM)	450
Choline sulphate (1.0 mM) + $\text{Na}_2\text{S}_2\text{O}_3$ (0.5 mM)	375
Choline sulphate (1.0 mM) + Na_2SO_4 (0.01 mM)	277
Choline sulphate (1.0 mM) + Na_2SO_4 (0.1 mM)	286
Choline sulphate (1.0 mM) + Na_2SO_4 (1.0 mM)	281
Choline sulphate (1.0 mM) + Na_2SO_4 (10 mM)	361
Choline sulphate (1.0 mM) + Na_2SO_4 (100 mM)	377

Table 1. *Growth of A. nidulans and its mutants on different sulphur sources*

Fungi were grown in 100 ml. of medium in static culture at 28° for 9 days. Yields of mycelia are the average values for six experiments. The sulphur sources added contained 3.2 mg. of S. Other details are given in the Experimental section.

Sulphur source	Strain ... A69 (wild-type)	Dry wt. of mycelium (mg.)							
		<i>gamma</i>	<i>iota</i>	<i>eta</i>	<i>A</i> *	<i>C</i> *	<i>alpha</i>	<i>beta</i>	<i>zeta</i>
SO_4^{2-}	210	25	15	6	15	20	5	2	8
SO_3^{2-}	259	332	290	270	266	191	4	2	4
$\text{S}_2\text{O}_3^{2-}$	253	266	311	227	128	98	214	235	194
Choline sulphate	260	21	10	10	215	150	5	27	—
Dimethylaminoethanol sulphate	252	21	8	25	181	105	3	30	—
Monomethylaminoethanol sulphate	235	20	25	21	89	110	9	31	—
Aminoethanol sulphate	230	20	12	30	193	154	3	34	—
<i>p</i> -Nitrophenyl sulphate	172	8	—	5	6	—	—	—	—
Sinigrin	285	11	—	20	7	—	—	—	—
Chondroitin sulphate A	170	6	—	7	5	—	—	—	—
Cysteine	226	242	235	261	228	211	230	192	215

* Biotin ($1 \mu\text{g}$ /100 ml. of medium) was included.

Later it was found that, of eight further biotin-requiring sulphateless mutants supplied by Professor Roper, four (mutants S_8 , S_9 , S_{12} and $Cys 2$) belong to group I and four (mutants S_3 , S_5 , S_{10} and S_{11}) to group II.

When mutant *A* was grown on choline sulphate (1mM) in the presence of various amounts of SO_4^{2-} (0.01–1mM) little or no effect on the final growth yield was observed compared with that obtained when choline sulphate was used alone. When 1mM- SO_3^{2-} or 0.5mM- $S_2O_3^{2-}$ was used to supplement the choline sulphate the growth yields were almost the sum of the yields obtained with choline sulphate and SO_3^{2-} (or $S_2O_3^{2-}$) separately (Table 2). These results ruled out the possibility that SO_4^{2-} acted as an inhibitor of spore germination or growth of the group II mutants and emphasized that the growing mycelium of these mutants could not use inorganic sulphate as a sulphur source for growth.

Table 3. *Choline sulphate synthesis in vivo by A. nidulans and its mutants from endogenously produced sulphate*

Mycelia were grown for 2 or 4 days at 27° with shaking on the modified Czapek–Dox medium with taurine (25mM) as the sole sulphur source. Biotin (2μg./100ml.) was included in the medium for mutant *A*. Choline sulphate in the mycelium was estimated as described in the Experimental section.

Strain	Choline sulphate (% of dry. wt. of mycelium)	
	2 days	4 days
A 69 (wild-type)	0.14	0.56
<i>A</i>	0.19	0.37
<i>gamma</i>	0	0
<i>iota</i>	0	0
<i>eta</i>	0.25	0.37

When much larger supplements of inorganic sulphate (10 and 100mM) were used to supplement the choline sulphate (1mM) some additional growth occurred (Table 2). That this is due to a slight 'leakiness' in the mutant is shown below.

The wild-type, but none of the mutants, was able to utilize the sulphate esters sinigrin, *p*-nitrophenyl sulphate and chondroitin sulphate as sulphur sources, suggesting that these esters must be hydrolysed to inorganic sulphate before the sulphur can be used for growth.

Heterokaryosis tests. In confirmation of the heterokaryosis test carried out by Hockenull (1949) on the A 69 mutants, four classes were recognized; I, mutant *gamma*; II, mutant *iota*; III, mutant *eta*; IV, mutants *alpha*, *beta* and *zeta*. The two sulphateless mutants, *A* and *C*, showed no heterokaryosis and appeared to be variants of the same mutant. Both mutants *A* and *C* formed heterokaryons with all the other mutants and thus form a fifth class.

Enzyme experiments. As judged from the density of the radioautograph spots, the wild-type (A 69) and mutants *alpha*, *beta*, *zeta* and *eta* synthesized approximately equal amounts of choline [^{35}S]-sulphate *in vivo* when grown on the glucose–peptone medium containing $Na_2^{35}SO_4$ as a supplement to cysteine as the main sulphur source. Extracts of acetone-dried mycelia of the wild-type and these mutants synthesized [^{35}S]PAPS and choline [^{35}S]-sulphate *in vitro* from $^{35}SO_4^{2-}$, ATP and choline in the presence of Mg^{2+} . Mutants *A* and *C* showed full synthesis of [^{35}S]PAPS and choline [^{35}S]sulphate *in vitro*, but *in vivo* the amount of choline [^{35}S]-sulphate formed by these mutants from $Na_2^{35}SO_4$ added to the medium, though just detectable by radioautography and thus showing a slight 'leakiness', was negligible compared with the amounts formed by the wild-type. However, the

Table 4. *Activity of ATP sulphurylase, choline sulphotransferase and choline sulphatase in extracts of mutants of A. nidulans*

For details of methods see the Experimental section.

Strain	ATP sulphurylase (μmoles of P_i liberated/mg. of protein/hr.)	Choline sulphotransferase (μμmoles of $^{35}SO_4^{2-}$ trans- ferred from [^{35}S]PAPS to choline/mg. of protein/hr.)	Choline sulphatase (μμmoles of SO_4^{2-} liberated/mg. of protein/hr.)
A 69 (wild-type)	1.4	20.6	1.9
<i>alpha</i>	1.3	30.8	—
<i>beta</i>	1.1	27.4	5.9
<i>gamma</i>	0.05	17.8	10.9
<i>zeta</i>	1.2	32.0	—
<i>iota</i>	1.3	24.2	—
<i>eta</i>	1.3	26.6	2.8
<i>A</i>	1.2	24.2	0.7
<i>C</i>	1.2	18.2	0.9

Table 5. *APS kinase activity in A. nidulans extracts*

The assay was carried out as described by Orsi & Spencer (1964). Fungal extracts were incubated with [^{35}S]APS in the presence of ATP, Mg^{2+} and F^- and the incubation mixtures subjected to electrophoresis. The extracts contained choline sulphotransferase, and some of the [^{35}S]PAPS formed by the kinase was used to form choline [^{35}S]sulphate from the exogenous and endogenous choline. The [^{35}S]APS was contaminated with $^{35}\text{SO}_4^{2-}$ to the extent of 7% of the total radioactivity (equivalent to 4.7 counts/min. in the Table). The extra $^{35}\text{SO}_4^{2-}$ found on the electrophoretograms is due to desulphation of [^{35}S]APS or [^{35}S]PAPS by enzymes in the fungal extract.

Strain	Fungal protein ($\mu\text{g./100}\mu\text{l.}$ of incubation mixture)	$10^{-2} \times$ Radioactivity of specific spots after electrophoresis of the incubation mixture ($100\mu\text{l.}$) (counts/min.)			
		Choline [^{35}S]sulphate	[^{35}S]PAPS	$^{35}\text{SO}_4^{2-}$	[^{35}S]APS
A 69 (wild-type)					
(medium complete)	41.16	13	26.3	27.9	0
(medium minus choline)	49.8	4.9	29.1	30.4	0
(medium minus choline and ATP)	49.8	0	0	52.8	13.1
(medium complete but enzyme extract heated at 100° for 90sec. before incubation)	41.6	0	0	7.3	58.5
<i>alpha</i>	24.5	16.4	23.8	23.0	2.5
<i>beta</i>	24.1	15.3	17.5	23.1	9.2
<i>gamma</i>	41.9	13.5	30.7	15.9	9.2
<i>zeta</i>	25.2	14.7	7.2	33.5	11.2
<i>iota</i>	26.7	0	0	29.2	35.6
<i>eta</i>	41.5	10.1	19.2	25.2	12.7
<i>A</i>	35.7	13.4	13.2	35.3	0
<i>C</i>	34.9	15.7	15.9	24.9	11.5

mycelium of mutant *A* (mutant *C* was not tested) contained amounts of non-labelled choline sulphate comparable with those found in the wild-type or mutant *eta* when grown in the minimal medium with taurine as the sulphur source (Table 3). Mutants *gamma* and *iota* contained no choline sulphate under these conditions.

All mutants possessed choline sulphotransferase activity (Table 4) and the inability of mutants *gamma* and *iota* to synthesize choline sulphate *in vivo* or *in vitro* was shown to be due to an inability to synthesize PAPS.

Since mutants *gamma* and *iota* form heterokaryons it seemed possible that one mutant would lack ATP sulphurylase and the other APS kinase. Mutant *gamma* possessed negligible ATP sulphurylase, whereas mutant *iota*, the other mutants and the wild-type contained appreciable and approximately the same activity of this enzyme (Table 4). Mutant *iota* appeared to be completely devoid of any APS kinase activity, as shown by the failure to synthesize [^{35}S]PAPS or choline [^{35}S]sulphate from [^{35}S]APS, 96% of the total radioactivity being accounted for as residual [^{35}S]APS and inorganic $^{35}\text{SO}_4^{2-}$ (Table 5). The wild-type, *iota* and all other mutants synthesized [^{35}S]PAPS and choline [^{35}S]sulphate from [^{35}S]APS, indicating the presence of APS kinase. Specific activities for this enzyme could not

be calculated owing to the production of large amounts of $^{35}\text{SO}_4^{2-}$ by APS sulphatase (Lewis & Spencer, 1962) and possibly by reversal of ATP sulphurylase activity in the presence of endogenous pyrophosphate.

These results established mutant *gamma* as being blocked at ATP sulphurylase and mutant *iota* at APS kinase. The lack of these enzymes prevented both mutants from growing on SO_4^{2-} (Table 1) and from producing the PAPS necessary for the formation of choline sulphate *in vitro* or *in vivo* (Table 3). The fact that these mutants could not use choline sulphate as a sulphur source suggested that in the wild-type the ester must first be hydrolysed to give inorganic sulphate before the sulphur becomes available (Scheme 1).

That choline sulphate must enter the pathway before SO_3^{2-} is confirmed by the behaviour of mutant *eta*, which grows on SO_3^{2-} but cannot utilize either sulphate or choline sulphate yet can synthesize choline sulphate *in vitro* and *in vivo*. The behaviour of mutants *A* and *C* now seemed anomalous. Though both mutants grew on choline sulphate and the sulphate esters of the demethylated cholines and could synthesize choline sulphate from SO_4^{2-} *in vitro*, they could not utilize SO_4^{2-} for growth. However, the fact that they did not synthesize choline [^{35}S]sulphate from exogenous

$\text{Na}_2^{35}\text{SO}_4$ *in vivo* except in trace amounts, yet the amount of choline sulphate synthesized from SO_4^{2-} produced endogenously from taurine was similar to that produced in the wild-type, suggested that these mutants failed to transport SO_4^{2-} from the medium.

Transport of sulphate and sulphate esters. The uptake of $^{35}\text{SO}_4^{2-}$ from a medium by fungi shows a triphasic pattern (Scott & Spencer, 1965b) in which radioactivity is taken up by the mycelium at a rapid rate, then partially eliminated and later gradually taken up again. The first and second phases represent the transport and elimination of sulphate, whereas the third phase is due to fixation of transported sulphate into organic combination. The duration of the phases varies considerably, depending on several undefined conditions, and they cannot always be separated so as to give a true value for sulphate transport (Scott & Spencer, 1965b). Table 6 shows the amounts of $0.05\ \mu\text{M}\text{-SO}_4^{2-}$ taken up by sulphur-starved mycelia after 10 and 60 min. and, although it is not possible to place exact quantitative significance in terms of sulphate transport, it is clear that the wild-type and all mutants, except mutant *A*, can transport sulphate. Mutant *S*₁₁, which was also unable to transport sulphate, is a biotin-requiring mutant with the same sulphur nutrient requirements as mutants *A* and *C* (see the section on nutritional studies above). The block in sulphate transport in mutants *A* and *S*₁₁ did not affect uptake of $4\ \mu\text{M}\text{-choline}\ [^{35}\text{S}]\text{-sulphate}$ compared with the wild-type and other mutants, and uptake of the sulphate esters of the demethylated cholines was actually greater in mutant *S*₁₁ than in the wild-type.

Choline sulphatase. The results indicated that choline sulphate is utilized by being hydrolysed and the liberated sulphate subsequently reduced via

PAPS and SO_3^{2-} (Scheme 1). In searching for the postulated choline sulphatase it was found that during sulphur sufficiency the enzyme is repressed and undetectable, but that de-repression occurs after a period of sulphur starvation (Scott & Spencer, 1965a). With mycelia that had been grown on a sulphur-sufficient medium for 4 days and then transferred to a sulphur-deficient medium for a further 4–5 days it was shown that the wild-type and all mutants tested possessed choline sulphatase activity (Table 4). The activity of the choline sulphatase was markedly lower than that of choline sulphotransferase (Table 4), but subsequently (Scott & Spencer, 1968) it was found that the choline sulphatase assays had been carried out at a concentration much below the maximum.

Choline sulphate as a source of choline and sulphur for cholineless mutants of N. crassa

The method used for the assay of choline sulphatase *in vitro* did not distinguish between liberation of sulphate by simple hydrolysis or liberation after degradation of the choline moiety. To decide this point and its relevance *in vivo* the ability of choline sulphate to serve as a source of choline for cholineless mutants of *N. crassa* was determined by growing the *Neurospora* mutants on a medium from which choline was omitted and replaced by the sulphate esters of choline, dimethylaminoethanol, monomethylaminoethanol and aminoethanol. Mutant 14078, which grew well when the medium was supplemented with choline, dimethylaminoethanol or monomethylaminoethanol but not with aminoethanol, also grew well with choline sulphate and dimethylaminoethanol sulphate as supplements but not with monomethylaminoethanol sulphate or aminoethanol sulphate.

Table 6. Uptake of $\text{Na}_2^{35}\text{SO}_4$, choline $[^{35}\text{S}]\text{sulphate}$ and analogues by *A. nidulans* mutants

Fungi used in this experiment were grown on a sulphur-sufficient medium for 4 days and transferred to a sulphur-deficient medium for 2 days. The harvested mycelia (1 g. wet wt.) were suspended in 5 ml. of uptake medium as detailed in the Experimental section.

Strain	Uptake (%)									
	$0.05\ \mu\text{M}\text{-Na}_2^{35}\text{SO}_4$		$4\ \mu\text{M}\text{-Choline}\ [^{35}\text{S}]\text{sulphate}$		$4\ \mu\text{M}\text{-Dimethylaminoethanol}\ [^{35}\text{S}]\text{sulphate}$		$4\ \mu\text{M}\text{-Monomethylaminoethanol}\ [^{35}\text{S}]\text{sulphate}$		$4\ \mu\text{M}\text{-Aminoethanol}\ [^{35}\text{S}]\text{sulphate}$	
	10 min.	1 hr.	10 min.	1 hr.	15 min.	1 hr.	15 min.	1 hr.	15 min.	1 hr.
A 69 (wild-type)	50	73	36	24	9	25	4	10	18	31
<i>gamma</i>	25	17	12	27	—	—	—	—	—	—
<i>iota</i>	82	7	8	10	—	—	—	—	—	—
<i>A</i>	2.5	2.5	22	29	—	—	—	—	—	—
<i>alpha</i>	17	19	25	35	—	—	—	—	—	—
<i>beta</i>	48	40	64	96	—	—	—	—	—	—
<i>S</i> ₁₁	3.5	6.5	35	40	22	30	15	11	22	26

Table 7. *Utilization of choline sources by cholineless mutants of N. crassa*

The wild-type and mutants were grown in 50 ml. of medium (Beadle & Tatum, 1945) at 29° for 5 days in static culture. Inorganic sulphate (6.4 mg. of S/flask) was the sulphur source and 10 μ moles of the choline source was added to each flask. Results quoted are the means of three experiments.

Choline source	Strain	Dry wt. of mycelium from 50 ml. of medium (mg.)		
		A.T.C.C. 9279 (wild-type)	A.T.C.C. 14078	A.T.C.C. 14714
Choline		270	338	259
Dimethylaminoethanol		225	339	239
Monomethylaminoethanol		239	343	24
Aminoethanol		278	37	20
Choline sulphate		283	306	265
Dimethylaminoethanol sulphate		233	106	109
Monomethylaminoethanol sulphate		233	40	31
Aminoethanol sulphate		239	36	20
Control (no choline source)		275	41	30

Table 8. *Utilization of sulphur sources by cholineless mutants of N. crassa*

Details are the same as in Table 7 but, in the preparation of the medium, chloride salts were substituted for the sulphates. Choline (10 μ moles) was included in each flask and the various sulphur sources were added to give 3.2 mg. of S/flask.

Sulphur source	Strain	Dry wt. of mycelium from 50 ml. of medium (mg.)		
		A.T.C.C. 9279 (wild-type)	A.T.C.C. 14078	A.T.C.C. 14714
Choline sulphate		214	258	295
Dimethylaminoethanol sulphate		103	59	72
Monomethylaminoethanol sulphate		213	155	142
Aminoethanol sulphate		215	205	194
SO ₄ ²⁻		242	293	262
Choline (100 μ moles) + SO ₄ ²⁻ (100 μ moles)		228	287	276
Control (no sulphur source)		2	7	8

Mutant 14714, blocked between monomethylaminoethanol and dimethylaminoethanol, showed good growth on choline, dimethylaminoethanol and the corresponding sulphate esters but failed to grow on monomethylaminoethanol, aminoethanol or their sulphate esters. The wild-type (A.T.C.C. 9279) grew well on choline, all the demethylated derivatives and their sulphate esters (Table 7).

Choline sulphate and the sulphate esters of the demethylated derivatives acted as good sulphur sources for *N. crassa* and the two cholineless mutants. Growth on dimethylaminoethanol sulphate was less than half that on the other sulphate esters (Table 8).

DISCUSSION

The results of the growth studies and enzyme experiments are compatible with the siting of mutant enzyme deficiencies as shown in Scheme 1.

The demonstration of the existence of an active choline sulphatase in the wild-type and all mutants

after de-repression by sulphur starvation confirmed the proposed pathway of choline sulphate metabolism. Hydrolysis of this ester by the sulphatase releases inorganic sulphate, which is then assimilated by the normal pathway of sulphate reduction. That this is the only available pathway of choline sulphate assimilation is evidenced by the inability to utilize choline sulphate found in all mutants unable to reduce sulphate, even though some of these mutants accumulated the ester *in vitro*. Scheme 1 summarizes the findings.

In the absence of any demonstrable reversal of the choline sulphotransferase activity of fungi (Kaji & Gregory, 1959; Orsi & Spencer, 1964) it follows that the energy used for the synthesis of choline sulphate is dissipated when choline sulphate is hydrolysed before utilization of its sulphate content. Though the view that choline sulphate is a store of readily assimilated sulphate still appears correct, it can no longer be regarded as existing in an activated state analogous to phosphagens in animals (Spencer &

Scheme 1.

Harada, 1960). In this and other respects, the synthesis and degradation of choline sulphate in fungi shows many similarities to those of inorganic polyphosphate in bacteria (Harold & Harold, 1965).

Mutants *A* and *C* showed trace synthesis of choline [^{35}S]sulphate from exogenously supplied $^{35}\text{SO}_4^{2-}$ and the blockage in their SO_4^{2-} transport mechanism must be slightly 'leaky'. The 'leakiness' is insufficient to allow new growth when SO_4^{2-} is supplied as the sole sulphur source, but when substantial amounts (10mM) of SO_4^{2-} are added to a medium containing 1mM-choline sulphate slight extra growth does occur (Table 2). Though the 'leakiness' could be due to incomplete blockage of the enzymically controlled active transport of SO_4^{2-} , some passive diffusion of SO_4^{2-} across the mycelial wall undoubtedly occurs and could account for the two results quoted.

There is evidence to suggest that $\text{S}_2\text{O}_3^{2-}$, an analogue of SO_4^{2-} , is transported by the same mechanism as SO_4^{2-} . In *Salmonella typhimurium*, cryptic SO_4^{2-} mutants do not transport $\text{S}_2\text{O}_3^{2-}$ (Dreyfuss, 1964) and in mitochondria $\text{S}_2\text{O}_3^{2-}$ is a competitive inhibitor of sulphate transport (M. H. R. Lewis & B. Spencer, unpublished work). The growth of the two cryptic *A. nidulans* mutants *A* and *C* on $\text{S}_2\text{O}_3^{2-}$ as the sole sulphur source was only about half that on cysteine, whereas all other mutants grew well on $\text{S}_2\text{O}_3^{2-}$ (Table 1). It is possible that fungal transport of $\text{S}_2\text{O}_3^{2-}$ is mediated through the same mechanism as SO_4^{2-} transport and that in mutants *A* and *C* the slow growth on $\text{S}_2\text{O}_3^{2-}$ is due to the 'leakiness' in the transport blockage or to rapid diffusion of $\text{S}_2\text{O}_3^{2-}$ across the mycelial wall.

The growth of the wild-type, but not the mutants, on *p*-nitrophenyl sulphate, chondroitin sulphate and sinigrin as the sole sulphur sources can be explained

as utilization of SO_4^{2-} liberated from these compounds. The cryptic mutants *A* and *C* might have been expected to utilize these sulphate esters, and their failure to do so suggests that in non-cryptic mutants these sulphate esters are transported across the mycelial membrane by the same mechanism as SO_4^{2-} or that they are hydrolysed exogenously and the SO_4^{2-} liberated is taken up by the mycelia. It is worth noting that inorganic sulphate and the sulphate esters (apart from choline sulphate, which is neutral) carry a strong negative charge. The wild-type and the cryptic mutants *A* and *C*, but not the other mutants, could also utilize the sulphate esters of dimethylaminoethanol, monomethylaminoethanol and aminoethanol. These sulphate esters, like choline sulphate, are internally compensated salts and essentially neutral. They are not affected by the SO_4^{2-} -transport block in mutants *A* and *C* and are readily transported (Table 6).

The results with the *N. crassa* cholineless mutants showed that choline could be split off from choline sulphate *in vivo* and that liberation of SO_4^{2-} from choline sulphate was due in part or in whole to simple hydrolysis of the sulphate ester. The ability of dimethylaminoethanol sulphate to serve as a choline source for mutant 14078 could be due either to the hydrolysis of this sulphate ester followed by the methylation of dimethylaminoethanol or to methylation of the sulphate ester to give choline sulphate, which was then hydrolysed. Whatever the mechanism may be, the enzymes concerned do not appear to act on monomethylaminoethanol sulphate, since this compound does not act as a choline source for mutant 14078 whereas monomethylaminoethanol itself is effective. The ability of the sulphate esters of choline and all the demethylated cholines to act as good

sulphur sources for *N. crassa* and its two cholineless mutants means, at the least, that SO_4^{2-} can be released from monomethylaminoethanol sulphate and aminoethanol sulphate by degradation of the carbon chains of these compounds. Liberation of SO_4^{2-} from monomethylaminoethanol sulphate by simple hydrolysis, as seems likely with the dimethyl and trimethyl analogues, would seem to be ruled out from the results of the choline experiment (Table 8).

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