The Role of Choline Sulphate in the Sulphur Metabolism of Fungi

By B. SPENCER AND T. HARADA*

Department of Biochemistry, University of Wales, Newport Road, Cardiff

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The sulphate ester, choline sulphate, is somewhat of a biological curiosity. It was first isolated by Woolley & Peterson (1937) from *Aspergillus sydowi* in a yield greater than 0.2% of the dry weight of the mycelium, and similar high concentrations were later found in *Penicillium chrysogenum* (De Fliès, 1955; Stevens & Vohra, 1955), in lichens of the *Rocella* species (Lindberg, 1955a) and the red alga *Gelida cartilaginum* (Lindberg, 1955b). Recent studies on the distribution of choline sulphate production (Harada & Spencer, 1960; Ballio *et al.* 1960) have shown a high degree of concurrence between the presence or absence of the compound and accepted taxonomic division. Synthesis of choline sulphate appeared to be limited to the higher fungi since it was not found in any Phycomycetes, and, of the Ascomycetes, the Endomycetales, including baker's yeast, did not accumulate this sulphate ester. Other Ascomycetes, including the Sphaeriales, produced choline sulphate as also did all the Basidiomycetes and Fungi Imperfecti. One or two exceptions to this taxonomic division were noted, particularly amongst the Fungi Imperfecti, but these exceptions were in confirmation of previous morphological evidence which re-locates the fungi in classes which do not produce choline sulphate. None of the bacteria tested produced choline sulphate (Harada & Spencer, 1960).

The relatively large amounts of choline sulphate found in the fungal mycelia would suggest that the ester is an end product or by-product of sulphur or choline metabolism rather than an active intermediate; it could also be envisaged as a waste or storage product. However, the compound can be used as the sole source of sulphur for the growth of various fungi and also as a source of choline by the choline-less strain of *Neurospora crassa* (Stevens & Vohra, 1955; Harada & Spencer, 1960). Furthermore, choline sulphate and inorganic sulphate must possess some common metabolic pathway since mutant strains of *Aspergillus nidulans* and *Penicillium notatum* could utilize neither inorganic sulphate nor choline sulphate as sulphur sources but the parent strains grew readily on either com-

pound (Hockenhull, 1948, 1949). Egami & Itahashi (1951) have shown that *Aspergillus oryzae* utilized choline sulphate for growth much more readily than inorganic sulphate, and competitive-metabolism experiments with the two sulphur sources (Itahashi, 1954) were interpreted as showing that choline sulphate was an intermediate in the utilization of the sulphur of inorganic sulphate. Similar experiments with *P. chrysogenum*, on the other hand, have shown that inorganic sulphate was used preferentially to choline sulphate for penicillin production (Stevens & Vohra, 1955). The evidence is thus somewhat controversial.

Harada & Spencer (1960) concluded that there were two possible ways in which choline sulphate could be connected with the utilization of the sulphur of inorganic sulphate for growth purposes by fungi; firstly, that the choline sulphate-producing and non-producing fungi metabolized sulphate in the same manner and that choline sulphate formation was just a side reaction in the former class of mould, and, secondly, that the two classes of fungi metabolize sulphate differently, choline sulphate formation being an obligatory stage only in the choline sulphate-producing fungi. It was the purpose of the present study to investigate these two alternatives.

As a first objective an attempt was made to find out why some fungi produced choline sulphate and others did not. Choline sulphate synthesis by *A. sydowi* is thought to involve the enzymically catalysed transfer of sulphate from adenosine 3'-phosphate 5'-sulphatophosphate to choline (Kaji & McElroy, 1958). It is further known that formation of adenosine 3'-phosphate 5'-sulphatophosphate in yeast involves a reaction between adenosine triphosphate and SO_4^{2-} ions to give adenosine 5'-sulphatophosphate and pyrophosphate and that this is followed by 3'-phosphorylation of the adenosine 5'-sulphatophosphate to give adenosine 3'-phosphate 5'-sulphatophosphate (Robbins & Lipmann, 1957). There are several places in this overall scheme where choline sulphate production could be stopped. One possibility, the absence of choline, has already been ruled out by two different methods (Harada & Spencer, 1960; Ballio *et al.* 1960), and it is hardly likely that there is insufficiency of adenosine triphosphate. The other

* Present address: Department of Applied Microbiology, Institute of Scientific and Industrial Research, University of Osaka, Osaka, Japan.

possibilities have therefore been examined. A preliminary communication has appeared (Spencer & Harada, 1959).

EXPERIMENTAL

Materials

$\text{Na}_2^{35}\text{SO}_4$ (code SJSI, specific activity greater than 100 mc/mg. of S) was obtained from The Radiochemical Centre, Amersham, Bucks. Adenosine 3'-phosphate 5'-[^{35}S]-sulphatophosphate was prepared by a biosynthetic method (Spencer, 1960). Choline [^{35}S]sulphate was synthesized from $\text{H}_2^{35}\text{SO}_4$ (The Radiochemical Centre) by the method of Schmidt & Wagner (1904), and had a specific activity of 14 000 counts/sec./mg. Adenosine 5' [^{35}S]-sulphatophosphate was synthesized by the method of Reichard & Ringertz (1959) and had a specific activity of 7000 counts/sec./mg.

The choline sulphate-producing fungi used were: *Aspergillus oryzae*, CMI: 17299; *Aspergillus nidulans*, CMI: 16643; *Aspergillus terreus*, LSH: Ab2; *Aspergillus sydowi*, CMI: 63904; *Penicillium aurantio-brunneum*, LSH: P2; *Penicillium notatum*, LSH: P45; *Penicillium chrysogenum*, CMI: 62728, *Penicillium citreo-roseum* LSH: P22.

The fungi not producing choline sulphate were: *Rhizopus stolonifer* (+), CMI: 57761; *Eremothecium ashbyii*, CMI: 31269; *Nematospora gossypii*, CMI: 31279; *Mucor hiemalis*, CMI: 21217; *Torulopsis utilis*, CMI: 23311; baker's yeast (Harada & Spencer, 1960).

Culture conditions. Fungi were grown at 27° either in static culture in 100 ml. of medium contained in 250 ml. conical flasks or shaking-culture in 200 ml. of medium in 1 l. flat-bottomed flasks. Vessels were inoculated with 0.5 ml. of spore suspension containing 10^7 – 10^8 spores/ml., prepared by washing spores from maltose-agar or glucose-peptone-yeast extract-agar with sterile water.

The following media were used: the Czapek-Dox liquid medium, containing 50 g. of glucose, 2.0 g. of NaNO_3 , 0.4 g. of KH_2PO_4 , 0.5 g. of KCl, 0.5 g. of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.01 g. of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 l. of water; a glucose-asparagine medium containing 10 g. of glucose, 1.5 g. of asparagine, 1.5 g. of $(\text{NH}_4)_2\text{H}_2\text{PO}_4$, 1.0 g. of KH_2PO_4 , 0.5 g. of MgSO_4 , 0.5 g. of NaCl, 20 mg. of inositol, 1 mg. of thiamine and 1 μg . of biotin in 1 l. of water; a glucose-peptone-yeast extract medium containing 20 g. of glucose, 10 g. of Bacto-peptone, 3 g. of Bacto-yeast extract and 0.6 g. of Na_2SO_4 in 1 l. of water. All media were adjusted to pH 5.4 with HCl or NaOH before sterilization for 20 min. at 15 lb./in.². Sulphate esters, apart from choline sulphate, which is stable under the sterilization conditions, were Seitz-filtered and added to sterilized media.

In experiments where the weight of the dried mycelium was measured, the mycelium was harvested by filtering through muslin, washed well with water and dried *in vacuo* over P_2O_5 for 2 days. The cells of some fungi, such as *E. ashbyii*, *T. utilis* and baker's yeast, were harvested by centrifuging.

Competitive metabolism. The complex glucose-asparagine medium was supplemented with normal or radioactive Na_2SO_4 or choline sulphate as shown in the legend to Fig. 1. The radioactive materials were diluted with the appropriate carrier compounds to give activities of the order of

94 counts/sec./ml. of medium for Na_2SO_4 and 112 counts/sec./ml. for choline sulphate.

The fungi were grown in 200 ml. of medium at 27° with shaking. At the commencement of the experiment, and thereafter every 24 hr., 5 ml. of the culture medium was aseptically removed, centrifuged to remove the mycelium, and 1 ml. of the supernatant was diluted to 5 ml. with water. A portion (0.8 ml.) of the diluted material was dried on stainless-steel planchets (5 cm.²) and the radioactivity measured (G.M. mica end-window). Corrections were made for dead time of the instrument, background and decay.

After good growth had been observed (3, 5 or 6 days, Fig. 1) the mycelium was harvested, washed well with water and dried *in vacuo* over P_2O_5 . A portion (200 mg.) of each mycelium was hydrolysed by heating with 20 ml. of 20% (w/v) HCl in 50% (w/v) formic acid at 90° for 5 hr. The hydrolysate was evaporated to dryness and the residue taken up in 10 ml. of 0.1 N-HCl. The solution was filtered and the precipitate washed with water. The filtrate and washings were made up to 50 ml. with water and the combined cystine and cysteine content was estimated by the method of Sullivan, Hess & Howard (1942). To 20 ml. of the solution was added 30 mg. of carrier cystine in 1 ml. of 0.02 N-HCl and the total cystine was then isolated in approximately 50% yield by the mercaptide method (Stekol, 1957). A portion (2.5 mg.) of the isolated cystine was weighed on to a planchet (5 cm.²) and dissolved in 0.5 ml. of 0.1 N-NaOH. After drying the sample, the radioactivity was counted as before. No correction was made for self-absorption and the values in Table 2 should be considered only relative.

A preliminary experiment with *A. oryzae* and *A. nidulans* was carried out with Czapek-Dox medium and higher sulphur concentrations. The sulphur sources were $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (200 mg. of SO_4^{2-} ions/l.) with and without choline sulphate (200 mg. of SO_4^{2-} ions/l.). $\text{Na}_2^{35}\text{SO}_4$ was incorporated into both media to give 220 counts/sec./ml. Samples of the culture media were removed and the radioactivity was assessed as before.

Acetone-dried fungal preparations. The fungus was grown on Czapek-Dox or glucose-peptone-yeast-extract media for 3–4 days with shaking. The mycelium was harvested, washed well with water and aerated in a large volume of tap water for 4 hr. (cf. Shepherd, 1956). After removal of as much water as possible by filtration, the mycelium was ground in a cooled mortar with ice-cold acetone and allowed to stand in acetone at 0° for 1 hr. The acetone was then quickly removed by filtration under suction and the precipitate washed well with acetone at 0°. All operations in which acetone was present were carried out in a cold room. The mycelium was finally dried *in vacuo* over P_2O_5 and stored at –20° in tightly stoppered bottles. For enzyme experiments a crude suspension of the dried material was made by homogenizing 25 mg. with 0.5 ml. of water in a small test tube and with a Teflon pestle. Occasionally an aqueous extract of the dried material was prepared by centrifuging the crude suspension and pouring off the supernatant.

Synthesis of choline sulphate in vitro. To 40 μl . of crude suspension of the acetone-dried preparation was added 10 μl . of a solution which had been adjusted to pH 7.3 with NaOH and which contained 1 μmole of adenosine triphosphate (ATP), 1 μmole of PO_4^{2-} ion, 0.3 μmole of MgCl_2 , 0.5 μmole of choline chloride and 5 μC of $\text{Na}_2^{35}\text{SO}_4$. After

incubation at 38° for 90 min. the mixture was placed in a boiling-water bath for 2 min., cooled and centrifuged. A portion (20 μ l.) of the supernatant was applied to Whatman no. 1 paper and the chromatogram developed with acetone-water (9:1) for 3-4 hr. Radioautographs were prepared with Ilford Industrial B X-ray film and an exposure time of 7-10 days. A spot on the radioautograph at R_F 0.26 indicated the synthesis of choline sulphate (Harada & Spencer, 1960). Occasionally, choline sulphate formation was verified chromatographically with *iso*-butyric acid-aq. 0.3N-NH₃ soln. (5:3) for 16 hr. (R_F 0.46). Relative assessment of the amount of choline sulphate formed in different systems was obtained by elution of the radioactive choline sulphate spot with water, drying the eluate on a planchet (5 cm.²) and measuring the radioactivity as before.

Synthesis of adenosine 3'-phosphate 5'-sulphatophosphate in vitro. The incubation mixture was the same as that used for choline sulphate synthesis but choline chloride was omitted. After incubation for 1 hr. at 38° the mixture was heated at 100° for 2 min., cooled and centrifuged. The presence of adenosine 3'-phosphate 5' [³⁵S]-sulphatophosphate in the supernatant was shown by paper electrophoresis as detailed below. None of the compound was formed in controls with boiled fungal preparations.

The supernatant (40 μ l.) was applied to Whatman no. 3MM paper and subjected to electrophoresis for 16 hr. at 4 v/cm. in 0.1M-ammonium acetate buffer, pH 5.6. The adenosine 3'-phosphate 5' [³⁵S]-sulphatophosphate and authentic compound run simultaneously were revealed by radioautography as spots with approximately 60% of the mobility of ³⁵SO₄²⁻ ions. Occasionally, a faint spot of choline [³⁵S]sulphate was observed at the origin. This was presumably formed by transfer of ³⁵SO₄²⁻ ions from adenosine 3'-phosphate 5' [³⁵S]-sulphatophosphate to endogenous choline.

The presence of adenosine 3'-phosphate 5' [³⁵S]-sulphatophosphate in the supernatant was confirmed by transfer of ³⁵SO₄²⁻ ions from the adenosine 3'-phosphate 5' [³⁵S]-sulphatophosphate to *p*-nitrophenol under the influence of phenolsulphokinase. The source of phenolsulphokinase was a particle-free iso-osmotic KCl (11.4 g./l.) preparation of rat liver (Spencer, 1960) and 0.08M-ethylenediaminetetraacetic acid (EDTA) was incorporated to inhibit completely both stages of the adenosine 3'-phosphate 5'-sulphatophosphate-synthesizing system which is present in the preparation (Brunngraber, 1958). To 30 μ l. of the supernatant was added 10 μ l. of a *p*-nitrophenol solution at pH 7.3 containing 1 μ mole of *p*-nitrophenol and 10 μ l. of rat-liver preparation. After incubation for 1 hr., protein was denatured by heating at 100° for 2 min. and, after centrifuging, 20 μ l. of the supernatant was placed on Whatman no. 1 paper and chromatographed. In the radioautographs a spot at R_F 0.85 (acetone-water) or 0.65 (*isobutyric acid*-aq. NH₃ soln.) indicated the formation of *p*-nitrophenyl sulphate and thus the presence of adenosine 3'-phosphate 5' [³⁵S]-sulphatophosphate in the supernatant from the incubation of the fungal extract. No *p*-nitrophenyl sulphate was formed in controls in which the supernatant from the incubation of boiled fungal extract was used. The arylsulphatase which is present in most fungi and which is capable of hydrolysing *p*-nitrophenyl sulphate was destroyed when the fungal incubation mixture was boiled.

Choline sulphokinase. To 10 μ l. of fungal preparation was

Table 1. *Synthesis of choline sulphate in vitro by Aspergillus oryzae*

To 25 μ l. of crude aqueous suspension of acetone-dried *A. oryzae* was added 5 μ l. of water and 10 μ l. of a solution adjusted to pH 7.3 with NaOH and containing 1 μ mole of ATP, 1 μ mole of KH₂PO₄, 0.3 μ mole of MgCl₂ and 5 μ C of Na₂³⁵SO₄. After incubation at 38° for 90 min., the protein was denatured at 100° and 10 μ l. of a clear supernatant obtained by centrifuging was chromatographed with the acetone-water (9:1) solvent. The choline sulphate spot at R_F 0.26 was eluted and the radioactivity of the eluate determined. A control in which the Na₂³⁵SO₄ was added immediately before heating at 100° was included. The various compounds added were dissolved in water, adjusted to pH 7.3 and substituted for the 5 μ l. of water.

Incubation mixture	Choline [³⁵ S]sulphate (counts/min.)
Complete	1940
Control	120
Minus ATP	180
Minus MgCl ₂	105
Plus 0.01 M-choline chloride	3780
Plus 0.01 M-choline chloride but with the aqueous extract of the fungi	3220
Plus 0.01 M-choline chloride and 0.0125 M-cysteine hydrochloride	4430
Plus 0.01 M-choline chloride, M-cysteine hydrochloride and 0.08 M-EDTA but minus MgCl ₂	150

added 10 μ l. of adenosine 3'-phosphate 5' [³⁵S]-sulphatophosphate solution (approx. 50 μ m-moles and 100 counts/sec.) and 10 μ l. of a solution containing 0.5 μ mole of choline chloride and 1 μ mole of PO₄²⁻ ions adjusted to pH 7.3. Incubation was for 1 hr. at 38° and enzyme action was stopped by heating at 100° for 2 min. After centrifuging, the presence of choline [³⁵S]sulphate was shown by chromatography and radioautography. Boiled fungal extracts did not produce choline [³⁵S]sulphate under these conditions.

RESULTS

The synthesis of choline [³⁵S]sulphate from ³⁵SO₄²⁻ ions by crude suspensions of the dried mycelia of *A. oryzae*, *P. chrysogenum*, *A. nidulans* and *A. terreus* required the presence of ATP and Mg²⁺ ions. A representative experiment is shown in Table 1. In these crude suspensions there was considerable synthesis of choline sulphate in the absence of added choline owing to the endogenous free and combined choline in the mycelia. Addition of 0.01 M-choline chloride and 12.5 mM-cysteine increased the amount of choline sulphate produced whereas 0.08 M-EDTA totally inhibited the synthesis. The enzyme system required for the choline sulphate synthesis was readily extracted from the acetone-dried mycelia by homogenizing with water. The crude suspensions and aqueous extracts

catalysed the transfer of $^{35}\text{SO}_4^{2-}$ ions from adenosine 3'-phosphate 5' [^{35}S] sulphatophosphate to choline chloride. The transfer was enzymic since boiled preparations were inactive in this respect. The specificity of the transferase, choline sulphokinase, has been examined and will be reported separately. Transfer of $^{35}\text{SO}_4^{2-}$ ions to choline from adenosine 5' [^{35}S] sulphatophosphate in the absence of added ATP could not be demonstrated.

The acetone-dried mycelia of the fungi which accumulate choline sulphate in their mycelia during growth (Harada & Spencer, 1960) were all able to synthesize choline sulphate *in vitro* in the obligatory presence of added ATP and Mg^{2+} ions. All these fungi possessed choline sulphokinase and the adenosine 3'-phosphate 5'-phosphatosulphate-synthesizing activity (Table 2). The fungi without choline sulphate (Harada & Spencer, 1960) all

produced adenosine 3'-phosphate 5' [^{35}S] sulphatophosphate but did not transfer the $^{35}\text{SO}_4^{2-}$ ions of the compound to choline (Table 2). This was ascribed to a lack of choline sulphokinase rather than to the presence of an inhibitor of the transferase, since extracts of baker's yeast, *R. stolonifer* and *E. ashbyii* did not inhibit the synthesis of choline [^{35}S] sulphate from adenosine 3'-phosphate 5' [^{35}S] sulphatophosphate and choline chloride by the choline sulphokinase of *A. oryzae*.

Competitive metabolism. Although the existence of the adenosine 3'-phosphate 5'-sulphatophosphate-synthesizing system in both the choline sulphate-producing fungi and the choline sulphateless fungi tended to support the hypothesis that the two classes of fungi had a common method of sulphate utilization, i.e. through adenosine 3'-phosphate 5'-sulphatophosphate, the position of

Table 2. Adenosine 3'-phosphate 5'-sulphatophosphate-synthesizing system and choline sulphokinase in fungi

Aqueous suspensions of the fungi were tested by the methods described in the text.

	Choline sulphate formation		Synthesis of adenosine 3'-phosphate 5'-sulphatophosphate <i>in vitro</i>	Choline sulphokinase <i>in vitro</i>
	<i>In vivo</i> *	<i>In vitro</i>		
<i>A. oryzae</i>	+	+	+	+
<i>A. nidulans</i>	+	+	+	+
<i>A. terreus</i>	+	+	Not tested	+
<i>A. sydowi</i>	+	+	+	+
<i>P. aurantio-brunneum</i>	+	+	+	+
<i>P. notatum</i>	+	+	+	+
<i>P. chrysogenum</i>	+	+	+	+
<i>P. citreo-roseum</i>	+	+	+	+
<i>R. stolonifer</i>	-	-	+	-
<i>E. ashbyii</i>	-	-	+	-
<i>N. gossypii</i>	-	-	+	-
<i>T. utilis</i>	-	-	+	-
<i>Baker's yeast</i>	-	-	+	-

* Harada & Spencer (1960).

Table 3. Competitive utilization of sodium sulphate for cystine synthesis by baker's yeast and *Aspergillus nidulans*

The fungi were grown on media containing different sources of sulphur as indicated. After growth for 3 days (baker's yeast) or 5 days (*A. nidulans*) the mycelia were harvested, hydrolysed, the radioactive cystine was isolated after the addition of carrier cystine and the specific activity measured. Details were as described in the text.

Fungus	Sulphur source	Radioactivity of cystine isolated from the mycelium (counts/sec./mg.)
Baker's yeast	$\text{Na}_2^{35}\text{SO}_4$	126
	$\text{Na}_2^{35}\text{SO}_4$ + choline sulphate	109
	[^{35}S]Choline sulphate	92
	[^{35}S]Choline sulphate + Na_2SO_4	1
<i>A. nidulans</i>	$\text{Na}_2^{35}\text{SO}_4$	138
	$\text{Na}_2^{35}\text{SO}_4$ + choline sulphate	120
	Choline [^{35}S]sulphate	121
	Choline [^{35}S]sulphate + Na_2SO_4	46

choline sulphate remained unclarified. Before further progress could be made it was essential to establish whether or not choline sulphate was an obligatory intermediate in sulphate utilization. Competitive-metabolism experiments were therefore carried out by growing fungi on media containing normal and radioactive sodium sulphate or choline sulphate, alone or in combinations, as the only sulphur sources. The rate of disappearance of ^{35}S from the media (Figs. 1, 2) and the specific

activity of the cystine isolated from the mycelia were then measured (Table 3).

The six fungi chosen (three choline sulphate producers and three non-producers) individually appeared to utilize the sulphur of $\text{Na}_2^{35}\text{SO}_4$ or choline [^{35}S]sulphate, when present singly, to about the same degree, as shown by the rate of disappearance of ^{35}S from the media (Figs. 1, 2) and the weights of mycelia produced (Table 4). In the presence of non-radioactive choline sulphate the

Fig. 1. Competitive utilization of Na_2SO_4 and choline sulphate by choline sulphate-producing fungi: (a) *A. nidulans*; (b) *P. chrysogenum*; (c) *P. aurantio-brunneum*. Fungi were grown on 200 ml. of the glucose-asparagine medium containing sulphur-containing supplements as indicated. $\circ$, Medium containing $\text{Na}_2^{35}\text{SO}_4$; $\bullet$, choline [^{35}S]sulphate; $\triangle$, Na_2SO_4 plus choline sulphate; $\blacktriangle$, Na_2SO_4 plus choline [^{35}S]sulphate. Na_2SO_4 was incorporated in all cases at a concentration of 34.5 mg. of SO_4^{2-} ions/l. and the choline sulphate at a concentration equivalent to 25 mg. of SO_4^{2-} ions/l.

Fig. 2. Competitive utilization of Na_2SO_4 and choline sulphate by choline sulphate-less fungi: (a) baker's yeast; (b) *Torulopsis utilis*; (c) *Rhizopus stolonifer*. Details were as given in Fig. 1.

Table 4. *Dry weights of mycelia produced in the competitive-utilization experiment*

Fungi were grown in 200 ml. of glucose-asparagine medium with sulphur-containing supplements as indicated and harvested on the final day. Details were as described in the text and Figs. 1 and 2. Mycelial dry weights are recorded in milligrams.

Sulphur source	<i>A. nidulans</i>	<i>P. chrysogenum</i>	<i>P. aurantio-brunneum</i>	Baker's yeast	<i>T. utilis</i>	<i>R. stolonifer</i>
$\text{Na}_2^{35}\text{SO}_4$	467	561	304	666	484	407
$\text{Na}_2^{35}\text{SO}_4$ + choline sulphate	538	753	306	667	547	397
Choline [^{35}S]sulphate	544	685	411	683	511	321
Choline [^{35}S]sulphate + Na_2SO_4	512	746	350	612	521	405

uptake of $\text{Na}_2^{35}\text{SO}_4$ was the same as in the absence of the sulphate ester. The rate of uptake of ^{35}S from the media containing choline [^{35}S]sulphate plus Na_2SO_4 could not be distinguished from the rates of uptake from the other media with *A. nidulans* and *P. chrysogenum* but *P. aurantio-brunneum* and the choline sulphate-less *T. utilis* showed a much slower rate of uptake. Baker's yeast and *R. stolonifer* showed no uptake of ^{35}S from this medium.

These results suggested that Na_2SO_4 was used preferentially to choline sulphate as a source of sulphur by *P. aurantio-brunneum*, *T. utilis*, *R. stolonifer* and baker's yeast. *A. nidulans* and *P. chrysogenum* showed an anomalous behaviour in that they both appeared to take up choline sulphate and Na_2SO_4 at approximately the same rate from the medium containing both sulphur sources. It should be pointed out that in experiments of this type only uptake of ^{35}S into the mycelium, and not utilization of ^{35}S , was being measured. *A. nidulans* and *A. oryzae* are both known to store relatively large amounts of choline sulphate in their mycelia (Harada & Spencer, 1960), whereas *P. aurantio-brunneum* contains only small amounts of the ester. It is also significant that although the data in Fig. 1 would suggest that *A. nidulans* and *P. chrysogenum* took up almost all the choline sulphate and Na_2SO_4 when both sulphur sources were present together, there was very little difference between the weights of mycelia formed when the two sulphur sources were present either together (equivalent to 59.5 mg. of SO_4^{2-} ions/l.) or singly (34.5 mg. of SO_4^{2-} ions/l. as Na_2SO_4 or 25.0 mg. of SO_4^{2-} ions/l. as choline sulphate) (Table 4). One interpretation of the results for *A. nidulans* and *P. chrysogenum* was that they showed only high rates of uptake and storage, rather than utilization, of choline sulphate and that although choline sulphate could be utilized as a sulphur source Na_2SO_4 was used preferentially.

Proof of this interpretation was provided by the specific radioactivities of the cystine isolated from the cells of baker's yeast and the mycelia of *A. nidulans* grown on the various media (Table 3). The radioactivity of the cystine from baker's yeast grown on choline [^{35}S]sulphate plus Na_2SO_4 was less

Fig. 3. Competitive utilization of MgSO_4 and choline sulphate by (a) *A. oryzae* and (b) *A. nidulans*. Fungi were grown on 200 ml. of the glucose-asparagine medium and harvested after 7 days' growth. O, MgSO_4 (198 mg. of SO_4^{2-} ions/l.) plus an insignificant amount of $\text{Na}_2^{35}\text{SO}_4$; Δ , MgSO_4 (198 mg. of SO_4^{2-} ions/l.) plus choline sulphate (at a concentration equivalent to 198 mg. of SO_4^{2-} ions/l.) and an insignificant amount of $\text{Na}_2^{35}\text{SO}_4$. The dry weights of mycelia were: *A. oryzae*, 675 mg. on MgSO_4 medium, 703 mg. on MgSO_4 + choline sulphate medium; *A. nidulans*, 650 mg. on MgSO_4 medium, 635 mg. on MgSO_4 + choline sulphate medium.

than 1 % of that grown on either $\text{Na}_2^{35}\text{SO}_4$ alone or $\text{Na}_2^{35}\text{SO}_4$ plus non-radioactive choline sulphate. The comparable figure for *A. nidulans* was approximately 30 %.

It was concluded that whereas baker's yeast can grow on either sulphur source independently it utilizes Na_2SO_4 to the exclusion of choline sulphate

when both sulphur sources are present; *A. nidulans* can use both sources when they are present simultaneously but Na_2SO_4 was the more readily assimilated. This suggests that the method of utilization of the sulphur of choline sulphate may be different in yeast and *A. nidulans*, but in neither case would it appear that choline sulphate was an obligatory intermediate in the utilization of inorganic sulphate.

With this knowledge it now became possible to explain the competitive-metabolism experiment of Itahashi (1954), in which the sulphate concentration was much higher than in the present series. A preliminary experiment which was carried out with 198 mg. of SO_4^{2-} ions/l. closely repeated the results of Itahashi (Fig. 3). The rate of uptake of ^{35}S by *A. oryzae* was slower from the $\text{Na}_2^{35}\text{SO}_4$ plus choline sulphate medium than from that containing $\text{Na}_2^{35}\text{SO}_4$ alone as the sulphur source. With *A. nidulans* the uptake of radioactivity from the medium containing $\text{Na}_2^{35}\text{SO}_4$ plus choline sulphate was barely detectable (Fig. 3). Both these fungi store choline sulphate and when $\text{Na}_2^{35}\text{SO}_4$ was used as the sole sulphur source a large amount of the $^{35}\text{SO}_4$ was presumably used for the formation and storage of choline sulphate. When $\text{Na}_2^{35}\text{SO}_4$ plus choline sulphate was present in the medium the choline sulphate was absorbed and stored directly and the $^{35}\text{SO}_4^{2-}$ ion was then only required for growth purposes. With these high sulphur concentrations the amount of $^{35}\text{SO}_4^{2-}$ ion used for growth was only a small percentage of the added amount and thus little uptake of $^{35}\text{SO}_4^{2-}$ ions was observed.

Utilization of sulphate esters as sulphur sources for fungal growth. *A. oryzae* grew well on media containing various aliphatic and aromatic sulphate esters as the sole sources of sulphur (Table 5). A certain amount of growth occurred in the absence of added sulphur, and this can probably be attributed to the presence of small sulphur reserves in the spores or to sulphur compounds introduced in the inoculum or as trace impurities in the media.

One way in which the sulphur of sulphate esters could have been made available to the fungi was by

release of SO_4^{2-} ions through the action of a sulphatase or by degradation of the organic moiety of the compounds followed by the further metabolism of the liberated SO_4^{2-} ions. For example, *A. oryzae* contains an arylsulphatase and during growth on nitrocatechol sulphate the medium turned red, indicating that nitrocatechol had been released. The red subsequently disappeared as the nitrocatechol was metabolized. It would be expected that the utilization of the sulphur of sulphate esters in this way would be strongly inhibited by the incorporation of BaCl_2 into the media to precipitate the liberated SO_4^{2-} ions. This experiment was carried out (Table 5) and, whereas the growth of *A. oryzae* on choline sulphate was unaffected by barium, growth on the other sulphate esters, apart

Table 5. *Utilization of sulphate esters as sulphur sources for Aspergillus oryzae in the presence and absence of barium chloride*

A. oryzae was grown in 200 ml. of Czapek-Dox medium in which MgSO_4 was replaced by an equivalent amount of MgCl_2 . Sulphur sources were incorporated at a concentration of 192 mg. of SO_4^{2-} ion/l. and BaCl_2 (1.0 g./l.) was added to one series of media. The mycelial dry weights are quoted as percentages of the dry weight of the mycelium when cysteine was the sulphur source.

Sulphur source	Dry wt. of mycelium	
	BaCl_2 absent	BaCl_2 present
None	10	5
Choline sulphate	101	105
Na_2SO_4	113	11
Sinigrin	80	13
Cortisone 21-sulphate	78	20
Phenyl sulphate	60	21
Nitrocatechol sulphate	101	25
Ethyl sulphate	83	30
Chondroitin sulphate A	51	69
2:4-Dichlorophenoxyethyl sulphate	87	78
Cysteine hydrochloride	100	100
Weight of mycelium with cysteine hydrochloride as sulphur source (mg.)	1.46	1.05

Table 6. *Utilization of different sulphur sources by fungi in the presence and the absence of barium chloride*

Fungi were grown in 200 ml. of Czapek-Dox medium in which MgSO_4 was replaced by an equivalent amount of MgCl_2 . Sulphur sources were incorporated at a concentration of 192 mg. of SO_4^{2-} ions/l. and BaCl_2 (1.0 g./l.) was added to one series of media. The mycelial dry weights are given as percentages of the dry weight of the mycelium when cysteine was the sulphur source.

	Dry wt. of mycelium							
	Sulphur sources, BaCl_2 absent				Sulphur sources, BaCl_2 present			
	None	Na_2SO_4	Choline sulphate	Nitrocatechol sulphate	None	Na_2SO_4	Choline sulphate	Nitrocatechol sulphate
<i>R. stolonifer</i>	16	121	89	126	10	22	62	81
Baker's yeast	11	100	100	79	13	97	102	77
<i>T. utilis</i>	7	77	83	100	6	20	103	100
<i>E. ashbyii</i>	20	176	121	83	20	19	133	87

from chondroitin sulphate and 2:4-dichlorophenoxyethyl sulphate, was strongly inhibited.

The experiment was repeated in part with several choline sulphate-less fungi with cysteine, SO_4^{2-} ions, nitrocatechol sulphate or choline sulphate as the sole sulphur source (Table 6). Both sulphate esters were good sources of sulphur for all the fungi in the presence as well as in the absence of barium. With baker's yeast, the good growth on Na_2SO_4 in the presence of excess of BaCl_2 can be explained as the utilization of the very small concentration of BaSO_4 which remains in solution (2.3 mg./l. at 18°). The similar good growth on the sulphate esters would therefore have been expected. The high growth rate of *R. stolonifer*, *T. utilis* and *E. ashbyii* on the sulphate esters in the presence of BaCl_2 was unexpected since there was very little growth on Na_2SO_4 in the presence of BaCl_2 . Two explanations may be advanced: (1) that the sulphur of choline sulphate and nitrocatechol is converted into a reduced form of organically combined sulphur without appearing as SO_4^{2-} ions; (2) that SO_4^{2-} ions are produced from the two sulphate esters but are further metabolized so rapidly at low concentration that BaSO_4 does not form. This could also happen if the mycelial membranes are not permeable to Ba^{2+} ions.

Sulphur storage in fungi. Much of the evidence indicated that choline sulphate could act as a store of sulphur. To test this suggestion fungi were grown for 4 days in shaking culture in 200 ml. of the glucose-asparagine medium. The mycelia of each species was harvested on sterilized muslin and the excess of water was removed from the collected mycelia by pressing between sterilized filter papers. The semi-dry mycelial mass was weighed and divided into three approximately equal portions, each of which was then weighed. One portion was used as the inoculum for 200 ml. of the glucose-asparagine medium, another portion was used as the inoculum for 200 ml. of the same

medium in which the MgSO_4 had been replaced by MgCl_2 and the remaining portion was dried *in vacuo* over P_2O_5 for the calculation of the dry weights of the mycelia used as the inocula. After 24 hr. further growth in the new media the mycelia were reharvested, dried and weighed. With *P. chrysogenum* and *R. stolonifer* a division of the collected mycelia into five portions was made so as to allow collection after 13 and 24 hr. further growth in the new media. Although absolute asepsis was impracticable during the transfer operations no contamination during the second period of growth was observed.

The results (Table 7) showed that the choline sulphate-producing fungi *A. oryzae* and *P. chrysogenum* grew well on sulphur-deficient media. After 13 hr. further growth there was no difference in the weights of new mycelia between that grown on the sulphate-containing medium and that grown on the sulphate-deficient medium. After 24 hr. the growth on the sulphate-containing medium exceeded that on the sulphate-deficient medium, indicating that the sulphur reserves of the mycelial inoculum in the latter case had been used. However, these reserves were sufficient to support a 150% increase in growth of *A. oryzae* and 74% increase of *P. chrysogenum*. On the other hand, very little further growth of the choline sulphate-less fungi occurred on the sulphate-deficient medium, indicating that these fungi did not contain any substantial sulphur reserves.

Chromatography of extracts of *A. oryzae* and *P. chrysogenum* (cf. Harada & Spencer, 1960) showed that when these fungi were grown in Czapek-Dox medium containing $^{35}\text{SO}_4^{2-}$ ions they stored ^{35}S both as $^{35}\text{SO}_4^{2-}$ ions and as choline [^{35}S]sulphate. The latter compound predominated in the ratio of approximately 10:1 when all nutrients in the medium were present in excess. On transfer of the fungi to media deficient in sulphate and when the fungi were grown to exhaustion, chromatography

Table 7. *Sulphur storage by fungi*

Fungi were grown in 200 ml. of the glucose-asparagine medium for 4 days. Portions of the mycelia were then transferred to fresh media, which was either complete or totally deficient in sulphate, and the increase in weight of the mycelium after 13 hr. or 24 hr. further growth was measured.

		Increase in dry wt. of mycelium (%)	
	Sulphate in medium	13 hr.	24 hr.
Choline sulphate-producing fungi			
<i>A. oryzae</i>	—	.	151
	+	.	277
<i>P. chrysogenum</i>	—	62	73
	+	67	182
Choline sulphate-less fungi			
<i>R. stolonifer</i>	—	.	14
	+	.	144
<i>M. hiemalis</i>	—	.	5
	+	.	44

of mycelial extracts showed that the mycelial store of both $^{35}\text{SO}_4^{2-}$ ions and choline [^{35}S]sulphate had been completely used.

DISCUSSION

Kaji & McElroy (1958) had previously shown that extracts of *P. sydowi* formed choline sulphate in the obligatory presence of ATP and SO_4^{2-} ions. From this and other results they suggested that adenosine 3'-phosphate 5'-sulphatophosphate was formed and that this acted as a donor of sulphate for the synthesis of choline sulphate under the influence of a transferase, choline sulphokinase. This suggestion has now been confirmed by the demonstration that a number of fungi can produce adenosine 3'-phosphate 5'-sulphatophosphate and that they can transfer sulphate from the exogenous compound to choline. No transfer of sulphate from adenosine 5'-sulphatophosphate was observed. Kaji & Gregory (1959) have also recently confirmed that transfer of sulphate from adenosine 3'-phosphate 5'-sulphatophosphate to choline takes place in extracts of *P. sydowi*.

All the fungi tested (Table 2) produced adenosine 3'-phosphate 5'-sulphatophosphate but the fungi which did not accumulate choline sulphate *in vivo* (Harada & Spencer, 1960) lacked the enzyme choline sulphokinase. It would seem a reasonable generalization to suggest that all fungi which can utilize inorganic sulphate as a source of sulphur for growth can produce adenosine 3'-phosphate 5'-sulphatophosphate. The distribution of choline sulphate synthesis in fungi can therefore be discussed in terms of the presence or absence of choline sulphokinase (cf. Harada & Spencer, 1960; Ballio *et al.* 1960).

Work with mutant strains of *A. nidulans* (Hockenull, 1949; Shepherd, 1956) has shown

that the reduction of sulphate to sulphite is an obligatory stage in the formation of cysteine from inorganic sulphate. The reaction

is an endergonic reaction with a standard free energy change of +14 kcal. (Postgate, 1956), and it is therefore not surprising to learn that reduction of sulphate *in vitro* by yeast requires ATP, Mg^{2+} ions and reduced triphosphopyridine nucleotide (TPNH) (Wilson & Bandurski, 1958; Hilz & Kittler, 1958). The ATP and Mg^{2+} ions can be replaced by adenosine 3'-phosphate 5'-sulphatophosphate (Wilson & Bandurski, 1958) or by *p*-nitrophenyl sulphate plus adenosine 3':5'-diphosphate in the presence of phenolsulphokinase (Hilz & Kittler, 1958), and it is further known that adenosine 3'-phosphate 5'-sulphatophosphate can be reduced by TPNH (Wilson & Bandurski, 1958). The evidence strongly suggests that adenosine 3'-phosphate 5'-sulphatophosphate must be formed before sulphate can be reduced although it does not rule out the possibility that the immediate precursor of SO_3^{2-} ions is adenosine 5'-sulphatophosphate. Yeast and other fungi are known to contain enzymes which degrade adenosine 3'-phosphate 5'-sulphatophosphate to adenosine 5'-sulphatophosphate (B. Spencer, unpublished work), and recently Peck (1959) and Ishimoto (1959) have shown that adenosine 5'-sulphatophosphate and not the former compound is the substrate which is reduced by extracts of *Desulphovibrio desulphuricans*. Whether the same reducing system exists in yeast, where its purpose is sulphate assimilation, and in *Desulphovibrio*, where sulphate serves as the terminal electron acceptor, is not known.

The following tentative scheme for sulphate utilization in fungi can be proposed.

In this scheme choline sulphate is envisaged as a storage compound containing sulphate in a high-energy state. On demand the sulphate can be transferred to adenosine 3':5'-diphosphate to give adenosine 3'-phosphate 5'-sulphatophosphate which can form sulphite. If, in the reduction, adenosine 3':5'-diphosphate is regenerated, as envisaged by Hilz & Kittler (1958), then only a catalytic amount of adenosine 3':5'-diphosphate would be required for the utilization of large amounts of choline sulphate.

Some evidence has been obtained to support the position of choline sulphate in this scheme. It has long been known (Egami & Itahashi, 1951) that choline sulphate can be utilized as a sulphur source without the appearance of inorganic sulphate, and this was confirmed in the present investigation when it was shown that in the presence of Ba^{2+} ions choline sulphate remains a good source of sulphur for growth but that several other sulphate esters do not. Another fact supporting the view that sulphate is transferred from choline sulphate to an acceptor rather than sulphate being liberated by degradation of the carbon chain of the choline is that choline sulphate can act as a source of choline for the choline-less mutant of *N. crassa* (Stevens & Vohra, 1955; Harada & Spencer, 1960). Furthermore, the choline-less mutant of *N. crassa* and the fungi listed in Table 2 did not possess any sulphatase activity *in vitro* towards choline sulphate when tested by a sensitive radioactive method (B. Spencer, unpublished work).

The results of the competitive-metabolism experiment are also consistent with the proposed scheme. In the choline sulphate-less fungi, choline sulphate did not affect the conversion of inorganic sulphate into cysteine and this is simply explained from the scheme as being due to the lack of choline sulphokinase. In the choline sulphate-producing fungi the finding that inorganic sulphate and choline sulphate can act as alternative and competing sources of sulphur for cysteine production is compatible with the proposed scheme. The fungi which accumulate choline sulphate in their mycelia during growth on a medium containing excess of sulphate could utilize the stored sulphur of the sulphate ester for further growth when transferred to a medium lacking any sulphur source. The choline sulphate-less fungi showed little further growth under these circumstances. These facts are again readily explained by the scheme. An important confirmation of the proposed method of utilization of choline sulphate would be the demonstration of the formation *in vitro* of adenosine 3'-phosphate 5'-sulphatophosphate from choline sulphate and adenosine 3':5'-diphosphate under the influence of choline sulphokinase. No evidence of this has yet been obtained (Kaji & Gregory, 1959). However, similar reactions

are known to occur with other sulphate esters (Lipmann, 1958).

If the proposed scheme should prove correct choline sulphate will assume a somewhat unique role in fungi as a store of easily assimilated sulphate existing in an activated state. The position would be somewhat akin to the role of guanidinium phosphates in animals, although the parallel should not be drawn too closely. In conditions of adequate sulphur nutrition choline sulphate would accumulate in the mycelia and then be re-utilized when sulphur supplies became subminimal. This property could be considered to have been of evolutionary advantage since it is limited to the higher orders of fungi (Harada & Spencer, 1960; Ballio *et al.* 1960). Some confirmation of the idea of choline sulphate as a storage compound has come from the work of Takebe & Yanagita (1959), who found an unidentified sulphur-containing compound in the conidiospores of *Aspergillus niger* which was utilized for the formation of sulphur-containing amino acids during germination. This compound has now been shown to be choline sulphate (I. Takebe, personal communication) and it occurred to the extent of 1.4% of the fresh spore weight.

SUMMARY

1. Choline sulphate synthesis *in vitro* by extracts of a number of fungi in the presence of adenosine triphosphate and Mg^{2+} ions was shown to involve the intermediate formation of adenosine 3'-phosphate 5'-sulphatophosphate followed by the enzymic transfer of sulphate from this compound to choline. Adenosine 3'-sulphatophosphate could not act as a sulphate donor.

2. All fungi tested produced adenosine 3'-phosphate 5'-sulphatophosphate *in vitro* but those fungi which did not accumulate choline sulphate in their mycelia *in vivo* could not transfer sulphate from adenosine 3'-phosphate 5'-sulphatophosphate to choline. The distribution of choline sulphate production can therefore be discussed in terms of the presence or absence of the transferase, choline sulphokinase.

3. In competitive-metabolism experiments choline sulphate did not affect the utilization of SO_4^{2-} ions by choline sulphate-less fungi without choline sulphate, including baker's yeast. In the choline sulphate-producing fungi choline sulphate and SO_4^{2-} ions were competing sources of sulphur for cysteine synthesis.

4. Choline sulphate served as a good source of sulphur for the growth of choline sulphate-producing fungi both in the absence and the presence of excess of Ba^{2+} ions. Other sulphate esters, apart from chondroitin sulphate and 2:4-dichlorophenoxyethyl sulphate, were poor sources of

sulphur in the presence of Ba^{2+} ions. Choline sulphate and nitrocatechol sulphate were good sulphur sources for the growth of choline sulphate-less fungi both in the absence and the presence of Ba^{2+} ions.

5. When choline sulphate-producing fungi were grown on an adequate sulphur medium a store of choline sulphate accumulated in the mycelia which could be used to maintain further growth when the fungi were transferred to a medium totally deficient in sulphur of any form. In the same circumstances no further growth of choline sulphate-less fungi occurred on the new sulphur-deficient medium.

6. Choline sulphate in fungal mycelia is envisaged as a store of easily assimilated sulphate existing in an activated state. The pathway of utilization is thought to involve transfer of sulphate from choline sulphate to adenosine 3':5'-diphosphate with the formation of adenosine 3'-phosphate 5'-sulphatophosphate and the subsequent reduction of adenosine 3'-phosphate 5'-sulphatophosphate to give SO_3^{2-} ions and eventually cysteine.

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Condensed Tannins

6. BIOCHEMICAL AND STEREOCHEMICAL INTERRELATIONSHIPS OF (+)-FUSTIN FROM BLACK-WATTLE HEARTWOOD (*ACACIA MOLLISSIMA*)*

BY D. G. ROUX AND E. PAULUS

Leather Industries Research Institute, Rhodes University, Grahamstown, South Africa

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Condensed tannins from the heartwood of *Acacia mollissima* consist of a molecular gradation (290–1650) of polyphenolic compounds (Roux & Evelyn, 1960) in which (+)-7:3':4'-trihydroxy-flavan-3:4-diol [(+)-mollisacacidin, Keppler (1957)] is the predominant single component. Polymeric

leuco-fisetinidin tannins form the major fraction of the heartwood polyphenols and appear to originate from this monomeric 'leuco-fisetinidin' flavan-3:4-diol (Roux, 1958). Other monomeric flavonoid substances of similar structural pattern are also present. The provisional identification of fustin and fisetin (Roux & Evelyn, 1960) is confirmed in this study, and their probable mechanism of biogenesis

* Part 5: Evelyn, Maihs & Roux (1960).