Choline Sulphate in Fungi

BY T. HARADA* AND B. SPENCER

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

SUMMARY: The occurrence of choline sulphate in 32 fungi and 9 bacteria was investigated by incorporating $\text{Na}_2^{35}\text{SO}_4$ into culture media and subsequently identifying radioactive choline sulphate in cell extracts by chromatography and radioautography. The production of choline sulphate appears to be restricted to higher fungi since it was not found in any of the tested Phycomycetes. Of the Ascomycetes, the Endomycetales did not synthesize choline sulphate but the mycelia of the Sphaeriales did accumulate the compound. The Basidiomycetes and all the Fungi Imperfecti, except *Torulopsis utilis*, were choline sulphate positive. The choline sulphate negative organisms, including all of the bacteria tested, could not be induced to produce choline sulphate by adding large amounts of choline to the culture media or by growing on chemically defined media in which inorganic sulphate was the sole sulphur source. On the other hand, *Aspergillus oryzae* produced choline sulphate on a minimal medium containing inorganic sulphate, cysteine, cysteic acid or taurine as sulphur sources. The distribution of choline sulphate production has been discussed in terms of the absence or presence of the enzyme choline sulphokinase.

The role of choline sulphate, $-\text{O}_3\text{S} \cdot \text{O} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{N}^+(\text{CH}_3)_3$, in the sulphur metabolism of fungi is still a matter of controversy. This sulphate ester has been isolated from *Aspergillus sydowi* (Woolley & Peterson, 1937) and *Penicillium chrysogenum* (De Flines, 1955; Stevens & Vohra, 1955) in yields greater than 0.2% of the dry weight of the mycelium; similar high concentrations have also been found in lichens of the *Rocella* spp. (Lindberg, 1955*a*) and the red alga *Gelida cartilaginum* (Lindberg, 1955*b*). The accumulation of such relatively large amounts of choline sulphate suggests that it is a storage compound, an end- or by-product of sulphate or choline metabolism, or an excretory compound, rather than an active intermediate in the utilization of the sulphur of inorganic sulphate. However, from the results of competitive metabolism experiments, it has been stated (Egami & Itahashi, 1951; Itahashi, 1954) that choline sulphate is indeed an intermediate in the utilization of sulphate by *Aspergillus oryzae*. On the other hand, similar experiments with *P. chrysogenum* (Stevens & Vohra, 1955) led to the opposite conclusion, since the sulphur of inorganic sulphate was used preferentially to that of choline sulphate for the synthesis of penicillin. It seems clear, however, that choline sulphate and inorganic sulphate do share some common metabolic pathway since mutant strains of *Aspergillus nidulans* and *P. notatum* which did not utilize inorganic sulphur as the sole sulphur source also failed to utilize choline sulphate, while the parent strains grew readily on either sulphur source (Hockenhull, 1948, 1949). The detection of 'ester sulphate' in the mycelia of

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

various *Penicillium* spp. and *Aspergillus* spp. by Raistrick & Vincent (1948) suggests that choline sulphate or some similar compound might be produced by more fungi than is now known. Since the extent of the occurrence of choline sulphate should indicate its relative importance, a survey of representative fungi has now been made as the first stage of a further investigation of the metabolism of this compound.

METHODS

Culture media. Organisms were grown mainly in Czapek-Dox medium in which inorganic sulphate (plus 100 μ c. carrier-free $\text{Na}_2^{35}\text{SO}_4$ /l. in some experiments) was the only sulphur source; or in a glucose peptone yeast-extract medium containing (g./l. water): 20, glucose; 10, Bacto-peptone; 3, Bacto yeast extract; 0.6, Na_2SO_4 ; 100 μ c. $\text{Na}_2^{35}\text{SO}_4$. The latter medium was sometimes supplemented with a total of 1.2 g. choline/l. added daily in 0.4 g./l. amounts. A more complex chemically defined medium than Czapek-Dox was used for certain fungi; this contained per litre: glucose, 10 g.; asparagine, 1.5 g.; $\text{NH}_4\text{H}_2\text{PO}_4$, 1.5 g.; KH_2PO_4 , 1.0 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; NaCl , 0.5 g.; inositol, 20 mg.; thiamine, 1 mg.; biotin, 1 μ g.; carrier-free $\text{Na}_2^{35}\text{SO}_4$, 100 μ c. Cultures (200 ml.) were grown for 4–6 days at 27° with shaking.

Preparation of mycelial extracts. The harvested mycelia were washed well with water and dried *in vacuo* over P_2O_5 for 2 days. Extracts of the dried mycelia were prepared by suspending 200 mg. mycelium in 10 ml. water and heating in a boiling water bath for 5 min. After cooling, 200 mg. alumina was added and the mixture ground in a glass homogenizer. To the supernatant fluid obtained after centrifugation was added 2 ml. *n*-butanol; the mixture was then shaken for 30 min., centrifuged and the protein-free aqueous solution freeze-dried. The dry material was dissolved in 0.2 ml. water. In cases where high concentrations of polysaccharide made the extract slimy, the freeze-dried material was dissolved in 0.1 ml. water and the polysaccharides precipitated by adding 0.1 ml. ethanol. For chromatography 10 and 20 μ l. samples of the final extract was used routinely in spotting on the papers.

Preparation of extracts of culture media. Fungi were grown on the complex chemically defined medium but containing 0.4% glucose and 600 μ c. $\text{Na}_2^{35}\text{SO}_4$ /200 ml. After growth for 4 days, the mycelium was separated and 200 ml. of the clear supernatant fluid was freeze-dried. The dried material was dissolved in 5 ml. water and desalted by using the Wood dialyser (Wood, 1956); choline sulphate does not migrate in an electric field. The solution was then freeze-dried and dissolved in 50 μ l. water. For chromatography a 10 μ l. sample was used.

Chromatographic detection of choline sulphate. Using Whatman no. 1 papers, three solvent systems were tested for the degree of separation of choline sulphate from other sulphur-containing compounds likely to be found in the mycelial extracts (Table 1). As well as the more obvious S-containing compounds, glyceryl taurine, monomethyl taurine and cysteinolic acid were also tested since they have been found with choline sulphate in extracts of certain algae (Wickberg, 1956, 1957). Of the systems tested, acetone/water (9:1) for

3 hr. was subsequently used for the routine testing of the fungal extracts. In this rapidly moving solvent, $^{35}\text{SO}_4^{--}$, which was sometimes present in the extracts in high concentration, remained at the origin and choline sulphate could be clearly distinguished from the other S-containing compounds. It is interesting that Strauss & Minagawa (1959) noted a non-ninhydrin staining spot containing radioactive ^{35}S at R_f 0.96 in phenol/water chromatograms of a methionine-requiring mutant of *Neurospora crassa*. This unknown spot might be choline sulphate (cf. Table 1).

Table 1. R_f values of S-containing compounds in different solvent systems, on Whatman no. 1 papers

	Phenol/water (80:20) for 16 hr.	Acetone/water (9:1) for 3 hr.	Butanol/ ethanol/water (4:1:5) for 16 hr.
	R_f values		
Na_2SO_4	0.05	0	0
Choline sulphate	0.94	0.26	0.15
Taurine	0.89	0.08	0.11
Cysteine	0.80	—	—
Cystine	0.23	0.17	0.21
Methionine	0.83	0.10	0.30
Glyceryl taurine	0.59	0.08	—
Monomethyl taurine	0.71	0.13	—
Cysteinolic acid	0.33	0.10	—
Glutathione	0.11	—	0.03

Choline sulphate was detected on the chromatograms by spraying with 5% (v/v) perchloric acid and heating at 70° for 5 min.; at higher temperatures the paper was destroyed. The location of inorganic sulphate liberated was detected as a yellow spot on a pink background by using the barium chloride + sodium rhodizonate technique of Schneider & Lewbart (1956). The lower limit of detection of choline sulphate by this method was 4 $\mu\text{g.}$, corresponding to a concentration of 100 $\mu\text{g.}$ choline sulphate/g. dried mycelium when 40 $\mu\text{l.}$ of extract was spotted. In experiments using $^{35}\text{SO}_4^{--}$, radioautograms of the chromatograms were prepared by using Ilford Industrial B X-ray film and an exposure time of 5–10 days. Under the conditions used and with a 10 $\mu\text{l.}$ sample for chromatography the limit of detection of choline sulphate by the radioactive method was better than 1 $\mu\text{g./g.}$ dry mycelium. When extracts of the culture medium were used the limit of detection was 0.5 $\mu\text{g./100 ml.}$

RESULTS

With the acetone/water solvent typical chromatograms of mycelial extracts showed a radioactive trail, or occasionally distinct spots, between the sulphate spot at the origin and the region R_f 0.20. This region of the chromatogram also showed a number of ninhydrin-staining spots. A clearly distinguished spot, R_f 0.260, occurred when choline sulphate was present. No other radioactivity was observed on the chromatograms except in the case of *Pichia membranaefaciens* which showed a strong spot at R_f 0.56. The latter material did not

appear to be a sulphate ester since chromatograms of the *Pichia* extract after hydrolysis with 2N-HCl for 1 hr. at 100° still showed a radioactive spot at R_f 0.56. In cases where the radioactive choline sulphate spot was strong, the presence of ester sulphate on the chromatogram was confirmed by hydrolysis and detection of the liberated sulphate by the BaCl_2 + rhodizonate technique. Further identification of choline sulphate in the extracts was made in a few cases by the observation of a radioactive spot R_f 0.94 on phenol/water chromatograms.

A total of 32 fungi and 9 representative bacteria were tested for choline sulphate production (Table 2). The concentration of choline sulphate in the mycelia of the 20 fungi which produced this compound differed considerably. By far the most potent producers were *Aspergillus nidulans*, *A. oryzae* and *Neurospora crassa* (Commonwealth Mycological Institute (CMI) 53238); *Penicillium notatum* and *P. aurantiobrunneum* produced small amounts only detectable by the radioautographic technique. Choline sulphate appeared to occur mainly in the mycelia and only small amounts were found in the culture filtrates.

Choline sulphate was formed in the mycelium of *Aspergillus oryzae* after 5 days growth in Czapek-Dox medium when cysteine, taurine or cysteic acid were substituted for inorganic sulphate. When these organic sulphur compounds served as the sole sulphur source a yellow spot, R_f 0.0, was shown by the BaCl_2 + rhodizonate technique, suggesting that inorganic sulphate had been formed. The cholineless strain of *Neurospora crassa*, CMI 19419 (Horowitz & Beadle, 1943), produced choline sulphate in the presence of added choline (Table 2). Choline sulphate could replace choline as the essential micro-nutrient for this fungus (cf. Stevens & Vohra, 1955).

Some fungi did not appear to accumulate choline sulphate in their mycelia when grown on the glucose peptone yeast-extract medium. The lack of choline was not a restricting factor since, with selected strains, the addition of 0.4 g. choline/l. to the glucose peptone yeast-extract medium on four occasions during growth failed to initiate choline sulphate production (Table 2). The use of the complex chemically defined medium in which inorganic sulphate was the only significant source of sulphur was also ineffective (Table 2). Since the permeability of these fungi might be different from those in which choline sulphate occurs in the mycelium the culture media from *Rhizopus stolonifer* and baker's yeast were tested for choline sulphate; these were negative. None of the bacteria produced choline sulphate (Table 2).

One further point which was established was that representative fungi from both the choline sulphate-producing and non-producing groups could utilize inorganic sulphate as the sole sulphur source. As well as those fungi listed in Table 2, which were grown on Czapek-Dox or the complex chemically defined medium, *Rhizopus stolonifer* (—) *Eremothecium ashbyi*, *Nematospora gossypii*, *Mucor hiemalis*, *Cunninghamella echinulata*, *Saccharomyces cerevisiae*, *Neurospora crassa* (CMI 53238) were all able to grow on the complex defined medium. Both strains of *Aerobacter aerogenes* tested utilized the sulphur of inorganic sulphate.

DISCUSSION

The synthesis of choline sulphate by *Penicillium chrysogenum* is known to involve the transfer of sulphate from 3'-phosphoadenosine-5'-phosphosulphate (PAPS) to choline under the influence of a specific transferase, choline sulphokinase (Kaji & McElroy, 1958). With baker's yeast it has been shown that the formation of PAPS is a two-stage process; the first stage involves the reversible action of an ATP-sulphurylase to give adenosine-3'-phosphosulphate (APS) and pyrophosphate and this is followed by a non-reversible phosphorylation of APS by a specific APS-kinase to give PAPS (Bandurski, Wilson & Squires, 1956). The presence of an efficient pyrophosphatase is necessary to remove pyrophosphate which tends to reverse the first reaction.

The inability of a fungus to produce choline sulphate might therefore be due to several reasons. One factor, the insufficient supply of choline, has been ruled out in the present investigation. Another point which was considered was that the PAPS and choline sulphate systems may be 'dormant' when the organism has an adequate supply of easily assimilated sulphur, such as cysteine. Against this possibility is the fact that the fungi which did not synthesize choline sulphate on a glucose peptone yeast-extract medium grew on a medium in which inorganic sulphur was the only significant sulphur

Table 2. *A survey of the occurrence of choline sulphate in some fungi*

Culture media were: (1) glucose peptone yeast extract; (2) glucose peptone yeast-extract + 1.2 g. choline chloride/l.; (3) Czapek-Dox medium; (4) chemically defined medium containing asparagine, inositol, biotin, etc. For details see text.

	Medium	Presence of choline sulphate
Phycomycetes		
Mucorales		
<i>Rhizopus stolonifer</i> (+) CMI 57761	1, 2, 4	—
<i>R. stolonifer</i> (—) CMI 57762	1, 2	—
<i>Mucor hiemalis</i> (—) CMI 21217	1	—
<i>Phycomyces blakesleanus</i> CMI 21156	1	—
<i>Cunninghamella echinulata</i> CMI 44534 (a)	1	—
Ascomycetes		
Endomycetales		
<i>Eremothecium ashbyi</i> CMI 31269	1, 2	—
<i>Nematospora gossypii</i> CMI 31279	1, 2	—
<i>Saccharomyces cerevisiae</i> CMI 19391	1	—
<i>S. cerevisiae</i> var. <i>ellipsoideus</i> NCYC 57	1	—
Baker's yeast	1, 2, 4	—
<i>Pichia membranaefaciens</i> NCYC 51	1	—
Sphaeriales		
<i>Neurospora crassa</i> CMI 19419	1, 2	+
<i>N. crassa</i> CMI 53238	2	+
<i>N. sitophila</i> (+) CMI 63919	1, 2	+
<i>N. sitophila</i> (—) CMI 63920	2	+
Basidiomycetes		
Ustilaginales		
<i>Ustilago scitaminea</i> CMI 35616	1	+

Table 2 (cont.)

	Medium	Presence of choline sulphate
Fungi Imperfecti		
Moniliales		
Sporobolomycetaceae		
<i>Sporobolomyces roseus</i> CMI 43529	2	+
<i>S. salmonicolor</i> CMI 56578	2	+
Moniliaceae		
<i>Aspergillus terreus</i> LSHTM Ab 2	3	+
<i>A. oryzae</i> CMI 17299	3	+
<i>A. sydowii</i> CMI 63904	3	+
<i>A. nidulans</i> CMI 16643	3	+
<i>Penicillium citreo-roseum</i> LSHTM P 22	3	+
<i>P. chrysogenum</i> CMI 62728	3	+
<i>P. notatum</i> LSHTM P 45	3	+
<i>P. aurantio-brunneum</i> LSHTM P 2	3	+
<i>Oedocephalum glomerulosum</i> CMI 60070	2	+
<i>Trichoderma viride</i> sp.	2	+
Pseudosaccharomycetaceae		
<i>Torulopsis utilis</i> CMI 23311	1, 2, 4	—
<i>Rhodotorula rubra</i> CMI 38784	1	+
Tuberulariaceae		
<i>Fusarium</i> sp.		+
Dermatiaceae		
<i>Cladosporium cladosporioides</i> CMI 49625	1	+
Bacteria		
<i>Pseudomonas pyocyaneus</i> NCTC 8097	2	—
<i>P. fluorescens</i> NCTC 4725	2	—
<i>Escherichia coli</i> NCTC 8196	2	—
<i>Aerobacter aerogenes</i> ATCC 9621	1, 2	—
<i>A. aerogenes</i> ATCC 7256	1, 2	—
<i>Staphylococcus aureus</i> NCTC 6571	2	—
<i>Streptococcus faecalis</i> NCTC 8966	2	—
<i>Bacillus subtilis</i> NCTC 2116	2	—
<i>Streptomyces albus</i> sp.	1	—

ATCC = American Type Culture Collection.

CMI = Commonwealth Mycological Institute.

LSHTM = London School of Hygiene and Tropical Medicine.

NCTC = National Collection of Type Cultures.

NCYC = National Collection of Yeast Cultures.

source, but no choline sulphate synthesis resulted (Table 2). Many other factors have been investigated in a separate investigation (Spencer & Harada, 1959) which shows that the fungi which utilize the sulphur of inorganic sulphate all synthesize PAPS and that the failure to produce choline sulphate is due to the absence of choline sulphokinase.

The systematics of choline sulphate synthesis can therefore be related to the presence or absence of choline sulphokinase (see Table 2). This enzyme would appear to be absent from the lower fungi since none of the Phycomycetes produced choline sulphate. In the Ascomycetes, none of the Endomycetales possess choline sulphokinase but the enzyme is present in the Sphaeriales. All the Fungi Imperfecti, except *Torulopsis utilis*, were choline sulphate positive. The degree of concurrence between the presence or absence of choline

sulphokinase and accepted taxonomic division is high, and the exception of *T. utilis* (syn. *Candida utilis*) becomes rather outstanding (Table 2). An unconfirmed observation of Windisch (1948) that *T. utilis* shows ascospore formation would place this organism in the Endomycetales, a group which does not synthesize choline sulphate. It is also possible to place the *Sporobolomyces* in the Basidiomycetes, and many of the aspergilli and penicillia as Eurotiales in the Ascomycetes, without affecting the relationship between choline sulphate production and taxonomy. However, by extension of this type of argument the position of *Rhodotorula rubra* might well become ambiguous with respect to the systematics of choline occurrence.

Both choline sulphate-producing and non-producing fungi can utilize the sulphur of inorganic sulphate for growth purposes. Two alternative schemes for sulphate assimilation can therefore be proposed: (i) that both classes of fungi metabolize SO_4^{--} in the same manner and that choline sulphate formation is only a side reaction; (ii) that the two classes metabolize sulphate differently, choline sulphate formation being an obligatory stage but only in the choline sulphate-producing fungi. *Aerobacter aerogenes*, *Pseudomonas pyocyaneus*, *P. fluorescens* and *Escherichia coli*, which are known from the literature to be capable of using inorganic sulphate, may possess a sulphate-metabolizing system like that of the fungi which do not form choline sulphate.

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