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Synthesis from Sulphate and Accumulation of *S*-Sulphocysteine by a Mutant Strain of *Aspergillus nidulans*

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It is well established that the activation of sulphate by adenosine triphosphate and subsequent reduction of the active sulphate to sulphite constitute the initial steps of sulphate assimilation in micro-organisms (Hilz, Kittler & Knape, 1959; Ragland, 1959; Wilson, Asahi & Bandurski, 1961; Asahi, Bandurski & Wilson, 1961). No agreement has, however, been reached on the pathway by which sulphite sulphur is incorporated into sulphur-containing amino acids.

Work with mutant strains of *Aspergillus nidulans* by Hockenhull (1949) has shown that thiosulphate is the form in which sulphate sulphur enters into organic linkages. Hockenhull has further obtained evidence that thiosulphate reacts with serine to form *S*-sulphocysteine (cysteine-*S*-sulphonate) as the immediate precursor of cysteine. Horowitz (1950) has also postulated the formation in *Neurospora crassa* of a sulphur–carbon bond at the thiosulphate stage of sulphate reduction. The occurrence in *A. nidulans* of the thiosulphate–sulphocysteine pathway of sulphate assimilation has recently been confirmed by biochemical genetical studies in this Laboratory (Nakamura & Sato, 1960; Nakamura, 1962), in which heterokaryosis was extensively used to elucidate the metabolic sequence. However, *S*-sulphocysteine, the key compound of the postulated pathway, has not yet been found in Nature, except for a report by Cavallini, de Marco, Mondovi & Tentori (1960) that this compound occurs among the products of cysteine metabolism *in vivo*.

This paper reports that a cysteine-less mutant, strain 793, of *A. nidulans* accumulates radioactive *S*-sulphocysteine when grown in the presence of [^{35}S]sulphate. Strain 793 has been shown to require cysteine, cystine or methionine for growth and to be incapable of utilizing *S*-sulphocysteine as well as inorganic forms of sulphur to fulfil its sulphur requirements (Nakamura & Sato, 1960; Nakamura, 1962). It is also reported that the two sulphur atoms in the molecule of accumulated sulphocysteine were equally labelled and therefore both must have been derived from the sulphate added in the medium. A preliminary account of this work has been published (Nakamura & Sato, 1962).

MATERIALS AND METHODS

Organism. The wild-type strain of *Aspergillus nidulans* IFO 5713 was supplied from the Institute for Fermentation, Osaka. The production, isolation and nutritional requirements of the mutant strains employed in this work were described by Nakamura (1962). These strains were maintained on agar slants containing L-methionine as the sulphur source.

Synthesis of S-*sulphocysteine.* Monosodium *S*-sulpho-L-cysteine was synthesized by a modification of the procedure of Kolthoff & Stricks (1951). L-Cystine (12 g.), 63 g. of Na_2SO_3 and 0·5 g. of $CuSO_4,5H_2O$ were dissolved in 800 ml. of aq. 0·2 N-NH_3 soln. and the solution was aerated for 9 hr. at room temperature. To this mixture was further added 63 g. of Na_2SO_3 and aeration was continued for 9 hr. The mixture was then adjusted to pH 6·8 with 2 N-acetic acid and evaporated *in vacuo* (below 40°) to about 400 ml. After removal of the precipitate by filtration, both SO_4^{2-} and SO_3^{2-} ions were precipitated with a slight excess of Ba^{2+} ions; both $BaCl_2$ and $Ba(OH)_2$ were used for this purpose to avoid acidification of the fluid. The insoluble barium salts were removed by centrifuging and the supernatant was again evaporated *in vacuo* to dryness. The residue was treated with 100 ml. of cold ethanol and the ethanol-insoluble fraction was collected by centrifuging, washed with a small amount of cold ethanol and dried in a vacuum desiccator. The dry material (about 20 g.), still contaminated by a large amount of inorganic salts, was dissolved in 20 ml. of water and insoluble impurities were removed by centrifuging. The clear solution was then passed through a column (3·2 cm. × 32·5 cm.) of Amberlite CG-120 (H^+ form; 100–200 mesh) to remove cations and the effluents were adjusted to pH 5·4 with dilute NaOH. This solution containing *S*-sulphocysteine was applied to a column (3·5 cm. × 57·5 cm.) of Dowex 1 (formate form; X8; 100–200 mesh), and eluted with pyridine–formate buffer, pH 5·4 (44·6 ml. of 85 %, w/v, formic acid and 162 ml. of pyridine diluted to 1 l. with water, according to Waley, 1959, who purified *S*-sulphoglutathione). Each fraction from the column was assayed by paper chromatography for *S*-sulphocysteine (by ninhydrin reaction and R_F) and the fractions containing the compound were combined, evaporated *in vacuo* to remove volatile material (formic acid, pyridine etc.) and neutralized with 2 N-NaOH. The neutralized solution was further concentrated *in vacuo* and the concentrate treated with 95 % (w/w) ethanol in the cold. Crystals of monosodium *S*-sulpho-L-cysteine which separated on this treatment were collected by filtration and washed with a small amount of cold ethanol. They were finally recrystallized from 80 % (w/w) ethanol, washed and dried in a vacuum desiccator. Yield, 7·0 g., m.p. 154–155° (decomp.) (Found: C, 15·8;

H, 2·6; N, 6·4; S, 28·1. Calc. for $C_3H_6NNaO_5S_2$: C, 16·1; H, 2·7; N, 6·3; S, 28·7%).

Other chemicals. Choline sulphate was prepared as described by Schmidt & Wagner (1904). Choline [^{35}S]-sulphate was similarly synthesized from conc. [^{35}S]sulphuric acid. Performic acid was prepared according to the directions of Schramm, Moore & Bigwood (1954). *N*-Ethylmaleimide was synthesized by the method of Marrian (1949). Carrier-free sodium [^{35}S]sulphate was purchased from Commissariat à l'Énergie Atomique, Scalay, France.

Production of mycelia. Conidiospores of the strain to be tested were inoculated into the basal sulphur-deficient medium (Nakamura, 1962) supplemented with L-methionine (25 mg./l.) and choline sulphate (100 mg./l.). With strain 793 as the test organism, $NaNO_3$ in the basal medium was replaced by L-asparagine (2·5 g./l.) because better growth of this strain was obtained on the latter nitrogen source. The cultivation was carried out at 33° for 40 hr. in shaking flasks (500 ml. capacity), each containing 100 ml. of the supplemented medium. Shaking conditions were 100 strokes/min. with an amplitude of 8 cm. The mycelia from five flasks were collected by filtration, washed with 2 l. of 0·9% NaCl soln. on the filter, and resuspended in 2 l. of fresh 0·9% NaCl soln. The suspension was gently stirred for 10 min. and filtered. The mycelia were washed again on the filter with 1 l. of 0·9% NaCl soln. to remove methionine and other medium constituents as completely as possible. Incomplete washing resulted in poor incorporation of [^{35}S]sulphate during replacement cultivation.

Replacement cultivation. The vegetative mycelia (wet wt. 30–40 g.), obtained as described above, were suspended in the basal sulphur-deficient medium to which were added carrier-free inorganic [^{35}S]sulphate (2 mc/l.), DL-serine (50 mg./l.) and choline sulphate (50 mg./l.); about 6 g. of wet mycelia was used per flask containing 150 ml. of medium. L-Asparagine was again used instead of $NaNO_3$ as the nitrogen source for strain 793. The mycelial suspensions were then shaken at 33° for 7 hr., during which time most of the radioactivity added in the replacement medium was taken up into the mycelia.

Extraction of radioactive metabolites. The mycelia of strain 793 that had incorporated [^{35}S]sulphate during replacement cultivation (wet wt. 35 g.) were collected by filtration and immediately suspended in 400 ml. of ethanol containing 0·02 M-*N*-ethylmaleimide. The suspension was vigorously stirred for 10 min. to convert intracellular free thiols into corresponding *N*-ethylsuccinimide derivatives (Marrian, 1949). The mycelia were then separated by filtration from ethanol and dried *in vacuo* over P_2O_5. To the dry material (7·5 g.) was added 140 mg. of authentic monosodium *S*-sulpho-L-cysteine as carrier. The mixture was then ground in a mortar with 37·5 g. of quartz sand and extracted with 100 ml. of water. The grinding was carried out for 20 min. at room temperature and the extract was freed from quartz sand and mycelial residues by centrifuging at 10 000 *g* for 10 min. The aqueous extract (95 ml.) thus obtained was then subjected to paper chromatography for radioactive substances or to column chromatography for purification of the accumulated *S*-sulphocysteine. Mycelia of the other strains were treated similarly.

Paper chromatography. One-dimensional paper chromatograms were developed on Toyo Roshi no. 51 paper by the ascending procedure. The following five solvent systems were used: (1) butan-1-ol–pyridine–water (1:1:1, by vol.); (2) methanol–pyridine–water (20:1:5, by vol.); (3) butan-1-ol–propan-2-ol–pyridine–water (4:5:3:6, by vol.); (4) propan-2-ol–water (7:3, v/v); (5) upper layer of butan-1-ol–acetic acid–water (5:1:4, by vol.). Amino acids were located on the chromatograms by the conventional ninhydrin reagent. When thiosulphate was to be detected, the paper was sprayed with an iodine reagent (0·3 g. of I_2 and 2·0 g. of KI were dissolved in 100 ml. of water), which stained the paper pale yellow and left thiosulphate-bearing spots colourless. Labelled compounds were detected on the paper by means of a radiometric chromatogram scanner (Nuclear–Chicago Corp., Actigraph 11). R_F values of *S*-sulphocysteine and other compounds were determined with authentic samples, labelled compounds being used for this purpose with inorganic and choline sulphates.

Column chromatography. Three types of columns were used for the purification of *S*-sulphocysteine from mycelial extracts of strain 793. A column (4·2 cm. × 12·0 cm.) of Amberlite CG-120 (H^+ form) was used for removing cationic impurities from the extracts. The cation-free extracts were concentrated to about 50 ml. by freeze-drying and then chromatographed on a column (2·2 cm. × 33·0 cm.) of an anion-exchange resin Daiaion SA no. 100 (Mitsubishi Chemical Industries Ltd., Tokyo), formate form, and eluted with pyridine–formate buffer, pH 5·4 (Waley, 1959). The resin used here is similar to Dowex 1 (X8). For further purification a third column (3·2 cm. × 48·0 cm.) of cellulose powder (100–200 mesh) was used; butan-1-ol–propan-2-ol–pyridine–water (4:5:3:6, by vol.) was employed as eluent. Fractions (7 g.) were collected and analysed for amino acids and for radioactivity.

Amino acid analysis. To 0·2 ml. of sample was added 0·5 ml. of a ninhydrin reagent (Moore & Stein, 1954) and the mixture heated for 15 min. in a boiling-water bath. After cooling, the mixture was diluted to 5·0 ml. with aq. 50% (v/v) ethanol. The colour developed was measured at 570 mμ in a Coleman junior spectrocolorimeter.

Radioactivity measurements. An automatic gas-flow counter (Nuclear–Chicago Corp., model D 47) was regularly used, but a Geiger–Müller counter (Kobe Kogyo Co. Ltd., model PC 8) was also employed in some measurements. Portions of samples less than 0·1 ml. were placed on aluminium planchets, carefully dried with an infrared lamp, and then counted. No corrections were made for self-absorption, since errors introduced by ignoring the corrections were negligible.

Cleavage of disulphide bond of S-*sulphocysteine.* Two methods were adopted to split the disulphide bond of the labelled sulphocysteine accumulated by strain 793.

In the first method, the labelled sulphocysteine purified through cellulose-column chromatography (24 mg.) was dissolved in 2·0 ml. of 0·5 M-tris–HCl buffer, pH 9·0, and after removal of air from the reaction system H_2S was bubbled into the solution for 1 hr. at room temperature. To the solution was then added 0·5 ml. of an ethanolic solution containing 0·32 M-*N*-ethylmaleimide (1·6 equivalent to the sulphocysteine used) to block the free sulphydryl group of the cysteine formed. To separate the cleavage products, the solution was carefully streaked on two sheets of Toyo Roshi no. 50 paper (40 cm. × 40 cm.) and submitted to ascending chromatography with butan-1-ol–propan-2-ol–pyridine–water (4:5:3:6, by vol.) as solvent. A small strip was then cut from the paper along the direction of solvent flow and the bands bearing thiosulphate and amino acids

were located by the iodine and ninhydrin reagents respectively. The sheets of paper were then cut into six areas as indicated in Fig. 4 (A) and each part was extracted with aq. 50% (v/v) ethanol. The extracts thus obtained were separately concentrated and counted in the gas-flow counter.

In the second method, the disulphide bond was oxidatively cleaved by performic acid. Purified labelled *S*-sulphocysteine (6·0 mg.) was dissolved in 20 ml. of performic acid and the solution was allowed to stand at 0° for 3 hr. The mixture was then concentrated to less than 1 ml. in a rotary evaporator, streaked on a sheet of filter paper (40 cm. × 40 cm.) and chromatographed with propan-2-ol–water (7:3, v/v) in an ascending run. The paper was cut into eight areas, as shown in Fig. 4 (B). Each part was then extracted and counted as described above.

RESULTS

Accumulation of S-*sulphocysteine by strain* 793. Since strain 793 has been suggested to be genetically blocked in the reaction by which *S*-sulphocysteine formed from inorganic sulphate and a C_3 compound is converted into cysteine (Nakamura & Sato, 1960; Nakamura, 1962), it was expected that this mutant would accumulate *S*-sulphocysteine when allowed to metabolize inorganic sulphate. An experiment was therefore designed to demonstrate the expected accumulation.

The thoroughly washed mycelia of strain 793 were suspended in the fresh medium containing carrier-free [^{35}S]sulphate, unlabelled choline sulphate and DL-serine as described in the Materials and Methods section. The mycelial suspensions in the replacement medium were shaken so that the serine added would react with thiosulphate derived from [^{35}S]-sulphate to form labelled *S*-sulphocysteine as suggested previously (Hockenhull, 1949; Nakamura & Sato, 1960; Nakamura, 1962). Although choline sulphate is incapable of supporting the growth of this mutant as the sole sulphur source, this sulphate ester was included in the replacement medium, as well as in the growth medium, with the hope that this would saturate the intracellular pool of this compound with unlabelled ester and thus suppress the extravagant consumption of [^{35}S]-sulphate for the sulphurylation of choline (Kaji & Gregory, 1959; Spencer & Harada, 1960). It is known that many fungi, including *A. nidulans*, accumulate large quantities of choline sulphate in their mycelia (Harada & Spencer, 1960).

It was found that during the replacement cultivation practically all of the radioactivity had been incorporated into the mycelia; the radioactivity of the medium as measured by the Geiger–Müller counter was decreased from the original value of 264×10^6 counts/min. to only 4×10^6 counts/min. after cultivation. When the medium after cultivation was analysed by paper chromatography in propan-2-ol–water (7:3, v/v), only one radioactive spot (R_F 0·1), corresponding to inorganic sulphate, was detected. The radioactivity could also be precipitated by barium chloride. It is apparent that any metabolites formed in the mycelia from inorganic sulphate had not been released into the medium.

The mycelia recovered from the replacement culture were treated with ethanol containing *N*-ethylmaleimide. This treatment resulted in a release of 39×10^6 counts/min. or 15% of the mycelial radioactivity into the ethanolic solution. The released radioactivity was found to be mainly due to inorganic and choline sulphates by paper chromatography (R_F 0·12 and 0·55 in propan-2-ol–water). This treatment, which blocks most of the intracellular free thiols in the form of *N*-ethylsuccinimide derivatives (Marrian, 1949), was essential for successful detection of the accumulated sulphocysteine, probably owing to the instability of this compound in the presence of thiols (Kolthoff & Stricks, 1951).

The mycelia were then dried, mixed with authentic *S*-sulphocysteine, ground with quartz sand and extracted with water. The aqueous extract thus obtained contained 210×10^6 counts/min. or 81% of the radioactivity that had been incorporated into the mycelia.

The presence of labelled *S*-sulphocysteine in this extract could be demonstrated by paper chromatography with propan-2-ol–water (7:3, v/v) as solvent. As can be seen from Fig. 1, a radioactive spot corresponding to *S*-sulphocysteine (R_F 0·34) was detected on the chromatogram in addition to those of choline sulphate (R_F 0·56), inorganic sulphate (R_F 0·09) and an unidentified sulphur

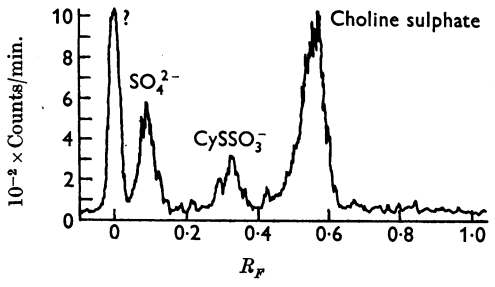


Fig. 1. Radiochromatogram of mycelial extract from strain 793 grown in the presence of [^{35}S]sulphate. About 0·05 ml. of extract was applied to a strip of Toyo Roshi no. 51 paper (2 cm. × 40 cm.) and the one-dimensional chromatogram was developed in an ascending run with propan-2-ol–water (7:3, v/v) as solvent; the solvent migrated about 20 cm. from the start line. The radioactivity scanning was made in a Nuclear-Chicago Actigraph 11. Collimeter, ¼ in.; scan speed, ¾ in./min.; time-constant, 2 sec.; count-rate range, 1000 counts/min. $CySSO_3^-$, *S*-Sulphocysteine.

compound remaining at the origin. The radioactive spot having R_F 0·34 developed a colour characteristic of *S*-sulphocysteine when stained with the ninhydrin reagent.

Purification of accumulated S-*sulphocysteine.* In order to further establish that the radioactive spot described above actually corresponds to *S*-sulphocysteine, the labelled compound from the aqueous mycelial extract was purified. The extract was freed from cationic impurities by passing it through a column of Amberlite CG-120 (H^+ form) and the cation-free effluents were concentrated by freeze-drying. This mixture was then applied to a column of Daiaion SA 100 (formate form) and eluted with pyridine–formate buffer. Fig. 2 shows the distribution of radioactivity and ninhydrin-reactive substances in the fractions obtained. On paper-chromatographic analysis of each fraction it was found that *S*-sulphocysteine was present in fractions around tube no. 75, which also corresponded to a small peak of radioactivity. The fractions from no. 70 to 89 (in total 140 g.) were combined and concentrated *in vacuo* at 40–45° to remove volatile substances. The residual liquid was neutralized with 2 N-sodium hydroxide and evaporated further *in vacuo* to yield 5·5 g. of dry residue. Since this material was still impure, 1·0 g. of dry material was placed on a column of cellulose powder and chromatography was carried out with butan-1-ol–propan-2-ol–pyridine–water (4:5:3:6, by vol.) as solvent. As shown in Fig. 3, the elution curve had only one radioactive and ninhydrin-positive peak at about fraction no. 63. The combined fractions from no. 59 to 72 were dried *in vacuo* and 80 mg. of dry material was obtained.

The purified sample thus obtained, though still contaminated by salts, gave only one radioactive and ninhydrin-positive spot when subjected to paper chromatography in the five solvent systems described in the Materials and Methods section. R_F values obtained in these solvent systems closely coincided with those of authentic *S*-sulphocysteine in corresponding chromatographic systems. The identity of purified product was confirmed further by co-chromatography with an authentic sample.

Radioactivity distribution between sulphur atoms of sulphocysteine. Since *S*-sulphocysteine contains two sulphur atoms in its molecule, it seemed desirable to determine the distribution of radioactivity between them to obtain information on the mechanism by which this compound had been synthesized from inorganic sulphate. It was necessary for this purpose to devise convenient methods permitting the cleavage of the disulphide bond of *S*-sulphocysteine. After a preliminary search, two methods were adopted and applied to the labelled sulphocysteine purified from the mycelial extract of strain 793.

The first method was based on a reversible cleavage of the compound by hydrogen sulphide to form cysteine and thiosulphate (Szczepkowski, 1958). To avoid the degradation during paper chromatography, the cysteine in the cleavage products was converted into its *N*-ethylsuccinimide derivative before chromatographic separation. Fig. 4 (A) shows the paper chromatogram of the reaction mixture thus treated. On the chromatogram were detected a band bearing thiosulphate (band *a*, R_F 0·11) and two ninhydrin-positive bands (bands *b* and *c*, R_F 0·26 and 0·67 respectively); the colour of band *c* was much more intense than that of band *b*. Of the last-named two, band *c* was identified as due to the *N*-ethylsuccinimide derivative of cysteine from its R_F value, whereas band *b* was assigned to *S*-sulphocysteine from its characteristic

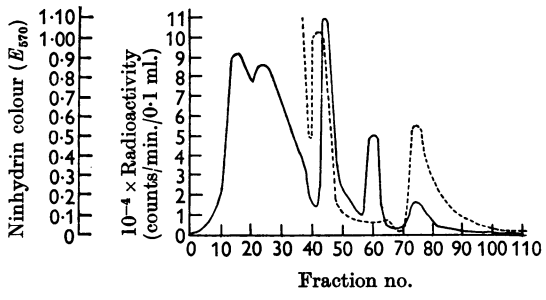


Fig. 2. Elution curve in Daiaion SA 100 (formate form) column chromatography of cation-free mycelial extract from strain 793. Column size, 2·2 cm. × 33·0 cm. Elution solvent, pyridine–formate buffer (pH 5·4). Flow rate, 17·5 g./hr. Fractions (7 g.) were collected. ——, Radioactivity; - - - - -, ninhydrin colour. The peak at fraction no. 75 was due to *S*-sulphocysteine. For further details see text.

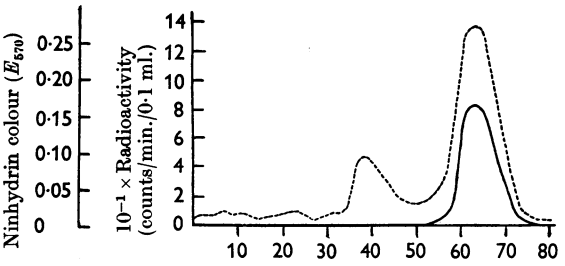


Fig. 3. Elution curve in cellulose-column chromatography of crude *S*[^{35}S]-sulphocysteine. The combined fractions from no. 70 to 89 obtained in chromatography shown in Fig. 2 were dried as described in the text and applied to the cellulose column. Column size, 3·2 cm. × 48·0 cm. Elution solvent, butan-1-ol–propan-2-ol–pyridine–water (4:5:3:6, by vol.). Flow rate, 14·0 g./hr. Fractions (7 g.) were collected. ——, Radioactivity; - - - - -, ninhydrin colour. For further details see text.

ninhydrin colour, though its R_F value was somewhat lower than expected (0·35). When the radioactivity of the six areas indicated in Fig. 4 (A) was measured, highest and nearly equal counts were detected in the areas corresponding to thiosulphate (band *a*) and the cysteine derivative (band *c*) (6904 and 7600 counts/min. respectively). The area bearing unchanged *S*-sulphocysteine (band *b*) also showed some activity. The relatively high counts detected in the area between bands *b* and *c* are difficult to interpret, but might be due to certain decomposition products formed from *S*-sulphocysteine through undesirable side reactions.

In the second method, which gave more satisfactory results than the first, the sulphocysteine was oxidized by performic acid. As shown in Fig. 4 (B), only one ninhydrin-positive band (band *d*, R_F 0·19) corresponding to cysteic acid could be detected on the paper chromatogram of the oxidation products. This indicates that the sulphocysteine had been almost quantitatively cleaved into cysteic acid and inorganic sulphate. Fig. 4 (B) also shows that the area bearing cysteic acid (band *d*) and that just beneath it (marked band *e*, R_F 0·11), which is an area corresponding to inorganic sulphate, possessed radioactivity of 1050 and 900 counts/min. respectively. The rest of the paper had only 13 % of the total radioactivity. It is certain from these results that the two sulphur atoms of the accumulated *S*-sulphocysteine were labelled to equal extents.

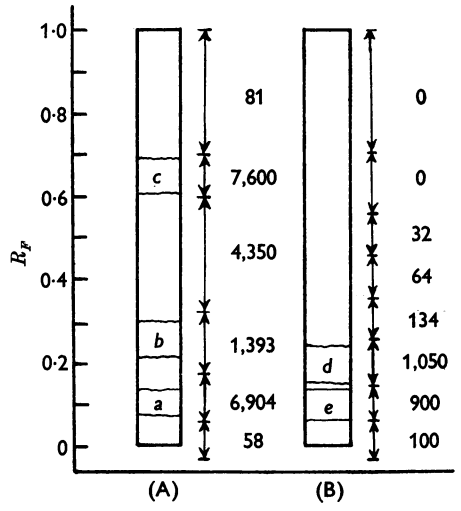


Fig. 4. Paper chromatograms of cleavage products of labelled *S*-sulphocysteine and distribution of radioactivity. Labelled *S*-sulphocysteine purified from mycelial extract of strain 793 was treated either with hydrogen sulphide (for A) or performic acid (for B) as described in the text. The reaction products were subjected to paper chromatography (ascending run) on Toyo Roshi no. 50 paper with butan-1-ol–propan-2-ol–pyridine–water (4:5:3:6, by vol.) for (A) or propan-2-ol–water (7:3, v/v) for (B) as solvent. Band *a*, thiosulphate; bands *b*, *c* and *d*, ninhydrin-positive substances; band *e*, sulphate. The papers were cut into areas indicated by arrows; each area was extracted and the extract counted. Figures shown at the side of chromatograms indicate the radioactivity (counts/min.) thus determined. For further details see text.

Experiments with strains other than 793. If sulphate assimilation in *A. nidulans* proceeds through the pathway suggested by Nakamura & Sato (1960) and Nakamura (1962), it would be expected that mutant strains of this fungus which are blocked at earlier steps than *S*-sulphocysteine would not accumulate this compound when they are grown in the presence of sulphate. To test this possibility, the mycelia of strains 954 (sulphite-less), 574 (thiosulphate-less) and 721 (*S*-sulphocysteine-less) as well as those of the wild-type strain were incubated aerobically for 7 hr. with a medium containing [^{35}S]sulphate, unlabelled choline sulphate and DL-serine. They were then treated with *N*-ethylmaleimide, dried and extracted as with strain 793. When the extracts were analysed for radioactive metabolites, it was in fact found that no radioactive spots corresponding to *S*-sulphocysteine could be detected in any of the mutants tested (Figs. 5–7). In the extract from the wild-type strain, on the other hand, a trace of *S*-sulphocysteine was detectable (Fig. 8). The radiochromatograms reproduced in Figs. 5–8, together with that in Fig. 1, further reveal several facts of interest. First, the unidentified radioactive sulphur compound (R_F 0·0) found in the extract from strain 793 (Fig. 1) could also be detected in the wild-type strain (Fig. 8), but the other mutants did not produce this compound (Figs. 5–7). Secondly, the sulphite-less mutant, strain 954, accumulated no labelled choline sulphate (Fig. 5). Finally, the synthesis of radioactive choline sulphate was significantly suppressed in the thiosulphate-less (strain 574) and *S*-sulphocysteine-less (strain 721) mutants (Figs. 6 and 7).

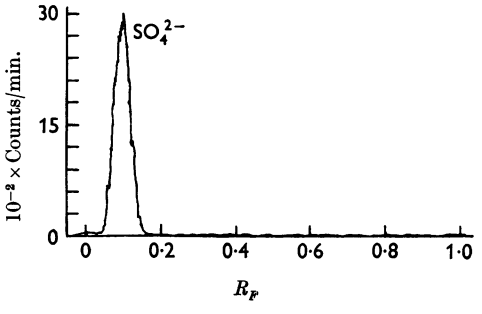


Fig. 5. Radiochromatogram of mycelial extract from strain 954 (sulphite-less mutant) grown in the presence of [^{35}S]-sulphate. The experimental conditions were the same as in Fig. 1 except that a count-rate range of 3000 counts/min. was used.

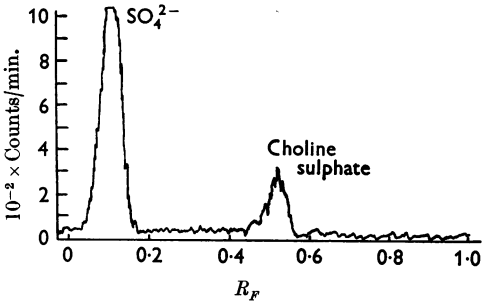


Fig. 6. Radiochromatogram of mycelial extract from strain 574 (thiosulphate-less mutant) grown in the presence of [^{35}S]sulphate. The experimental conditions were the same as in Fig. 1.

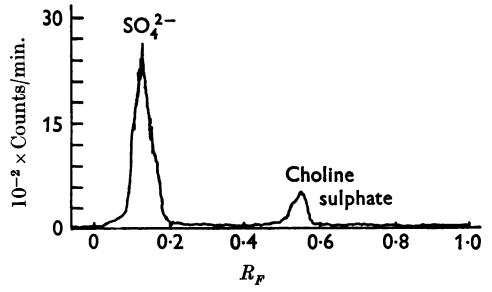


Fig. 7. Radiochromatogram of mycelial extract from strain 721 (*S*-sulphocysteine-less mutant) grown in the presence of [^{35}S]sulphate. The experimental conditions were the same as in Fig. 6.

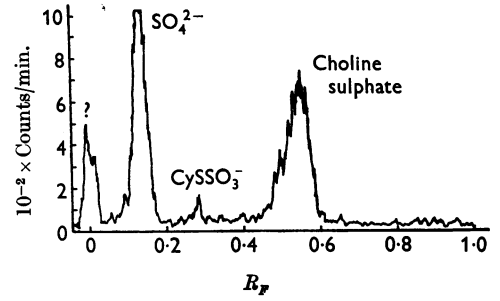
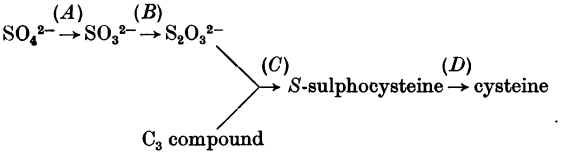


Fig. 8. Radiochromatogram of mycelial extract from the wild-type strain grown in the presence of [^{35}S]sulphate. The experimental conditions were the same as in Fig. 1. $CySSO_3^-$, *S*-Sulphocysteine.

DISCUSSION

Growth studies with biochemical mutants of *A. nidulans* (Hockenhull, 1949; Nakamura & Sato, 1960; Nakamura, 1962) have produced evidence indicating that the synthesis of cysteine from sulphate in this organism takes place through the pathway shown in Scheme 1. It has further been shown that, among the mutant strains isolated in this Laboratory, strain 793 exhibits nutritional requirements that are compatible with the assumption that this strain is genetically blocked at the reaction converting *S*-sulphocysteine into cysteine



Scheme 1

(reaction *D*). In the present investigation it was in fact shown that strain 793 accumulates radioactive *S*-sulphocysteine, the substrate of the blocked reaction, when it is grown in the presence of [^{35}S]-sulphate. Mutant strains genetically blocked at earlier steps than *S*-sulphocysteine (reaction *A*, *B* or *C*), on the other hand, did not accumulate this compound even when they were allowed to metabolize labelled sulphate. These facts are in full accord with the pathway illustrated in Scheme 1. The detection of a small amount of labelled *S*-sulphocysteine in the wild-type strain further supports the view that this compound is an obligatory intermediate of sulphate utilization in this organism.

Our results also indicate that the two sulphur atoms of the accumulated sulphocysteine are equally labelled and therefore both must have been derived from the sulphate added in the medium. This finding excludes the possibility that the sulphocysteine had been formed by a non-enzymic reaction between unlabelled cellular cystine and radioactive sulphite produced from the added sulphate. This process, which has been studied by Kolthoff & Stricks (1951), may be represented by $CySSCy + S^*O_3^{2-} \rightleftharpoons CySS^*O_3^- + CyS^-$, where Cy stands for the $-CH_2 \cdot CH(NH_2) \cdot CO_2H$ group and asterisks indicate the labelling. Another possible non-enzymic process (Kolthoff, Stricks & Kapoor, 1955; Szczepkowski, 1958) between preformed cold cysteine and labelled thiosulphate,

$$CyS^- + S^*S^*O_3^{2-} \rightleftharpoons CySS^*O_3^- + S^{*2-},$$

is also incapable of explaining the formation of doubly labelled *S*-sulphocysteine. It may therefore be concluded that the sulphocysteine had been synthesized by the condensation of a C_3 compound, probably serine (Hockenhull, 1949), with radioactive thiosulphate formed from sulphate via sulphite. This reaction, which may be written as

$$CyOH + S^*S^*O_3^{2-} \rightarrow CyS^*S^*O_3^- + OH^- \text{(CyOH, serine)},$$

seems to be enzymic in nature, since no reaction occurs chemically when DL-serine and thiosulphate are mixed at physiological pH values and this reaction, if actually occurring in *A. nidulans*, must be controlled genetically. Attempts are now being made to detect an enzyme responsible for this reaction in mycelial extracts of this fungus.

Nakamura & Sato (1960) briefly mentioned that the formation of *S*-sulphocysteine could be detected polarographically in a reaction mixture containing a yeast extract, thiosulphate, serine and pyridoxal phosphate. Later investigations have, however, disclosed that the polarographic wave thought to be due to *S*-sulphocysteine had in fact been caused by an artifact. It now seems more likely that sulphate assimilation in such organisms as yeast does not involve *S*-sulphocysteine as an intermediate and thus its pathway differs from that operative in *A. nidulans*. The findings from recent studies on yeast (Hilz *et al.* 1959; Lezius, 1959) may be explicable by a pathway in which sulphate is reduced to sulphide and the latter reacts with serine to form cysteine under the influence of a pyridoxal phosphate-dependent enzyme, serine sulphydrase (Schlossmann & Lynen, 1957).

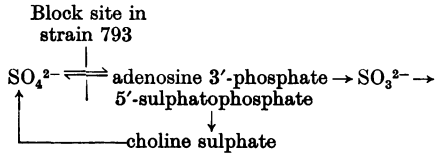
Harada & Spencer (1962) have recently reported that the synthesis of arylsulphatase in fungi is strongly inhibited by such sulphur sources as sulphate, sulphite and cysteine, but not by taurine, cysteate, choline sulphate etc. In interpreting this interesting phenomenon in terms of a diauxie effect, they have suggested that only those sulphur compounds that are direct intermediates in sulphate assimilation inhibit the synthesis. If this be the case, important information on the pathway of sulphate assimilation may be obtained by observing the effects of various sulphur sources on arylsulphatase synthesis. They have in fact found that both thiosulphate and *S*-sulphocysteine inhibited the synthesis in *A. nidulans* and implied that these compounds are normal intermediates in sulphate assimilation. However, there are organisms such as *Rhizopus stolonifer* in which *S*-sulphocysteine showed no inhibitory action. As these authors have pointed out, these observations may be regarded as an indication of the occurrence of different pathways of sulphate assimilation in different microorganisms.

The success in the present investigation in detecting the accumulation of labelled *S*-sulphocysteine by strain 793 was achieved only by introducing the *N*-ethylmaleimide pretreatment. Not even a trace of *S*-sulphocysteine could be found in the mycelial extracts when this treatment was omitted. It seems that the maleimide prevents the chemical decomposition of sulphocysteine during extraction by blocking sulphydryl groups of intracellular thiols. The pretreatment of mycelia with cupric salts was also found to be effective, though less satisfactory, in preventing the disappearance of *S*-sulphocysteine (T. Nakamura & R. Sato, unpublished work).

The inclusion of unlabelled choline sulphate in the replacement medium appeared favourable for the accumulation of labelled sulphocysteine. This may be accounted for by the suppression by the added choline sulphate of the consumption of labelled inorganic sulphate for the synthesis of this sulphate ester. It has been shown that *A. nidulans* produces large amounts of choline sulphate from inorganic sulphate (Hockenhull, 1949; Harada & Spencer, 1960). It does not seem likely that the added choline sulphate has any significant effects on the utilization of labelled inorganic sulphate, since Stevens & Vohra (1955) have shown that *Penicillium chrysogenum*, which is also a choline sulphate accumulator, preferentially utilizes inorganic sulphate as sulphur source when grown in a medium containing both inorganic and choline sulphates.

The nature of a sulphur compound remaining on the starting line on paper chromatography of the extract from strain 793 (Fig. 1) is not yet clear. This substance could also be detected in the wild-type strain (Fig. 8), but not in the mutants unable to synthesize *S*-sulphocysteine (Figs. 5–7). A relationship therefore seems to exist between the production of this substance and the ability to synthesize *S*-sulphocysteine. It might be suggested that this unidentified compound is formed from *S*-sulphocysteine by a branched pathway of sulphate assimilation. Another possibility is that it might be an artifact produced from the sulphocysteine during extraction.

It was somewhat unexpected to find that the sulphite-less mutant, strain 954, did not produce labelled choline sulphate from [^{35}S]sulphate, since the site of block in this strain had previously been assigned to the reaction by which the active sulphate, adenosine 3′-phosphate 5′-sulphatophosphate, is reduced to sulphite (Nakamura, 1962), and many fungi contain an active enzyme, choline sulphurylase, catalysing the transfer of sulphate from the 5′-sulphatophosphate to choline (Kaji & Gregory, 1959; Spencer & Harada, 1960). The absence of labelled choline sulphate in the extract from strain 954 now makes it necessary to locate anew the block site between sulphate and the 5′-sulphatophosphate as shown in Scheme 2. In this



Scheme 2

case, the fact that this strain is unable to utilize choline sulphate as sulphur source should be explained by the lack of reversal in the choline-sulphurylase reaction. Actually, Kaji & Gregory (1959) have failed to detect this reversal in a system *in vitro*. This lack of reversal in choline sulphurylation raises a question about the route by which choline sulphate is utilized by the wild-type strain. It has been established that many fungi, including *A. nidulans*, grow well with choline sulphate as the sole source of sulphur (Itahashi, 1959; Nakamura, 1962). No liberation of inorganic sulphate from choline sulphate has, however, been detected in fungi (Itahashi, 1959), and the only enzyme so far known to hydrolyse this sulphate ester is a specific choline sulphatase found in a pseudomonad (Takebe, 1961). It has therefore been suggested that choline sulphate is utilized by fungi through regeneration of the active sulphate (Spencer & Harada, 1960). It will be evident, however, from the considerations just described, that this mechanism of choline sulphate utilization is no longer tenable. It appears likely that choline sulphate is hydrolysed before its assimilation by a hitherto unknown mechanism.

The reasons for lower production of labelled choline sulphate in the thiosulphate-less and *S*-sulphocysteine-less mutants as compared with strain 793 and the wild-type strain are not yet known.

SUMMARY

1. When the vegetative mycelia of a cysteine-less mutant, strain 793, of *Aspergillus nidulans* were aerobically incubated with a medium containing [^{35}S]sulphate, unlabelled choline sulphate and DL-serine, practically all of the radioactivity was incorporated into the mycelia.

2. The mycelia, after pretreatment with *N*-ethylmaleimide, were extracted and the extract was analysed for radioactive metabolites by paper chromatography. Labelled *S*-sulphocysteine was detected, in addition to inorganic and choline sulphates and an unidentified sulphur compound. The sulphocysteine was purified by column chromatography and its identity was confirmed by paper chromatography in five different solvent systems.

3. The disulphide bond in the purified *S*-sulphocysteine was cleaved reductively with hydrogen sulphide or oxidatively with performic acid. By measuring the radioactivity of the cleavage products separately, it was concluded that the two sulphur atoms of the accumulated sulphocysteine are equally labelled.

4. Mutant strains with genetical blocking at earlier steps than *S*-sulphocysteine in the sulphate-assimilation pathway did not accumulate this compound when similarly incubated in the presence of [^{35}S]sulphate. These mutants accumulated labelled inorganic sulphate and choline sulphate, except for a sulphite-less mutant, strain 954, which accumulated only inorganic sulphate.

5. A trace of labelled *S*-sulphocysteine, together with relatively large amounts of inorganic and choline sulphates and an unidentified sulphur compound, was detected in the extract of wild-type strain grown in the presence of [^{35}S]sulphate.

6. These findings have been discussed and it has been concluded that the pathway of sulphate assimilation in *Aspergillus nidulans* involves *S*-sulphocysteine as an obligatory intermediate.

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