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Sulphate metabolism of moulds

By MARVIN J. JOHNSON, *Department of Biochemistry, University of Wisconsin, Madison, Wisconsin*

Most micro-organisms are able to use sulphate ion as a sole source of sulphur. During the last decade, much has become known about the mechanism of conversion of sulphate ion into sulphur amino acids and other organic sulphur compounds. Much more remains to be learned.

I should like to discuss some aspects of intermediary sulphur metabolism of micro-organisms in general, and of moulds in particular. Our laboratory first became interested in the sulphur metabolism of moulds through our work on penicillin formation. Tardrew & Johnson (1958) noted that a number of *Penicillium chrysogenum* mutants, selected for their ability to give high yields of penicillin, had an abnormal sulphur metabolism. They used about four times as much inorganic sulphate as unmutated strains, and produced about four times as much organic sulphur, even under conditions where penicillin production was low. Segel & Johnson (1961) showed that these strains had a much higher level of intracellular inorganic sulphate than normal strains. During growth of mycelium, the intracellular inorganic sulphate concentration reached 0·028 M, while the extracellular sulphate level was 0·005 M. The actual intracellular sulphate was higher than 0·028 M, because in our calculation we assumed that all of the water in the washed, blotted mycelium was inside the cells. The intracellular sulphate level of the unmutated strain, grown under similar conditions, at similar extracellular sulphate concentrations, was less than 0·005 M.

We decided to do further work on the mutated strain. We grew the mycelium on a mineral medium in which the sulphate content was low, but not low enough to limit growth. Then carrier-free radioactive sulphate was added and, after various time periods, measured in sec, a sample of the mycelium was quickly filtered and inactivated with boiling water. Before discussing the results of these experiments, it is

appropriate to review the pathway, as far as it is known, of sulphate utilization by micro-organisms.

In yeast (Hilz, Kittler & Knape, 1959; Wilson, Asahi & Bandurski, 1961), and probably in all other micro-organisms, the primary reaction undergone by sulphate ion is conversion into adenosine 5′-phosphosulphate (APS) by the following reaction:

$$ATP + SO_4^{2-} \longrightarrow APS + PP.$$

This reaction is strongly endergonic; the equilibrium constant is approximately 10^{-8} (Wilson & Bandurski, 1958; Robbins & Lipmann, 1958). It is caused to proceed in a forward direction only because both products are removed as quickly as formed. The pyrophosphate is removed by a pyrophosphate phosphohydrolase, and the APS is phosphorylated by ATP to produce 3′-phosphoadenosine-5′-phosphosulphate (PAPS). The sulphate group can be transferred, by ATP: sulphate adenylytransferase, from PAPS to a wide variety of compounds. The transferring reaction that interests us is the transfer of sulphate from PAPS to a heat-stable protein containing sulphydryl groups (Wilson *et al.* 1961). Hilz *et al.* (1959) found that lipoic acid could also function in the reaction, which may be written:

$$RSH + PAPS \longrightarrow R\text{-}S\text{-}SO_3^- + PAP.$$

The resulting thiosulphate ester then decomposes, and RSH is regenerated:

$$R\text{-}S\text{-}SO_3^- + RSH \longrightarrow RSSR + SO_3^{2-},$$
$$RSSR + TPNH \longrightarrow TPN^+ + 2RSH.$$

The net reaction is therefore the oxidation of TPNH and the reduction of sulphate to sulphite. The decomposition of the thiosulphate ester may not require enzyme catalysis. Segel & Johnson (1963*a*) studied the decomposition of cysteine-S-sulphate, which is also a thiosulphate ester. They found that the reaction

$$\text{cysteine-S-sulphate} + \text{cysteine} \longrightarrow \text{cystine} + \text{sulphite}$$

occurred very rapidly in neutral aqueous solution, without catalysis. Although the equilibrium constant was low (3×10^{-3} at pH 6·7) the reaction went almost to completion because of the precipitation of cystine. In biological sulphate reduction, the S-S compound is removed by reduction with TPNH. When Hilz *et al.* (1959) studied the production of sulphite by the above series of reactions, they found that in their yeast enzyme preparations, the sulphite was further reduced to sulphide. Wainwright (1962) has reported occurrence in yeast of a sulphite reductase system. He was able to fractionate the system into six protein components. The overall reaction appears to be the reduction of sulphite to H_2S by reduced pryidine coenzymes, but the sequence of reactions is not clear. Schlossmann & Lynen (1957) have purified from yeast an enzyme catalysing the following reaction:

$$\text{serine} + H_2S \longrightarrow \text{cysteine} + H_2O.$$

The enzyme requires pyridoxal phosphate, and the equilibrium of the reaction is such that cysteine is readily formed.

Because yeast preparations reduce sulphite to H_2S, and convert H_2S and serine into cysteine, it is generally assumed that these reactions constitute the main pathway of sulphate assimilation. However, Nakamura (1962) believes that in moulds, sulphite

is reduced to thiosulphate. This thiosulphate is then presumably esterified with serine to produce cysteine-S-sulphate, which is then hydrolysed to cysteine. Nakamura & Sato (1963) found that a mutant of *Aspergilus nidulans*, which required cystine or methionine as a sulphur source, accumulated cysteine-S-sulphate in mycelium incubated with inorganic sulphate and serine. They suggest that the mutant is unable to convert cysteine-S-sulphate to cysteine.

Leinweber & Monty (1963) have investigated thiosulphate utilization in *Salmonella typhimurium*. They present evidence that thiosulphate is not an intermediate in the utilization of sulphate by this organism.

It is difficult to rule out thiosulphate as an intermediate, but it is probable that the normal pathway of sulphate reduction does not involve thiosulphate. As a sole source of sulphur, moulds can use sulphate, sulphite, thiosulphate, sulphide, choline sulphate, cystine, methionine, and many other sulphur compounds. They therefore possess enzyme systems capable of interconversion of a large number of sulphur compounds. Hence, if one demonstrates that a given reaction can occur, one cannot conclude that this reaction is part of the normal sulphate utilization mechanism.

The short-time labelling experiments carried out in our laboratory (Segel & Johnson, 1963*b*) were undertaken in the hope of obtaining information about the actual in vivo events of sulphate utilization. Fig. 1 shows the results of an experi-

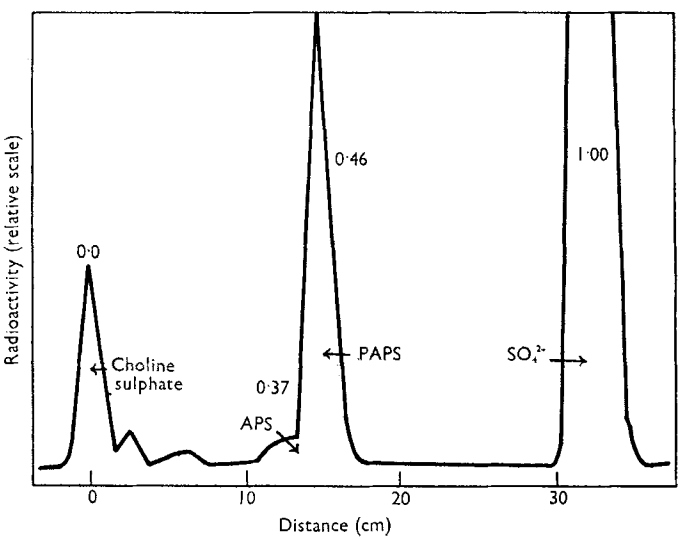

Fig. 1. Electrophoretic distribution of ^{35}S-labelled compounds in the hot-water extract of *Penicillium chrysogenum* mycelium. The mycelium was incubated with $^{35}SO_4^{2-}$ for 33 sec, then inactivated with boiling water. APS, adenosine 5′-phosphosulphate; PAPS, 3′-phosphoadenosine-5′-phosphosulphate.

ment in which growing mycelium was allowed to assimilate radioactive inorganic sulphate for 33 sec before being extracted with boiling water. The figure shows the radioactivity distribution obtained by paper electrophoresis. The mobility of inorganic sulphate was given an arbitrary value of 1·0. The numbers on the figure are mobilities of other compounds relative to that of inorganic sulphate.

The radioactive compound present in largest amount was PAPS. Only a small amount of labelled APS was present. This is to be expected from the unfavourable equilibrium constant for APS formation. The only other identified compound present is choline-O-sulphate. This compound will be discussed later. At the moment we will only note that it is formed very rapidly from inorganic sulphate. We carried out incubations with radioactive sulphate for intervals varying from 15 to 600 sec. The results of a 308 sec incubation are shown in Fig. 2.

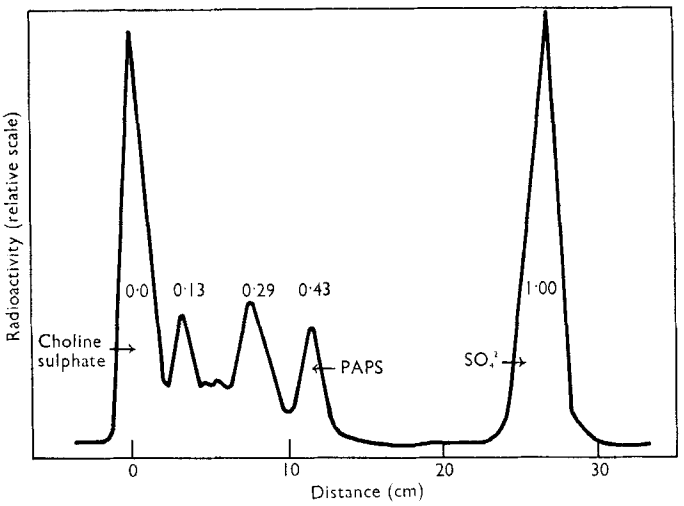

Fig. 2. Electrophoretic distribution of ^{35}S-labelled compounds in the hot-water extract of mycelium. Conditions as in Fig. 1, except that the incubation time was 308 sec. PAPS, 3′-phosphoadenosine-5′-phosphosulphate.

The relative amount of PAPS has decreased, and the relative amount of choline sulphate has increased. The compound with a mobility of 0·29 has been identified as glutathione, but the identity of the compound of mobility 0·13 is unknown. Inorganic sulphite and sulphide were also measured in experiments in which carrier sulphite or sulphide was added at the time of inactivation of the mycelium. Fig. 3 shows the relative amounts of radioactivity in various compounds as a function of time. It should be stressed that the total radioactivity increases steadily with time, but what is plotted in Fig. 3 is the distribution of label among the various compounds. It will be noted that the percentage of radioactivity in PAPS and in sulphite decreases with time, whereas the percentage of radioactivity in choline sulphate increases. The curve for sulphide also apparently shows a decrease, but the total amount is so low that the results may be equivocal. It will also be noted that, during the first 90 sec, label appeared in organic compounds other than PAPS and choline sulphate.

The labelling pattern is consistent with the pathway of sulphate reduction we have described, but it is also consistent with many other possible pathways. The striking features are the early appearance of PAPS and the steady accumulation of choline-O-sulphate. When our mould strain grows normally, about 10% of the soluble sulphur in the mycelium is choline sulphate. Choline sulphate was first isolated from moulds by Woolley & Peterson (1937–8). It has since been found widely distributed in fungi

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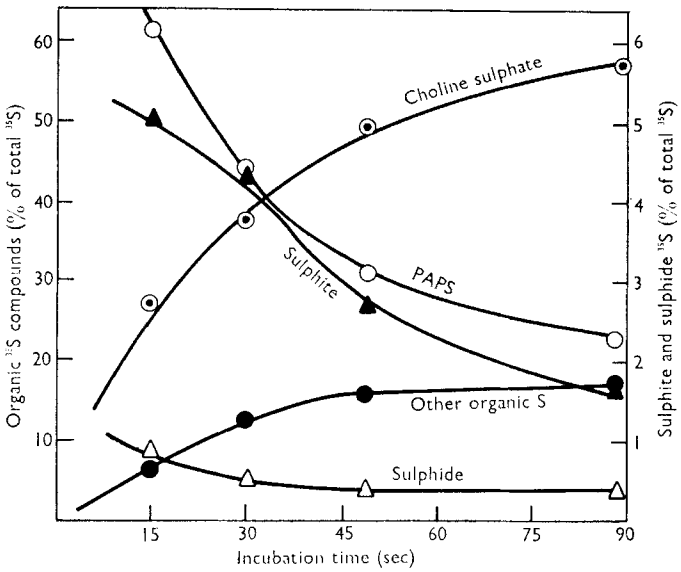

Fig. 3. Changes in the relative amounts of ^{35}S incorporated into various compounds as a function of incubation time. PAPS, 3′-phosphoadenosine-5′-phosphosulphate.

and green plants. Its role is apparently sulphur storage. Moulds that accumulate choline sulphate are able to use it as a sole sulphur source (Nakamura, 1962; Spencer & Harada, 1960).

The choline sulphokinase of moulds has been studied by Kaji & Gregory (1959) and by Orsi & Spencer (1962). This enzyme catalyses the transfer of sulphate from PAPS to choline. In this reaction, the equilibrium is overwhelmingly in favour of choline sulphate formation. Orsi & Spencer were unable to demonstrate any reverse reaction whatever. It is striking that all attempts to demonstrate an active choline sulphate-hydrolysing enzyme in the moulds that use choline sulphate have, with one exception (Segel & Johnson, 1963*c*), failed.

Choline sulphate cannot be utilized by transfer of its sulphate to PAPS, and is apparently not hydrolysed, yet is readily used as a sulphate source. We have attempted to determine the equilibrium constant for choline sulphate hydrolysis. Lacking an enzyme of sufficient activity, Richard Read, in our laboratory, incubated carrier-free ^{35}S(sulphate) with the chloride salt of choline and 0·1 N-HCl at 96°. He also incubated carrier-free ^{35}S(choline-O-sulphate) (Segel & Johnson, 1963*c*) with choline chloride and HCl. Essentially the same equilibrium point was reached from both directions. The results give for the hydrolysis reaction, calculated for pH 7, a free energy of hydrolysis of −10·4 kcal/mole. This figure is not accurate, because the effect of temperature on the equilibrium is not taken into account. However, it is far lower than the 18–19 kcal estimated by Robbins & Lipmann (1958) for PAPS hydrolysis. This is consistent with the observed irreversibility of the choline sulphokinase reaction. The hydrolysis energy of choline sulphate appears sufficiently high, however, to make possible transfer of sulphate from choline to the sulphydryl compound

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whose decomposition yields sulphite. One might therefore suggest that choline sulphate need not be hydrolysed to make its sulphur available for reduction. If this is found to be true, the hypothesis that choline sulphate is a sulphur-storage compound will be strengthened.

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