# ON THE PERMEABILITY OF *THIOBACILLUS NEAPOLITANUS* TO THIOSULPHATE

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[Manuscript received January 14, 1965]

#### Summary

Freshly prepared cells of T. neapolitanus had an effective thiosulphateimpermeable volume of  $2 \cdot 21$  ml/g dry weight; on treatment of the bacteria with n-butanol this value fell to 0.86 ml/g dry weight. T. neapolitanus catalysed an exchange of sulphur between sulphite and the inner-sulphur group of thiosulphate although the bacteria appeared to be impermeable to sulphite. The exchange reaction was inhibited by N-ethylmaleimide and iodoacetamide. No accumulation of the substrate or products within the bacterial cell occurred during the oxidation of thiosulphate. It is concluded that thiosulphate sulphur enters the bacterial cell by a process other than simple diffusion and that the exchange between sulphite and thiosulphate takes place at the bacterial cell membrane. A possible role of sulphenyl derivatives in the uptake of thiosulphate is discussed.

## I. INTRODUCTION

The thiobacilli oxidize thiosulphate to sulphate and Ostrowski and Krawczyk (1957) proposed that the initial reaction in this oxidation takes place at the bacterial cell membrane. Lees (1960) suggested that the initial reaction was between thio-sulphate and a disulphide group on the membrane. These hypotheses are largely unsupported by experimental evidence although recent studies with thiol-binding reagents have indicated that thiol groups are necessary for thiosulphate oxidation and that some of these groups are located at the membrane (Trudinger 1965).

This paper describes results indicating the existence in *Thiobacillus neapolitanus* of a permeability barrier to thiosulphate, and further evidence that the membrane is the site of some reactions involving thiosulphate.

# II. MATERIALS

## (a) Organism

The organism used in this study was the chemosynthetic sulphur autotroph T. neapolitanus<sup>†</sup> (Parker and Prisk 1953). The bacteria were grown in 200 ml of medium in  $1 \cdot 2$ -1. penicillin flasks as described previously (Trudinger 1961*a*). The bacteria were washed three times with  $0 \cdot 1$  potassium phosphate, pH 7, before use and were aerated in buffer for 30 min at 30°C between the first and second washes to exhaust endogenous substrates.

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#### (b) Labelled Thiosulphates

Sodium thiosulphate  $(Na_2S_2O_3)$  labelled with <sup>35</sup>S in either the inner (-SO<sub>3</sub>) or outer (-S-) positions was obtained from the Radiochemical Centre, Amersham, England.

# III. METHODS AND RESULTS

#### (a) Effective Cell Volumes

The effective thiosulphate-impermeable volumes of centrifuged *T. neapolitanus* were measured by the method of Mitchell (1953). Washed bacteria (approx. 100 mg dry wt.), suspended in  $1 \cdot 0$  ml of  $0 \cdot 1$ M potassium phosphate, pH 7, were mixed under nitrogen at 1°C with  $0 \cdot 2$  ml of  $0 \cdot 1$ M [ $^{35}$ S]Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>. No detectable metabolism of thiosulphate took place under these conditions. After 2 min the mixture was centrifuged at 0°C for 30 min at 13,000 r.p.m. (20,000 g) in the SS34 rotor of the refrigerated Servall centrifuge. The concentration of  $^{35}$ S in the supernatant (*A*) was determined by plating samples on stainless steel planchets and counting with a gas flow counter. The concentration of  $^{35}$ S in a mixture of 1 ml buffer with  $0 \cdot 2$  ml [ $^{35}$ S]Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (*B*) was also measured, and the effective cell volume (*V*) calculated from the equation  $B/A = (1 \cdot 2 - V)/1 \cdot 2$ .

The volume of fresh *T. neapolitanus* not penetrable by thiosulphate was  $2 \cdot 21 \pm 0.09 \text{ ml/g}$  dry weight of organisms (six determinations). After treatment of the bacteria with 5% (v/v) n-butanol, to destroy permeability barriers (Mitchell 1953), this value fell to  $0.86 \pm 0.08 \text{ ml/g}$  dry weight of organisms (five determinations). Thus the bacteria possess a permeability barrier to thiosulphate enclosing a volume of about 1.35 (2.21-0.86) ml/g. Similar results were obtained with both inner- and outer-labelled thiosulphate and when thiosulphate was determined by iodine titration. Since a significant amount of oxidation of thiosulphate occurred at  $10-30^{\circ}$ C it was not possible to determine the effective cell volumes at these higher temperatures.

## (b) Exchange of <sup>35</sup>S between Thiosulphate and Sulphite

Sulphite is oxidized to sulphate by extracts of a number of thiobacilli (Peck 1962), including *T. neapolitanus* (Hempfling 1964). Sulphite is, however, not oxidized by intact *T. neapolitanus* grown under the conditions used in this study (Trudinger, unpublished results). It may be concluded, therefore, that the intact bacteria are impermeable to sulphite. On the other hand sulphite inhibits the oxidation of thiosulphate by intact *T. novellus* (De Ley and van Poucke 1961) and by *T. neapolitanus* (Trudinger 1964d). *T. neapolitanus* also catalyses an exchange of sulphur between sulphite and the inner sulphur of thiosulphate as is shown by the following experiment. Washed cells of *T. neapolitanus* were incubated for 10 min at 30°C under oxygen-free N<sub>2</sub> with 30 µmoles of Na<sub>2</sub>SO<sub>3</sub> and 32 µmoles of inner-labelled [<sup>35</sup>S]Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (approx. 10<sup>6</sup> counts/min) in 2 ml of 0.12M potassium phosphate, pH 7. At the end of the incubation sulphite and thiosulphate were oxidized to sulphate and tetrathionate respectively by the addition of 1.5 ml of 0.1N iodine. There was no exchange of <sup>35</sup>S between sulphate and tetrathionate (cf. Elkeles 1953, 1954). Sulphate was separated from the mixtures by chromatography on Dowex 2X8

(Trudinger 1964b) and the amount of  $^{35}$ S determined. Control experiments, in which the sulphite and thiosulphate remaining at the end of the incubation period were separately determined by iodine titration, showed that the concentrations of the reactants changed by less than 3%.

The results (Table 1) show that the bacteria catalysed an incorporation of  $^{35}S$  from the inner-position of thiosulphate into sulphite. This reaction was inhibited by the thiol-binding reagents N-ethyl maleimide and iodoacetamide (Table 1, expt. 2);

#### TABLE 1

EXCHANGE OF <sup>35</sup>S BETWEEN INNER-LABELLED [<sup>35</sup>S]THIOSULPHATE AND SULPHITE The experimental procedure is described in the text. The dry weights of *T. neapolitanus* were 1.4 mg and 0.17 mg for experiments 1 and 2 respectively. In experiment 2 the inhibitors (2  $\mu$ moles) were incubated with the bacteria for 10 min before the addition of sulphite and thiosulphate. The results are expressed as the fraction (%) of the total <sup>35</sup>S present in sulphate: the theoretical value for complete exchange is 46.9%

| Expt.<br>No. | Reaction Mixture  | <sup>35</sup> S in<br>Sulphate<br>(%)                                |
|--------------|---|--|
| 1            | Na <sub>2</sub> SO <sub>3</sub> , Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub><br>Na <sub>2</sub> SO <sub>3</sub> , Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> , bacteria<br>Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> , bacteria<br>Na <sub>2</sub> SO <sub>3</sub> , Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> , heated bacteria*   | $0 \cdot 05 \\ 37 \cdot 5 \\ 0 \cdot 23 \\ 0 \cdot 14$               |
| 2            | Na <sub>2</sub> SO <sub>3</sub> , Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> , bacteria<br>Na <sub>2</sub> SO <sub>3</sub> , Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> , bacteria, <i>p</i> -chloromercuribenzoate<br>Na <sub>2</sub> SO <sub>3</sub> , Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> , bacteria, <i>N</i> -ethyl maleimide<br>Na <sub>2</sub> SO <sub>3</sub> , Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> , bacteria, iodoacetamide | $12 \cdot 3$<br>$12 \cdot 1$<br>$5 \cdot 4$<br>$7 \cdot 2^{\dagger}$ |

\* Bacteria heated for 10 min at 60 °C.

 $\dagger$  This value has been corrected for the incorporation of  $^{35}S$  into sulphate which occurred in the absence of bacteria. This incorporation was presumably due to the exchange between sulphite and the sulphenyl sulphite (Fava and Pajaro 1956) which is formed by a chemical reaction between thiosulphate and iodoacetamide (Trudinger 1965).

assuming that the reaction exhibits the normal kinetics of exchange (Mackay 1938) the inhibition by 1 mm N-ethyl maleimide and 1 mm iodoacetamide was 60% and 45% respectively. The lack of inhibition by the thiol-binding reagent *p*-chloromercuribenzoate may be due to the fact that this reagent reacts reversibly with thiol groups and with thiosulphate (Trudinger 1965).

#### (c) Oxidation of Outer-labelled [<sup>35</sup>S]Thiosulphate

To determine whether any concentration of the substrate or products occurred within the cell during the oxidation of thiosulphate, the following experiment was performed. Washed cells of *T. neapolitanus* (32 mg dry wt.) were shaken in air for 3 min at 30°C with 600  $\mu$ moles of potassium phosphate, pH 7, and 32  $\mu$ moles of outer-labelled [<sup>35</sup>S]Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (specific activity 159  $\times$  10<sup>3</sup> counts/min per  $\mu$ mole) in a

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volume of 6.5 ml. The mixture was cooled rapidly to 1°C and centrifuged at 0°C for 5 min at 27,000 g. The pellet was drained as much as possible, extracted with 2 ml of cold 10% (v/v) acetic acid in 50% ethanol for 5 min, and then made to 10 ml with water. The extracted bacteria were removed by centrifugation, washed twice with buffer, and digested with a mixture of HC1, HNO<sub>3</sub>, and Br<sub>2</sub> (Trudinger 1964a) for the determination of insoluble sulphur. The medium and the bacterial extract were analysed by chromatography on Dowex 1X2 (Trudinger 1964b). The results are shown in Table 2, and the following points may be noted:

## TABLE 2

| DISTRIBUTION   | OF | SULPE | UR | BETWEEN | BACTER | IA. | AND | MEDIUM | DURING | THE |
|--|----|-------|----|---------|--------|-----|-----|--------|--------|-----|
| OXIDATION OF OUTER-LABELLED [ <sup>35</sup> S]THIOSULPHATE |    |       |    |         |        |     |     |        |        |     |

|  | $^{35}{ m S~in}$ Medium $(10^{-3}~	imes~{ m counts/min})$ | <sup>35</sup> S in<br>Bacterial Extract<br>(10 <sup>-3</sup> × counts/min) |
|--|---|--|
| Sulphide + sulphite                        | 1.5   | 0  |
| Sulphate                                   | 765   | 26   |
| Thiosulphate                               | 3200  | 66   |
| (Specific activity of <i>inner</i> S)      | (24.7)  | $(24 \cdot 0)$   |
| Polythionates                              | 898   | 18   |
| Insoluble S                                | 10  | $\cdot 2$  |
| Total <sup>35</sup> S in soluble fractions | 4865  | 110  |

| Outer-labelled [35S]Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> (specific activity 159 | 9 	imes 1 | 0 <sup>3</sup> coun | ts/min per | µmole) |
|--|-----------|---------------------|------------|--------|
| was incubated with $T$ . <i>neapolitanus</i> as  | descr     | ibed in             | the text   | • •    |

- (1) There were no major differences between the medium and bacterial extract in the distribution of  $^{35}$ S.
- (2) The total <sup>35</sup>S in the bacterial extract, which also included material washed from the wall of the centrifuge tube, was about 2% of total <sup>35</sup>S used in the experiment. Since the volume of the bacterial pellet (in the order of 0.1 ml) was about 1.5% of the total volume it is clear that there was no marked accumulation of <sup>35</sup>S within the bacterial cell.
- (3) The outer sulphur of thiosulphate was oxidized and some <sup>35</sup>S became incorporated into the inner position of thiosulphate (cf. Trudinger 1964d). The specific activities of the inner sulphur groups of thiosulphate in the bacterial extract and in the medium were almost equal; thus the product of the oxidation of the outer sulphur of thiosulphate from which the <sup>35</sup>S in the inner sulphur group arose exchanged freely with the substrate in the external medium.

#### IV. DISCUSSION

During the metabolism of thiosulphate by T. neapolitanus the substrate and the products of its metabolism rapidly equilibrate between the sites of metabolism and the external medium (Table 2). On the other hand the effect of n-butanol on

the effective thiosulphate-impermeable volume of T. *neapolitanus* shows the existence at 1°C of a permeability barrier to thiosulphate, which, by analogy with other bacterial systems, is probably the bacterial cell membrane. Assuming that the permeability barrier also exists at physiological temperatures, the transfer of thiosulphate sulphur across the membrane must take place by a mechanism other than simple diffusion.

Lees (1960) suggested that this sulphate reacts with a disulphide group on the membrane to form a sulphenyl this sulphate [equation (1)]:

$$R-S-S-R+S_2O_3^{2-} \longrightarrow R-S-S-SO_3^{-}+RS^{-}, \qquad (1)$$

and the effects of thiol-binding reagents on the oxidation of thiosulphate to tetrathionate by intact cells and extracts of T. *neapolitanus* are consistent with this reaction being involved in the uptake of thiosulphate into the bacterial cell (Trudinger 1965). Since T. *neapolitanus* appears to be impermeable to sulphite, the inhibition of thiosulphate oxidation and the exchange between sulphite and the inner sulphur group of thiosulphate possibly occur at the membrane. Sulphite reacts readily with disulphide groups to form sulphenyl sulphites [equation (2); Milligan and Swan 1962] and is a more powerful S-nucleophile than thiosulphate (Parker and Kharasch 1959):

$$R-S-S-R+SO_3^{2-} \longrightarrow R-S-SO_3^{-}+RS^{-}.$$
 (2)

Sulphite would, therefore, be expected to displace thiosulphate from a sulphenyl thiosulphate; this may account for the inhibition of thiosulphate oxidation by sulphite. The bacteria-catalysed exchange between sulphite and thiosulphate is also sensitive to thiol-binding reagents (Table 1). It is possible, therefore, that the uptake of thiosulphate, the inhibition of thiosulphate oxidation by sulphite, and the exchange reaction are all consequences of reactions of thiosulphate and sulphite with disulphide groups at the bacterial cell membrane.

Whether or not the thiosulphate molecule undergoes further transformation at the membrane is not certain. Ostrowski and Krawczyk (1957) suggested that the sulphur-sulphur bond of thiosulphate is cleaved and that only the outer sulphur is taken into the bacterial cell. Peck (1960, 1962) and others have demonstrated that extracts of thiobacilli in the presence of excess glutathione catalyse the reductive scission of thiosulphate to sulphide and sulphite. The oxidation of thiosulphate to tetrathionate by intact thiobacilli, however, is apparently catalysed by a soluble enzyme located within the bacterial cell (Trudinger 1961b). Moreover the oxidation of this sulphate to sulphate by T. neapolitanus appears to involve the formation of intermediates containing sulphur from both groups of thiosulphate (Trudinger 1964c), and there is evidence that some of the thiol groups necessary for this oxidation are situated internally (Trudinger 1965). These results imply that thiosulphate sulphur is taken into the bacterial cell with the sulphur-sulphur bond intact. This conclusion is supported by recent results of London and Rittenberg (1964) who have prepared bacterial extracts which oxidize thiosulphate in essentially the same manner as intact bacteria.

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