[^{35}S]Thiosulphate Oxidation by Rat Liver Mitochondria in the Presence of Glutathione

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1. Rat liver mitochondria incubated in oxygen with glutathione and [^{35}S]-thiosulphate produced labelled sulphate. 2. Inner-labelled thiosulphate $(S \cdot {}^{35}SO_3)^{2-}$ was converted into [^{35}S]sulphate more rapidly than outer-labelled thiosulphate $({}^{35}S \cdot SO_3)^{2-}$. 3. Thiosulphate labelled in both sulphur atoms was formed during $({}^{35}S \cdot SO_3)^{2-}$ oxidation; the outer sulphur atom before oxidation to sulphate was incorporated into the inner position. 4. A thiosulphate cycle in the metabolic pathway of sulphate formation in animal tissues is discussed.

Thiosulphate may be oxidized to sulphate in whole animals (Nyiri, 1923; Skarzynski, Szczepkowski & Weber, 1959) or in slices from rat liver (Pirie, 1934). The mechanism of this oxidation was not elucidated until observations by Sörbo (1964) showed that addition of some thiols greatly increased sulphate production from thiosulphate incubated with rat liver homogenate in an oxygen atmosphere. This author postulated the reductive breakdown of thiosulphate in the presence of dihydrolipoate or glutathione and the enzyme (rhodanese or thiosulphate reductase) and further oxidation of sulphite to sulphate. We were able to confirm this fully during comparative studies of thiosulphate oxidation in mitochondria isolated from livers of different animal species (Koj & Frendo, 1967). The purpose of the investigations reported here was to compare the velocity of the oxidation of the two sulphur atoms of thiosulphate in rat liver mitochondria enriched with glutathione.

MATERIALS AND METHODS

General procedure. Mitochondria were isolated from rat liver by centrifuging in iso-osmotic sucrose solution according to Weinbach (1961), then suspended in 0·05M-diammonium hydrogen orthophosphate and immediately used in the experiment. The protein content of the preparation was measured by the method of Lowry, Rosebrough, Farr & Randall (1951). Incubation was carried out in Warburg vessels in an atmosphere of O_2 with constant gentle shaking. A 2ml. sample contained the mitochondrial preparation, 3–15μmoles of $Na_2S_2O_3$ (FOCH, Gliwice), and an equimolar amount of reduced glutathione (British Drug Houses Ltd., Poole, Dorset) at the final pH7·9. [^{35}S]Thiosulphate labelled in the inner ($-SO_3$) or outer (-S) atom (The Radiochemical Centre, Amersham, Bucks., sp. activity 12·8–26·6mc/m-mole) was added to unlabelled thiosulphate solution; the final concentration was 0·1–0·3μc of thiosulphate in a 2ml. sample. Control samples contained all the components but the mitochondria were inactivated by 3min. heating on a boiling-water bath. After 20–60min. of incubation the contents of the Warburg vessels were transferred into centrifuge tubes and the proteins were precipitated by adding 8ml. of ethanol. The supernatant obtained after centrifuging was used for the estimation of thiosulphate and sulphate.

Thiosulphate estimation and analysis. The amount of thiosulphate remaining in the samples after incubation was measured by the colorimetric method proposed by Sörbo (1957*a*) as described by Koj & Frendo (1967).

The alkaline cyanolysis of thiosulphate in the presence of copper ions was also employed for measurements of the distribution of radioactivity within both sulphur atoms of thiosulphate. The sample of ethanol supernatant was treated with NH_3, KCN and $CuCl_2$ as for thiosulphate estimation but, instead of $Fe(NO_3)_3$ reagent, 1ml. of 2N-HNO_3 and 20μmoles of carrier sulphate were added after incubation. As was demonstrated by Sörbo (1957*a*), the outer atom of thiosulphate is converted in this reaction quantitatively into rhodanide while the inner atom appears in the form of sulphate. The sulphate was precipitated by an excess of $Ba(NO_3)_2$, the $BaSO_4$ crystals obtained were centrifuged, washed with 0·2N-HCl, water and ethanol and transferred to a planchet. The supernatant left after sulphate precipitation contained the rhodanide deriving from the outer sulphur atom. Its activity was estimated after drying 0·2ml. of the solution on the planchet and then multiplying the net counts by the total volume of the supernatant.

All the measurements of radioactivity were carried out in a windowless gas-flow counter (Frieseke-Hoepfner) and corrected for background radiation. Solutions of ^{35}S compounds were dried on 2·5cm.-diam. aluminium planchets, usually to give infinitely thin samples. When thick samples were used, corrections for self-absorption were applied by reference to infinitely thin samples. Time of counting was selected to reduce standard error to $\pm 3\%$.

When analysis of labelled thiosulphate was carried out in samples containing also [^{35}S]sulphate the activity of this had to be taken into account in evaluating specific

activity of the inner sulphur atom. The standard procedure in our experiments was based on the use of 2ml. of ethanol supernatant to determine the activity contained both in sulphate and the inner atom of thiosulphate and then subtracting the activity of the sulphate present in 2ml. of supernatant after its separation on a Dowex column.

Sulphate estimation. The formation of labelled sulphate in the incubated samples was measured after separation of the sulphate on a Dowex 2 column in the acetate form according to Trudinger (1961). Sulphate was eluted from the column with 2M-ammonium acetate, pH5, then precipitated with $BaCl_2$ and its activity measured as described above. The labelled sulphate production (in μmoles) was calculated by dividing the total sulphate activity in the sample by thiosulphate specific activity expressed in counts/min./μmole (assuming the label of thiosulphate to be limited to the inner or outer position and regarding the labelled atom of thiosulphate as the sole precursor of sulphate).

Sulphate formation in the samples incubated was also measured by means of the chemical method described by Spencer (1960) by using barium chloranilate prepared from chloranilic acid (British Drug Houses Ltd). The details of the procedure employed are given by Koj & Frendo (1967).

RESULTS

Rat liver mitochondria incubated during 1hr. with glutathione and [^{35}S]thiosulphate produce an appreciable amount of labelled sulphate (Table 1): 1μmole of utilized thiosulphate yields 1·7μmoles of sulphate—that is slightly below the expected ratio assuming total oxidation of the two sulphur atoms. Isotopic measurements, however, do not coincide with chemical estimations and indicate that the inner atom of thiosulphate is converted into sulphate much more easily than the outer one. Moreover, the apparent discrepancy exists between the amount of labelled sulphate produced from $(S \cdot {}^{35}SO_3)^{2-}$ and the amount of thiosulphate utilized (one inner labelled sulphur atom in thiosulphate can give rise to only one molecule of sulphate). Such results, however, were obtained repeatedly if a high concentration of thiosulphate was used. On the contrary, when experimental conditions are suitable for the almost total oxidation of thiosulphate, namely by using minimal amounts of thiosulphate and excess of glutathione, the difference between the outer and the inner sulphur atoms regarded as precursors of sulphate becomes negligible (Table 2).

Explanation of this discrepancy is possible by assuming initial reductive breakdown of thiosulphate in the presence of glutathione followed by oxidation of sulphite to sulphate, while sulphide is oxidized to another molecule of thiosulphate according to equations (1), (2) and (3).

$$(S \cdot SO_3)^{2-} + 2\,GSH \rightarrow HS^- + HSO_3^- + GSSG \quad (1)$$

$$SO_3^{2-} + 1/2\,O_2 \rightarrow SO_4^{2-} \quad (2)$$

$$2\,HS^- + 2\,O_2 \rightarrow (S \cdot SO_3)^{2-} + H_2O \quad (3)$$

Therefore in a case when only a small fraction of thiosulphate present in the sample is utilized, sulphate derives mainly from the inner atom and labelled sulphate production exceeds the apparent thiosulphate utilization (Table 1, sample 2) because of the regeneration of $S \cdot SO_3^{2-}$ from the outer atom.

In our previous set of experiments (Koj & Frendo, 1967) we were able to detect the labelled sulphide deriving from $(^{35}S \cdot SO_3)^{2-}$ when mitochondria were incubated in anaerobic conditions with thiosulphate and glutathione. During incubation in an oxygen atmosphere, however, no sulphide could be demonstrated; this fact may be explained by the immediate oxidation of sulphide non-enzymically or by sulphide oxidase found in rat liver mitochondria (Sörbo, 1956; 1958; 1960; Baxter, van Reen, Pearson & Rosenberg, 1958; Baxter & van Reen, 1958*a*; Baxter & van Reen, 1958*b*). If this is the case, oxidation of $(^{35}S \cdot SO_3)^{2-}$ must lead to formation of thiosulphate labelled in both sulphur atoms. This was demonstrated in an experiment when outer labelled thiosulphate was incubated for different periods with mitochondria enriched with glutathione and then analysis of ^{35}S distribution within the thiosulphate molecule was carried out (Table 3).

It may be seen that thiosulphate decomposition

Table 1. *Oxidation of labelled thiosulphate to sulphate by rat liver mitochondria*

Samples (2ml.) containing rat liver mitochondria (13mg. of protein), 15μmoles of GSH and 15μmoles of thiosulphate labelled in the outer atom (sample 1) or the inner atom (sample 2), were incubated for 60min. in an oxygen atmosphere, and then the reaction was stopped by addition of 8ml. of ethanol. Thiosulphate utilized was measured by the colorimetric procedure; sulphate formed was estimated by colorimetric and isotopic methods.

Sample	Thiosulphate utilized (μmoles)	Sulphate formed (μmoles)	
		Chemical method	Isotopic method
(1) 15μmoles of $(^{35}S \cdot SO_3)^{2-}$	2·0	3·4	0·2
(2) 15μmoles of $(S \cdot {}^{35}SO_3)^{2-}$	2·1	3·4	3·1

Table 2. *Oxidation of labelled thiosulphate to sulphate when small amounts of substrate are used*

Samples (2ml.) containing rat liver mitochondria (39mg. of protein) were incubated for 60min. in oxygen in the presence of 10μmoles of GSH and 3μmoles of thiosulphate labelled in the outer (sample 1) or the inner sulphur atom (sample 2).

Sample	Thiosulphate utilized (μmoles)	Sulphate formed (μmoles)	
		Chemical method	Isotopic method
(1) 3μmoles of $(^{35}S \cdot SO_3)^{2-}$	2·8	4·5	2·0
(2) 3μmoles of $(S \cdot {}^{35}SO_3)^{2-}$	2·8	4·4	2·5

Table 3. *Production of sulphate from thiosulphate labelled in the outer sulphur atom*

Samples (2ml.) containing rat liver mitochondria (19mg. of protein) were incubated in oxygen for 20, 40 and 60min. respectively, in the presence of 10μmoles of GSH and 10μmoles of $(^{35}S \cdot SO_3)^{2-}$ (final sp. activity 43000 counts/min./μmole). Labelled sulphate was isolated on a Dowex 2 column; radioactive thiosulphate was analysed for distribution of ^{35}S after its degradation in the presence of cyanide and copper ions.

Incubation time (min.)	Thiosulphate utilized (μmoles)	Sulphate formed (μmoles)		Sp. activity (counts/min./μmole)	
		Chemical method	Isotopic method	Sulphate	Inner atom thiosulphate
0	0	0	0·01	—	480
0–20	1·2	2·3	0·15	2340	5300
20–40	0·8	1·4	0·25	6420	9080
40–60	0·5	0·6	0·17	10900	12500

and sulphate production measured by chemical methods decrease slowly during the incubation and the specific activity of the sulphate formed increases continually, as does the activity appearing in the inner atom of thiosulphate.

It should be pointed out, however, that when thiosulphate labelled in the inner sulphur atom was incubated with rat liver mitochondria no appreciable increase of activity in the outer sulphur atom was found.

DISCUSSION

A preferential formation of sulphate from the inner atom of thiosulphate, shown in Table 1, has also been observed by some authors with autotrophic bacteria (Peck & Fisher, 1962; Trudinger, 1964; Kelly & Syrett, 1966) and in the whole animal (Skarzynski *et al.* 1959). The delayed appearance of the labelled outer atom in the form of sulphate obviously depends on the intermediate steps of oxidation of bivalent sulphur. The results in Table 3 clearly indicate that during oxidation of thiosulphate by rat liver mitochondria the outer sulphur atom is promptly incorporated into the inner position. This reaction seems to be rather a general rule during enzymic oxidation of thiosulphate since different authors (Trudinger, 1964; Kelly & Syrett, 1966) have also observed the appearance of thiosulphate labelled in both sulphur atoms when chemoautotrophic bacteria were incubated with $(^{35}S \cdot SO_3)^{2-}$.

Our experimental data suggest that thiosulphate labelled in the inner atom is the main intermediate accumulating during the oxidation of outer sulphur atom. The existence of other intermediates as sulphite, elementary sulphur, polysulphide or polythionate cannot be ruled out, however, because the amount of sulphate produced, measured by the chemical method, is always lower than the amount of thiosulphate decomposed, assuming that both sulphur atoms of thiosulphate are finally oxidized to sulphate. As an average value 1μmole of utilized thiosulphate yields in our experiments 1·5–1·7μmoles of sulphate. The fate of the residual sulphur is not quite clear, but our previous observations (Koj & Frendo, 1967) indicate that sulphite found in the sample incubated in oxygen comprises only a fraction of this sulphur. We could also demonstrate that organic persulphide, being a hypothetical intermediate in the reaction between a thiol compound and thiosulphate (Sörbo, 1962; Villarejo & Westley, 1963), does not accumulate during incubation in oxygen (Koj & Frendo, 1967). On the other hand it is possible that products of thiosulphate decomposition are bound to proteins (Szczepkowski, 1963).

From Table 3 it may be concluded that the inner

atom of thiosulphate behaves as a precursor of sulphate, taking into account the specific activities of thiosulphate and sulphate and the conditions corresponding to precursor–product relationships. This may be surprising at a first glance because apparently sulphite originating from thiosulphate should be a direct precursor of sulphate. We have demonstrated, however, that the free sulphite pool is relatively small (Koj & Frendo, 1967), and moreover it is known that sulphite may be in equilibrium with the inner atom of thiosulphate because of the exchange reaction catalysed by rhodanese (Sörbo, 1962). The postulated relationship between thiosulphate, sulphide, sulphite and sulphate is presented in Scheme 1.

At present it is impossible to decide whether the outer atom of thiosulphate is incorporated into the inner position only by direct oxidation to thiosulphate (reaction 4, Scheme 1) or whether an alternative way is possible: sulphide oxidation to sulphite (reaction 3) and then exchange reaction with the inner atom (reaction 2). In this case sulphite could be regarded as a precursor of both sulphate and the inner atom of thiosulphate.

The metabolic model presented in Scheme 1 emphasizes the hypothetical central position of thiosulphate during sulphate formation in animal tissues. It is well known that thiosulphate may be formed in the animal organism in many different reactions involving β-mercaptopyruvate (Sörbo, 1957*b*), organic persulphide (Szczepkowski, 1961; De Marco, Coletta & Cavallini, 1962), alanine thiosulphonate and thiotaurine (De Marco, Coletta, Mondovi & Cavallini, 1960) or in other ways (Sörbo, 1956; Sörbo, 1958; Baxter *et al.* 1958; Baxter & van Reen, 1958*a*; Baxter & van Reen, 1958*b*; De Marco & Coletta, 1961; De Marco, Borydo & Coletta, 1962). Decomposition of cysteine by cystathionase (reaction 5, Scheme 1) leads to formation of sulphide (Smythe, 1942; Koj & Frendo, 1962), and transamination of cysteinesulphinic acid is the main source of sulphite reaction 6, Scheme 1) (Singer & Kearney, 1956). Both these products can enter the thiosulphate cycle before complete oxidation to sulphate occurs. The physiological role of the proposed thiosulphate cycle in sulphur metabolism may depend on a slowing down of the irreversible oxidation of sulphide and sulphite to sulphate.

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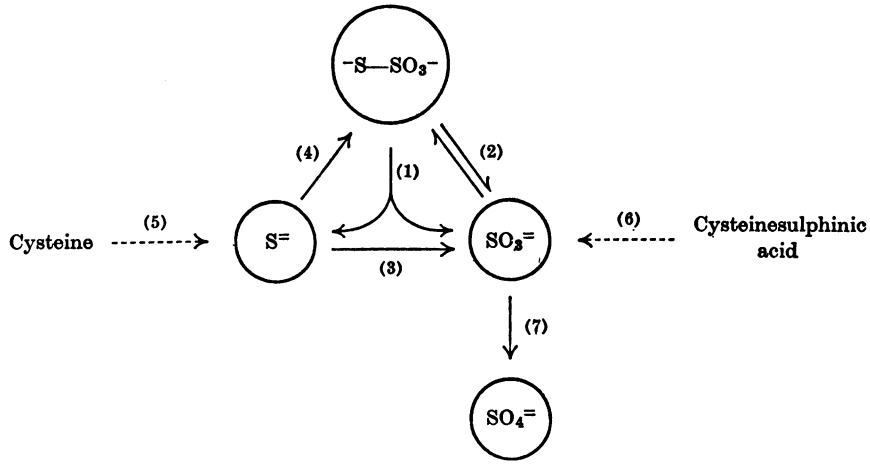

Scheme 1. Thiosulphate cycle during sulphate formation in animal tissues. (1) Thiol-dependent reductive breakdown of thiosulphate (rhodanese, thiosulphate reductase). (2) Sulphite exchange with inner atom of thiosulphate (rhodanese). (3) Sulphide oxidation to sulphite (non-enzymic, sulphide oxidase). (4) Sulphide oxidation to thiosulphate (haematin derivatives, sulphide oxidase, metalloproteins, non-enzymic). (5) Sulphide production from cysteine (cystathionase). (6) Sulphite production from cysteinesulphinic acid (transaminase, desulphinase). (7) Sulphite oxidation to sulphate (sulphite oxidase).

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