A POSSIBLE INTERMEDIATE IN THE REDUCTION OF 3'-PHOSPHORYL-5'-ADENOSINEPHOSPHOSULFATE TO SULFITE*

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The mechanism of the enzymatic formation of 3'-phosphoryl-5'-adenosinephosphosulfate (PAPS) and the subsequent reduction of this substance to sulfite have recently been reviewed (Gregory and Robbins, 1960; Wilson, 1962; Black, 1963). Evidence adduced in this laboratory using an enzyme system from yeasts (Wilson et al., 1961; Asahi et al, 1961) suggested a two step reductive sequence as shown in equations 1 and 2:

$$1.\quad H^+ + TPNH + C\text{-}SS \xrightleftharpoons{\text{Enzyme A}} TPN^+ + C(SH)_2$$

$$2.\quad C(SH)_2 + PAPS \xrightleftharpoons{\text{Enzyme B}} PAP + SO_3^{=} + C\text{-}SS$$

Enzyme A is a flavoprotein diaphorase; C-SS is a low-molecular weight protein, which upon reduction to $C(SH)_2$, is the reductant for PAPS; and PAP is 3'-phosphoryl-5'-adenosinephosphate.

In the present paper, we present evidence that the reaction product is not free inorganic sulfite, but is rather a bound form of sulfite, $X\text{-}SO_3^-$, where X is, most probably, a protein thiol group. Wortman (1963) has previously observed an unidentified product of PAPS in corneal extracts, but his substance differs in electrophoretic properties from those here described.

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That free S^{35}-labeled inorganic sulfite is not the product of the enzymatic reduction of $PAPS^{35}$ is shown by the data of Fig. 1. Carrier unlabeled sulfite was added after incubation of the enzyme reaction mixture, followed by acid to liberate sulfite as volatile SO_2. The specific activity of the SO_2 collected was not constant, as would be expected for a mixture of S^{35}-$SO_3^=$ and unlabeled carrier $SO_3^=$. Instead, a slow increase in specific activity occurs, this increase being a measure of the time required for exchange equilibration:

$$3.\quad X\text{-}S^{35}O_3^- + SO_3^= \rightleftharpoons X\text{-}SO_3^- + S^{35}O_3^=$$

The later decline in specific activity is probably due to loss of unlabeled $SO_3^=$ from the acidified reaction mixture to a concentration below that permitting exchange equilibration.

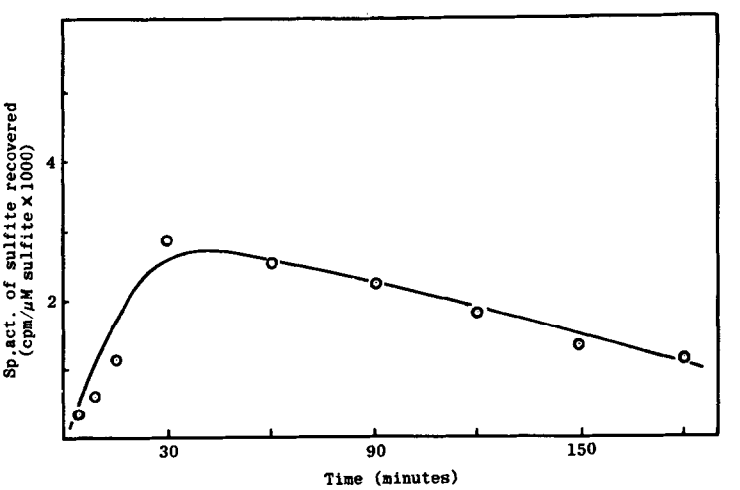

Figure 1. Time course of exchange between X-$S^{35}O_3^-$ and carrier sulfite. The incubation mixture contained in μmoles: Tris-HCl, pH 7.5, 25; $MgCl_2$, 2.5; glucose-6-P, 2.5; $PAPS^{35}$, 0.05; TPN, 0.3; EDTA, 0.5; NaF, 50; Enzyme A, 0.2 mg; Enzyme B, 0.2 mg; in a volume of 0.75 ml. Incubation was for 1 hour at 37° under hydrogen in a Warburg vessel. The reaction was terminated by the addition of 2.5 μmoles of Na_2SO_3 followed by H_3PO_4 and after shaking for the time periods indicated, volatile radioactive substances which had distilled into NaOH in the center well were assayed for radioactivity and sulfite. Enzymes and assay procedures were as previously described (Wilson, et al, 1961).

Paper electrophoretic analysis of the products of the enzymatic reduction of $PAPS^{35}$ provided further proof of the existence of a bound form of sulfite.

As demonstrated by the data of Fig. 2 (b,c), an S^{35}-labeled, non-dialyzable substance, with low electrophoretic mobility accumulates in reaction mixtures not containing carrier inorganic sulfite. This substance, $X\text{-}SO_3^-$, upon elution from the origin of the electrophoretogram, yields S^{35}-labeled SO_2 in the presence of carrier sulfite and acid. When carrier unlabeled sulfite is present during incubation as in Fig. 2a, the $X\text{-}SO_3^-$ does not accumulate and only residual $PAPS^{35}$ and S^{35}-labeled sulfite and sulfate, which migrate conincidently, remain. Dialysis of reaction mixtures containing carrier sulfite results in almost complete loss of radioactivity from the dialysis tube. Thus, when carrier sulfite is present during incubation all of the reduced sulfur occurs as inorganic sulfite, but when carrier sulfite is absent during incubation, reduced sulfur accumulates as $X\text{-}SO_3^-$.

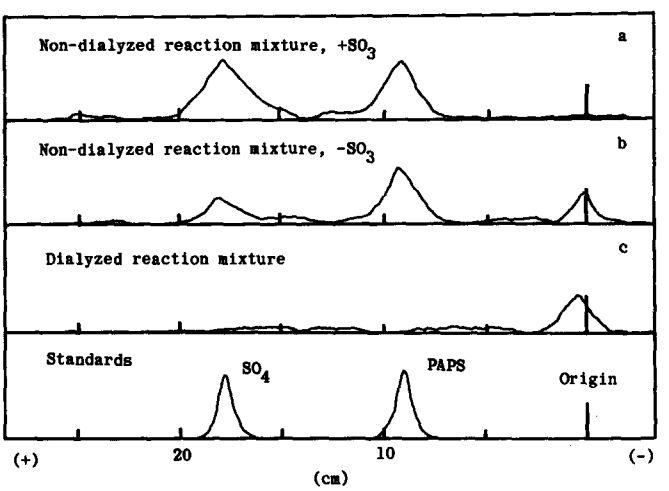

Figure 2. Electrophoretograms of radioactive products of $PAPS^{35}$ reduction. Electrophoresis was carried out in 0.1 M acetate buffer, pH 4.5 on HCl-EDTA washed, Whatman 3 MM, previously moistened with buffer. The current applied was 10 V/cm for 4 hours at 1°. Radioactivity was located with a chromatogram scanner. Incubation mixture as for Fig. 1. a) incubation mixture contained 2.5 μM of Na_2SO_3 during incubation. b) incubation mixture contained no carrier sulfite. c) incubation mixture of b) dialyzed against Tris-EDTA buffer pH 7.0 for 90 minutes after incubation.

Further investigation of this non-dialyzable fraction indicated that 40 to 50% of the radioactivity could be volatilized by the addition of carrier sulfite and acid. Storage for three days at room temperature resulted in the conversion of this substance to a form which is not volatile in the presence of carrier sulfite and acid; storage of the non-dialyzable fraction in the presence of carrier unlabeled sulfite preserves the acid-volatile radioactivity by converting it to inorganic sulfite.

Fig. 3 shows the rate of accumulation of total and non-dialyzable S^{35}-labeled acid-volatile sulfite. The reaction mixture used for the measurement of total S^{35}-sulfite contained carrier unlabeled sulfite. In the assay for non-dialyzable S^{35}, carrier sulfite was added following dialysis and immediately before the addition of acid. Less volatile radioactivity is recovered when carrier sulfite is added following dialysis, probably owing to the number of possible side reactions for mμmolar amounts of $X\text{-}SO_3^-$.

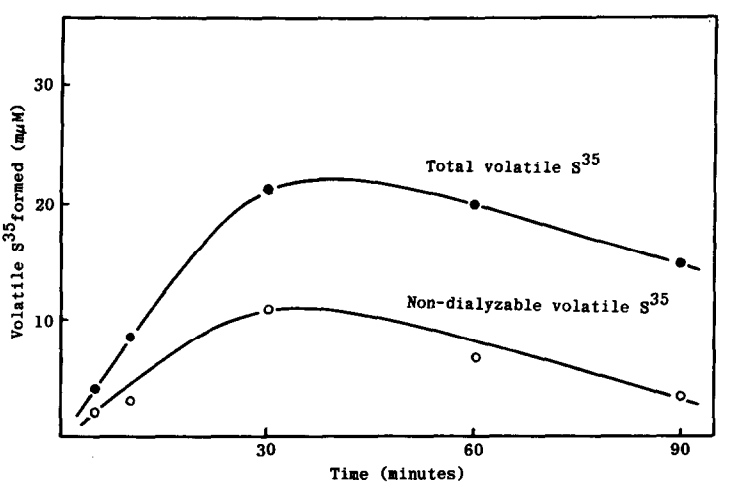

Figure 3. Formation of total and non-dialyzable volatile radioactivity as a function of time of incubation. Incubation mixture and assay methods as for Fig. 1 and 2.

As shown in Fig. 4, incubation of Enzymes A and B in the presence of dithionitrobenzoate (DTNB) and TPNH lead to the appearance of sulfhydryl (SH). The appearance of SH is markedly reduced by the addition of PAPS to the incubation mixture. This result indicates an interaction between PAPS and sulfhydryl of an enzyme protein.

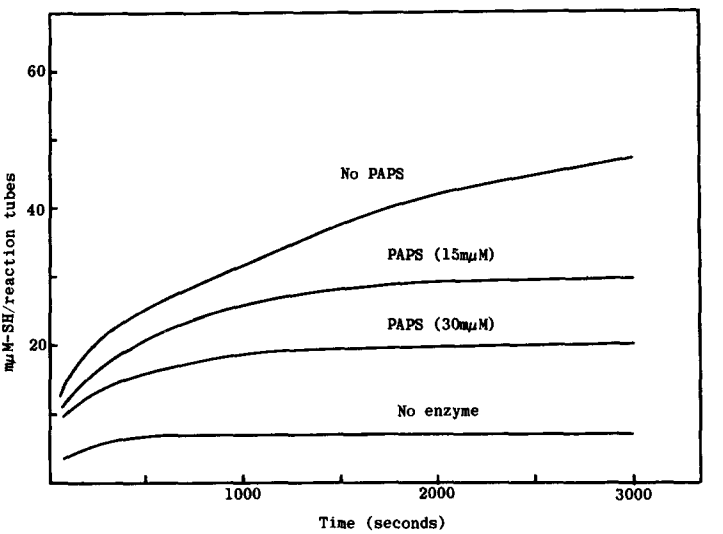

Figure 4. Sulfhydryl formation as a function of time. Each cuvette contained in umoles: DTNB, 0.1; Tris-HCl, pH 8.0, 90; $MgCl_2$, 5; TPNH, 0.2; Enzyme A, 0.5 mg; Enzyme B, 0.5 mg; and PAPS as indicated. Incubation was at room temperature. The optical density change at 412 mμ, due to release of thionitrobenzoate anion, is a measure of SH formation (Ellman, 1959).

The data presented in this paper demonstrate that an, as yet, uncharacterized intermediate, $X\text{-}SO_3^-$, is the product of the enzymatic reduction of PAPS. We, propose the following reaction sequence as a working hypothesis:

$$4.\quad H^+ + TPNH + PAPS \xrightarrow{A,\ B,\ C\text{-}SS} PAP + X\text{-}SO_3^- + TPN^+$$

where X may be Enzyme A, B, or Fraction C-SS. It is thus possible that free sulfite is never formed in the reduction of inorganic sulfate to sulfhydryl compounds in a yeast enzyme system.

Acknowledgments

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