ENZYMIC TRANSFER OF SULFUR FROM MERCAPTOPYRUVATE TO SULFITE OR SULFINATES

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Thiosulfate is known to be a normal constituent of urine in man[1] and higher animals[2], but its mode of formation has up to now been unknown. It has been suggested that thiosulfate is formed by oxidation of hydrogen sulfide, the latter being liberated by intestinal microorganisms or formed through the cysteine desulfhydrase reaction. We have briefly reported[3] that the ability of tissues to oxidize hydrogen sulfide to thiosulfate is a general heme-catalyzed reaction which probably bears no physiological importance. However an enzymic reaction was discovered, by which thiosulfate is formed from β-mercaptopyruvate and sulfite. When sulfite is replaced in this reaction by the structurally related sulfinates, thiosulfonates are formed instead of thiosulfates. The present paper deals with the transsulfuration reactions of mercaptopyruvate.

MATERIALS

Ammonium mercaptopyruvate, commercially available from Bios Inc., was used in some experiments. It was twice recrystallized from an acetone-water mixture, as the original product was found to be decomposed to some extent. In later experiments sodium mercaptopyruvate prepared according to PARROD[4] was used. L-Cysteinesulfinic acid was prepared according to LAVINE[5] and hypotaurine[6] was a gift from Dr. F. KIEFFER of this laboratory. Water homogenates of rat tissues were prepared with a Bühler or Turmix blender.

METHODS

Thiosulfate was determined by a recently developed colorimetric procedure[7] based on the conversion of thiosulfate to thiocyanate by cyanide and cupric ions, and a subsequent colorimetric determination of the thiocyanate with ferric ions. Since mercaptopyruvate interferes with this determination, it was removed by precipitation with cadmium ions. This step was carried out under slightly acid conditions, as considerable amounts of thiosulfate were carried down with the precipitate under more alkaline conditions. Since the assay systems also contained phosphate buffer, the main component of the precipitate was cadmium phosphate. Cadmium ions predominantly precipitate the secondary and tertiary phosphate ions; thus the desired slightly acid medium was directly produced in this step by the primary phosphate remaining in the supernatant. The determination was carried out as follows. To 3.0 ml reaction mixture, containing 0.1 *M* phosphate buffer at pH 7.4, 0.5 ml 1 *M* cadmium acetate was added at the end of reaction. The precipitate was centrifuged off and to 0.50 ml of the supernatant 0.40 ml 1 *M* ammonia, 3.30 ml water, 0.5 ml 0.1 *M* KCN, 0.30 ml 0.1 *M* cupric chloride and 0.50 ml ferric nitrate reagent (100 g $Fe(NO_3)_3$–$9H_2O$ and 200 ml HNO_3, sp.gr. 1.40 and water to 1000 ml) were added. The additions were made in the order indicated and the samples were thoroughly mixed before each new addition. The optical density in a 1 cm cuvette was then determined at 460 mμ. Recovery

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experiments demonstrated that 94% of thiosulfate added to the complete system was recovered in the final test and the values were correspondingly corrected. Thiosulfonates do not interfere with this determination of thiosulfate if they are not present in too large an excess (demonstrated with ethanethiosulfonate and *p*-toluenethiosulfonate). Pyruvate was determined in the cadmium supernatants by the salicylaldehyde method[8]. Cyanide labile sulfur (from thiosulfonates) was determined in the sulfinate systems by the following method. The remaining mercaptopyruvate was removed by cadmium precipitation as in the thiosulfate determination. To 0.50 ml supernatant was added 0.20 ml 1 *M* ammonia and 0.70 ml 0.1 *M* KCN and the mixture was heated for 30 min in boiling water. To the cooled sample 0.50 ml ferric nitrate reagent was then added, the sample was made up to 5 ml and the optical density was determined at 460 mμ. As sulfinic acids give a red colour with ferric ions, a blank determination was carried out, in which the ferric nitrate was added to the sample before the cyanide, and the heating step omitted.

Paper chromatography was carried out directly on the sulfinate systems after removal of proteins by heat denaturation (1 min at 100° C) and centrifugation. 10 μl was put on Whatman paper No. 1 and the chromatograms were obtained by descending development with water-saturated phenol and the spots were revealed with ninhydrin.

RESULTS

Preliminary experiments demonstrated that 89% of 0.01 *M* mercaptopyruvate was converted to thiosulfate and pyruvate when incubated with sulfite (0.02 *M*) and liver homogenate (2%) for 30 min at 37° C and pH 7.4. The reaction studied was thus the following one:

$$SCH_3COCOO^- + SO_3^{2-} = CH_3COCOO^- + S_2O_3^{2-}.$$

The factor in the liver homogenate was heat-sensitive (complete inactivation occurred in 5 min at 55° C, whereas 20% of the activity remained after 5 min at 50° C) and nondialyzable. Experiments were then undertaken in order to develop a suitable assay system. As the enzyme was highly active it was decided to lower the temperature to 20° C and to shorten the reaction time to 10 min. The effect of pH on the system under these conditions is shown in Fig. 1. It is evident that the enzyme has an optimum around pH 7.4–8, and the following experiments were consequently carried out at this pH. In Fig. 2 is demonstrated the effect of sulfite concentration. Maximum activity was obtained with 0.02 *M* sulfite; higher concentrations were slightly inhibitory. Fig. 3 demonstrates the effect of mercaptopyruvate concentration. Saturation of the enzyme with mercaptopyruvate was not obtained in this experiment. In order to save the valuable mercaptopyruvate it was decided to use this compound at 0.01 *M* concentration in the standard test. Fig. 4 finally shows the relation between thiosulfate formation and enzyme concentration in this standard test. The activities of different rat tissues were studied by means of this test. The results are shown in Table I. The most active tissue is liver, followed by kidney and blood. The activity of blood is confined to the cell fraction, whereas blood serum is practically inactive. Brain and muscle showed some activity but this was attributed to the blood content of these tissues.

Sulfinates as sulfur acceptors

When mercaptopyruvate and L-cysteinesulfinate were incubated together with rat liver homogenate, only insignificant amounts of thiosulfate were formed. A certain degree of formation of thiosulfate was in fact expected, as cysteinesulfinate yields sulfite when incubated with liver[9]. Paper chromatography of the reaction mixture demonstrated not only the expected cysteinesulfinate spot with an R_F of 0.14 but

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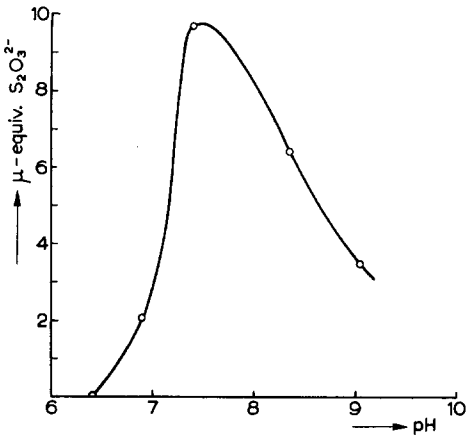

Fig. 1. Effect of pH on thiosulfate formation. Test conditions: Mercaptopyruvate 0.01 *M*, sulfite 0.02 *M*, phosphate 0.1 *M*, pH in the whole system as indicated. Each sample contained 0.5 ml 2% rat liver homogenate, final volume 3.0 ml. After 10 min at 20° C the reaction was stopped by the addition of 0.5 ml 1 *M* cadmium acetate and the necessary amount of NaOH or HCl to bring the pH back to 7.4 (as measured before the cadmium acetate addition). All samples were then brought to a final volume of 4.1 ml before centrifugation. Thiosulfate is determined in the supernatant as described in METHODS.

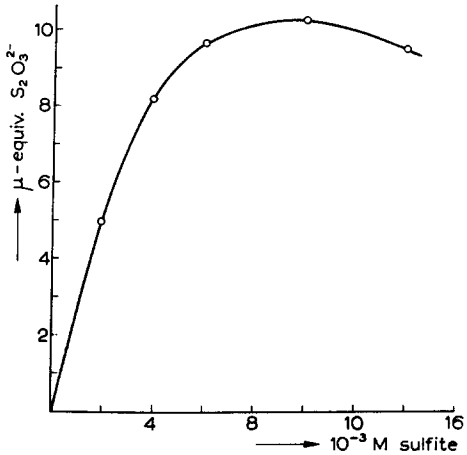

Fig. 2. Effect of sulfite concentration. Mercaptopyruvate 0.01 *M*, phosphate 0.1 *M*, pH 7.4, reaction time 10 min and temperature 20° C. Each sample, with a final volume of 3.0 ml contained 0.5 ml 2% liver homogenate.

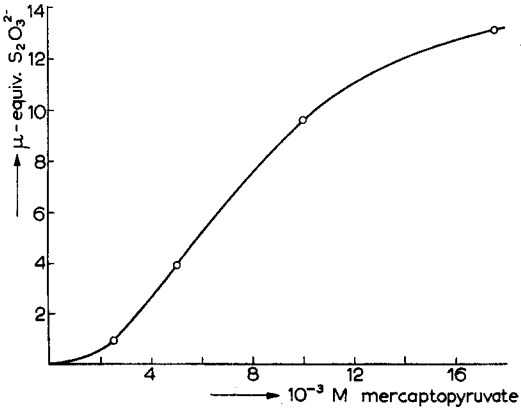

Fig. 3. Effect of mercaptopyruvate concentration. Sulfite 0.02 *M* and mercaptopyruvate as indicated, other conditions as in Fig. 2.

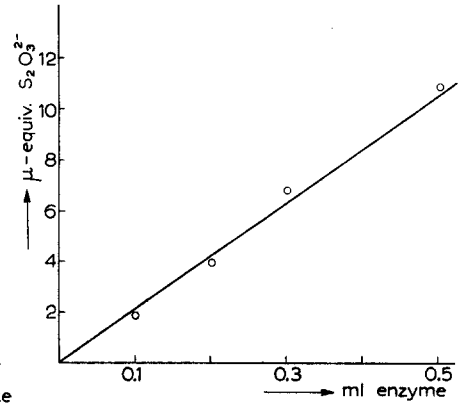

Fig. 4. Effect of enzyme concentration. Mercaptopyruvate 0.01 *M*, sulfite 0.02 *M* and indicated amount of 2% liver homogenate. Other conditions as in Fig. 2.

also a ninhydrin-positive unknown spot with an R_F of 0.06. When heat-denatured liver homogenate was used, only the cysteinesulfinate spot was detected. Sulfinates can add on sulfur, yielding thiosulfonates, and it was therefore expected that the unknown compound was a thiosulfonate. When thiosulfonates are heated with cyanide, they are converted to the corresponding sulfinate and thiocyanate[10] and this reaction was used for identifying the unknown compound. When 0.5 ml of the deproteinized reaction mixture was heated with 1.0 ml 0.1 *M* KCN for 30 min af

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TABLE I

ENZYME ACTIVITY OF RAT TISSUES

Tissue	*Activity* μ*equiv.* $S_2O_3^{2-}$ *formed per mg fresh weight*
Liver	0.87 (0.62–1.12)
Kidney	0.46 (0.29–0.70)
Blood	0.33 (0.26–0.45)
Serum	0.01
Blood cells	0.40
Muscle	0.06 (0.03–0.08)
Brain	0.05 (0.04–0.06)

The standard assay system contained 0.5 ml of a 2% homogenate in the case of liver, kidney and blood, and 0.5 ml of a 10% homogenate in the case of brain and muscle. All values, except those for serum and blood cells are the averages of 3 determinations on tissues from different animals. Ranges indicated in brackets.

100° C considerable amounts of thiocyanate were formed. Paper chromatography showed that the unknown spot had by that time disappeared and that the cysteinesulfinate spot was more intense. Consequently, the unknown compound was the thiosulfonate analogue of cysteinesulfinate. The name of the corresponding acid, $HS_2O_2CH_2CH(NH_2)COOH$, would be alaninethiosulfonic acid* according to existing rules. As the thiosulfonate group is about as polar as the sulfonate group, alaninethiosulfonate should have on a paper chromatogram an R_F value similar to the R_F value of cysteate, which was actually observed. When the sodium salt of hypotaurine (aminoethanesulfinic acid) was used instead of cysteinesulfinate, a similar reaction occurred. Paper chromatography demonstrated two ninhydrin-positive spots, one from hypotaurine with an R_F of 0.65 and one from an unknown more polar compound with an R_F of 0.36. After heating with cyanide, only a more intense hypotaurine spot remained and concomitantly the formation of thiocyanate was demonstrated. The unknown compound was thus aminoethanethiosulfonate $NH_2C_2H_4S_2O_2^-$. The amount of thiosulfonate formed in these reactions could be obtained be determining cyanide-labile sulfur. When 0.5 ml of a 10% liver homogenate was incubated with 30 μ-moles each of mercaptopyruvate and cysteinesulfinate (in the presence of 0.1 *M* phosphate buffer pH 7.4 and in a final volume of 3.0 ml), 7.4 μmoles of thiosulfonate had been formed after 30 min at 30° C. When cysteinesulfinate was replaced by aminoethanesulfinate under otherwise equal conditions, 16.8 μmoles of thiosulfonate were formed. Aminoethanesulfinate is thus more active as sulfur acceptor than cysteinesulfinate.

DISCUSSION

GAST *et al.*[1] have found that the sulfur-containing amino acids in the diet are the source of excreted thiosulfate. As the enzymic formation of thiosulfate from mercaptopyruvate and sulfite has now been demonstrated, it is possible to formulate a sequence of established reactions, through which one molecule of thiosulfate can be produced from 2 molecules of cysteine. One cysteine molecule is transaminated, yielding mercaptopyruvate[11]. Another cysteine molecule is first oxidized to cysteinesulfinate and sulfite is then formed through the desulfinase system[9,12]. Sulfur is finally transferred from mercaptopyruvate to sulfite. It is of interest, that FROMAGEOT AND ROYANÉ[13] observed an increased excretion of thiosulfonate after the administration of cysteinesulfinate to rabbits.

* Cysteinesulfinic acid is more appropriately called alaninesulfinic acid; however, the established name cysteinesulfinic acid is used in this paper.

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An enzymic desulfuration of mercaptopyruvate was first demonstrated by MEISTER *et al.*[11], who observed that pyruvate and free sulfur were formed when mercaptopyruvate was incubated with a liver preparation. This reaction was slow and incomplete even in the presence of large amounts of liver preparation, but in the presence of certain thiol compounds the reaction became rapid and complete. Under these conditions hydrogen sulfide is formed instead of free sulfur. It is probable that in this case sulfur is first transferred from mercaptopyrucate to the thiol compound, resulting in a persulfide formation. Persulfides are however very unstable and will react with the excess thiol, giving the corresponding disulfide and hydrogen sulfide. Another desulfuration reaction of mercaptopyruvate was discovered by WOOD AND FIEDLER[14], who found that thiocyanate was formed when mercaptopyruvate was incubated with liver in the presence of cyanide. This reaction was attributed to rhodanese, an enzyme catalyzing the formation of thiocyanate from thiosulfate and cyanide. Experiments by the present author[15] demonstrated that the two reactions are catalyzed by different enzymes, and this was recently confirmed by FIEDLER AND WOOD[16]. The present work has demonstrated a third type of desulfuration reaction of mercaptopyruvate in which sulfite or sulfinates are the sulfur acceptors. It is of interest that all the sulfur acceptors that are active in these reactions (thiol, compounds, cyanide, sulfite or sulfinates) have strong nucleophilic properties. It is consequently possible that these desulfuration reactions, or better transsulfuration reactions, involve a nucleophilic attack on the sulfur-carbon bond in mercaptopyruvate. The adjacent carbonyl group in mercaptopyruvate will make this bond more susceptible to this attack, in contrast to the stabilizing effect of the amino group in cysteine. Thus an electronic interpretation is offered for the fact that cysteine is inactive in these transsulfuration reactions and must first be transaminated before the sulfur can be detached. An objection to this theory seems to be that cysteine yields hydrogen sulfide when incubated with liver[17], but evidence has recently been obtained by CHATAGNER AND SAURET-IGNAZI[18] that a transamination is the first step in this reaction.

The demonstration of an enzymic formation of thiosulfonates from cysteine-derived metabolites, which are normally formed in the body, is of interest, since it is the first indication of the biological occurrence of thiosulfonates. These compounds have not yet been directly demonstrated in the body, but an observation of SMITH AND TULLER[19] might be mentioned in this connection. In a study of sulfur-containing compounds in human blood serum by a paper-chromatographic technique, these authors observed an unknown spot with an R_F value similar to that of taurine. However, the unknown compound contained reducing sulfur, in contrast to taurine. This unknown compound might well be aminoethanethiosulfonic acid, for which we suggest the shorter name of *thiotaurine*, in analogy with the name of hypotaurine for aminoethanesulfinic acid. (Alaninethiosulfonic acid may then be called thiocysteic acid; however, this name could also be given to the compound $COSH(NH_2)CHCH_2SO_3H$.) Further studies are being made at present on the synthesis and metabolism of these thiosulfonates. The activity of other thiosulfonates as sulfur donors in the rhodanese reaction has previously been demonstrated[20].

ACKNOWLEDGEMENT

The author is very grateful to Professor C. FROMAGEOT for his kind interest in this work.

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SUMMARY

An enzyme has been found in rat tissues, which forms thiosulfate from mercaptopyruvate and sulfite. Liver, kidney and blood are the most active tissues. When sulfite is replaced by sulfinates, thiosulfonates are formed. The formation of the salts of two new compounds, aminoethanethiosulfonic acid (thiotaurine) and alaninethiosulfonic acid has been demonstrated. The significance of these reactions in sulfur metabolism is discussed.

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Received November 26th, 1956

DIALYZABILITY OF HISTONES*

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Recent investigations by ultracentrifugation[1–4], electrophoresis[3–8], salting-out[8,9], isoelectric precipitation[8], or chromatography[5,10,11] have shown that histone is a complex which can be resolved into several fractions. The findings differ in regard to the number and ratio of histone components and in regard to their amino acid content. The inconsistencies appear to be due in part to the natural tendency of histones to form aggregates, and in part to the application of non-quantitative fractionation procedures. For example, it has been our experience[2] and also that of DAVISON *et al.*[4] and CRAMPTON[11] that dialysis has marked effects upon histone preparations and seems to

* Aided by research grants from the National Cancer Institute of the U.S. Public Health Service (C-1249) and the American Cancer Society (BCH-55), and by an institutional grant of the American Cancer Society.

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