ISOLATION AND PROPERTIES OF A β-MERCAPTOPYRUVATE-CLEAVING COPPER ENZYME

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(Received June 23rd, 1958)

SUMMARY

1. The distribution and isolation of β-mercaptopyruvate transsulfurase is described. The enzyme was isolated from rat liver by $(NH_4)_2SO_4$ fractionation and repeated preparative electrophoresis.

2. The enzyme was characterized by constant specific activity, copper content and its absorption spectrum.

3. The mechanism of action of 2-mercaptoethanol and Na_2SO_3 on the enzymic formation of pyruvate from β-mercaptopyruvate was determined. While 2-mercaptoethanol acts as a reducing and stabilizing agent, $SO_3^=$ reacts as a second substrate to accept S from β-mercaptopyruvate.

4. The catalytically active group of the enzyme, which participates in the removal and transfer of S from the substrate, was identified as a thiol-disulfide-Cu system. A mechanism of transsulfuration based on analytical and kinetic measurements is described. This mechanism is restricted to the description of the reaction of the mercapto group of the substrate with the enzyme.

INTRODUCTION

Both "desulfurase" and "transsulfurase" type of enzymic cleavage of β-mercaptopyruvate have been observed in tissue extracts and homogenates (MEISTER *et al.*[1] and SÖRBO[2]). The enzymes responsible for these reactions were not identified. We have recently reported that a copper-protein, obtained from rat liver, catalyses both the removal and transfer of sulfur from β-mercaptopyruvate (KUN AND FANSHIER[3]). The present paper deals with the isolation and properties of this enzyme. A probable reaction mechanism will be discussed.

EXPERIMENTAL

Chemical preparations and analytical methods

The ammonium salt of β-mercaptopyruvic acid was prepared as previously described (KUN[4]; KUMLER AND KUN[5]). The recrystallized salt was stored in 2 to 3 g

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portions under vacuum at 4°. This precaution minimized auto-oxidation. Pyruvic and β-mercaptopyruvic acids and the disulfide form of β-mercaptopyruvic acid were identified by chromatographic analyses (KUN AND GARCIA-HERNANDEZ[6]). It was found that the 2,4-dinitrophenylhydrazone of β-mercaptopyruvic acid was unstable under alkaline conditions (in 10 % Na_2CO_3) and yielded small amounts of hydroxypyruvic acid which appeared as the bis-2,4-dinitrophenylhydrazone on paper chromatograms[6]. The disulfide form of β-mercaptopyruvic acid and its 2,4-dinitrophenylhydrazine derivative were prepared according to PARROD[7]. The free disulfide-dicarboxylic acid has a pH-dependent enol band at 290 mμ, similar to that of the mercaptopyruvate ion[4]. Pyruvic acid was determined as described by FRIEDEMANN AND HAUGEN[8], and also by the method of BUNTSSON[9]. Protein analysis was carried out in trichloroacetic acid (TCA) precipitates of crude tissue preparations by the method of LOWRY *et al.*[10], and in purified samples according to WARBURG AND CHRISTIAN[11]. 2-mercaptoethanol, used as a reducing agent during enzyme purification, interfered with the colorimetric and spectrophotometric protein assays and was removed by reprecipitation of the protein by $(NH_4)_2SO_4$ or by washing the TCA precipitate with 10 % TCA. The nitrogen content of the purified enzyme was determined by a modified method of NESSLER[12]. Copper was determined by a dithizon procedure[13] and by the catalytic method of WARBURG[14].

Enzyme assay

The measurement of enzymic activity was based on optimal experimental conditions. 1.5 ml of 0.02 *M* potassium phosphate buffer + 0.5 ml of a freshly prepared solution of ammonium β-mercaptopyruvate (50 μmoles) + distilled water to a final volume of 2.5 ml + 0.1 ml of 2-mercaptoethanol were pipetted into a test tube. The mixture was chilled to 0 to 4° for 5 min. To this chilled solution 0.1 ml of the enzyme preparation was added, the reaction started by immersion of the tubes in a water bath of 30° and allowed to incubate for 10 min. The reaction was stopped by the addition of 1 ml of a saturated solution of cadmium acetate. The tubes were then thoroughly shaken and allowed to stand for 10 min at room temperature. The precipitate was removed by centrifugation and a 1 ml sample of the supernatant fluid analyzed for pyruvate. Reagent blanks were set up with each series and blank values subtracted from those obtained by enzymic reactions. Reagent blanks were found to vary between 0.18 to 0.2 μmoles of pyruvate per test system. The rate of enzymic formation of pyruvate was linear during the first 20 min and directly proportional to the amount of enzyme protein.

Distribution of the enzyme

All tissues tested exhibited enzymic activity. Since the enzyme was found to be very sensitive to oxidation, the tissues were homogenized in phosphate buffer containing 1 % 2-mercaptoethanol. The enzyme content of various tissues is summarized in Table I. A homogenate of rat liver, prepared in 0.25 *M* sucrose (containing 2-mercaptoethanol) was subjected to fractionation by differential centrifugation, according to SCHNEIDER AND HOGEBOOM[12]. Results are shown in Table II.

Since none of the fractions were further purified, only tentative conclusions could be drawn. The enzyme appeared to be present in highest concentrations in the "nuclear" and "soluble" fractions, the latter containing microsomes. When homog-

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enates were prepared in a salt solution (0.15 *M* KCl) the particle-free centrifugal supernatant fluid represented only ¼ to ½ of the total enzyme content, which suggests that some particulate elements (nuclei and microsomes) bind the enzyme. The enzyme can be quantitatively extracted from an acetone powder of rat livers.

TABLE I

DESULFURASE ACTIVITY OF TISSUES

Tissue	*S.A.**
Mouse red corpuscules	1.2
Mouse plasma	0.0
Rat heart	5.5
Rat brain	2.1
Rat kidney	21.0
Rat muscle	4.0
Rat liver	10.0
Human red corpuscules	1.0
Human plasma	0.1**

* S.A. = Specific activity: μmoles of pyruvate formed in 10 min at 30° (pH 7.45) per mg protein.

** A trace of haemolysis cannot be excluded.

TABLE II

DESULFURASE ACTIVITY OF CENTRIFUGAL FRACTIONS OF RAT LIVER

Cell fraction	*S.A.*	*Total activity*
Rat-liver homogenate	10.7	4,000
Nuclear fraction	11.6	2,350
Mitochondria	3.1	290
Supernatant*	16.7	1,600

* Contains soluble proteins and microsomes.

Isolation of the enzyme

All operations were carried out in a cold room at 0 to 4°. Two freshly removed rat livers (wet weight 13.0 g) were homogenized in a blendor for 50 sec in about 50 ml of 0.02 *M* phosphate buffer (pH 7.45, containing 1 % 2-mercaptoethanol). The homogenate was poured, with stirring, into 1 l of acetone previously cooled to —20°. The temperature was allowed to rise to —5°, whereupon the precipitate was quickly filtered through a Büchner funnel, washed with cold acetone (—5°) and dried under vacuum. The acetone powder, together with the filter paper, was rehomogenized in a blendor with 100 ml of 0.02 *M* phosphate buffer (containing 1 % 2-mercaptoethanol) for 50 sec. The insoluble material was spun down at 8,000 × *g* for 20 min at 0°. To the clear reddish-yellow supernatant fluid 0.2 g/ml $(NH_4)_2SO_4$ (solid) was then added with stirring at 0°. The precipitate formed after 5 min (F_I) was centrifuged down and discarded. Enzymic activity was quantitatively recovered in F_{II} by the addition of a second portion of 0.2 g per ml of $(NH_4)_2SO_4$ (solid). The amount of $(NH_4)_2SO_4$ added was based on the original volume of the extract of the acetone powder.

Subsequent purification was carried out by repeated preparative electrophoresis of the F_{II} fraction on a polyvinyl chloride resin (Geon 426*). The commercial resin was prepared by suspending it in 2 *N* HCl (50 g resin in 2 l 2 *N* HCl), filtering and washing with de-ionized water until the effluent became neutral. The last washing was carried out with 500 ml of 0.01 *M* phosphate buffer (pH 7.45). Finally, the thick suspension of resin in phosphate buffer containing 1 % 2-mercaptoethanol was stored at 4°. A plastic trough (50 cm long, 5 cm wide with a depth of 3 cm) was filled with a slurry of the resin, and the excess buffer blotted off by means of strips of filter paper. The electrode vessels were then connected to the moist resin bed by wicks of thick

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filter paper, the resin trough covered and current allowed to pass through the system for 30 min. The current density used for electrophoresis was 40 mA with a potential gradient of 5 to 7 V/cm. The electrode vessels contained 1 l of 0.01 *M* phosphate buffer at pH 7.45 (+ 1 % 2-mercaptoethanol). At the end of equilibration, a segment (2 to 3 cm long) was cut out and replaced by a slurry of the F_{II} fraction and the resin. For this purpose it was convenient to precipitate F_{II} with $(NH_4)_2SO_4$ and redissolve it in a small volume of buffer. About 150 to 200 mg of protein was put on a column. Each electrophoretic separation was carried out for 3 to 4 h at 0 to 4°. At the end of each run the segment, containing the enzyme, was extracted with 2-mercaptoethanol containing phosphate buffer, and refractionated with $(NH_4)_2SO_4$ as described above. Results of an electrophoretic fractionation are shown in Fig. 1.

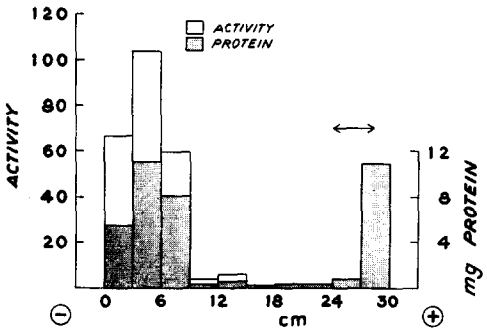

Fig. 1. Electrophoretic fractionation of F_{II} on Geon 426 resin. The arrow refers to the position of F_{II} at 0 time.

TABLE III

PURIFICATION AND RECOVERY OF THE ENZYME FROM 2 RAT LIVERS

$F_{II}A$, $F_{II}B$ and $F_{II}C$ are consecutive purified fractions obtained by electrophoresis of F_{II}.

Preparation	*Protein (mg)*	*Specific activity*	*Total activity*
Rat-liver homogenate	1,800	10.3	18,500
Extract of acetone powder	635	39.0	24,700
F_{II}	424	45.5	19,100
$F_{II}A$	276	62.3	17,200
$F_{II}B$	194	86.0	16,700
$F_{II}C$	—	86.5	—

Quantitative recovery of the enzyme and concentration of its activity as the result of purification are summarized in Table III.

The first electrophoretic fraction, as estimated by its specific activity, was about 80 % pure. In the analytical ultracentrifuge this fraction was found to be 80 to 85 % homogenous. Repeated electrophoretic fractionation resulted in a protein which was at least 90 % homogenous as determined by electrophoretis (at pH 7.4 and 8.4) and by the analytical ultracentrifuge. Another indication of purity was established by specific activity measurements of fractions separated from the enzyme-containing protein peak, obtained by preparative electrophoresis. The third electrophoresic fraction was divided into three segments on the column, each cut out, extracted and tested separately. The specific activity was found to be the same in all three segments.

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Sedimentation constants were determined in phosphate and Tris buffers (0.01 *M*, pH 7.45). It was found that at a protein concentration of 1 mg protein/ml, S_{20} was 2.72 and 2.86 at 3.7 mg/ml. As reported earlier, a molecular weight of 35,000 to 40,000 was calculated from the sedimentation and diffusion constants[3]. A turnover number of 350 (moles of pyruvate formed per mole of enzyme per minute) was calculated at pH 7.4, and 30°. A value for molecular weight was also calculated from the copper content. The purest enzyme preparations contained no trace metals except copper (Fig. 2). It it is assumed that the molecular weight is 35,000 to 40,000, copper analysis indicates that the purest preparation contains 1 atom of Cu per mole of enzyme (0.17 % Cu). This is shown in Table IV. The enzyme preparation used for Cu analyses was that described in Table III. Copper content and specific activity remain constant in the purified preparations. In earlier experiments, we did not routinely use 2-mercaptoethanol as a stabilizing agent and experienced considerable variation in both enzymic activity and Cu content[3]. It was concluded that 2-mercaptoethanol serves to protect the protein from these variations.

The absorption spectrum of the pure enzyme was determined and is shown in Fig. 3. The small band at 415 mμ could indicate contamination with a heme-protein; however, the preparation of a pyridine hemochrome derivative was unsuccessful. It is of interest that certain complexes of cuprous ion, *e.g.* with 2,9-dimethyl-1,10-phenanthroline (SMITH AND MACCURDY[15]) in the presence of cysteine or a protein containing free SH groups exhibit and absorption band in the 450 mμ region (FRIEDEN[16]). The 415 mμ band of the enzyme may indicate a similar type of complex of copper. The broad band in the 600 mμ region is characteristic of copper proteins[22–25]. More detailed spectrophotometric studies will be published elsewhere.

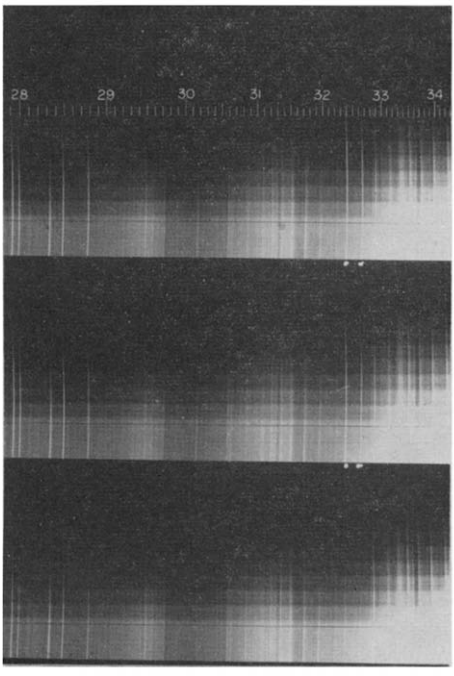

Fig. 2. Emission spectrum of the purified enzyme. The sample shown on the bottom is the solvent (phosphate buffer). The characteristic spectrum lines in the region of 3247 Å and 3274 Å identify the copper. Measurements were carried out with the Hilger medium quartz spectrograph.

TABLE IV

THE CONCENTRATION OF THE ENZYME AND Cu IN FRACTIONS OBTAINED DURING PURIFICATION

(See Table III)

Preparation	*μmoles/mg protein*	
	Enzyme	*Copper*
Rat-liver homogenate	$3 \cdot 10^{-3}$	$12.6 \cdot 10^{-3}$
Extract of acetone powder	$11 \cdot 10^{-3}$	$8.5 \cdot 10^{-3}$
Ammonium sulfate F_{II}	$13 \cdot 10^{-3}$	$8.0 \cdot 10^{-3}$
Electro. $F_{II}A$	$18 \cdot 10^{-3}$	$8.2 \cdot 10^{-3}$
Electro. $F_{II}B$	$25 \cdot 10^{-3}$	$30.0 \cdot 10^{-3}$
Electro. $F_{II}C$	$25 \cdot 10^{-3}$	$29.0 \cdot 10^{-3}$

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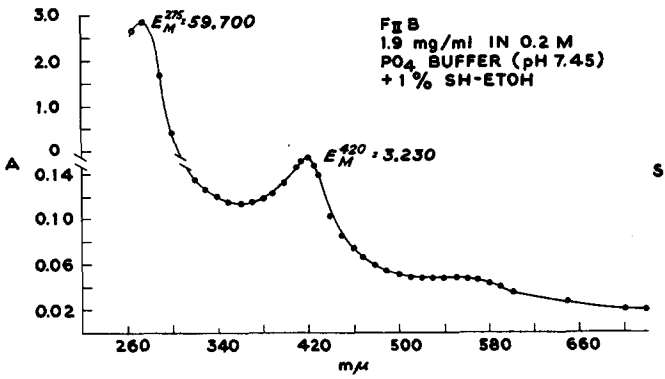

Fig. 3. Absorption spectrum of the purified enzyme.

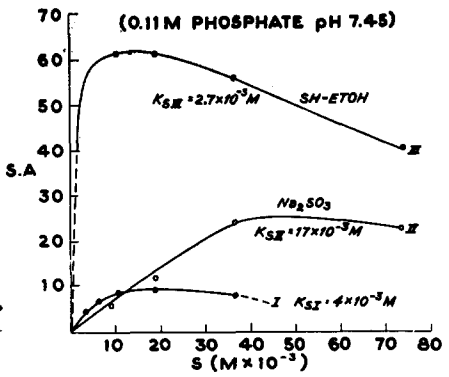

Fig. 4. The effect of substrate concentration on enzymic activity, expressed in specific activity. I. No additives; II. + Na_2SO_3 (10^{-2} *M*); III. + 2-mercaptoethanol (0.8 *M*).

Kinetic measurements

The rate of pyruvate formation from β-mercaptopyruvate was studied under various conditions, as shown in Fig. 4. As reported previously[3], both 2-mercaptoethanol and Na_2SO_3 increase this rate. Addition of 2-mercaptoethanol to fresh enzyme preparations (after reprecipitation with $(NH_4)_2SO_4$ from buffer containing 2-mercaptoethanol) increased the rate of reaction six-fold, but did not markedly influence the K_s value of β-mercaptopyruvate ($2.7 \cdot 10^{-3}$ and $4.0 \cdot 10^{-3}$ or $2.5 \cdot 10^{-3}$; see Fig. 4 and Fig. 5B). On the other hand, Na_2SO_3, at a concentration of 10^{-2} *M*, markedly changed this value (see Fig. 4). Detailed studies of this phenomenon revealed that at a low concentration range of β-mercaptopyruvate (below 2 μmoles) $SO_3^=$ was inhibitory (apparent K_s is $15 \cdot 10^{-3}$), while above a concentration of 5 to 10 μmoles of β-mercaptopyruvate, $SO_3^=$ had an activating effect and reduced the K_s to $0.5 \cdot 10^{-3}$ (Fig. 5). A more complete kinetic study of these interactions was difficult due to technical obstacles, *e.g.*, lengthy enzyme assay, instability of the enzyme, etc.; nevertheless, the available data were taken as an indication, suggesting that both β-mercaptopyruvate and $SO_3^=$ interact with the enzyme as substrates. This conclusion was strengthened by a quantitative study of the activating effects of both 2-mercaptoethanol and $SO_3^=$. It is shown in Fig. 6 that a concentration of 2-mercaptoethanol, which is 80 times higher than that of $SO_3^=$, is required to bring about maximal activation. The saturation curve of Na_2SO_3 follows a normal Michaelis-Menten type of substrate-activity relationship. It is suggested that the role of 2-mercaptoethanol is that of a reducing agent, maintaining optimal conditions for catalysis. This substance may protect the two SH groups of the enzyme[3] from oxidation as well as keep the copper atom of the enzyme in the cuprous form. The exact mechanism of this reduction is difficult to ascertain at present. It is probable that the rapid reversible inactivation is connected with the valence change of copper, while the slower irreversible inactivation may be due to the formation of a disulfide or mercaptide. A study of the effect of 2-mercaptopyruvate on thiosulfate formation in the presence of $SO_3^=$ and β-mercaptopyruvate meets technical obstacles since 2-mercaptoethanol interferes with the $S_2O_3^=$ analysis. However, the activation of the transsulfurase reaction by 2-mercaptoethanol would be expected. This assumption is based on the fact that a 1:1

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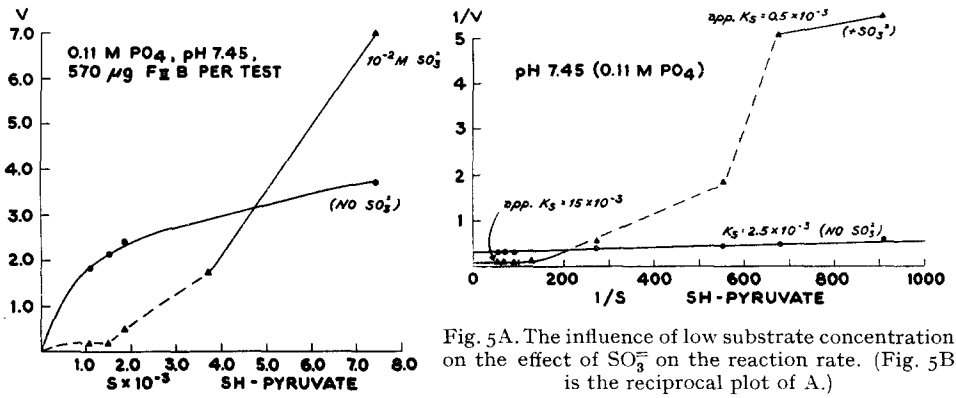

Fig. 5A. The influence of low substrate concentration on the effect of $SO_3^=$ on the reaction rate. (Fig. 5B is the reciprocal plot of A.)

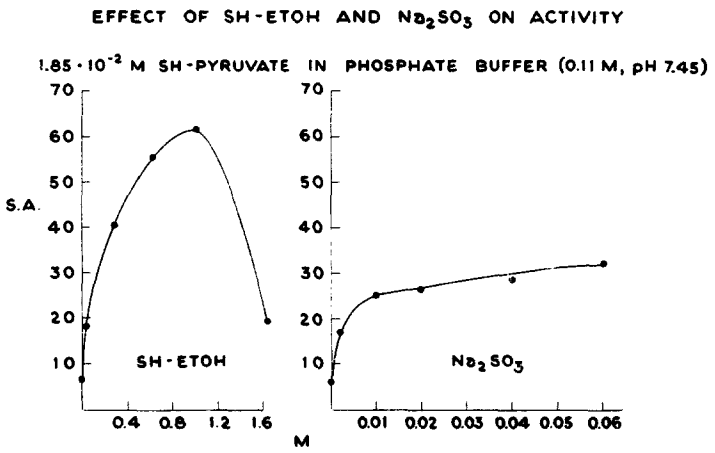

Fig. 6. Effect of SH-ETOH and Na_2SO_3 on activity.

ratio exists between pyruvate and $S_2O_3^=$ formation, as determined in the absence of 2-mercaptoethanol.

The effect of hydrogen ion concentration on enzymic desulfuration is shown in Fig. 7. The maximum velocity of the reaction in the presence of 2-mercaptoethanol increased with alkalinity. The curve obtained coincides with the titration curve of the mercapto group of the β-mercaptopyruvate ion[5]. The concentration of β-mercaptopyruvate required to saturate the enzyme increased with higher OH^- concentration. While at pH 7.4, 50 μmoles per test system saturated the enzyme, 200 μmoles are needed at pH 9 to 10. The non-enzymic decomposition of the substrate in this range is negligible and shows no marked pH dependence. In addition, it was found that the enzyme has a maximum affinity for the substrate at pH 7.4, as expressed as the reciprocal of K_s. This pH value corresponds to the isoelectric point[3] of the enzyme. It is clear that both the substrate and the enzyme possess pH dependent molecular properties. The kinetic treatment of such a pH effect on enzymic reactions is very difficult at this time (ALBERTY[17]). The conclusion can be drawn, however, that the true substrate is the ionized form of β-mercaptopyruvate; and further, that the net charge of the enzyme protein itself must be zero for optimal conditions of catalysis. MEISTER *et al.*[1] reported that at 37° the pH optimum of enzymic desulfuration by liver extracts

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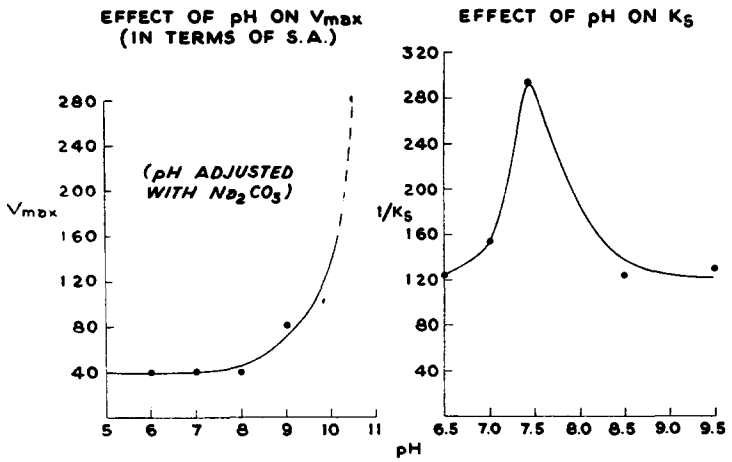

Fig. 7. No external buffer was used.

is approximately 8. These workers used phosphate buffer in the pH range of 5.5 to 8 and borate buffer in the pH range of 8 to 10. In our experiments dealing with the effect of pH on desulfuration (measured at 30°) no buffer was used, but each incubation mixture was adjusted to the appropriate pH by means of a glass electrode. The interference of borate ions with the enzymic reaction is one of the possible explanations of this discrepancy. In addition, at higher pH values the amount of substrate used by MEISTER *et al.*[1] may not have saturated the enzyme. The pH optimum of transsulfuration is 7.4 (SÖRBO[2]). This value coincides with the pH at which the enzyme has greatest affinity for β-mercaptopyruvate.

Catalytically active groups of the enzyme

Elementary analysis of the protein revealed four atoms of sulfur per mole of enzyme[3]. When a freshly prepared enzyme (freed of 2-mercaptoethanol by repeated precipitation with $(NH_4)_2SO_4$) was reacted with *p*-chloromercuriphenylsulfonate according to BOYER[18], the reaction went to completion in about 30 min and showed two SH groups per mole of enzyme. It was found that reagents which combine with SH groups inhibit enzymic activity; Cu^{++} at $3.7 \cdot 10^{-4}$ *M* inhibits 81 % and Fe^{+++} at the same concentration inhibits 23 %. Versene was found to reverse the inhibition by these metals. Inhibition by cupric ions had been observed earlier by MEISTER *et al.*[1]. NaN_3 at $5 \cdot 10^{-2}$ *M* inhibits 67 % and Tris at 0.1 *M* inhibits 45 %. Loss of copper due to the exposure of the enzyme to pH below 5 irreversibly inactivated the enzyme. From these observations it was concluded that both the SH groups and protein-bound copper participate in enzymic catalysis.

Mechanism of enzymic catalysis

The copper protein isolated from rat liver was found to catalyse at least one distinct enzymic reaction: the removal and transfer of atomic sulfur to an acceptor. So far we have only studied $SO_3^=$ as a sulfur acceptor. In the absence of an acceptor, the enzyme catalyses desulfuration which may be considered to be a half reaction. In this case the enzyme itself is the S acceptor and is progressively inactivated. It was found in the course of further kinetic studies that in the presence of 10^{-2} *M* substrate

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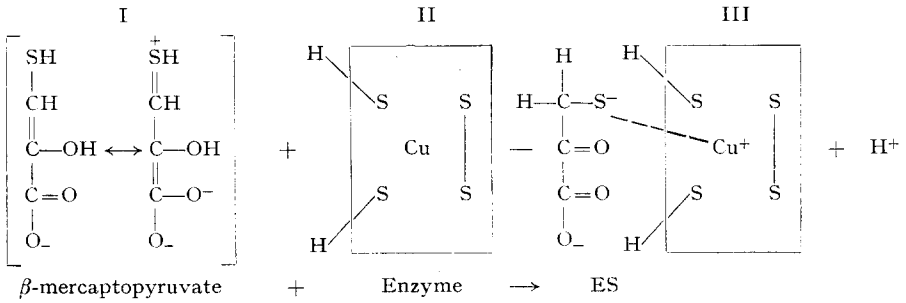
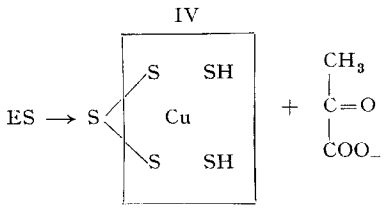
the rate of enzymic pyruvate formation deviated from linearity after about 20 min incubation time. This apparent inhibition of desulfuration, which increased with time, was absent when $SO_3^=$ was present as a sulfur acceptor. This suggested that the accumulation of a product caused enzyme inhibition. In agreement with MEISTER *et al.*[1] it was found that pyruvate, even at 10^{-2} *M* concentration was not inhibitory, therefore it was concluded that sulfur was responsible for the product inhibition. The inhibitory effect of sulfur is readily explained by the reaction of sulfur with the SH groups of the enzyme, which takes place in the absence of a more effective sulfur-reducing substance. The substrate, β-mercaptopyruvate is, itself, a reducing agent and may protect the enzyme in the initial phase of the reaction. The mercapto group of β-mercaptopyruvate is 3 to 5 times less reactive than the SH group of cysteine. Cysteine is a good reagent for the determination of free sulfur, since a rapid reduction to H_2S takes place in its presence (SMYTHE[19]). The H_2S formation which occurs during the enzymic desulfuration of β-mercaptopyruvate may be explained by a similar non-enzymic mechanism. As expected, no H_2S is evolved when $SO_3^=$ is present as an S acceptor. It should be mentioned that cysteine itself is not acted upon by the β-mercaptopyruvate-cleaving enzyme. High concentrations of cysteine (10^{-2} *M*) are noticeably inhibitory.

A series of reactions may be written which represent a summary of our present knowledge of the mechanism of enzymic transsulfuration.



As the result of enzyme–substrate complex (ES) formation, resonance of the enol form of the substrate (KUMLER AND KUN[5]) is eliminated. This is followed by ketonization with the resulting labilization of the sulfur atom. The interpretation of the following step is based on the assumption that there is a possibility of electron transfer between the dithiol and disulfide groups of the enzyme. The copper atom may serve as a mediator of electron transfer. A similarity between mixed copper complexes and the enzyme provides ample grounds for this assumption (MARTELL AND CALVIN[20]). As the result of catalysis, the enzyme–substrate complex (III) rearranges, yielding an enzyme–sulfur compound of a trisulfide type (IV). Simultaneously pyruvate is formed.



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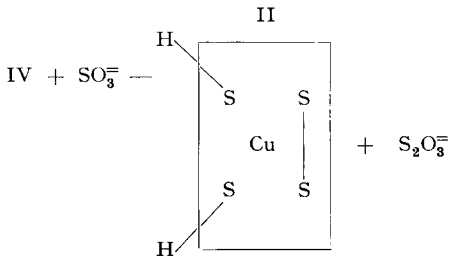
This enzyme-product complex (IV) may then react with the second substrate, $SO_3^=$. This is an S acceptor which may react with the enzyme-product complex (IV) in a manner similar to the reaction of $SO_3^=$ with cystine (CHAPVILLE AND FROMAGEOT[21]).



While the products of the reaction of cystine and $SO_3^=$ are cysteine and alaninethiosulfate, the enzymic reaction above yields reduced enzyme (II) and thiosulfate. When no sulfur accepting substrate is present, the atomic sulfur of the complex (IV) may undergo reduction in the presence of reducing agents (to H_2S), or decomposition, and the polymerization of atomic S to elementary sulfur will occur. The enzyme sulfur complex may be regarded as a reactive form of sulfur, capable of entering various types of reactions.

DISCUSSION

It is of interest to compare the copper protein which acts on β-mercaptopyruvate with other copper proteins of animal origin. MANN AND KEILIN[22] identified a copper protein fraction from red corpuscules. Initially, these investigators obtained two protein fractions: "solution F", containing 0.17 % Cu and "solution G" with 0.24 % Cu. "Solution G" was crystallized, whereupon the Cu contents increased to 0.34 %. This protein, termed haemocuprein, had a molecular weight of 35,000. Haemocuprein from human red cells is known to cyntain from 0.18 to 0.21 % copper. MANN AND KEILIN isolated another copper protein from liver, hepatocuprein, which was found to be very similar to crystalline haemocuprein. Recently MOHAMED AND GREENBERG[23] crystallized a copper protein from liver which appeared to be similar or identical with hepatocuprein. PORTER AND FOLCH[24] isolated several copper-containing protein fractions from brain tissue. A molecular weight of 30,000 to 40,000 was calculated by these workers for the purest fraction. The copper content varied between 0.14 to 0.3 %. None of these copper proteins were identified as enzymes.

The copper enzyme, acting on β-mercaptopyruvate, and these copper proteins have certain striking physical similarities. Their molecular weight is the same although other properties (isoelectric point[3] and copper contents) are different. It is not unlikely that a family of copper proteins exists. Since the isolation procedures described by the previous workers would yield complete inactivation of the β-mercaptopyruvate-cleaving enzyme, a direct comparison with the copper proteins mentioned is difficult.

The copper protein of blood serum, called coeruloplasmin, of HOLMBERG AND LAURELL[25], has a molecular weight of 150,000 and contains 8 copper atoms per mole of protein. Coeruloplasmin oxidizes *p*-phenylene-diamine, hydroquinone, catechol, pyrogallol, dopa and epinephrine.

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The interrelationship of various copper proteins and their physiological role in intermediary metabolism is unknown. The identification of a copper protein as a participant in an enzyme reaction involved in sulfur metabolism was unexpected. The pursuit of this problem may lead to a clearer understanding of the role of copper in mammalian physiology.

NOTE ADDED IN PROOF

In the course of recent work done since the acceptance of this paper it was found that copper analyses of crude liver fractions with 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline (Table IV: homogenate, extract, and F_{II}) gave much lower values than obtained with the catalytic method of WARBURG. Since the colorimetric method is more specific it is probable that values for crude tissue preparations as reported in Table IV are too high. Colorimetric analyses with dithizone and with the catalytic method were in closer agreement in purified protein fractions.

(Received February 16th, 1959)

ACKNOWLEDGEMENTS

This investigation was supported by grants of the U.S. Public Health Service (Heart H-2897 and Cancer C-3211).

The authors are grateful to Mr. M. K. HRENOFF for spectrographic analyses and to Dr. LOUIS A. STRAIT for valuable advice. Our thanks are due to Dr. JOHN B. NEILANDS for a gift of *p*-chloromercuriphenylsulfonate and for this help during the determination of the diffusion constant of the enzyme. Ultracentrifugal analyses were kindly performed by Miss PEARL APPEL.

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