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Crystalline Rhodanese

II. The Enzyme Catalyzed Reaction

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In the preceeding paper the preparation and physicochemical characterisation of crystalline rhodanese was described [1]. In the present investigation the effect of different factors upon the enzyme catalyzed reaction has been studied. As the question of the reversibility of the reaction has got different answers [2-4] the problem was now reinvestigated. In a study of the substrate specifity of rhodanese we recently [5] found that *p*-toluene thiosulfonate could replace thiosulfate as sulfur donor, and this investigation has now been extended to other thiosulfonates. Inhibitor experiments [6,7] have suggested that the active group in the enzyme is a disulfide bond, and the crystalline enzyme was now consequently tested for these groups with the nitroprusside reaction. The reaction mechanism [7] of the enzyme is also discussed.

EXPERIMENTAL

Materials. Crystallized rhodanese was used throughout this part of the investigation. The sodium thiosulfonates listed in Table 2 were prepared from the corresponding sulfonyl chlorides and sodium sulfide following the directions given by Troeger and Linde [8]. Each was crystallized at least twice from ethanol, after which all but two gave satisfactory analytical values for sulfur. In the case of ethane and *n*-butane thiosulfonate however, even repeated recrystallisations from ethanol did not give more than about 90 % of the theoretical value. Difficulties in obtaining the aliphatic thiosulfonates in a pure form have previously been noted [9].

Methods. In studying the rhodanese catalyzed reaction between cyanide and thiosulfate, the enzyme was always diluted in the presence of 0.0125 *M* thiosulfate and 0.025 % albumin. The other conditions are evident from the figure legends. The obtained thiocyanate was determined as in the standard test [1].

The sensitive pyridine-pyrazolone method as described by Boxer and Rickards [10] was used in an attempt to demonstrate a formation of cyanide from thiocyanate and sulfite in the presence of rhodanese. The concentration of thiocyanate and sulfite was 0.05 *M* and the reaction mixture of total volume 2.1 ml contained also 0.05 *M* phosphate buffer, pH 7.4. After 15 minutes incubation in the "oxidation tube", described by Boxer and Rickards [11], the reaction mixture was acidified to pH 4 with 2 ml 0.05 *M* sulfuric acid, and any hydrogen cyanide formed was removed by aeration and collected in 1 ml 0.1 *M* sodium hydroxide. As sulfur dioxide is also liberated from the acid reaction mixture and interferes with the final color development [10] it had to be removed by oxidation. This

was accomplished by passing the gas stream from the reaction mixture first through an empty trap containing glass wool, and then through a trap containing 2 ml 0.1 *M* potassium permanganate and 2 ml 0.5 *M* sulfuric acid. The purpose of the empty trap was to prevent any spray from the reaction mixture reaching the permanganate, as otherwise cyanide would be formed by oxidation of the thiocyanate present in the reaction mixture. The colorimetric determination of cyanide was carried out as in the original method [10].

The different thiosulfonates were analyzed for sulfur according to Grothe-Krekeler and their content of thiosulfonate was determined by a colorimetric modification of the titrimetric procedure given by Gutman [12]. In this method the thiosulfonate is treated with cyanide in the heat, giving the corresponding sulfinate and thiocyanate. In the original method the sulfinate was titrated iodometrically, but in the present modification the thiocyanate is determined with ferric ions in the presence of formaldehyde. The determination was carried out as follows: To 1 ml of a thiosulfonate solution, containing up to 10 μ-mole thiosulfonate, was added 0.5 ml 0.25 *M* potassium cyanide and the mixture evaporated to dryness on a steam bath. The residue was dissolved in 2.0 ml distilled water and 0.5 ml 38 % formaldehyde, 2.5 ml ferric nitrate reagent [1], and 25 ml water was added. The yield of thiocyanate formed was then obtained from the optical density at 460 mμ.

The ability of the different thiosulfonates to function as sulfur donors in the rhodanese reaction was examined in the following test system, which is an improvement of the earlier method [5]. 1 ml of a 0.125 *M* thiosulfonate solution containing 0.20 *M* phosphate buffer pH 7.4 and 1.0 ml 0.125 *M* potassium cyanide (adjusted to pH 7.4 with hydrochloric acid) were mixed and 0.5 ml of enzyme added to start the reaction. The thiosulfonate was dissolved in the phosphate buffer in order to diminish decomposition of the salt. In the case of the naphtalene salts nevertheless a decomposition of 10 % was obtained in dissolving the salt. The enzyme was always diluted in the presence of the sulfur donor and phosphate buffer pH 7.4 (0.0125 and 0.02 *M* resp.) and 0.025 % albumin. The reaction was stopped after 5 minutes at 20° C by the addition of 0.5 ml formaldehyde and 2.5 ml ferric nitrate reagent. A heavy precipitate now appeared and was left for 30 minutes in order to obtain complete precipitation. 25 ml of distilled water was then added and the precipitate centrifuged off. The thiocyanate formed was then obtained as usual from the light absorption at 460 mμ.

The nitroprusside reaction for sulfhydryl and disulfide groups was carried out on filter paper according to Mirsky and Anson [13].

RESULTS

Effect of pH. The influence of pH on the rate of thiocyanate formation from thiosulfate and cyanide is shown in Fig. 1. A broad pH-optimum between pH 8 and 9 is observed.

Effect of enzyme concentration. As shown in Fig. 2 the thiocyanate formed in 5 minutes was proportional to the amount of enzyme in the range giving not more than 7.5 μ-equivalents of thiocyanate. This value corresponds to 6 % conversion of the substrate.

Rate of thiocyanate formation. The results are shown in Fig. 3. Also here a linear relation was obtained until about 7 μ-equivalents of thiocyanate had been formed.

Effect of substrate concentration. The initial reaction rate was studied at pH 8.5 and varying thiosulfate concentrations, as shown in Fig. 4. The inhibitory effect of sulfite [3,6] is also shown in the same figure. The effect of varying thiosulfate concentrations was also studied at pH 7.4 (Fig. 5) and also the rate dependence on the cyanide concentration. From Fig. 4 it is evident that raising the concentration of cyanide and thiosulfate from 0.05 to 0.10 *M* has only a slight effect on the rate, in disagreement with the results obtained by Saunders and Himwich [14]. When the curves in Figs. 4, 5 and 6 were replotted

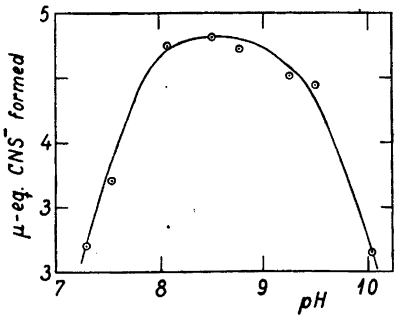

Fig. 1. Effect of pH on rhodanese activity. 0.05 M thiosulfate and cyanide, 0.10 M phosphate, final volume 2.5 ml. 5 minutes incubation at 20° C, pH measured on the complete system.

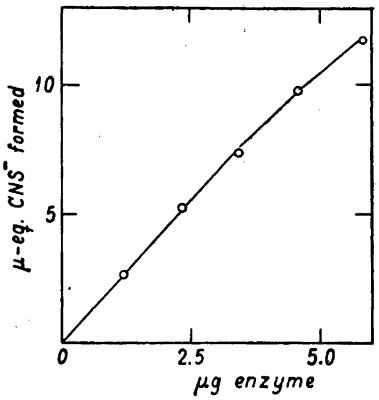

Fig. 2. Relation of enzyme concentration to activity. 0.05 M thiosulfate and cyanide, 0.04 M phosphate, pH 8.5, final volume 2.5 ml. 5 minutes incubation at 20° C.

according to Lineweaver and Burk [15] (the reciprocal of reaction velocity plotted against reciprocal of substrate concentration) approximately straight lines were obtained for the values in Figs. 4 and 6 but not for Fig. 5. The deviations occur at the lower thiosulfate concentrations and are attributed to the inhibitory effect of cyanide [6,14,16]. Consequently no Michaelis constants for thiosulfate and cyanide have been calculated, as the kinetics for the rhodanese reaction are more complex than the simple Michaelis-Menten theory [17] permits. Not only one of the initial reactants (cyanide), but also at least one of the reaction products (sulfite), inhibits the enzyme. The effect of an excess of the other reaction product (thiocyanate) on the velocity could not be investigated, as no satisfactory analytical method for following the initial reaction rate was in this case available.

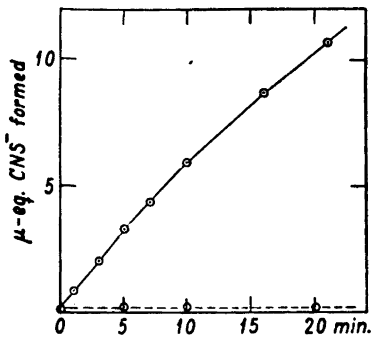

Fig. 3. Reaction velocity and time. 0.05 M thiosulfate and cyanide, 0.04 M phosphate, pH 8.5, final volume 2.5 ml. Temperature 20° C. ⊙ 1.2 μg enzyme. ○ Blank, containing albumin, no enzyme.

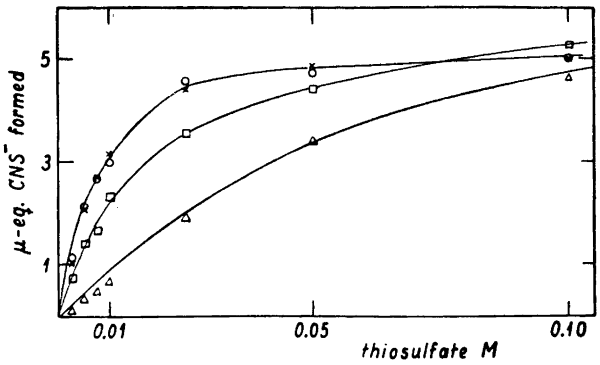

Fig. 4. Relation of thiosulfate concentration to activity at pH 8.5. □ 0.10 M cyanide, 0.08 M phosphate. ⊙ 0.05 M cyanide, 0.04 M phosphate. × 0.025 M cyanide, 0.02 M phosphate. △ 0.05 M cyanide, 0.04 M phosphate and 0.05 M sulfite. Final volume 2.5 ml, 5 minutes incubation at 20° C.

Inhibition with cysteine. It was previously reported by us [6] that cysteine did not inhibit rhodanese but on the contrary could prevent the inhibition with cyanide. These findings were in disagreement with the results obtained by Saunders and Himwich [14] who observed a strong inhibition with cysteine. That this compound failed to inhibit the enzyme in our experiments could be due to a too high proportion between substrate and inhibitor (0.04 and 0.02 *M* resp.), but it was then to be expected that an inhibition should be obtained if the substrate concentration was lowered sufficiently. This is in fact the case, as shown in Table 1. If only the thiosulfate concentration was lowered, no inhibition was observed, presumably because the inhibitory effect was cancelled by the protective effect of the cysteine against cyanide. The demonstration of a cysteine inhibition supports the theory of a disulfide bond as the active group in rhodanese. As previously [7] pointed out, the complex formed between rhodanese and cysteine must be freely dissociable and the cysteine easily displaced by thiosulfate, as preincubation of the enzyme and cysteine has no apparent effect on the inhibition [6]. It must be mentioned that there is still an unexplained discrepancy between our results and those of Saunders and Himwich, as they found 83 % inhibition with 0.04 *M* cysteine using the high substrate concentration of 0.42 *M* thiosulfate and 0.14 *M* cyanide.

Table 1. Inhibition with cysteine. The test system contained except indicated constituents 0.05 M phosphate and 4.3 μg rhodanese in a final volume of 2.5 ml. pH 7.4.

Cysteine *M*	Thiosulfate *M*	Cyanide *M*	CNS^- formed. μ-eq.	Inhibition %
—	0.005	0.005	3.10	—
0.05	0.005	0.005	2.81	9.4
—	0.0025	0.0025	1.74	—
0.10	0.0025	0.0025	1.13	35
—	0.0025	0.05	1.29	—
0.10	0.0025	0.05	1.31	0

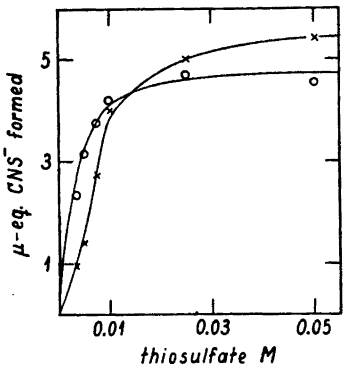

Fig. 5. Relation of thiosulfate concentration to activity at pH 7.4. × 0.05 M cyanide, 0.05 M phosphate. ⊙ 0.01 M cyanide, 0.05 M phosphate. Final volume 2.5 ml, 5 minutes incubation at 20° C.

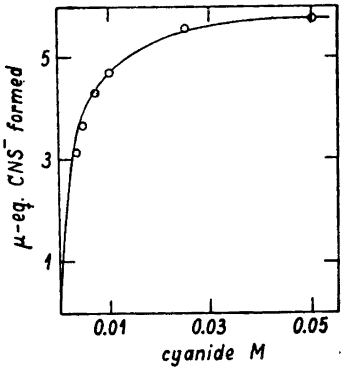

Fig. 6. Relation of cyanide concentration to activity at pH 7.4. 0.05 M thiosulfate, 0.05 M phosphate, final volume 2.5 ml. 5 minutes incubation at 20° C.

Effect of temperature. The relation between reaction rate and temperature was studied between 0° and 55° C (Fig. 7). A temperature optimum of about 50° C was observed (the value 38—40° C was reported by Saunders and Himwich [14]). When the logaritm of initial rate was plotted against the reciprocal of temperature according to Arrhenius [18], a straight line was obtained between 0° and 45° C, Fig. 8. Above this temperature inactivation occurs [6]. The corresponding value for the apparent activation energy was 7 900 cal.

Reversibility. Lang [2] could not demonstrate any reversibility of the rhodanese reaction, but Rosenthal [3] attributed the decrease of thiocyanate formation obtained with excess sulfite to a reversibility effect. As sulfite is a strong inhibitor of the enzyme [6] this conclusion was probably erroneous, and Goldstein and Rieders [4] reported also recently that the reaction was irreversible. Similar investigations had independently been carried out by us, and since we used the crystalline enzyme and a more sensitive method for determining cyanide than the one used by Goldstein and Rieders, our results are reported here. When rhodanese (90 RU or a 100-fold excess of the amounts used in following the forward reaction) was incubated with thiocyanate and sulfite, as described in "Methods", about 0.001 μ-equivalents of cyanide, corresponding to a concentration of $0.5 \cdot 10^{-6}$ *M* in the test, were obtained. The equilibrium constant $K = \frac{[CNS^-][SO_3^{2-}]}{[CN^-][S_2O_3^{2-}]}$ is then of the magnitude $1 \cdot 10^{10}$ and the reaction thus practically irreversible.

Substrate specifity. The ability of different thiosulfonates to replace thiosulfate in the rhodanese reaction is shown in Table 2. The results indicate that the activity of the thiosulfonate is roughly correlated with the electropositivity of the radical attached to the hexavalent sulfur atom, as the activity is increased when the hydroxyl group in the thiosulfate is replaced by an aromatic radical and further increased, when instead an aliphatic radical is introduced.

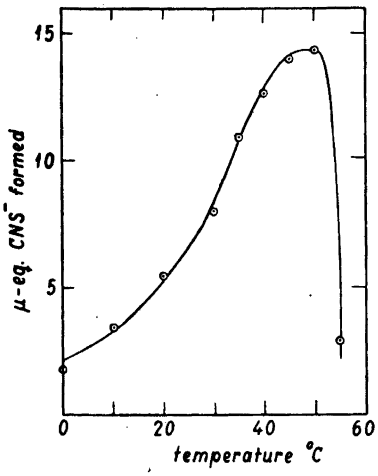

Fig. 7. Effect of temperature on rhodanese activity. 0.05 M thiosulfate and cyanide, 0.04 M phosphate, pH 8.5, final volume 2.5 ml. 5 minutes incubation at temperature indicated. At 0—30° C 3 μg rhodanese, at 35—55° C 1.5 μg rhodanese in the test. Activity values from the latter temperature interval corrected to correspond with the higher enzyme concentration.

Substitution in the benzene ring of the aromatic thiosulfonates seems on the other hand to have only a small effect on the activity. The difference between the previously reported value for *p*-toluene thiosulfonate versus thiosulfate and the value now obtained (457 and 389 % resp.) is ascribable to the change in the test system. It must be added that no studies on the kinetics of the thiosulfonate reactions have been carried out, and the values presented in Table 2 merely indicate the magnitude, but may not represent the initial reaction velocity in each case.

Sulfur groups in the enzyme. The native enzyme (recrystallized and in the form of crystals from ammonium sulfate) gave a strong positive reaction

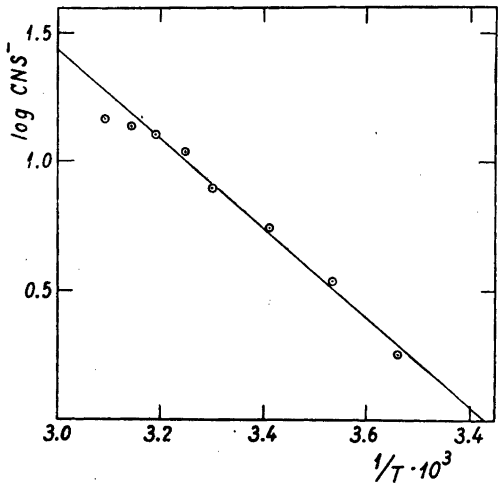

Fig. 8. Arrhenius equation plot of activity values from Fig. 7. Inactivation occurs above 45° C.

Table 2. Thiosulfonates as substrates for rhodanese. The test system as described in the text contained 1.5 μg crystalline rhodanese, except in the case of butane thiosulfonate, where the test contained 0.75 μg enzyme.

Compound			CNS^- formed μ-eq.	Activity of thiosulfate system %
Thiosulfate			1.46	100
Benzene thiosulfonate			4.44	304
p-Chlorobenzene	»		5.59	383
p-Bromobenzene	»		4.80	329
p-Toluene	»		5.67	389
(1)-Naphtalene	»	*	1.64	113
(2)-Naphtalene	»	*	1.93	132
Etane	»		15.4	1 054
n-Butane	»		10.2	1 396

* Partial decomposition occurred when the compound was dissolved.

with nitroprusside + cyanide, but with nitroprusside + ammonia a negative reaction appeared. Rhodanese thus contains disulfide bonds, but no "unmasked" sulfhydryl groups. A negative sulfhydryl reaction was however obtained with the enzyme in the presence of thiosulfate. This is apparently not in agreement with the reaction mechanism proposed for the enzyme [7], as the active disulfide bond in the enzyme is here supposed to react with thiosulfate under formation of a sulfhydryl group. Control experiments showed that thiosulfate had no inhibiting effect on the nitroprusside reaction given by cysteine. It is of interest in this connection that papain, which is activated by cyanide and then gives a positive nitroprusside reaction [19] is also activated by thiosulfate, but then gives a negative nitroprusside reaction [20]. Heat denatured rhodanese gave a strong positive reaction with nitroprusside and ammonia, demonstrating the presence of "masked" sulfhydryl groups in the enzyme. This may be related to the inhibition observed with certain sulfhydryl reagents [6,14].

DISCUSSION

The reaction mechanism for rhodanese referred to before was developed from the fact that rhodanese is inhibited by cyanide in the absence of thiosulfate, which makes the assumption of a primary enzyme-thiosulfate complex more likely than a primary enzyme-cyanide complex. As inhibition data indicated that rhodanese contained an active disulfide group, it was consequently assumed that thiosulfate reacts with the enzyme through this group under the formation of a sulfenyl thiosulfate according to

$$\left[\text{Enzyme}\right]\begin{matrix}-S\\ |\\ -S\end{matrix} + S_2O_3^{2-} \rightarrow \left[\text{Enzyme}\right]\begin{matrix}-S-S_2O_3^-\\ \\ -S^-\end{matrix} \qquad (1)$$

This compound then decomposes in the presence of cyanide according to

$$\left[\text{Enzyme}\right]\begin{matrix}-S-S_2O_3^-\\ \\ -S^-\end{matrix} + CN^- \rightarrow \left[\text{Enzyme}\right]\begin{matrix}-S\\ |\\ -S\end{matrix} + SO_3^{2-} + CNS^- \qquad (2)$$

The latter reaction probably takes place in two steps as

$$\left[\text{Enzyme}\right]^{-S-S_2O_3^-}_{-S^-} + CN^- \rightarrow \left[\text{Enzyme}\right]^{-S-SCN}_{-S} + SO_3^{2-} \qquad (2a)$$

giving a sulfenyl thiocyanate, which then reacts according to

$$\left[\text{Enzyme}\right]^{-S-SCN}_{-S^-} \rightarrow \left[\text{Enzyme}\right]^{-S}_{-S} + CNS^- \qquad (2b)$$

if the free enzyme is regenerated, or according to

$$\left[\text{Enzyme}\right]^{-S-SCN}_{-S^-} + S_2O_3^{2-} \rightarrow \left[\text{Enzyme}\right]^{-S-S_2O_3^-}_{-S^-} + SCN^- \qquad (2c)$$

if the enzyme-thiosulfate complex is regenerated. Sulfenyl thiocyanates do in fact rapidly condense with thiols [21] under formation of thiocyanate and a disulfide in support of equation 2 b (or 2 c). When analogies are sought for equation 2 a, one objection against the proposed reaction mechanism arises. Cyanide does not split off sulfite from sulfenyl thiosulfates. Instead thiosulfate[22] is formed according to

$$RS-S_2O_3^- + CN^- \rightarrow RSCN + S_2O_3^- \qquad (3)$$

If reaction 3 occurred instead of reaction 2 a, the enzyme would be without catalytical effect. The activation of thiosulfate brought about by combination with rhodanese may however weaken the sulfur-sulfur bond in thiosulfate, with the result that sulfite instead of thiosulfate is split off from the intermediate compound. The disulfide bond in rhodanese must be endowed with unique properties in this respect, as no other disulfide containing protein has the catalytical properties of rhodanese. Another example of such specific catalytic properties of a protein bound group is afforded by the sulfhydryl group in different dehydrogenases [23].

SUMMARY

The effect of different factors on the rhodanese-catalyzed reaction between thiosulfate and cyanide has been studied. From the relation between temperature and activity the apparent heat of activation was calculated to 7 900 cal. The reaction is inhibited by cysteine if the concentration of substrate is sufficiently low. The irreversibility of the reaction was confirmed. Different thiosulfonates were investigated with respect to their ability to function as sulfur donors in the enzyme reaction. The crystalline enzyme contains disulfide and sulfhydryl groups, as shown with the nitroprusside reaction. The reaction mechanism is discussed.

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