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On the Substrate Specificity of Rhodanese

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Lang [1] showed in 1932 that cyanide in the presence of thiosulfate and liver tissue was converted to thiocyanate. An enzyme present in the liver and named rhodanese was shown to be responsible for the reaction. Lang also found that liver tissue catalyzed the formation of thiocyanate from colloidal sulfur and cyanide and assumed that rhodanese was responsible also for the latter reaction. Rhodanese was later studied by Saunders and Himwich [2,3] and Bénard *et al.*[4], using tissue homogenates as a source of the enzyme. Different sulfur containing compounds were investigated with respect of their ability to replace thiosulfate as sulfur donor in the enzyme system. The French workers claimed that sodium hydrosulfite $Na_2S_2O_4$ was active in this respect, other compounds were found to be practically inactive. The present study was undertaken in order to reinvestigate the results reported for colloidal sulfur and hydrosulfite with the use of a purified enzyme preparation. In connection with the work the efficiency of some other compounds as sulfur donors in the rhodanese system was investigated.

METHODS

Materials. Colloidal sulfur solution was prepared as follows: 40 mg sulfur was dissolved in 100 ml hot 96 % ethanol, the solution was rapidly cooled to room temperature, diluted to 500 ml with distilled water and then evaporated *in vacuo* to a volume of 200 ml. The solution had to be prepared fresh each day because sulfur gradually precipitated out of the solution.

Sodium ethyl thiosulfate (Bunte's salt) was prepared according to Otto and Troeger [5] and recrystallized from ethanol.

Sodium *p*-toluene thiosulfonate was prepared from *p*-toluene sulfonchloride and sodium sulfide according to the directions of Troeger and Linde [6]. It was recrystallized from ethanol.

Sodium hydrosulfite, potassium ethyl xanthate and sodium diethyldithiocarbamate were commercial products and used without further purification.

Rhodanese was purified from beef liver using an improvement of the method described in a preceding paper [7]. The liver was extracted, treated with lead acetate and fractionated with ammonium sulfate as described before. It was then dialyzed against 0.02 *M* sodium acetate, the pH adjusted to 5.0 and ethanol added to 10 % by volume. The solution was shaken in the cold room for two minutes with chloroform and the precipitated proteins and excess chloroform was centrifuged off. The supernatant was then fractionated with acetone at −5° C and the precipitate appearing between 25 and 45 % acetone was collected and dissolved in *M*/15 disodium phosphate. The pH was adjusted to 8.0 and the enzyme precipitated by raising the acetone concentration to 50 %. The enzyme was dissolved in *M*/150 phosphate buffer pH 7.4 and again precipitated by raising the acetone concentration to 50 %. The last two steps gave only an insignificant purification and can advantageously be omitted. The acetone remaining in the precipitate was now removed by evaporation at −18° C and the precipitate was then dissolved in distilled water. The pH was adjusted to 4.6 and the solution fractionated with ammonium sulfate. The precipitate appearing between 7.5 and 46 % saturation was collected and dissolved in *M*/15 disodium phosphate. The preparation thus obtained represented a 400-fold purification from the starting material and had a specific activity over twice that of the preparation obtained by the original method. It was stored for 3 months at −18° C without any loss of activity. When a 0.5 % solution of this preparation was examined in the "Spinco ultracentrifuge" a homogenous boundary was observed with $s_{20} = 2.76$ *S*. A similar preparation was then studied with respect to solubility in ammonium sulfate solutions of different concentrations and appeared homogenous. When the concentration of ammonium sulfate was brought to incipient precipitation of the enzyme, the latter crystallized in small plates. Further details concerning the new method of purification will be published in the near future.

Liver homogenates were prepared by disintegrating frozen, thawed beef liver with 3 volumes distilled water in an "Atomix" blender and filtering the obtained suspension through glass wool in order to remove coarse particles.

Determinations. The formation of thiocyanate from an equimolar mixture of the sulfur donor and cyanide was with two exceptions studied in the following test system. To 1.0 ml of a 0.125 *M* solution of the sulfur donor studied (in the case of hydrosulfite this solution was 0.08 *M* with respect to NaOH in order to obtain the desired pH in the test) was added 0.5 ml of a 0.40 *M* phosphate buffer (equimolar mixture of disodium and monopotassium phosphate) and 0.5 ml of the properly diluted enzyme. The reaction was started by adding 0.5 ml of a 0.25 *M* KCN solution and stopped after 5 minutes at 20° C by adding 2.5 ml of a ferric nitrate-nitric acid reagent. The latter contained 100 g $Fe(NO_3)_3$ 9 H_2O and 200 ml 65 % HNO_3 per 1 000 ml. The test solution was diluted to 30 ml and in the case of ethyl xanthate and diethyldithiocarbamate centrifuged, as the latter compounds gave precipitates with the ferric nitrate reagent. 30 minutes after the addition of ferric nitrate the optical density at 4 600 Å was determined in the Beckman spectrophotometer. The values obtained were compared with those obtained using a standard solution of thiocyanate in the test system. A blank determination was always carried out, using 0.5 ml distilled water instead of enzyme. The pH in this test system was 8.75 which was found to be the pH-optimum for the enzyme when thiosulfate was the substrate. In the case of *p*-toluene thiosulfonate preliminary experiments showed, however, that the spontaneous reaction between this compound and cyanide was so rapid at pH 8.75, that any effect of the enzyme would be imperceptible. By lowering the pH to 7.4 better conditions were obtained and the test

was in this case carried out as follows. At the start of reaction 1.0 ml of a solution, which was 0.125 *M* with respect to cyanide and 0.20 *M* with respect to phosphate and adjusted to pH 7.4 with HCl was added to 1.0 ml of a 0.125 *M* solution of *p*-toluenethiosulfonate and 0.5 ml enzyme. After 5 minutes at 20° C 2.5 ml ferric nitrate reagent and 0.5 ml 38 % formaldehyde were added and the test solution diluted to 30 ml. A precipitate was then centrifuged off, and the optical density at 4 600 Å was determined 30 minutes after the addition of ferric nitrate. Formaldehyde was added in this test in order to remove the interfering colour, which *p*-toluene thiosulfonate and *p*-toluene sulfinate (formed during the enzyme reaction) give with ferric ions. The activity of rhodanese in the *p*-toluene thiosulfonate test was compared with the activity obtained in a similar test system, which contained thiosulfate instead of *p*-toluene thiosulfonate. In the case of colloidal sulfur it was for practical reasons not possible to use a higher concentration of this compound than equivalent to 0.005 *M*. The same concentration of of cyanide and pH 8.75 were used. The components in the test were the following: 2.0 ml colloidal sulfur solution, 0.4 ml enzyme and 0.1 ml of a solution of pH 8.75, containing 0.125 *M* cyanide and 0.20 M phosphate. After 5 minutes incubation at 20° C, 2.5 ml ferric nitrate reagent was added and the test solution diluted to 30 ml. 30 minutes later the optical density at 4600 Å was determined. Turbidity was removed by a preceding centrifugation.

In these investigations the effect of dilution on the enzyme was considered. During the purification experiments it was found that the purified enzyme was considerably inactivated when it was diluted with distilled water or buffer before the assay. We never observed this inactivation with crude enzyme preparations in contrast to Saunders and Himwich [3] and Rosenthal *et al.*[8], who observed a rapid inactivation of rhodanese when tissue homogenates were diluted over a certain range. These authors also reported that the inactivation could be prevented by diluting the enzyme with a solution of thiosulfate. Thiosulfate could in fact prevent the inactivation observed with the purified beef liver enzyme. In consequence of this the assay method as described by Cosby and Sumner [9] was abandoned and the purification of rhodanese was followed with a new method of assay. The cyanide concentration was here 0.05 *M*, the thiosulfate concentration 0.10 *M*, the pH was 8.75 and the enzyme was always diluted with thiosulfate before assay. One μq protein of our purified enzyme as described above was in this test able to form 1.8 μ equivalents CNS^- during 5 minutes incubation at 20° C. In the experiments with the different sulfur donors the enzyme was diluted before incubation with a 0.01 *M* solution of the sulfur donor (in the case of hydrosulfite this solution was neutralized with NaOH). The only exception was colloidal sulfur in which case the enzyme was diluted with distilled water. The decrease in activity was here compensated for by comparing the obtained results with those obtained using the diluted enzyme in a simultaneous test in which colloidal sulfur was replaced by thiosulfate in the same molar concentration.

RESULTS

The results obtained with the purified enzyme are summarized in Table 1.

Because no activity was obtained with hydrosulfite and colloidal sulfur in contrast to previous reports, the effect of a crude liver homogenate on these systems was investigated. In the hydrosulfite system 0.5 ml homogenate di-

luted 1—20 gave 0.17 μeq. CNS^-, in the corresponding thiosulfate system the same amount of homogenate gave 7.02 μ eq. CNS^-. The activity obtained with hydrosulfite was thus 2.5 % of that obtained with thiosulfate. The colloidal sulfur and corresponding thiosulfate system contained 0.4 ml undiluted homogenate, which was adjusted with NaOH to pH 8.75 due to the weak buffer capacity of the system. With thiosulfate 4.35 μ eq. and with colloidal sulfur 1.96 μ eq. CNS^- were obtained. As the kinetics of the thiocyanate formation in these systems does not follow the zero order curve, the fractional activity can in this case not be expressed in %, but the activity of the colloidal sulfur is evident.

Table 1. Specificity of purified rhodanese

The test systems as described in the text contained 7.5 μg enzyme protein with the exception of *p*-toluene thiosulfonate and corresponding thiosulfate system, which contained 3 μg protein, and colloidal sulfur and corresponding thiosulfate system, which contained 60 μg protein.

Sulfur donor	Concentration of sulfur donor *M*	pH	CNS^- formed μeq.	Activity of thiosulfate system %
Thiosulfate	0.05	8.75	10.9	100
Ethyl thiosulfate	0.05	8.75	0	0
Ethyl xanthate	0.05	8.75	0.18	1.7
Diethyldithiocarbamate	0.05	8.75	0	0
Hydrosulfite	0.05	8.75	0.51	4.7
Thiosulfate	0.05	7.40	2.54	100
p-Toluene thiosulfonate	0.05	7.40	11.6	457
Thiosulfate	0.005	8.75	6.55	100
Colloidal sulfur	0.005	8.75	0	0

DISCUSSION

The results obtained with purified rhodanese showed that thiosulfate and *p*-toluene thiosulfonate but not ethyl thiosulfate could function as sulfur donor in the enzyme system. Considering the structure of these compounds [10a,11] it is seen that thiosulfate and *p*-toluene thiosulfonate contain a free thiol group. In ethyl thiosulfate this is blocked and the activity seems thus to be connected with the presence of this thiol group in the substrate molecule.

$^-S-SO_2-O^-$	$^-S-SO_2-C_6H_4CH_3$	$C_2H_5-S-SO_2-O^-$
Thiosulfate	*p*-Toluene thiosulfonate	Ethyl thiosulfate

Gutman [12] also found that no thiocyanate was formed in the spontaneous reaction between ethyl thiosulfate and cyanide under conditions when thiosulfate or aryl thiosulfonates gave a high yield of thiocyanate. In a recent communication it was concluded from inhibition data that the active group in rhodanese was a disulfide bond and a possible reaction mechanism was proposed [13]. Thiosulfate was assumed to form an intermediate enzyme-substrate compound, which decomposes in the presence of cyanide giving thiocyanate, sulfite and the free enzyme. This enzyme-substrate compound would, in its molecule, contain a chain of three sulfur atoms. The lability of such sulfur chains in the presence of cyanide is well known, for instance in the case of polythionates. In consequence of this all compounds containing a thiol group linked to sulfur would be expected to function as substrate for rhodanese. Sodium disulfide was then studied as a possible substrate. It was found, however, that the rate of the spontaneous reaction between the disulfide and cyanide was so high even at pH 7, that it was impossible to investigate the effect of the enzyme. But as a solution of sulfur in sodium sulfide with a composition corresponding to Na_2S_2 contains an equilibrium mixture [14] of Na_2S and Na_2S_4 these results are easily explained. Salts of dithioacids, with the group $-CSS^-$, in which, however, the two sulfur atoms are separated by a carbon atom, were also of interest as possible substrates. Ethyl xanthate and diethyldithiocarbamate were studied but found to be inactive. The latter compound was of special interest as its disulfide, tetraethylthiuram-disulfide is known to react spontaneously with cyanide, giving thiocyanate [15]. Previous workers have established [2,4] that compounds, containing a thiol group not linked to sulfur, are devoid of activity as substrates for rhodanese. Their stabilizing and protecting effect on the enzyme [2,7,13] must, however, be interpreted as a reaction between them and the enzyme.

These investigations do not confirm the claim of Bénard *et al.*[4] that hydrosulfite can function as a sulfur donor in the rhodanese system. Only insignificant amounts of thiocyanate was formed in the presence of the purified enzyme or a liver homogenate. As solutions of hydrosulfite rapidly decompose with the formation of thiosulfate [10b], the results obtained here are attributable to impurities of thiosulfate. This decomposition is retarded in alkali, and the high pH used in the present investigation was thus an advantage.

In the case of colloidal sulfur no formation of thiocyanate was observed with the purified enzyme, but with a liver homogenate a strong reaction was observed. Further experiments showed that this reaction obtained in the presence of liver homogenates was caused by a heat labile factor, also present in blood serum and muscle homogenates, which are entirely devoid of the catalytic activity on cyanide and thiosulfate. The enzyme catalyzing the

reaction between colloidal sulfur and cyanide must thus be different from that catalyzing the reaction between thiosulfate and cyanide. We suggest that the first enzyme is called rhodanese S, while the old name rhodanese is kept for the latter enzyme. The properties of rhodanese S will be reported in a following paper.

SUMMARY

1. Different sulfur containing compounds were investigated with respect to their efficiency as sulfur donors in the rhodanese system.

2. *p*-Toluene thiosulfonate was more active than thiosulfate. Ethyl thiosulfate, ethyl xanthate, diethyldithiocarbamate, hydrosulfite and colloidal sulfur were inactive.

3. The catalytic effect of liver homogenates on the formation of thiocyanate from colloidal sulfur and cyanide is due to an enzyme different from rhodanese.

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