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Crystalline Rhodanese

I. Purification and Physicochemical Examination

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The purification of rhodanese, an enzyme catalyzing the reaction between thiosulfate and cyanide to form sulfite and thiocyanate, was attempted by Lang [1], who obtained a 20-fold purification from liver and by Cosby and Sumner [2], who reported a 100-fold purification from beef liver. The present author [3] later obtained a 150-fold purification from the same source.

In this paper the isolation of rhodanese in the crystalline state from beef liver and some physico-chemical measurements on the enzyme are described. A preliminary report of a part of this work has already been published [4].

EXPERIMENTAL

Determination of Rhodanese Activity and Purity

The successful isolation of the enzyme was made possible only after a suitable assay system had been worked out. None of the earlier methods for determination of rhodanese [1,2,5,6] was found to be applicable to the purified enzyme. Rhodanese is to a large extent inactivated, if diluted with water or buffer before assay, but this inactivation is prevented in the presence of thiosulfate [5-7]. The purified rhodanese is, however, also partly inactivated if incubated with thiosulfate of too high concentration, and it was not found permissible first to mix the diluted enzyme with thiosulfate of the high concentration used in the test and then start the reaction by adding cyanide as in earlier tests [5,6]. Thiosulfate and cyanide had thus to be mixed before the enzyme was added. The enzyme was diluted with dilute thiosulfate containing serum albumin, as the latter was found to increase the activity of the purified enzyme. The effect of albumin may be attributable to a removal of trace metals or a protection against surface denaturation.

The enzyme was assayed with the following test system. After dilution in the presence of 0.0125 *M* thiosulfate and 0.025 % bovine albumin (Armour) 0.5 ml enzyme was added to a mixture of 1.0 ml 0.125 *M* $Na_2S_2O_3$, 0.5 ml 0.20 *M* KH_2PO_4 and 0.5 ml 0.25 *M* KCN. The conditions in the test system were then: thiosulfate 0.052 *M*, cyanide 0.05 *M*, phosphate 0.04 *M* and pH 8.6 and were optimal for the beef liver enzyme. The reaction vessels were 50 ml Erlenmeyer flasks kept in a thermostate at 20° and the enzyme was added from a 0.5 ml transfer pipette, treated with silicone in order to minimize emptying time. After 5 minutes at 20° C the reaction was stopped by the addition of 0.5 ml 38 % formaldehyde, whereupon 2.5 ml of a ferric nitrate reagent (containing 100 g $Fe(NO_3)_3 \cdot 9\ H_2O$ and 200 ml HNO_3, sp.g. 1.40, and distilled water to 1 000 ml) was added. After dilution with 25 ml distilled water, the optical density at 460 mμ in a 1 cm cuvette was determined in the

Beckman DU spectrophotometer. The use of formaldehyde in the colorimetric determination of thiocyanate was found to be a great improvement. When ferric ions are added to a solution containing thiocyanate in an excess of thiosulfate, a blue colour due to a complex of ferric ions and thiosulfate first develops, but rapidly fades, whereupon the red colour of the iron-thiocyanate complex becomes visible. As also the latter colour is unstable, previous investigators have usually determined the red colour after a fixed time interval after the addition of ferric nitrate. But in the presence of formaldehyde no blue colour develops and the colour of the iron-thiocyanate complex is completely stable for at least one hour even in bright daylight.

The test system was calibrated with an argentometrically standardized thiocyanate solution. A plot of thiocyanate formed against amount of enzyme in the test gave an approximately straight line. The relative error of the method is less than 3 %. One rhodanese unit (RU) was taken as the amount of enzyme, which in the test system formed 10 μ-equivalents of thiocyanate, and was equivalent to the density reading 1.08. The purity of the different enzyme preparations was expressed in RU per mg of dry weight. The latter was sometimes determined by drying a sample to constant weight at 105—110° C after removal of salt impurities by dialysis against distilled water. As rhodanese is inactivated by dialysis against distilled water [1], protein determinations were mostly carried out instead of dry weight determinations, in order to avoid losses of valuable material. Protein was determined with the turbidimetrical method given by Bücher [6]. This method was found not to be influenced by the presence of moderate amounts of salts or organic solvents in the sample. The determination was carried out as follows: To the sample, containing about 1 mg of protein, was added 1.0 ml 0.67 % aqueous ammonium sulfate solution and the sample made up to 9.5 ml with distilled water. After the addition of 0.5 ml 1.5 *M* trichloroacetic acid the optical density at 350 mμ in a 1 cm cuvette was determined in the Beckman spectrophotometer. The test was calibrated with a solution of crystalline rhodanese, the dry weight of which was determined after dialysis against 0.01 *M* sodium sulfate and correction of the obtained dry weight for the ash content. The optical density was found to be proportional to the protein content in the test up to 1.6 mg protein and 1 mg of crystalline rhodanese was equivalent to the density reading 0.235 at 1 mm slit width.

Purification

Extraction. Beef liver was reported to be the best source of the enzyme [3], but only 50 % of the total activity in the liver could be extracted, as the rest was bound to sedimentable particles, presumably mitochondria fragments. Different extraction procedures have now been tried in order to improve the yield in the extraction step. The butanol treatment of Morton [9], reported to release many mitochondria linked enzymes into true solution, was found to inactivate a large part of the rhodanese activity. Extraction of minced liver with acetate buffer at pH 5 and 37° C, as described by Kerr and Levy [10] for β-glucuronidase, was found to release about 80 % of the rhodanese, and similar results were obtained by using acetone dried liver as the starting material. However, no improvement in purity was reached after the following step, and none of these time-consuming procedures were consequently used in the final fractionation scheme. Treatment of liver homogenate with Teepol (a surface active agent), reported by Walker and Levvy [11] to release β-glucuronidase, was found to destroy the rhodanese. Autolysis of liver brei at an alkaline pH, which was found by Baumann *et al.*[12] to solubilize liver esterase, had no effect on rhodanese. The extraction procedure used in the previous work [3] was consequently retained. It consists of homogenizing beef liver (usually first frozen and then thawed, as this treatment was found to increase the yield, *cf.* Walker and Levvy [11]) with water and then precipitation of some impurities by the addition of a small amount of basic lead acetate. After centrifugation a deep red, usually clear extract is obtained. Any turbidity, sometimes encountered when the liver was stored a long time before use, was removed during the following steps, and did not affect these.

Ammonium sulfate fractionation. The fractionation was carried out as described before [3] first at pH 3.8 in the cold room, and collecting the enzyme between 1.36 and 1.91 *M* ammonium sulfate. The precipitates obtained during fractionation were separated from the solutions either by centrifugation in a refrigerated centrifuge or by suction filtration with the aid of Hyflo Super Cel. The yields obtained with the latter procedure were

usually 10—20 % lower than with the first, presumably due to surface-denaturation of the enzyme during the filtration. The precipitate, containing the enzyme, was dissolved in a solution containing secondary phosphate and thiosulfate, the latter present in order to stabilize the enzyme during the subsequent fractionation with ammonium sulfate, carried out at pH 7.7. The enzyme was here collected between 1.95 and 2.55 *M* concentration of ammonium sulfate.

Dialysis. Before the obtained preparation could be fractionated with organic solvents, the remaining ammonium sulfate had to be removed by dialysis. Rhodanese is partly inactivated by dialysis against distilled water, but can be dialyzed against buffers in a certain pH-range without inactivation. After the appropriate conditions for the following acetone fractionation step had been established, it was found convenient to remove the remaining ammonium sulfate by dialysis against 0.01 *M* sodium acetate. No appreciable inactivation was encountered in this step.

Organic solvent fractionation. The conditions for fractionation with organic solvents at low temperature according to the principles given by Cohn *et al.*[13] were now investigated. In preliminary experiments the solubility of the salt fractionated enzyme, freed from ammonium sulfate by dialysis against distilled water, was studied in 20 % ethanol by volume at varying pH. The desired pH and ionic strength was obtained with acetate buffers; the pH of the system measured in the absence of ethanol at room temperature. At an ionic strength of 0.0015 a pronounced solubility minimum at pH 6.0 was observed, at the ionic strength 0.01 the solubility curve was more flat. Better separation of rhodanese and impurities was obtained on the acid side of the solubility minimum and at the higher ionic strength. The enzyme could be freed from some impurities by fractionation with ethanol at pH 5, ionic strength 0.01, but attemps to precipitate out the enzyme from the supernatant by raising the ethanol concentration resulted in an appreciable inactivation of rhodanese, even when all the precautions given by Deutsch [14] were followed. Other organic solvents were then tried, and it was found that rhodanese could be precipitated at low temperature with acetone or methanol without any appreciable inactivation. It was then decided to use acetone as precipitant, and the conditions finally adopted were as follows: Impurities were removed at pH 4.9, ionic strength 0.006, temperature —5° C and acetone concentration 35 % by volume, and the rhodanese then precipitated by raising the acetone concentration to 50 %. Rhodanese has thus a considerable solubility in solutions containing organic solvents, which may be connected with its high optical density at 280 mμ, corresponding to a high content of aromatic amino acids. The solubility of rhodanese in organic solvents could be depressed by the addition of Zn-ions, and preliminary experiments were carried out according to the directions given by Cohn *et al.*[15], but no purification was obtained. The solubility was also depressed in the presence of sulfate ions, but no systematic survey of the purification possibilities in this system was undertaken.

Further fractionation with ammonium sulfate. The acetone remaining in the preparation was removed by dialysis against 0.01 *M* sodium acetate, and the enzyme was further purified by precipitation with 1.75 *M* ammonium sulfate at pH 4.5. The enzyme had now a purity of about 200 RU/mg and was ready for crystallization, if a solution of the enzyme had only a faint yellow colour. Any remaining hemoglobin is difficult to separate from the enzyme by crystallization, but can conveniently be removed by fractionation with ammonium sulfate at pH 7.8, in which case the hemoglobin separates with the precipitate first obtained. When the precipitate from the acid ammonium sulfate was suspended in water, 0.01 *M* sodium acetate or dilute ammonium sulfate pH 7.8, an insoluble fraction sometimes remained, which after centrifugation was obtained as a gelatinous pellet (with a brown precipitate at the bottom). This gelatinous pellet was soluble in 0.1 *M* sodium thiosulfate, giving a solution containing rhodanese of high purity. From this solution the enzyme could be crystallized as usual. The "thiosulfate soluble rhodanese" may represent a polymer in which the enzyme molecules are held together with disulfide bonds, which are broken in the presence of thiosulfate. Its formation could be reduced by carrying out the acid ammonium sulfate precipitation at room temperature and rapidly (in a few hours) collecting the precipitate.

THE CRYSTALLINE ENZYME

Crystallisation. It has thus far been possible to obtain rhodanese as microscopically visible crystals only from ammonium sulfate at pH 7.8. At pH 5.5 a pronounced prethixotropy could be obtained by a gradual addition of ammonium sulfate to the purified enzyme, but no visible crystals were obtained. From ammonium sulfate at pH 7.8 the enzyme crystallized either as rectangular plates (Fig. 1) or as elongated prisms (Fig. 2) or as mixtures of both. They were composed of the same protein as a preparation, which first crystallized as elongated prisms, was recrystallized as plates, and another preparation which first crystallized as plates, was recrystallized as prisms. No conclusions could be drawn concerning the factors governing the appearance of the different crystal forms. Similar results has been obtained with other enzymes [16,17]. The best way to crystallize the enzyme was to precipitate it with ammonium sulfate at pH 7.8 and then dissolve the precipitate in a small volume of about 1 *M* ammonium sulfate of the same pH, when the enzyme immediately crystallized. Crystallization was also accomplished by gradually adding ammonium sulfate to the solution, or even by precipitating the pure enzyme with ammonium sulfate and leave the precipitate in the refrigerator, whereupon the amorphous precipitate in a few days was converted into crystals. Rhodanese could also be crystallized by dialysis against ammonium sulfate of increasing concentration, but no advantage was found in the latter method. The enzyme was sometimes unstable during the crystallisation, as losses up to 25 % were encountered.

Isolation experiment. A typical preparation of crystalline rhodanese is described as follows: 6.70 kg frozen, thawed beef liver was disintegrated in a Turmix blendor together with tap water, 2.5 l water used for each kg liver. The homogenate contained 600 000 RU with a purity of 0.288. 670 ml 20 % basic lead acetate (Sw. P.) was then added to the homogenate and the suspension left over night in the cold room. The suspension was then centrifuged at room temperature, giving 16.0 l turbid extract, containing 396 000 RU of purity 1.07. The extract was brought to + 4° C and to it was added 876 g ammonium sulfate and the pH adjusted to 3.8 with 720 ml 1 *M* HCl. After the precipitate had settled 2 450 g ammonium sulfate was added. (Better yields are, however, obtained if the first precipitate is removed before the ammonium sulfate concentration is increased [3]). After 2 hours the precipitate was removed by suction filtration with the aid of 1 200 g Hyflo Super Cel. 14.4 l turbid filtrate was obtained, to which was added 1 225 g ammonium sulfate and the precipitate was left over night to settle. The precipitate was filtered by suction with the aid of 144 g Hyflo Super Cel and the filtrate discarded. The enzyme was eluted from the filter cake with 700 and 300 ml of a solution 0.05 *M* with respect to Na_2HPO_4 and $Na_2S_2O_3$. 875 ml clear, reddish-brown solution was obtained, containing 177 000 RU of purity 16.8. To this solution was added 490 g ammonium sulfate, dissolved in 875 ml water and containing 4.2 ml ammonia. After 3 hours at room temperature the precipitate was removed by centrifugation and to the 1 960 ml clear supernatant obtained 196 g ammonium sulfate was added. The precipitate was removed by centrifugation in a refrigerated centrifuge after 12 hours at + 4° C and dissolved in 150 ml 0.01 *M*

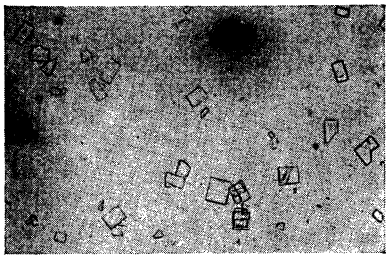

Fig. 1. Crystalline rhodanese as plates, × 150.

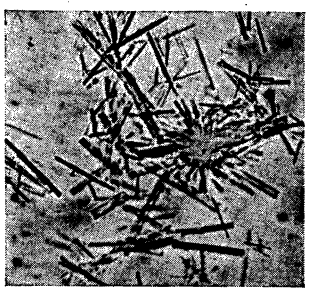

Fig. 2. Crystalline rhodanese as elongated prisms, × 300.

sodium acetate, giving 218 ml deep red solution, containing 130 000 RU of purity 34.2. The remaining ammonium sulfate was removed by dialysis at + 4° C against large volumes of 0.01 *M* sodium acetate, and to the 245 ml solution, still containing 130 000 RU, was added 25 ml 0.1 *M* acetic acid. The solution, which now had a pH of 4.9 was cooled until ice formation was obtained, whereupon 140 ml acetone was gradually added. After 3 hours at −5° C the suspension was centrifuged at the same temperature in a refrigerated centrifuge. From the 370 ml supernatant obtained, the enzyme was precipitated by the addition of 110 ml acetone. After an additional 3 hours at −5° C the precipitate was separated as before and dissolved in 100 ml cooled 0.01 *M* sodium acetate. 110 ml turbid, pink solution, containing 86 700 RU of purity 77.6 was obtained. The remaining aceton was removed by dialysis against 4 l 0.01 *M* sodium acetate at + 4° C. A precipitate appearing in the dialysis bag was centrifuged off, whereupon 122 ml solution, containing 82 700 RU was obtained. The pH was adjusted to 4.5 with 25 ml 0.1 *M* acetic acid and 126 ml 3.78 *M* ammonium sulfate was added. After 3 hours at + 4° C, the precipitate was centrifuged off in a refrigerated centrifuge and suspended in 15 ml 1 *M* ammonium sulfate, pH 7.8. The insoluble portion (containing 13 300 RU "thiosulfate soluble rhodanese") was centrifuged off and 17.3 ml of a solution containing 58 100 RU of purity 201 was obtained. The pH was adjusted to 7.8 with 0.2 ml 1 *M* ammonia, and the enzyme precipitated by the addition of 10.2 ml 3.78 *M* ammonium sulfate pH 7.8. The precipitate was centrifuged off and suspended in 4 ml 0.95 *M* ammonium sulfate pH 7.8. Beautiful crystals in the form of plates were immediately obtained, and the crystallisation was completed by the addition of 0.5 ml 3.78 *M* ammonium sulfate pH 7.8. The crystals were centrifuged down and dissolved in 5 ml distilled water, giving 6.2 ml solution containing 45 800 RU of purity 257. To this solution was added 3 ml 3.78 *M* ammonium sulfate pH 7.8 and an amorphous precipitate was obtained. This was put in the refrigerator and was found next day to have changed into needle-like prisms. 0.5 ml 3.78 *M* ammonium sulfate pH 7.8 was added in order to complete crystallisation. The crystals were separated by centrifugation and dissolved in 5 ml distilled water, giving 6.5 ml solution containing 33 500 RU of purity 267, correspond-

ing to 125 mg of the purified enzyme. The total purification from the starting material was 930 with a total yield of 5.6 %.

Absorption spectrum. The absorption spectrum of crystalline rhodanese is shown in Fig. 3 as determined in the Beckman DU spectrophotometer. The spectrum was not changed in the presence of thiosulfate or cyanide, and no formation of an enzyme-substrate compound could thus be demonstrated spectrophotometrically.

Stability. The crystalline enzyme was more unstable than impure preparations. The enzyme lost about 10 % of its activity in 4 days, when stored as a 1 % solution in phosphate buffer in the refrigerator. It could be frozen without inactivation and is best stored in this condition.

Electrophoresis. Recrystallized rhodanese was investigated in the analytical Tiselius electrophoresis apparatus. The long section cell was used and measurements were carried out in phosphate buffers of ionic strength 0.1 at pH 5.52, 6.53 and 7.44. At the last pH the enzyme appeared homogenous, but at pH 6.53 two components and at pH 5.52 four components were obtained. The enzyme was, however, unstable during the preliminary dialysis in the latter two experiments, thus at pH 6.53 a turbidity and at pH 5.52 a precipitate appeared, which were removed by centrifugation prior to electrophoresis. No definite conclusions concerning the homogenity of the crystalline enzyme could then be drawn from these experiments, as the enzyme was unstable. Nor was it possible to determine the isoelectric point of rhodanese, but even at pH 5.52 all the components migrated anodically, which means that the isoelectric point must be lower, in accordance with observations on impure enzyme [3]. It is of interest that all the patterns obtained in the descending limb with the crystalline enzyme showed a "spike", similar to the β-anomaly of blood serum and also observed with crystalline aldolase [17]. This spike was in the case of aldolase connected with a precipitation of protein, observed in the descending boundary.

Ultracentrifugation. The sedimentation constant for the recrystallized enzyme was determined in the Spinco analytical ultracentrifuge, Model E. Measurements were made at room temperature (20—23° C) in the analytical rotor, type A, at a speed of 59 780 rpm. The sedimentation constants were calculated as described by Shulman [18], and corrected to standard conditions according to Svedberg and Pedersen [19]. The sample contained except rhodanese phosphate buffer pH 7.44, ionic strength 0.1 and 0.17 *M* NaCl. Two determinations were made at 0.32 and 0.20 % protein concentration, and the values obtained for S_{20} were 3.03 and 2.91 · *S* respectively. The value 3.0 · *S* was taken as the sedimentation constant for rhodanese. Only one component was observed at pH 7.4, 6.5 and 5.5 in contrast to the electrophoresis experiments.

Diffusion. The diffusion constant was determined in the long section electrophoresis cell as described by Longsworth [20]. The boundary was, however, observed by the schlieren method and the diffusion followed only in that limb, into which the protein was displaced during the compensation, as the boundary in the other limb was found to be distorted. The diffusion constant was calculated according to the maximum ordinate-area method from the slope of the straight line, obtained by plotting $1/H_m^2$ (H_m:maximum ordinate) against time. The values were reduced to standard conditions (water at 20° C)

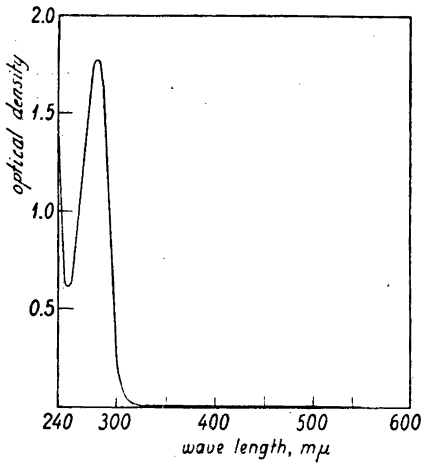

Fig. 3. Absorption spectrum of crystalline rhodanese: D = 1 cm., 0.1 % rhodanese in phosphate buffer pH 7.4.

using the formula given by Sumner *et al.*[21]. Two experiments were carried out in phosphate buffer of ionic strength 0.1 and pH 7.44. In one experiment the protein concentration was 0.176 % and the diffusion was allowed to proceed for 110 000 seconds at + 0.3° C giving $D_{20} = 7.36 \cdot 10^{-7}$ and in the other the diffusion of a 0.338 % rhodanese solution was followed for 140 000 seconds at + 1.1° C giving $D_{20} = 7.66 \cdot 10^{-7}$. The mean value $7.5 \cdot 10^{-7}$ cm^2 sec^{-1} was taken as the diffusion constant for rhodanese.

Molecular weight. The apparent partial specific volume of the recrystallized enzyme was determined pycnometrically at 25° on a solution of the recrystallized enzyme in phosphate buffer pH 7.44, ionic strength 0.1. The obtained value was 0.742. The molecular weight for the enzyme could now be calculated and found to be 37 100. The frictional ratio f/f_0 was 1.28.

Turnover number. The mean value for the specific activity of four different preparations of recrystallized rhodanese was 270 (range 259—286) RU/mg. The corresponding value for the turnover number of the enzyme is of the magnitude 20 000 molecules of thiocyanate per minute per molecule enzyme formed from thiosulfate and cyanide at 20° and pH 8.6.

SUMMARY

The enzyme rhodanese has been purified from beef liver and crystallized. The colorless enzyme was electrophoretically homogenous at pH 7.4, at lower pH:s denaturation occurred. Rhodanese appeared homogenous in the ultracentrifuge with a sedimentation constant of $3.0 \cdot S$.. The diffusion constant was $7.5 \cdot 10^{-7}$ and the partial specific volume 0.74. The molecular weight was 37 000 and the turnover number in the standard test about 20 000 molecules of thiocyanate formed per minute per molecule enzyme.

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