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# The Enzymic Hydrolysis of S-Phosphorylated Thiols by Bovine Brain

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In a previous communication [1] it was shown that an enzyme hydrolyzing the S—P bond of cysteamine S-phosphate (S-(2-aminoethyl) phosphorothioate) is present in the human erythrocyte. Since a number of S-phosphorylated thiols are now available [2] it was of interest to study the relative rates of enzymic hydrolysis of the S—P bond for a variety of these compounds. Information regarding the effect of substituents on the rate of enzymic hydrolysis could thus be obtained. The effect of substituents on the nonenzymic rate of hydrolysis of the corresponding compounds has been determined earlier [3].

Bovine brain was chosen as an easily accessible source of relatively stable enzyme for these investigations. Brain was chosen as an enzyme source also because of its low blood content. Interference by erythrocyte phosphatases [1] could thus be easily eliminated.

For the preliminary investigations cysteamine S-phosphate was used as a substrate. Pilot experiments with whole brain homogenate showed a pH optimum for hydrolysis of cysteamine S-phosphate around pH 7. The products of hydrolysis were cysteamine and orthophosphate. $Mg^{2+}$ ions were strongly stimulatory. Of various brain fractions tested, the insoluble part of brain homogenate was found to account for 93 % of the total cysteamine S-phosphate hydrolyzing activity present in the homogenate. The insoluble brain fraction was therefore used in all subsequent experiments.

The cysteamine S-phosphate hydrolyzing activity was heat sensitive. 5 min at 50° destroyed 34 % of the initial activity; 5 min at 100° destroyed 84 % and 10 min at 100° destroyed all the initial activity. The hydrolyzing activity thus appeared to be enzymic in nature.

*Effect of pH.* The effect of pH on the cysteamine S-phosphate hydrolyzing enzyme was determined in the presence of $Mg^{2+}$ ions (5 mM). An optimum was found at pH 7.0. At pH 8.0 the rate of enzymic hydrolysis was 67 % and at pH 6.0 it was 78 % of the rate at pH 7.0.

The enzymic formation of cysteamine from cysteamine S-phosphate was linear with time for about 60 min in a system as described in the legend to Table 2.

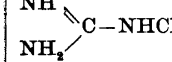
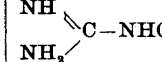
*Effect of metal ions.* Of the metal ions tested $Mg^{2+}$ ions were found to be the most efficient ones in stimulating the cysteamine S-phosphate hydrolyzing activity. The optimum $Mg^{2+}$ ion concentration was 5 mM, which increased the original activity by 210 % at pH 7.0. Of other metal ions tested only $Fe^{2+}$ had an effect comparable to that of $Mg^{2+}$ (Table 1). In contrast to the other cations, nickel ions were found to catalyze the nonenzymic hydrolysis of cysteamine S-phosphate at an extremely high rate (Table 1).

When enzyme was added in the presence of $Ni^{2+}$ ions the amount of cysteamine formed was actually less than in the blank containing no enzyme. A sorbtion of $Ni^{2+}$ ions on the protein probably accounts for this phenomenon.

*Table 1.* Effect of metal ions on the cysteamine S-phosphate hydrolyzing activity. Test system: cysteamine S-phosphate 5.0 μmoles; metal chloride 5.0 μmoles; enzyme corresponding to 60 mg of fresh brain and tris buffer pH 7.0 90 μmoles in 1.0 ml of water. The enzyme was omitted from the blanks (nonenzymic hydrolysis). Incubation: 30 min at 35°.

| Metal ion added | μmoles of cysteamine formed | |
|---|---|---|
| | enzymically | nonenzymically |
| 0 | 0.41 | 0.00 |
| $Mg^{2+}$ | 0.86 | 0.00 |
| $Ca^{2+}$ | 0.30 | 0.00 |
| $Fe^{2+}$ | 0.78 | 0.04 |
| $Co^{2+}$ | 0.06 | 0.06 |
| $Ni^{2+}$ | — | 3.02 |

*Table 2.* Hydrolyzing activity of the enzyme on various S-phosphorylated thiols. Test system: S-phosphorylated thiol 10.0 μmoles; $MgCl_2$ 5.0 μmoles; enzyme corresponding to 60 mg of fresh brain and tris buffer pH 7.0 90 μmoles in 1.0 ml of water. Incubation: 30 min at 35°. The enzyme was omitted from blanks (nonenzymically formed thiol).

| Compound $RSPO_3^{2-}$ (R listed) | Formed thiol, μmoles | |
|---|---|---|
| | enzymically | nonenzymically |
| $NH_2CH_2CH_2$ | 1.10 | 0.00 |
| $CH_3NHCH_2CH_2$ | 0.57 | 0.00 |
| $(CH_3)_2NCH_2CH_2$ | 0.56 | 0.00 |
| + $(CH_3)_3NCH_2CH_2$ | 1.19 | 0.03 |
| $[(CH_3)_2CH]_2NCH_2CH_2$ | 0.71 | 0.00 |
| $NH_2CH_2CH_2CH_2$ | 0.32 | 0.25 |
| $(CH_3)_2NCH_2CH(CH_3)$ | 0.70 | 0.00 |
|  | 1.35 | 0.12 |
|  | 0.84 | 0.60 |
| $NH_2COCH_2$ | 1.31 | 0.03 |
| $HOCH_2CH_2$ | 0.77 | 0.64 |
| $HOCH_2CH_2CH_2$ | 0.79 | 1.00 |

*Effect of cystemaine S-phosphate concentration.* The cysteamine S-phosphate concentration was varied from 0 to 15 mM. The hydrolyzing enzyme was saturated with substrate at about 10 mM of cysteamine S-phosphate.

*Effect of the hydrolyzing enzyme on other S-phosphorylated thiols.* In the presence of $Mg^{2+}$ ions the protein fraction used in the study of the enzymic hydrolysis of cysteamine S-phosphate also catalyzed the hydrolysis of the S—P bond in a number of other S-phosphorylated thiols. A thiol and orthophosphate was formed. The results are presented in Table 2. (Only enzyme preparations with the same hydrolytic activity on cysteamine S-phosphate were used to obtain these values).

It is evident from Table 2 that all tested substances were hydrolyzed at a relatively slow rate. Individual variations in the rate of formation of thiol were not found to be very great. It can therefore be concluded that the enzyme(s) responsible for the observed reaction is a relatively non-specific one.

In Table 2 the formation of thiols from S-phosphorylated thiols at pH 7.0 in the presence of $Mg^{2+}$ ions are also listed. No correlation is found between the nonenzymic and the enzymic hydrolysis.

In an earlier investigation on an enzyme from bovine brain, Feuer and Wollemann [4] demonstrated the enzymic hydrolysis of coenzyme A S-phosphate to coenzyme A and orthophosphate. The enzyme was active at pH 8 in the presence of $Mg^{2+}$ ions.

*Experimental. Preparation of enzyme.* 20 ml of a 30 % homogenate of 0° (Potter-Elvehjelm homogenizer) of calves brain (the enzyme activity was found to be about the same in different parts of brain) was centrifuged for 5 min at 700 *g* at 0°. The supernatant was poured off. The precipitate was washed twice with 20 ml of ice cold distilled water followed each time by centrifuging for 5 min at 700 *g* at 0°. The supernatant precipitate was stirred up in ice cold water (final volume 20 ml) and was used within a few hours.

*Determination of thiols in the presence of S-phosphorylated thiols.* The described method is a modification of an earlier described procedure [1]. To 1.0 ml of incubated solution was added 1.0 ml of an ice cold 10 % solution of trichloroacetic acid in water. After rapid mixing, the precipitated protein was centrifuged down during 2 min at about 700 *g*. 1.0 ml of clear supernatant was immediately adjusted to about pH 7 by the addition of 0.8 ml of a 1 M phosphate buffer of pH 9.1. The hydrolysis of residual S-phosphorylated thiol during this procedure was less than 1 %.

The amount of thiol present was determined according to Grunert and Phillips [5]. The color yield of the various thiols had earlier been determined by hydrolysis of the corresponding pure S-phosphorylated thiols, prepared accord ing to Ref[2]. A correction was made for the small amount of thiol sometimes formed during storage of the S-phosphorylated thiols. In all experiments where the enzymic hydrolysis of S-phosphorylated thiols was determined a correction was made for the nonenzymic hydrolysis of the compounds under identical conditions.

The enzyme preparation used did not contain detectable amounts of nitroprussiate reactive substances.

*The S-phosphorylated thiols used* were prepared according to Ref[2]. The compounds that

were isolated as barium salts in Ref[2]. were converted to sodium salts before use by trituration with a slight excess of sodium sulfate (molar ratio 1:1.25).

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5. Grunert, R. R. and Phillips, P. H. *Arch. Biochem. Biophys.* **30** (1951) 217.

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# Identification of An Impurity in Chlorine Gas from Mercury Cells

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When brine is electrolyzed in technical cells with mercury cathodes and graphite anodes chlorine gas is obtained which is saturated with water vapor at 60–70°C, and which also contains carbon dioxide, air, hydrogen, and traces of other impurities. The moist gas is first cooled, either directly with water or indirectly, then dried with sulfuric acid, and finally condensed after compression and cooling.

In the gas ducts leading to the drying equipment and in the drying equipment itself various kinds of impurities are deposited, thus interfering with the operation. Part of the impurities generally accompanies the chlorine to the condensation department, and then becomes dissolved or dispersed in the liquid phase, causing trouble in the customers' bleaching equipment, etc.

For these reasons it would be valuable to identify the compounds in order to, *e.g.*, find suitable methods for an easy removal of the deposits, but also to elucidate the mechanism of formation.

*Earlier investigations.* In some technical reports, solid impurities in chlorine gas are mentioned. Billiter[1] states, *e.g.*, that salt particles might be entrained in the gas stream at high loads. In some plants in USA glass fiber filters are employed in the chlorine pipes from the cells.

Wranglén[2] investigated corrosion products from hard rubber (ebonite) linings on cell covers and frames. In the deposits examined the chlorine content reached about 65 %. Age determination according to the $^{14}C$ method showed that up to 70 % of the impurities might originate from graphite, whereas only 30 % of the carbon was "fresh".

Penfield and Cushing[3] describe a fractionating column for removal of chlorinated organic compounds, the concentration of which is said to reach 0.2 % in liquid chlorine. The impurities had a "taffy-like" consistency and such a high vapor pressure that they sublimated at room temperature. About 1/3 was identified as chloroform and 1/6 as insoluble in chloroform, consisting mainly of hexachloroethane and hexachlorobenzene. By fractionating the chlorine in a column the impurity content decreased to less than 0.01 %.

Finally in a US patent[4] a method is described for purifying moist chlorine gas by injection of liquid chlorine. The hydrate thereby formed is said to bind such impurities as carbon tetrachloride, chloroform and hexachloroethane.

*Chemical analysis of a sample.* In connection with cleaning the drying towers in the Domsjö chlorine plant, which must be done at about 6 months' intervals, a sample of the deposits was taken. It had a fine crystalline structure, a pale yellow color, and a typical intense odor.

The crystals were only slightly soluble in ethanol and chloroform, but almost completely soluble in benzene. They sublimated upon slight heating. The melting point range of the sample was 220–260°C (the wide range indicates that the sample was impure).

The compound was purified by sublimation and chemically analyzed. The following result was obtained: C 27.8; H 0.5; O 10.5; Cl 59.6. (Sum 98.4 %). The corresponding molecular composition is represented by the formula $C_7H_{1.5}O_2Cl_5$. The high oxygen content is remarkable, since oxygen containing impurities have apparently not been reported in the literature earlier.