149. *The Enzymatic Degradation of Heme Proteins into Bile Pigments. VII*

The Stability of the Enzyme Protein

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In the previous communications,^{1,2)} the purification of the enzyme capable of degrading pyridine-hemichromogen to a direct precursor of biliverdin (656 m μ substance) was reported.

The present paper describes the enzyme stability and the effect of several substances to prevent the inactivation of the enzyme protein. This enzyme protein is discovered to be inactivated in the presence of air, and the inactivation was completely avoided when the enzyme was stored in a Thunberg tube evacuated and filled with nitrogen gas.

Materials and methods. 1) Enzyme source. From the results in the previous communication,³⁾ guinea-pig liver was used as an enzyme source.

2) Preparation of the crude enzyme preparation. Livers were homogenized in four volumes of 0.1 M phosphate buffer of pH 7.4 with a Potter-Elvehjem homogenizer. After a preliminary centrifugation at 10,000 $\times g$ for 15 minutes, the supernatant solution was further centrifuged at 105,000 $\times g$ for 60 minutes. In the supernatant finally obtained about 70 to 80 per cent of the activity of the original homogenate was recovered (Fraction A).

3) Partial purification of the enzyme. The supernatant solution was fractionated with neutral saturated ammonium sulfate solution, approximately 65 per cent of the initial activity being precipitated between 0.40 and 0.60 saturation. The preparation at this stage of the purification was referred to as the partially purified enzyme preparation (Fraction B).

4) Preparation of the substrate. The substrate employed was a solution of a crystalline hemin⁴⁾ in redistilled pyridine at a concentration of 4×10^{-4} M.

5) Preparation of the co-enzyme solution. The boiled extracts of liver¹⁾ were used as a co-enzyme solution.

6) Composition of the reaction mixture. The reaction mixture consists of each 2.0 ml of the enzyme solution, 0.1 M phosphate buffer of pH 7.4, boiled extracts of liver, and 4×10^{-4} M pyridine-hemi-

chromogen solution. The mixture was subjected to aerobic reaction in a 100 ml flask at 38° and pH 8.0. The control experiment with boiled enzyme was performed at the same time.

7) Assay. The activity was assayed by the determination of 656 m μ substance formed after the 20 minutes of incubation. The details of the assay method were described in the previous communication.²⁾ Protein was estimated by the spectrophotometric method of Warburg and Christian.⁵⁾

8) Chemicals. All of the chemicals were reagent grade chemicals from commercial sources.

Fig. 1. pH-stability of the enzyme. The enzyme preparation (Fraction B) was stored for 30 minutes at 20° and at different pH indicated above. The enzyme reactions were performed on the each preparation. The reaction mixture and the assay were the same as described in the text.

Results and discussions. This enzyme protein, either in a crude or partially purified state, was remarkably unstable. Even when stored at 0° in an open test tube (1 cm diameter, and 5 cm in height), Fraction A and Fraction B were inactivated to about 80 per cent and 70 per cent respectively of their original activities after 16 hours.

To stabilize the enzyme protein, the effect of adding several substances which were known to stabilize other enzymes or which were expected to prevent the inactivation was tested. The addition of various concentrations of glutathione, ascorbic acid, mercaptoethanol, and EDTA to the enzyme preparation did not prevent the inactivation. The alteration of ionic strength and enzyme concentration was quite ineffective. The enzyme was most stable at pH 7.4 in both

the crude and purified state as shown in Fig. 1. If the enzyme was stored in a Thunberg tube evacuated and filled with nitrogen gas or in an open test tube at 0° , the later was more stable than the former. After 24 hours the activity of Fraction B became 97 per cent in nitrogen gas and about 60 per cent in oxygen as shown in Fig. 2. When the enzyme was stored under nitrogen gas at -10° its activity remained 75 per cent of the original after 10 days. These facts

Fig. 2. Inactivation of the enzyme protein in the presence of oxygen.

indicate that the enzyme protein is inactivated in the presence of oxygen, one of the substrate of this enzyme. As described in the previous communication¹⁾ this enzyme was completely inactivated when treated at 80° for 5 minutes at pH 7.4.

Summary. 1) This enzyme was extremely unstable in the presence of oxygen, and this instability was completely avoided when stored in a Thunberg tube evacuated and filled with nitrogen gas.

2) This enzyme was most stable at pH 7.4 in both crude and purified state.

References

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