CCXXI. THE PREPARATION AND PROPERTIES OF A HIGHLY ACTIVE CATALASE FROM HORSE LIVER

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THE enzyme catalase has been investigated by a great number of workers. Zeile & Hellström's [1930] method of preparing horse liver catalase has been used with some slight modifications by most investigators. The most active preparation so far obtained is that of v. Euler & Josephson [1927] (Kat. f. 43,000). Sumner & Dounce [1937] have recently described a different method for the preparation of catalase from beef liver. Sumner's crystalline catalase showed the activity 28,000.¹ The present paper reports the preparation of an enzyme from horse liver, with an activity of 55,000–60,000.

The method of preparation

2 kg. horse liver are ground and extracted twice with 2 l. water for 6 hr. Haemoglobin and other inert proteins are precipitated with alcohol and chloroform according to Tsuchihashi [1923]. After centrifuging, the clear supernatant solution is evaporated at a low temperature down to 2 l. and mixed with 3 l. saturated ammonium sulphate solution. The precipitate is dissolved in 1.5 l. water after which an equal vol. of 95 % alcohol is added. Precipitated protein and salts are centrifuged down and 700 ml. alcohol more are added. The enzyme is precipitated and is separated from the solution after which it is dissolved in 1 l. water. 800 ml. saturated ammonium sulphate are added again. A compound with a high Fe-content (about 16 %) is thrown down. This compound accompanies the catalase, when preparations are made according to previously described adsorption methods, and is the cause of the widely different values reported for the iron content. This iron is easily split off and after acid hydrolysis gives the characteristic red colour with NH_4CNS . The ammonium sulphate concentration is raised to 58–60 % and the enzyme precipitate is filtered off. It is dissolved in water, reprecipitated with alcohol, and the preparation is then dialysed. The activity is 40,000–43,000. The preparation is stable for months at 0°. The yield is 0.8 g.

The preparation is further purified by adsorption on tricalcium phosphate. The diluted solution of enzyme (0.2 mg./ml.) is adjusted to about pH 5.5 by addition of KH_2PO_4 and is then filtered through a column of tricalcium phosphate as is usual with chromatographic analysis. The enzyme is adsorbed in a distinct layer at the top of the column and then eluted by a phosphate buffer at pH 8. The catalase solution is dialysed in cellophane bags.

¹ During Prof. Sumner's stay in Uppsala last winter I made a joint determination with him of Kat. f. of catalase, in the course of which we confirmed that our procedures were identical. The present values of Kat. f. are therefore directly comparable with those of Sumner & Dounce.

The activity of the preparation is 55,000–60,000. This procedure has been repeated a number of times with the same result.

The analyses show that the preparation contains 15.5% N (by micro-Kjeldahl) $0.085 \pm 0.005\%$ Fe (by the method of Jorpes), and 0.02–0.03% Cu [by the [1933] method of Eisler *et al.* 1936].

For the determination of the activity the method described by v. Euler & Josephson [1927] was used. $\text{Kat. f.} = \frac{\text{reaction constant}}{\text{weight of preparation in g.}}$.

Determination of the spectrum

The light absorption at different wave-lengths was estimated by a photo-electric apparatus like that described by Warburg & Negelein [1929].

Stern [1937] has recently reported a quantitative determination of a catalase spectrum in the visible range. His preparation does not seem, however, to have been highly purified.

Fig. 1.

Fig. 2. The approximate position of the coloured preparation after the cataphoresis.

The molecular absorption coefficients are calculated from $\beta = \frac{1}{d} \frac{1}{c} \ln \frac{I_0}{I}$.

d = the depth of the cell in cm.

$$c = \frac{\text{g. preparation}}{\text{ml.} \times \text{mol. weight}}.$$

I_0 represents the intensity of the incident light and I the intensity of the transmitted light.

Table I. *The molar coefficients at the maxima of absorption*

Wave-length mμ	Molar absorption coefficients
623	8.3×10^7
536	9.4×10^7
400	78.5×10^7
280	85.0×10^7

Cataphoresis experiments

The purity of the preparation was tested with the cataphoresis apparatus of Theorell [1934]. The boundaries between the catalase solution and the buffer solution were level with the middle disk at the start of the experiment. During

the experiment the preparation migrated towards the anode. The displacement was counteracted by transferring the liquid from one vessel to the other, according to the principle introduced by Tiselius [1937]. In this way it was possible to have the boundaries of the enzyme solution at the end of the experiment approximately in the same position as at the beginning.

Conditions: *pH* 6.99. Conductivity 1.37×10^{-3} . Current 5 mA. Time: 22 hr. and 40 min. Temp. 20°

8×2 ml. were brought from the "negative" electrode vessel to the "positive", corresponding to a displacement of the free boundaries of 8 cm.

The results of the analyses from the experiments are given in Table II.

Table II

		mg. prep. ml.	Kat. f.	% N	10 ⁻² k/ml.		10 ⁻¹ k/ml.		10 ⁻² k/ml.	
					N mg./ ml.	% Fe	Fe µg./ ml.	% Cu	Cu µg./ ml.	
The solution contained in the three cells above the middle disk	+ I	2.0	50,000	14.9	3.30	0.1	7.8	—	—	
Ditto below the middle disk	- I	5.0	51,000	15.5	3.47	0.082	6.6	0.044	1.15	
"	+ II	9.55	54,000	15.8	3.35	0.091	5.8	0.027	1.9	
"	- II	11.4	52,000	15.3	3.41	0.079	6.2	0.026	2.0	
The preparation in the U-tube		11.5	51,000	15.9	3.21	0.081	6.3	0.026	1.7	
Preparation not submitted to cataphoresis		12.5	56,000	15.7	3.62	0.082	6.8	0.023	2.4	

The contents of nitrogen and iron were both proportional to the activity of the solution in the cells. The copper also accompanied the activity, but in one cell (-I) the percentage was double as much as that usually found.

Ultracentrifuge experiments

Thanks are due to Prof. Svedberg for the opportunity of performing ultracentrifuge experiments at his Institute in co-operation with Nils Gralén. Using the light absorption method and light of wave-length $546\text{m}\mu$ the sedimentation constant of the iron-porphyrin-protein was found to be 11.2×10^{-13} . The preparation proved however not to be homogeneous. Ultraviolet light photographs, $250\text{--}290\text{m}\mu$, showed the presence of a component with a sedimentation constant of about 3.2×10^{-13} . From dry weight determinations of the content in the two compartments of a "separation cell" [Tiselius *et al.* 1937] the concentration of this component is calculated to be 15–20 % of the total protein.

The diffusion constant was measured by the method of Lamm & Polson [1936] and was found to be 4.3×10^{-7} while the partial specific volume was determined as 0.715. Using the formula $M = \frac{RT s_{20}}{D_{20}(1 - \bar{V}\zeta)}$ the molecular weight is calculated to be 225,000.

Stern & Wyckoff [1938] found the value 11×10^{-13} for the sedimentation constant of horse liver catalase.

Sumner & Gralén [1938] have found the sedimentation constant¹ for crystalline catalase from beef liver to be 11.3×10^{-13} and the diffusion constant 4.1×10^{-7} , which give a value of $M = 248,000$.

Separation of a copper-protein from the iron-porphyrin-protein

The preparation (2 mg./ml. in $M/15 \text{Na}_2\text{HPO}_4$) was mixed with 1/3 of its volume of a saturated solution of picric acid. Then $N/10$ acetic acid was added till a precipitate appeared at *pH* 5.2. This was centrifuged down and more

¹ Corrected values, personal communication.

acetic acid added to the solution to bring the *pH* to 4.6, at which another fraction precipitated. These two fractions were dissolved separately in $M/15$ Na_2HPO_4 and were dialysed free from picric acid.

Each fraction has separately a considerably lower activity than the preparation before separation, i.e. fraction 1: Kat. f. 6000 and fraction 2: Kat. f. 3500. If however the original preparation is mixed with an equal volume of picric acid and the precipitation carried out by the addition of enough acetic acid to precipitate both fractions together at *pH* 4.6, the preparation retains the greater part of its activity after removal of the picric acid. This makes it probable that the inactivation is not due to the slightly acid reaction or to the action of the picric acid. It is possible therefore that both components may be necessary for the catalase activity.

The fraction which is first precipitated has an Fe content about 0.1 % and almost no Cu, whilst the other fraction has a Cu content of 0.16 % and very little Fe.

The sedimentation constants of the two isolated components have been determined. The Fe-porphyrin-protein had a sedimentation constant of about 12×10^{-13} and that of the Cu-protein was 3.27×10^{-13} .

It is probable that the preparation by picric acid precipitation gives rise to the same two fractions which were observed in the earlier ultracentrifuge experiments, mentioned above.

Kubowitz [1937] has recently shown that an oxidative enzyme, polyphenol-oxidase, is a Cu-protein (0.165 %). Keilin & Hartree [1938] have shown that the Fe in catalase is reduced by hydrogen peroxide from the trivalent to the bivalent state. It is however immediately oxidized in the presence of molecular oxygen. It seems possible, that the Cu-protein in this highly active catalase preparation might—by analogy with Kubowitz' oxidative enzyme—be necessary for the re-oxidation of the bivalent Fe of the reduced catalase. The Cu-protein would in such a case be an integral part of a catalase system.

The activity was not, however, restored by a simple mixing of the two fractions nor was it raised by the addition of the Cu-protein to the original preparation. Further studies will however show, whether it is possible to separate the original preparation into two inactive fractions by other means than addition of picric acid and whether, in such a case the activity will be restored after mixing.

The differences in the activity of the preparation of Sumner & Dounce and that here described may be due not to impurities but to a difference in the structure of the protein (the preparations are made from different species of animal) or, if the Cu-protein is significant in the activity, to a different content of this substance. No information is given by Sumner & Dounce concerning the presence of copper.

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