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The Magnetic Properties of Crystalline Horse Erythrocyte Catalase and Some of its Derivatives

H. F. DEUTSCH* and A. EHRENBERG

Biokemiska avdelningen, Medicinska Nobelinstitutet, Stockholm, Sweden

The magnetic susceptibility as an adjunct to spectrophotometric technique is a powerful tool for the study of the nature of the heme-protein bonds in iron porphyrin proteins, as first shown by Pauling and his collaborators [1]. Previous work of the nature from this laboratory have been concerned with cytochrome c [2], horseradish peroxidase [3], horse liver catalase [4], and myoglobin [5]. The more recent investigations have utilized the apparatus described by Theorell and Ehrenberg [6]. This instrument provides a high degree of sensitivity and makes possible the accurate measurement of the magnetic susceptibility of small samples.

In connection with the systematic investigations of the magnetic properties of iron porphyrin proteins, it seemed opportune to investigate horse erythrocyte catalase. The study of this protein is somewhat more elucidative than the horse liver catalase studied previously by Theorell and Agner [4] since all the iron is present as hemin iron, and one is thus not faced with the problems introduced by the presence of biliverdin iron. The work reported here is concerned with the magnetic susceptibility of a highly active horse erythrocyte catalase as well as a number of its derivatives.

EXPERIMENTAL

A single sample of the enzyme was employed throughout the work. It represented the catalase preparation no. 8 Aq, described in the preceding article [7]. The enzyme had been crystallized from water three times and showed an initial Kat.F. of 125 000. Near the end of the investigations (approximately one month), the activity had fallen to 90 000.

* Rockefeller Foundation Fellow 1950—1951. Present address: Department of Physiological Chemistry, Medical School, University of Wisconsin, Madison.

This is somewhat characteristic of the high activity catalases previously reported [7,8]. The fall in activity did not appear to affect the magnetic susceptibility of the enzyme as measured during this interval.

The recrystallized enzyme was dialyzed exhaustively against distilled water and the crystals dissolved by addition of a trace of ammonia to give a 12.125% solution. The iron content of the protein was found to be 0.0937%, which if one assumes four atoms of iron per molecule of catalase corresponds to a molecular weight of 238 000.

Solutions for magnetic susceptibility measurements were made by mixing 0.3 ml of the above solution with 0.1 ml of buffer or a particular reagent while the sample was being stirred magnetically. All solutions measured thus contained 9.094% protein. The ionic strength was 0.25 except in the case of the studies on the fluoride derivative. In these experiments a fluoride concentration of 0.5 *M* and a total ionic strength of 0.65 was employed. In studies of the effect of pH on magnetic susceptibility, the salt consisted chiefly of sodium chloride solutions containing either dilute sodium hydroxide or acetic acid. All pH measurements were made by means of a glass electrode immediately after the removal of the sample from the measuring tube. The glass electrode had been previously carefully calibrated against a hydrogen electrode. The actual magnetic measurements and calculations of susceptibilities were carried out as described by Theorell and Ehrenberg [5,6].

The preparation of catalase derivatives was carried out in the usual manner as described in previous work.

RESULTS

The magnetic susceptibility data for catalase and the derivatives which were measured here are shown in Table 1. These values have been corrected for the diamagnetism of catalase. The ferriprotoporphyrin of catalase cannot be removed without denaturation of the protein. Neither can a diamagnetic compound of catalase such as a ferro-carbon monoxide derivative be prepared easily. However, the diamagnetic gram susceptibilities of myoglobin, peroxidase and cytochrome c did not vary much [5,3,2]. The mean value of their diamagnetism was therefore taken for our calculations.

Table 1. Magnetic susceptibility of catalase and derivatives.

Compound	$\chi_{Fe} \times 10^6$ cgs	μ (Bohr magnetons)	Number of unpaired electrons	μ theoretical (Bohr magnetons)
Catalase (pH 4.8—10.4)	13 390	5.60	5	5.92
Fluoride-Catalase	13 350	5.60	5	
Cyanide-Catalase	2 240	2.29	1	1.73
Hydrogen-Sulfide-Catalase	2 700	2.51	1	
Azide-Catalase	12 310	5.36	5	

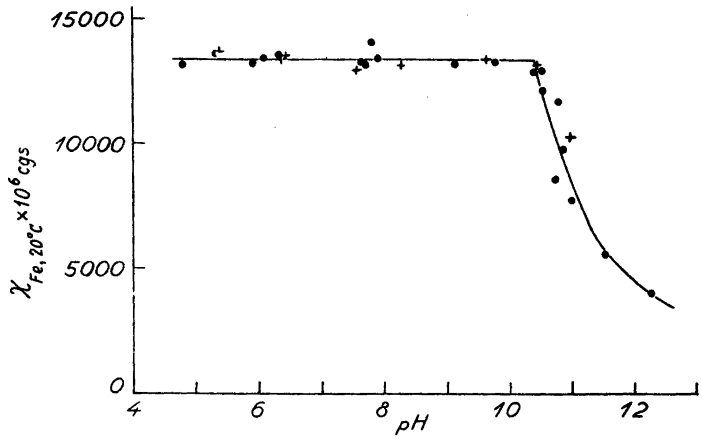

Fig. 1. Effect of pH on the paramagnetic susceptibility of the iron of catalase (•) and catalase fluoride (+).

Theorell and Agner's [4] diamagnetic correction, which contributes 30 % to the final χ_{Fe} of neutral catalase and fluoride catalase was probably somewhat too high. Our calculated diamagnetic contribution amounts to 23.6 % which accounts for practically the whole difference between the two sets of results for the strongly paramagnetic compounds of catalase. Our susceptibility values for the weakly paramagnetic cyanide and hydrogen sulfide compounds of about $2\,500 \times 10^{-6}$ cgs are to be expected from their spectral type. This is strongly in favour of the lower correction figure. A 30 % correction would give values of about $3\,700 \times 10^{-6}$ which are probably too high. It is interesting to note that if the susceptibilities obtained by Theorell and Agner [4] for the derivatives of horse liver catalase are corrected, first for the assumption that the fourth iron atom in the molecule is unaffected by the reagents, as described in their paper, and second for the too high diamagnetic correction, then their measurements are found to agree quite closely with our results.

No change in the magnetic susceptibility of catalase was found from pH 4.8 to approximately 10.4. At the latter point a sudden change is seen. Above this pH, horse erythrocyte catalase undergoes denaturative changes characterized by a marked increase in viscosity and loss of solubility at neutral pH. Almost identical results were obtained in the presence of 0.5 *M* fluoride over this pH range. This remarkably abrupt change in stability is very similar to that found by Theorell and Ehrenberg [5] in the case of myoglobin. At pH below 5.5, where acetate buffer was used, about 30 % of the catalase is combined with the acetate as was shown by Agner and Theorell [9]. Spectrophoto-

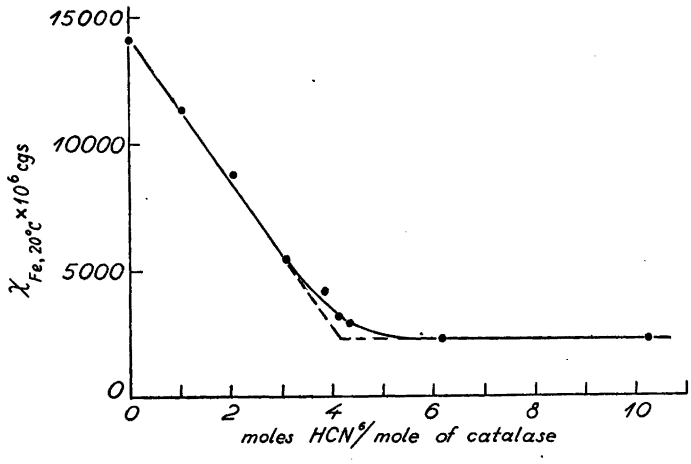

Fig. 2. Magnetometric titration of horse erythrocyte catalase with HCN at pH 7.75.

metric titration experiments with fluoride at pH values between 5.8 and 7.8 indicated that there is a pure competition between fluoride and hydroxyl ions. With fluoride ion concentration of 0.5 *M* there is present about 30 % catalase fluoride compound at pH 7.6 and about 80 % at pH 6.4. The two experiments at the latter pH were made with phosphate buffer so that the acetate could not interfere. Thus both the acetate and the fluoride compounds have about the same paramagnetic susceptibility as neutral catalase. The results for these experiments are plotted to give Figure 1. The mean value for the molar susceptibility (χ_{Fe}) of the iron in catalase for eleven observations between pH 4.8—10.4 is 13 390 $\times$ 10^{-6} ($\pm$ 100) cgs. (The iron of the denaturation product that has a typical hemichrome spectrum is found to have a molar susceptibility of 2 500 $\times$ 10^{-6} cgs as determined from extrapolation of the data of Figure 1.) The mean value of six observations of χ_{Fe} for catalase fluoride in the same pH range was 13 350 $\times$ 10^{-6} ($\pm$ 120) cgs.

In magnetometric studies of ferrimyoglobin fluoride, Theorell and Ehrenberg [5] noted three p*K* values in the region pH 6, 8, and 10, only two of which were spectrophotometrically operable. The magnetometric studies of catalase fluoride as shown in Figure 1 did not suggest any heme-linked groups to be dissociated over the pH range 4.8—10.4.

The magnetic susceptibility of the cyanide catalase derivative was determined for various ratios of cyanide to catalase at pH 7.75. The results for these experiments are plotted to give Figure 2. All of the iron atoms are titrated to the point of saturation when slightly over four (4.18) equivalents of cyanide per

molecule of catalase have been added. Assuming the dissociation constant of HCN to be 2×10^{-9} a $K_I = \frac{[HCN][FeOH]}{[FeCN]} = 18 \cdot 10^{-6}$ can be calculated from the data. This is only slightly higher than the values determined by Chance [10] in kinetic, spectrophotometric, and inhibition experiments. The susceptibility of the cyanide compound is determined to $2\,240 \times 10^{-6}$ cgs.

The hydrogen sulfide derivative of catalase gave a molar susceptibility of $2\,700 \times 10^{-6}$ cgs. This corresponds most closely to one unpaired electron for the iron atoms. The slightly higher value for hydrogen-sulfide-catalase as compared with the value found for cyanide catalase has been previously noted by Theorell and Agner [4].

The χ_{Fe} for azide catalase is close to that of catalasefluoride and free catalase.

From Table 1 it is apparent that the number of unpaired electrons for the iron of catalase and its fluoride and azide derivatives must be five. The measured susceptibility is somewhat lower than the theoretical value for five unpaired electrons, just as in ferri Hb[1], ferri Mb[5] and horse radish peroxidase[3].

The cyanide and sulfide derivatives show susceptibilities slightly higher than would be expected for one unpaired electron. However, when one considers that the orbital contributions may add about 0.5 Bohr magnetons to these values, the difference from 1.75 is not too great. The higher values may indicate some heme-heme interaction or a greater than usual orbital contribution.

The magnetic susceptibility data for horse erythrocyte catalase and derivatives as obtained in these studies support the results obtained earlier by Theorell and Agner [4] on horse liver catalase and proves the correctness of their assumption that biliverdin iron as present in liver catalase is held by essentially ionic bonds and does not take part in the formation of the covalent complexes so far studied.

SUMMARY

The magnetic susceptibilities of a sample of highly active crystalline horse erythrocyte catalase and some of its derivatives have been studied. The results obtained support most previous work on the electronic nature of the iron in this type of enzyme.

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