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The Properties of Various Crystalline Horse Erythrocyte Catalase Preparations

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It has been recently pointed out that various crystalline erythrocyte catalases do not show constant activity [1,2]. A series of molecules of varying reactivities appear to comprise the usual material. In further investigations of such variability a number of horse erythrocyte catalases were studied in terms of activity, iron and hemin contents and absorption coefficients. Since the possibility of molecular kinetic variation appeared likely, sedimentation study of four samples of rather widely different activity and properties was also carried out. From these and partial specific volume and diffusion constant measurements, a molecular weight was calculated and compared with those obtained from the iron and heme contents. The most striking differences noted among the preparations studied were the variations in activity and crystallizability.

EXPERIMENTAL

The catalases were prepared essentially by the method of Bonnichsen [3] as described previously [1]. Particular care was taken to make the period of the Tsuchihashi [4] treatment as short as possible. In this respect sufficient ethanol-chloroform mixture was always added to satisfactorily precipitate (denature) the hemoglobin at room temperature within 30—60 minutes after the completion of the addition of this reagent. Two preparations (nos. 5 and 8) were unusual in that most of the material readily crystallized from water after the acetone precipitation and pH 4 acetic acid buffer treatments. The crystals were readily soluble on addition of a trace of ammonia and would begin to recrystallize after only several hours dialysis against distilled water. Some of the aqueous supernatants to recrystallized catalase preparation number 8 were examined to determine the extent of solubility. This was found to be as low as 0.003 g/100 ml at 3—5° C and pH 5.9—6.0, the hydrogen ion concentration of the supernatant to the exhaustively dialyzed crystals. Dilution of a 11.4 % solution of this material (no. 8) with 250 volumes of water caused

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it to show a marked turbidity within 10 minutes at room temperature. This was followed by the formation of well-defined crystals within 30 minutes. The above solubility properties of catalase preparations numbers 5 and 8 contrast strongly with catalase from horse erythrocytes which have been described in the literature. In these cases crystallization from water occurs only after prolonged standing in the cold of dialyzed and concentrated solutions and only relatively small amounts of the protein crystallizes. Numerous other of our enzyme preparations could not be crystallized under aqueous conditions analogous to those used for preparations nos. 5 and 8 although they showed the usual crystallizability from strong ammonium sulfate solution at room temperature as well as from concentrated aqueous solution after prolonged standing in the cold. This solubility difference is further indication of the lack of uniformity of various horse erythrocyte catalases in addition to those previously noted [2].

All activity measurements were made by the method of Bonnichsen, Chance and Theorell [5] after it was demonstrated to our satisfaction that it was equivalent to the method of von Euler and Josephson [6]. Iron was determined as the sulfosalicylate in ammoniacal solution and heme as the alkaline pyridine hemochromogen. Absorption measurements were made with a Beckman spectrophotometer.

Sedimentation and diffusion analyses were carried out in the Institute of Physical Chemistry of the University of Uppsala under the direction of Dr. K. O. Pedersen. The partial specific volume measurement was carried out by Dr. Carl Drucker of the same laboratory.

RESULTS

The various analytical data for the enzyme preparations studied are given in Table 1.

Table 1. Properties of Various Catalase Preparations.

Preparation	Kat. F.	Per cent Fe	Per cent Hemin	Absortion Coefficient [1] $\times 10^{-7}$ (β)		s_{20w}
				405 mμ	280 mμ	
2[4]	117 000	0.097	1.04	89.4	68.8	
4A	97 000	0.098	1.04	92.7	71.4	11.61 [2]
5Aq[5]	110 000	0.098	1.04	96.0	68.0	11.61 [2]
5S	76 000	0.094	1.06	94.9	72.5	11.61 [2]
6A	69 000	0.097	1.04	91.6	69.7	
8Aq	125 000	0.094	1.06	90.9	68.0	11.64 [3]
Average		0.096	1.04			

[1] Calculated for a molecular weight of 225 000.

[2] Sedimentation constant for single runs at 0.8% protein.

[3] Sedimentation constant for single run at 0.6% protein.

[4] The properties of this preparation were presented in a previous report [1].

[5] This preparation was also assayed for activity by Dr. Britton Chance in Philadelphia and found to possess a Kat. F, of 107 000.

Activity: A relatively wide range of activity is noted. All of the samples studied also invariably showed decreases in activity during the assay by the rapid method [5] for determining the Kat. F. and the firstorder reaction constants were extrapolated to zero time as was done previously [1]. The values reported in Table 1 were obtained at temperatures between 18—20° C. Bonnichsen, Chance and Theorell [5] have reported a value for this constant of 3.5×10^7 liter $mole^{-1}$ $sec.^{-1}$ at 22.5° for a catalase of Kat. F. of 61 000. We have used this figure to calculate the Kat. F. values in this work and have not corrected for the differences in temperature at which the assays were conducted. However, the low temperature coefficient of catalase would raise these figures only slightly.

The catalase samples usually tended to show activity decreases upon standing in the cold. This was usually synchronous with the formation of small amounts of a greyish precipitate. Samples stored in the frozen state also usually gave small amounts of insoluble material on thawing. Preparation no. 8 with an initial Kat. F. of 125 000 showed a value of only 90 000 after storage in 12 % solution at 1—3° C for a two-week period. The activity then remained constant over a further three-week period. A similar decrease for a high activity catalase has been previously reported [1]. Such findings are further indication that at least a portion of the molecules of such highly active eryhthrocyte catalases are more labile than has been generally assumed for this enzyme. Of particular interest is the ability to consistently obtain material of higher activity than the generally accepted Kat. F. 60 000 material.

Yields: The amount of material recovered from horse erythrocytes varied greatly and usually amounted to 10—60 % of the yields experienced by Bonnichsen [3]. In about one out of four fractionations suitable crystalline material, that is crystals not contaminated by amorphous material, could not be obtained. The most successful preparation was no. 8 in which experiment 2.5 g of three times aqueous crystallized enzyme was recovered from 35 liters of washed erythrocytes. In the case of preparation no. 5 only half of the total recovered catalase crystallized on aqueous dialysis (5 Aq). The remainder (5 S) could, however, be crystallized from ammonium sulfate. As shown by the data of Table 1, the latter fraction possessed a considerable lower activity than 5 Aq.

Absorption spectra: These were determined by means of a Beckman spectrophotometer. Dry weight determinations (constant weight at 105° C) on aliquots of exhaustively dialyzed samples were used to standardize the solutions employed. The same maxima as reported by other workers were found but differences in extinction coefficients were noted. Average values for the molar extinction coefficient (β) at 405 and 280 mμ were 92.6 and 69.7 cm^2 $mole^{-1}$

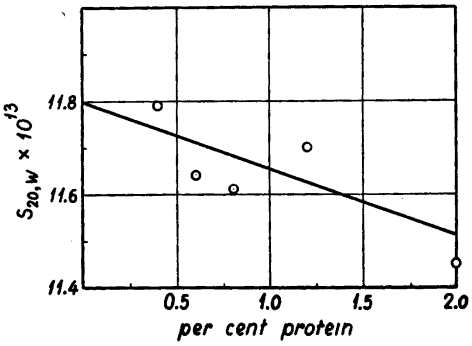

Fig. 1. Sedimentation constant of preparation 5 Aq at various protein concentrations.

respectively. The absorption coefficients (β) were calculated on the basis of a molecular weight of 225 000 since this is the value usually employed by other workers and hence will allow for direct comparison of results. Values for β may be readily calculated to the molecular weights reported in this paper. The results at 405 mμ in particular is considerably higher than that found by Bonnichsen [3] but is not reflected by a higher hemin content. Preparation 5 Aq while showing the same 280 mμ β reported by this worker gave a value at 405 mμ of 96 in contrast to the latter workers value of 86×10^7 cm^2 mole^{-1}. All of the preparations show a ratio of the 405 to the 280 mμ extinction of 1.30 or greater. A high value for this ratio should be synonomous with increased catalase purity in the absence of other proteins showing a higher absorption at 405 mμ than catalase. The low hemin content would appear to obviate the latter possibility.

Hemin and Iron Content: The hemin values determined for the various preparations were relatively constant. The average of 1.04 % is slightly lower than the value of 1.07—1.09 % reported by Bonnichsen [3], for horse erythrocyte catalse. A value for the molecular weight of 250 000 is obtained on the basis of the hemin content and the assumption of four hemins per mole of protein.

Some variation in the iron content was found for various samples, an average of 0.096 % obtaining. This is slightly higher than the value of 0.091—0.095 found by Bonnichsen [3]. From the iron content a molecular weight of 233 000 may be calculated.

Molecular Kinetic Studies: Three preparations, 4 A, 5 Aq and 5 S, which showed considerable variation in Kat. F. and absorption coefficients were first studied in the ultracentrifuge at a concentration of 0.8 %. A single symmetrical boundary was seen for preparations 4 A and 5 S. Sample 5 Aq showed a small amount of material of sedimentation constant of approximately 3 *S*. Identical average values of $s_{20w} = 11.61$ *S* were obtained in each case.

The spread in interval values during each experiment was sufficiently large to indicate that the remarkable close agreement of the individual experiments must be regarded as fortuitous. Nevertheless it indicates a close similarity in molecular mass for the above preparations of widely different activity. Further single sedimentation experiments were also carried out on preparations 8 Aq at 0.6 % protein, and 5 Aq at 0.4, 1.2 and 3.0 % protein. The value for the latter sample at the 3.0 % protein concentration was very low (10.45 S) and was not used for the extrapolation of the sedimentation constant to infinite dilution. The data for these experiments which are plotted to give Fig. 1 indicate that the sedimentation constant for horse erythrocyte catalase is close to 11.8×10^{-13} cm per sec. Our value for the sedimentation constant of a solution near 1 % protein is higher than the 11.0—11.3 S previously found for the main components of horse liver [7,8], and human erythrocyte and bacterial catalases [9].

Various diffusion experiments were carried out. The most technical perfect experiments performed on sample 8 Aq gave the following results.

Concentration	D_A	D_m
g/100 ml		
0.6	4.14	4.11×10^{-7} cm²/sec.
0.43	4.08	4.28×10^{-7} » »

The close agreement of the diffusion constant when calculated by both the height-area and moments method indicates that the enzyme was molecularly homogeneous.

After the diffusion experiments this material (8 Aq) was studied in the ultracentrifuge at a concentration of approximately 0.6 %. A single peak of sedimentation constant 11.64 S was observed. This result is again in remarkable agreement with the sedimentation results obtained for the other three catalase preparations studied in this concentration range.

Table 2. Physical constants of horse erythrocyte catalase.

Molecular weight:	Fe content	233 000
	Hemin content	250 000
	Molecular kinetic	269 000
V_{sp}		0.74
D_A		4.11×10^{-7} cm²/sec.
D_m		4.19×10^{-7} » »
s_{20w}		11.8×10^{-13} cm/sec.
f/f_0		1.17

The value of the partial specific volume was 0.74 which is near the 0.73 reported by Sumner and Gralén [10] for beef liver catalase, but considerably higher than the value of 0.715 found by Agner [7] for a horse liver catalase. From the above values of the sedimentation and diffusion constants and partial specific volume a molecular weight of 269 000 is calculated. This is considerably higher than the commonly accepted value of 225 000 for erythrocyte catalase and likewise higher than the values calculated from the hemin and iron contents. From the above data a dissymmetry factor (f/f_0) of 1.17 and an axial ratio near 4 is obtained if one assumes the molecule to be an elongated ellipsoid. The various physical data are summarized in Table 2.

SUMMARY

Variations in the analytical data for six crystalline horse erythrocyte catalase preparations indicate that this enzyme is not a uniformly pure protein as is ordinarily assumed. Support for this has already been presented in the form of denaturation studies on several preparations of this enzyme [2]. The variations in activity and conditions for crystallization are not reflected in any discernable molecular mass difference. It would appear that the present methods of preparation result in only slight modification of the catalase molecule but that these are synchronous with rather large changes in activity and in ease of crystallization.

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