# Crystalline Human Erythrocyte Catalase

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The enzyme catalase has been isolated in crystalline form from ox liver (Sumner & Dounce, 1937), horse liver (Agner, 1942), lamb liver (Dounce, 1942), ox erythrocytes (Laskowski & Sumner, 1941), human and horse erythrocytes (Bonnichsen, 1947). In the preceding paper (Herbert & Pinsent, 1948) we describe the isolation of crystalline catalase from a bacterium, *Micrococcus lysodeikticus*.

With the exception of human-blood catalase, all these enzymes have been reported to contain about 0·097 % Fe or 1·1 % Fe-porphyrin, corresponding to four iron-porphyrin groups in a molecule of molecular weight 230,000; in bacterial and erythrocyte catalases all four iron-porphyrin groups are haematin, while in liver catalases a varying proportion may be verdohaematin or a related compound. This similarity between the catalases of three mammalian and one bacterial species is remarkable, and the reported exception of human-blood catalase is unexpected. This enzyme was crystallized by Bonnichsen (1947), who found it to contain only 0·077 % Fe and 0·83% haematin, and stated 'if we assume the same molecular weight as for the other catalases, this would mean that this catalase contains only three haematin groups'.

We have isolated crystalline catalase from human red cells by a new method. Contrary to Bonnichsen's findings, it contained 1·15 % haematin, corresponding to four haematin groups/molecule, if the molecular weight is assumed to be 220,000. Since no ultracentrifugal measurements have hitherto been reported for erythrocyte catalases, our enzyme was submitted to Dr R. Cecil, who found it to have a sedimentation constant of $11{\cdot}26 \times 10^{-13}$ (Cecil & Ogston, 1948), identical with the recorded values for liver catalases. Our preparation had a *Kat.-f.* of 63,000 and a ratio *Kat.-f.*/percentage of haematin of 54,400, which are similar to the values reported for other mammalian catalases (see Table 2, Herbert & Pinsent, 1948).

These results show human erythrocyte catalase to be essentially similar in catalytic activity and molecular weight to all other erythrocyte catalases, and to have the same number (four) of haematin groups/molecule. As our method of isolation evidently produces a purer enzyme than that of Bonnichsen (1947), and also gives higher yields, the details were thought to be worth placing on record.

## EXPERIMENTAL

*Methods.* All methods used for determination of *Kat.-f.*, haematin content, etc., were exactly as described by Herbert & Pinsent (1948).

### *Isolation of crystalline human erythrocyte catalase*

No particularly novel methods have been used in our isolation procedure. The first step consists in shaking the haemolyzed red cells with ethanol and chloroform (cf. Tsuchihashi, 1923); this removes all the haemoglobin, leaving the catalase in the supernatant fluid. The catalase is then absorbed on calcium phosphate gel. It may be eluted from the gel with 0·05N-ammonium hydroxide or disodium phosphate, but several washes with rather large volumes are necessary for complete elution, so that the final eluate is very dilute. Instead, we decompose the gel by shaking with potassium oxalate and centrifuging off the precipitated calcium oxalate. This gives quantitative recoveries in a small volume.

These two steps effect a hundredfold purification and give an enzyme approximately 20 % pure, from which pure, crystalline catalase may easily be obtained by careful fractionation with ammonium sulphate. Controlled salt fractionation has not much been used by previous workers on catalase, who have mostly preferred fractionation with organic solvents; our experience shows it to be an efficient as well as a simple technique.

The following are the details of a typical preparation:

*Stage* 1. The red cells were removed from citrated human blood by centrifuging, and washed twice on the centrifuge with 0·9 % NaCl; 6·84 l. of packed red cells were laked with an equal volume of distilled water, giving a solution containing 1690 g. total protein and 2·71 g. pure catalase; *Kat.-f.* = 101 (Note 1).

*Stage* 2. The laked red cells were treated with 0·44 vol. of a 3:1 ethanol-chloroform mixture, and stirred vigorously for 15 min. The sticky mass of precipitated haemoglobin was removed by straining through muslin and well pressed out. The rather turbid solution was filtered through a pad of 'Hyflo Super-Cel,' giving 14·6 l. of a clear, pale yellow filtrate showing no haemoglobin spectrum; *Kat.-f.* = 1620, yield 70 %.

*Stage* 3. The above filtrate was treated (Note 2) with 2 l. of a 1·5 % suspension of $Ca_3(PO_4)_2$ gel (Keilin & Hartree, 1938) and stood 1 hr. to allow the gel to settle. Most of the supernatant fluid could then be decanted off and the re-

mainder separated by centrifuging. The gel had adsorbed a considerable quantity of protein but very little catalase. A second addition of 30 g. of $Ca_3(PO_4)_2$ (Note 2) adsorbed almost all the catalase, and was collected by decantation followed by centrifuging. The packed $Ca_3(PO_4)_2$ precipitate was shaken vigorously with 600 ml. of 0·5M-potassium oxalate in 0·5M-$NaH_2PO_4$ (Note 3). The calcium oxalate was centrifuged off and washed twice with water, the washings being added to the first eluate. This gave 1620 ml. of a brown solution with a clearly visible catalase spectrum; *Kat.-f.* = 10,400, overall yield 56%.

*Stage* 4. Solid $(NH_4)_2SO_4$ was added to the above solution (30 g./100 ml.), and the greyish brown precipitate collected by centrifuging and dissolved in 100 ml. water; 20 ml. of N-acetate buffer pH 4·0 were then added and the solution allowed to stand 2 hr. (cf. Bonnichsen, 1947). A flocculent greyish precipitate formed and was removed by centrifuging, leaving a clear greenish brown liquor which was adjusted to about pH 5·6 with $Na_2HPO_4$; *Kat.-f.* = 21,800, overall yield 36%.

*Stage* 5. The catalase solution from stage 4 was treated with 4M-$(NH_4)_2SO_4$, added drop by drop with good stirring. A small, greyish brown precipitate which appeared at an $(NH_4)_2SO_4$ conc. of 1·4M (Note 4) was removed by centrifuging. Careful addition of $(NH_4)_2SO_4$ was continued until a faint turbidity appeared; on standing at room temperature this rapidly increased and developed a silken sheen, noticeable on stirring, like a suspension of bacteria. Microscopic examination showed it to consist of very fine needle-shaped crystals. After standing 1 hr. these were centrifuged down, and a further crop of crystals obtained from the mother liquor by adding a little more $(NH_4)_2SO_4$. The two crops of crystals were pooled and dissolved in 50 ml. of water; total 710 mg. or 26% overall yield on the red-cell haemolysate. The *Kat.-f.* was 50,200 and the haematin content 1·05%. After two recrystallizations by the same method, the *Kat.-f.* was raised to 63,000 and the haematin content to 1·15%.

*Note* 1. This batch of red cells had been kept for 13 days at 2° before using. In another preparation where the blood was used fresh, the washed, laked red cells had a *Kat.-f.* of 166. Both batches were pools from several donors.

*Note* 2. The quantity of calcium phosphate required varies from one preparation to another, and should be determined by a small-scale trial, treating, e.g. 10 ml. samples of enzyme with 1, 2, 3 and 4 ml. of gel suspension, centrifuging, and estimating the catalase left in the supernatant fluid.

*Note* 3. When $Ca_3(PO_4)_2$ is decomposed with oxalate the solution becomes strongly alkaline through the formation of $K_3PO_4$; hence $NaH_2PO_4$ is added to keep the pH neutral.

*Note* 4. The exact $(NH_4)_2SO_4$ concentration cannot be standardized, as it depends on many factors, including the catalase concentration; it is necessary to proceed empirically (cf. Herbert & Pinsent, 1948).

### *Properties of the crystalline enzyme*

As already mentioned, the enzyme crystallizes from $(NH_4)_2SO_4$ in fine needles. Their refractive index is very similar to that of the mother liquor, which makes them difficult to photograph. On dialysis for many weeks against distilled water the enzyme crystallized in large irregular plates. It is more difficult to crystallize in this way than bacterial catalase; electrodialysis would probably be more efficient (cf. Bonnichsen, 1947). Solutions of the crystals in 0·05M-phosphate pH 6·8 are reddish brown, and show absorption bands at 630, 544 and 505 m$\mu$. (Hartridge reversion spectroscope). Treatment with pyridine, sodium hydroxide and sodium hyposulphite produces the characteristic spectrum of pyridine haemochromogen. On treating with acetone-hydrochloric acid (cf. Herbert & Pinsent, 1948) the prosthetic group is split off, and appears to consist entirely of haematin; no trace of biliverdin could be detected.

The purest sample obtained had a *Kat.-f.* of 63,000, and contained 1·15% haematin. If this is the pure enzyme, the molecular weight is $n \times 55{,}000$, where $n$ is the number of haematin groups/molecule; for four haematin groups, the molecular weight would be 220,000. This sample was examined in the ultracentrifuge by Cecil & Ogston (1948) who found it to consist in the main of a homogeneous protein with a sedimentation constant of $11{\cdot}26 \times 10^{-13}$. This is in good agreement with the values of $11{\cdot}2 \times 10^{-13}$ for beef-liver catalase (Sumner & Gralén, 1938) and $11{\cdot}2 \times 10^{-13}$ for horse-liver catalase (Agner, 1938). This component accounted for 72% of the total refractive increment, and traces of another coloured protein were present in the ultracentrifuge photographs. Unfortunately some time had elapsed between the preparation of this sample and its examination in the ultracentrifuge, during which period the activity had decreased from the original value of *Kat.-f.* = 63,000 to 44,300, a decrease of 29%. We suspect, therefore, that the coloured impurity present was denatured catalase.

A rough estimate of the minimum molecular weight from the sedimentation constant is 176,000, assuming a spherical unhydrated molecule. If the molecule is not spherical, which is certainly the case (Sumner & Frampton, 1940), the molecular weight would be higher. It was unfortunately impracticable to perform measurements of partial specific volume and diffusion constant.

## DISCUSSION

The results show that (within the limits of error of the various methods) human-blood catalase has essentially the same *Kat.-f.*, haematin content and molecular weight as other red-cell catalases, and like them, has four haematin groups/molecule. The reason for the discrepancy between our results and those of Bonnichsen (1947) is not clear. His preparations, however, had about the same *Kat.-f.*/percentage of haematin ratio as ours, although the absolute values of both were lower. The simplest explanation would be that his preparations were not pure; we found several recrystallizations necessary to obtain our purest enzyme. Our method of purification is simpler than any hitherto described, and should be applicable to the red cells of other species.

Crystalline erythrocyte catalase may now be considered a readily available enzyme.

It is interesting to calculate the catalase content of the human erythrocyte. This is obtained from the *Kat.-f.* of the pure enzyme (63,000) and of washed red cells, which in two different preparations was found to be 101 and 166. Hence catalase accounts for about 0·16–0·26 % of the total dry matter of the human red-blood corpuscle.

## SUMMARY

1. The isolation of crystalline catalase from human red cells is described. The method is simple and the yield of crystalline enzyme is about 25 % of that present in the red cell.

2. The crystalline enzyme had a *Kat.-f.* of 63,000 and a haematin content of 1·15%. The sedimentation constant was $11{\cdot}26 \times 10^{-13}$. The molecular weight was calculated to be 220,000 from the haematin content. There are four haematin groups/molecule; no verdohaematin is present.

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## REFERENCES

Agner, K. (1938). *Biochem. J.* **32**, 1702.
Agner, K. (1942). *Ark. Kemi Min. Geol.* **16** A, no. 6.
Bonnichsen, R. K. (1947). *Arch. Biochem.* **12**, 83.
Cecil, R. & Ogston, A. G. (1948). *Biochem. J.* **43**, 205.
Dounce, A. L. (1942). *J. biol. Chem.* **143**, 497.
Herbert, D. & Pinsent, J. (1948). *Biochem. J.* **43**, 193.
Keilin, D. & Hartree, E. F. (1938). *Proc. Roy. Soc.* B, **124**, 397.
Laskowski, M. & Sumner, J. B. (1941). *Science*, **94**, 615.
Sumner, J. B. & Dounce, A. L. (1937). *J. biol. Chem.* **121**, 417.
Sumner, J. B. & Frampton, O. D. (1940). *J. biol. Chem.* **136**, 343.
Sumner, J. B. & Gralén, N. (1938). *J. biol. Chem.* **125**, 33.
Tsuchihashi, M. (1923). *Biochem. Z.* **140**, 63.

# Examination of Crystalline Catalases in the Ultracentrifuge

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Three samples of crystalline catalase were supplied by Dr Denis Herbert (Herbert & Pinsent, 1948 *a*, *b*). They were examined in a Svedberg oil-turbine ultracentrifuge by the method of Philpot (1938). The solutions all contained approximately 0·5 % catalase in a buffer of composition sodium chloride 0·2M, disodium hydrogen phosphate 0·05M, sodium dihydrogen phosphate 0·05M.

The speed was approximately 1015 rev./sec., and the wave length of light used 620–660 mμ. For the calculation of the sedimentation constants, the partial specific volume was taken as 0·73 ml./g. (Sumner & Gralén, 1938),

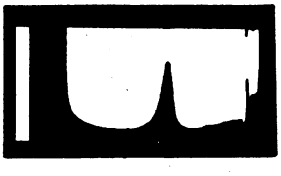

Fig. 1.

Fig. 2.

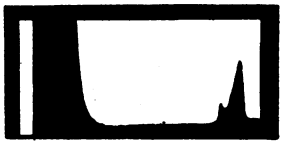

Fig. 3*a*.

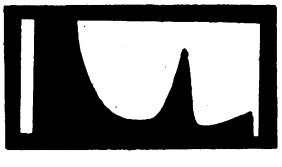

Fig. 3*b*.

Fig. 1. Sample AI (bacterial catalase) approx. 25 min. after reaching full speed.

Fig. 2. Sample AII (bacterial catalase) approx. 25 min. after reaching full speed.

Fig. 3*a*. Erythrocyte catalase immediately on reaching full speed, showing heavier component.

Fig. 3*b*. Erythrocyte catalase approx. 25 min. after reaching full speed.

*Note.* The different appearance of the meniscus in Figs. 1 and 2 was due to the use of a layer of paraffin in these two runs.