Studies of Human Erythrocyte Catalase by Fluorescent Antibody Technic
The Distribution of Catalase in Acatalasia and Hypocatalasia

By Takemitsu Hosoi, Seiji Yahara, Howard B. Hamilton, Norio Fujiki, Teruo Sasaki and Yoshinori Ishihara

Human hereditary acatalasia, the absence of catalase activity in erythrocytes and other tissues of the body, is transmitted by a codominant autosomal gene. Heterozygous carriers of this trait generally have enzyme activities about mid-way between zero and normal, a situation referred to as hypocatalasia.¹

A fluorescent antibody technic was used to study the distribution of catalase protein in human erythrocytes, since it has been reported that enzyme activity as well as the amount of catalase protein in hypocatalasic blood, determined chemically or immunologically, is approximately one-half of that in normal blood.

Materials and Methods

Venous blood, obtained from previously described acatalasic and hypocatalasic individuals, was heparinized and transported in iced containers to the laboratory. The acatalasic samples (TE II-2; Kcat = 0.0) were obtained from family TE and the hypocatalasic samples (OK III-1: Kcat = 2.37) from family OK, previously described by Hamilton et al.²
Normocatalasic control samples (Mean $K_{\text{cat}} = 5.38 \pm 0.73$) were obtained from 5 members of the laboratory group.

Crystalline human erythrocyte catalase was prepared from outdated blood bank blood by the method of Herbert and Pinsent\(^3\) and had a $K_{\text{at}}$ value of 59.500.

Rabbit anticatalase sera were prepared in 4 rabbits by five weekly intramuscular injections of 5–7 mg. of this purified catalase in Freund’s adjuvant. Two weeks after the final injection the rabbits were bled and the sera were separated from the cells.

The sensitivity of the anticatalase sera were ascertained by the capillary ring method. Dilutions of the antisera between 0 and 1:100 were tested against concentrations of purified catalase ranging from 1.0 to 0.001 mg./ml. The specificity of the anticatalase sera were tested using Ouchterlony’s double diffusion method.

Fluorochrome conjugation to the rabbit anticatalase serum was carried out as follows: the globulin fraction of the antisera was prepared by precipitating twice with one-third saturated ammonium sulfate; after elimination of ammonium sulfate by dialysis against 0.01 M phosphate buffered saline (pH 7.1) and adjustment to pH 9.3 with 0.5 M carbonate-bicarbonate buffer, fluorescein isothiocyanate (FITC) was added in a ratio of about 1:100 to the protein content and stirred for several hours at room temperature. Free fluorochrome was removed by filtration through a Sephadex G 50 column.

Thin smears were prepared from whole blood or from washed red cells, dried at room temperature and fixed with acetone for a few minutes.

The blood film was flooded with the fluorochrome conjugate and allowed to stand for about an hour at room temperature. After washing the slide with 0.01 M phosphate buffered saline (pH 7.1), the stained film was covered with Elvanol (commercial product, polyvinyl alcohol diluted with glycerine) and observed using a dark field microscope and mercury vapor fluorescence lamp.

## RESULTS

The sensitivity of our rabbit antihuman catalase serum to human purified catalase is shown in Table 1. The catalase-anticatalase reaction is more sensitive than hydrogen peroxide in detecting small amounts of catalase, i.e., 0.005 mg./ml. catalase gives a positive capillary precipitin ring test but fails to form bubbles with hydrogen peroxide.

Anticatalase serum reacts with human erythrocyte catalase, normal adult hemolysate and commercial beef liver catalase but not with human serum. As shown in Figure 1, the anticatalase serum also reacts with an hypocatalasic hemolysate but not with an acatalasic hemolysate. The reaction with the hypocatalasic hemolysate is weaker than that with normal adult hemolysate, al-

### Table 1.—Precipitin Reaction Between Increasing Dilutions of Rabbit Antihuman Erythrocyte Catalase Serum and Decreasing Concentrations of Purified Human Erythrocyte Catalase, Tested by the Capillary Precipitin Ring Method

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Fig. 1.—Agar double diffusion of anticalase serum (central well) and purified human catalase, normal adult hemolysate, hypocatalasic hemolysate and acatalasic hemolysate.

though both hemolysates were prepared in 7 Gm./100 ml. hemoglobin concentration. Moreover, additional thin precipitin bands formed near the center well containing anticalase; all hemolysates, including the acatalasic hemolysate, participated in this secondary precipitin reaction.

Anticalase serum labelled with FITC was used to stain normal adult, hypocatalasic and acatalasic blood smears. The fluorescence in normal adult red cells stained with FITC labelled anticalase serum appeared approximately homogeneous in each erythrocyte. In hypocatalasic cells fluorescence was also evenly distributed in the red cells, but was weaker than in normals. Acatalasic red cells did not show any fluorescence (Figs. 2A, B and C).

To eliminate the possibility of misinterpretation of variable staining that might occur from one smear to the next, artificial mixtures of various blood samples were prepared, and smears were stained with FITC labelled anticalase. Normal blood was mixed with acatalasic blood in ratios of 1:9 and 1:4 (both samples blood group A). Two populations of cells were present: brightly fluorescent and nonfluorescent cells, corresponding to normo-calasic and acatalasic cells respectively, and present in the approximate proportions of the original mixtures (Fig. 3A).

Normal adult blood (group O) was also mixed with hypocatalasic blood (group O) in ratios of 1:9 and 1:4 (Fig. 3B). The difference in fluorescence between normal and hypocatalasic red cells was not as distinct as in the experiments illustrated in Figure 3A.

When normal blood (group A) was mixed with hypocatalasic blood (group O), the agglutinated blood group A normal cells were brighter than unagglutinated blood group O hypocatalasic cells. In a similar experiment, fluorescent hypocatalasic cells could be distinguished from nonfluorescent acatalasic cells although the difference was not marked.
Fig. 2.—Photomicrographs of human erythrocytes stained with fluorescein-labelled anticalatalase.

A. Normal adult red cells.
B. Hypocalatalasic red cells.
C. Acatalasic red cells.
DISCUSSION

Subsequent to the discovery of acatalasia and hypocatalasia, a number of immunological studies of human erythrocyte catalase have been performed by others. Our results confirm the findings of these investigators that on Ouchterlony’s double diffusion and immunoelectrophoresis, rabbit antihuman erythrocyte catalase serum produces two precipitin bands, one with normal and hypocatalasic hemolysates only, and a second fainter band with acatalasic as well as hypocatalasic and normal hemolysates. While the former band exhibits catalase activity, the latter does not. Fusion of the bands indicates immunologic identity. Shibata et al. suggested that the enzymatically inactive component which precipitates with anticatalase may be either a catalase subunit or precursor.

Most heterozygous carriers of the acatalasia gene have blood catalase activity about one half of normal. Utilizing the quantitative precipitin method, Ogata and Shibata et al. also found that the precipitate of the hypocatalasic hemolysate with anticatalase was approximately one-half that of normal.

In the study reported here, the fluorescent antibody labelling technic was used to estimate the distribution of catalase in individual red cells from normal, hypocatalasic and acatalasic individuals. The amount of enzyme in the hypocatalasic cells was judged to be less than that in the normals. The cellular
distribution of the enzyme appeared to be fairly homogeneous in the normal and hypocatalasic cells, and was not demonstrable in acatalasic cells. Aebi et al., utilizing a method that measures the sensitivity of red cells to H₂O₂ have also demonstrated that in hypocatalasia there is but a single cell population with respect to catalase distribution. Aebi et al. further suggested that in blood smears from acatalasics, one out of 100-150 cells (i.e., 0.6-1.0 per cent) seemed to contain approximately the same amount of catalase as that of a normal red cell. We did not observe this phenomenon in our acatalasic smears, however. It is quite possible, as others have suggested, that the Swiss acatalasics and those studied by us differ somewhat from each other, and therefore, we might not necessarily expect to demonstrate “residual” catalase activity in our acatalasic samples.

Previously, one of the present authors studied the cellular distribution of hemoglobin F (HbF) in red cells by the fluorescent antibody technic and concluded that differences in fluorescent intensity might be interpreted as an indication of quantitative variation of HbF in the cells. Recently, we studied red cell A and B blood group substances using the same technic, and variation of fluorescent intensity in different cells was observed. It was tentatively assumed that the amount of A and B antigen varies from cell to cell. Cohen et al. using the immunofluorescence technic, stated that subgroup A₁ could be subdivided into “strong,” “intermediate” and “weak” A₁ according to the intensity of the fluorescence. In the present study, no such variation of fluorescein conjugated catalase was observed, and it is concluded that catalase is distributed approximately uniformly among the red cells in normal and hypocatalasic individuals.

**SUMMARY**

In order to investigate the cellular distribution of catalase in normal, hypocatalasic and acatalasic red blood cells, the fluorescent antibody labelling technic was employed. Sensitive anticatalase sera were produced in rabbits by immunization with purified catalase extracted from human erythrocytes. Specificity against human erythrocyte catalase was confirmed by Ouchterlony’s double diffusion method.

The distribution of catalase is fairly homogeneous in normal and hypocatalasic red cells, but in acatalasic cells fluorescence due to the presence of catalase was not observed.

By this method the amount of catalase in hypocatalasic red cells was judged to be between that of normal and acatalasic red cells.

**SUMMARIO IN INTERLINGUA**

Pro investigar le distribution cellular de catalase in erythrocytos normal, hypocatalasic, e acatalasic, le technica del marcation a anticorpore fluorescente eseva utilisate. Sensibile seros anti catalase eseva producite in conilios per immunisation con purifice catalase extrahite ab erythrocytos human. Specificitate contra catalase ab erythrocytos human eseva confirmate per le methodo de Ouchterlony a double diffusion.

Le distribution del catalase es satis homogenee in erythrocytos normal e hypocatalasic, sed in cellulas acatalasic nulle fluorescentia causate per le presentia de catalase eseva observate.
Per iste methodo le quantitate de catalase in erythrocytos hypocatalasic pareva esser intermediari inter illo de erythrocytos normal e illo de erythrocytos acatalasic.

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