Blood Catalase Polymorphism: Some Immunological Aspects

Abstract. *The immunological properties of the erythrocyte catalase of mice—normal (wild type) strain, one lacking catalase (acatalasemic), and four with only slight catalase activity (hypocatalasemic strains)—have been investigated. Agar-diffusion tests and antigen titration of red-cell lysates against rabbit antiserum to catalase from normal mouse blood showed that immunologically identical catalase protein was present in large amounts in the acatalasemic as well as in the hypocatalasemic mutant strains. Despite lack of catalatic activity, the erythrocytes lacking catalase as well as those with only a little catalase contain catalase protein that has been modified at the site of enzyme activity, although the antigenic determinants are identical with those of normal catalase protein. This mutation is purely structural, being characterized by modification of the enzyme active site but not of the antigenic site.*

The development of five strains of mice, mutant with respect to blood (*1*) and tissue (*2*) catalase, has been described. One strain, with blood catalase activity 1 to 2 percent of normal, is called the "acatalasemic" strain and is designated Cs^b. The other four strains, with blood catalase activity 20 to 30 percent of normal, are called "hypocatalasemic" and are noted as Cs^c, Cs^d, Cs^e, and Cs^f. The normal, wild-type strain from which the mutants were derived is known as Cs^a. The wild-type and five mutant strains have been shown to be multiple alleles at a single locus (*1*).

As judged by stability to heat, *p*H extremes, urea, formamide, and other physical and chemical agents, the various mutant blood catalases represent distinct and different molecular species (*2*). Hence this situation may be an instance of enzyme polymorphism, that is, a structural gene mutation.

Whether the acatalasemic mouse synthesizes any blood catalase at all or approximately normal amounts of an enzymatically inactive catalase protein has not been known. We now report on the immunological relationships between the catalase of the normal strain and that of the various mutants, and show that, while the site of enzyme activity differs between the former and the latter, the immune site is unchanged.

For this purpose, the six strains of mice described above were used: normal, Cs^a; acatalasemic, Cs^b; and hypocatalasemic, Cs^{c-f}. These strains were developed from progenitors (*3*) screened from a large number of mice whose blood had been assayed for catalase activity. Heparinized blood for antigen was collected from the orbital sinuses (*4*), the animals being exsanguinated and the blood being pooled when necessary. The blood was centrifuged, and the packed cells were washed twice with cold saline. They were then lysed by the addition of an appropriate amount (at least an equal volume) of cold 0.2 percent saponin and refrigerated for 30 minutes to permit lysis to proceed. For an antigen-titration experiment, or an Ouchterlony double-diffusion or immunoelectrophoresis test, the lysate was centrifuged to eliminate most of the cell debris. When the lysate was used to immunize mice or rabbits, the centrifugation was omitted.

Three antiserums were prepared: Cs^a mouse antiserum to Cs^b blood, Cs^b mouse antiserum to Cs^a blood, and rabbit antiserum to Cs^a blood. Mice were immunized with three series of injections at 45-day intervals. The first series consisted of three intramuscular injections, on alternate days, of 0.1 ml of an emulsion (1 : 1) of concentrated red-cell lysate and Freund's complete adjuvant; the second and third series consisted of three intraperitoneal injections, on alternate days, of 0.25 ml of the concentrated red-cell lysate without Freund's adjuvant. A week after the third series of injections, the mice were exsanguinated via the orbital sinus, and the plasma was separated and frozen. Rabbits were also immunized with three series of injections at 45-day intervals. The first series consisted of five injections of 1 ml of a 1 : 1 emulsion of concentrated red-cell lysate and Freund's complete adjuvant in the nuchal region on each of 3 days during the course of a week; the second and third series consisted of one injection into each footpad, thrice weekly, of 0.25 ml of the concentrated red-cell lysate without Freund's adjuvant. A week after the third series of injections, the rabbits were bled, and their serums were separated and frozen.

Immunoelectrophoresis and Ouchterlony double-diffusion tests were carried out on microscope slides coated with agar (Noble, Difco). The conditions for electrophoresis were: barbital buffer, *p*H 8.6, ionic strength 0.1; 3°C (cold room); 1 hour with an applied voltage of 250 volts. Diffusion was carried out at room temperature. The LKB apparatus and technique were used for both procedures. In some instances, instead of staining the slides with amido

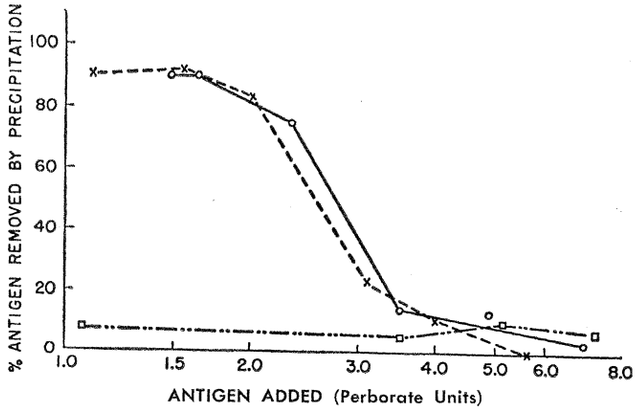

Fig. 1 (left). Antigen titration curves. Rabbit antiserum to Cs^a mouse blood is labeled against the following antigens: (circles) Cs^a mouse blood; (crosses), C3Hf mouse blood; squares) rat blood.

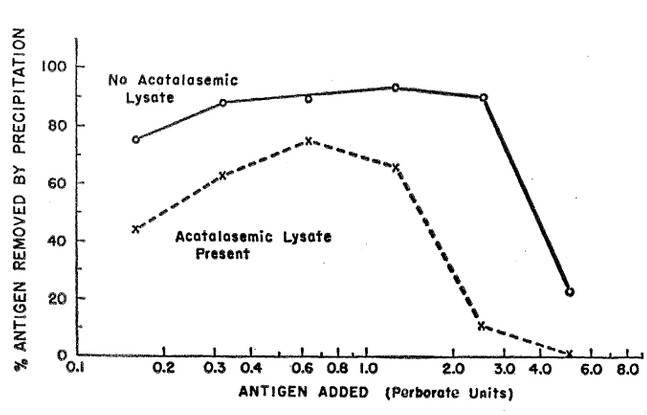

Fig. 2 (right). Effect of acatalasemic (Cs^b) mouse blood on Cs^a mouse blood titration curve; rabbit antiserum to Cs^a mouse blood.

black, they were flooded with 3 percent hydrogen peroxide for visualization of catalase by formation of oxygen bubbles (*5*).

For antigen titration, 0.5 ml of antigen, 0.5 ml of saline, and 0.5 ml of antiserum were mixed in a tube, incubated at 37°C for 30 minutes, and centrifuged. The supernatant was drawn off into a fresh, chilled container, diluted if necessary, and assayed for catalase activity. Controls were included to determine catalase activity added as antigen, and also to observe any possible catalase activity contributed by the antiserum. Rabbit erythrocytes are relatively rich in catalase, so that even slight, inadvertent laking of cells will require an appreciable correction for the value in the serum. The saline was included because sometimes we determined the effect of one cell lysate on the titration of another; in such a case, the second lysate replaced the saline.

Assays of catalase activity were done by the perborate method (*6*). Sodium perborate serves as substrate in a 5-minute incubation at 37°C in the presence of phosphate buffer at *p*H 6.8, and the amount of perborate destroyed is measured by permanganate titration.

The Cs^a blood lysate as antigen was titrated against rabbit antiserum to Cs^a blood, and 90 percent of the catalase activity was precipitated in the zone of optimum proportions. In another experiment, intended to quantify the antigen-antiserum relationship, it was found that doubling the antiserum concentration caused the precipitation, at the threshold of antigen excess, of twice the amount of catalase activity. Normal rabbit serum, on the contrary, precipitated only traces of the catalase.

Figure 1 compares the titration curves of rabbit antiserum to Cs^a-mouse blood against antigen lysates prepared from Cs^a mouse, C3Hf mouse (as example of a genetically different strain), and from rat red-blood cells. The precipitin curves indicate that C3Hf and Cs^a mouse antigens are identical, but are not related to rat red-blood cell lysate.

To determine whether or not there is any immunologically related but noncatalatic protein in the lysate from the acatalasemic mutants, we titrated Cs^a lysate in the presence of an equivalent amount of Cs^b lysate. As indicated by a shift of the threshold of antigen excess to the left in Fig. 2, the Cs^b lysate, though catalatically inactive, effectively acts as if the amount of antigen were doubled (*7*). The specificity of this cross-reaction is confirmed by the fact that the addition of rat red-cell catalase that is not cross-reactive is completely without effect on the Cs^a antigen titration curve. The addition of egg albumin, bovine serum albumin, or bovine α-globulin is similarly without effect.

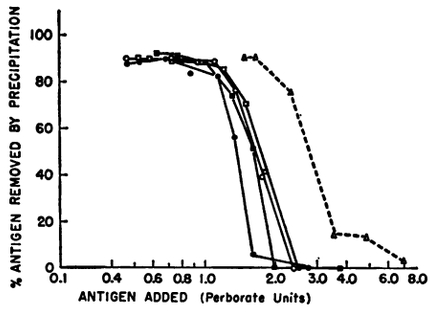

Fig. 3. Antigen titration curves, normal (Cs^a) mouse blood compared to four different hypocatalasemic mouse bloods. Antiserum for all is rabbit antiserum to Cs^a mouse blood. For the dashed line, antigen is Cs^a blood. Each solid line represents blood of one of the hypocatalasemic mutants as antigen.

Figure 3 shows antigen titration curves for normal (Cs^a) red-cell lysates, and also for cell lysates of the four hypocatalasemic strains (Cs^{c-f}). The same concentration of the same antiserum was used throughout. The threshold of antigen excess is approximately the same for all four mutants, but distinctly different for the normal. In addition, the peak of maximum precipitation is 90 percent for the mutants as well as the normal. This entire series of titrations has been repeated, with essentially the same results.

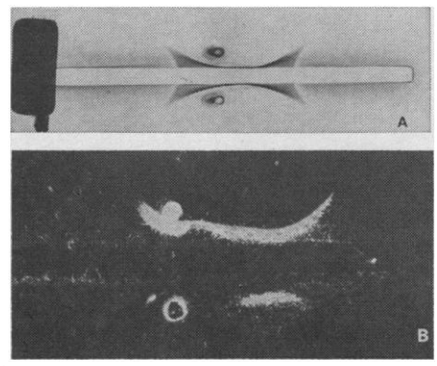

Fig. 4. Immunoelectrophoresis. (A) stained for insoluble protein. (B) Replicate of A, but unstained and flooded with H_2O_2 to detect the line due to catalase and its antibody. Antiserum (central trough) is rabbit antiserum to Cs^a mouse blood. Antigens are: (top) Cs^a blood lysate; (bottom) Cs^c blood lysate.

Results of immunoelectrophoresis are shown in Fig. 4, A and B. These are replicate slides, but in Fig. 4A the insoluble proteins were stained with amido black, whereas the slide of Fig. 4B was flooded with H_2O_2 to localize the line of catalase with its antibody. The normal antigen (upper well in each case) produces proteins that stain exactly like those of the hypocatalasemic antigen (lower well), but whose catalatic activity is much greater for the former than the latter. In Fig. 4B, the Cs^a (normal) lysate apparently exhibits two electrophoretically different species of catalase. One of the active areas is at the origin and presumably represents catalase bound to cell debris which was not completely removed by the initial centrifugation of the blood lysate. As the reaction proceeded further than that shown in Fig. 4B, the lower portion (hypocatalasemic blood) also expanded into a double scallop, with the second curve centered on the origin.

Comparisons by the double-diffusion method of Ouchterlony were also made of normal, acatalasemic, and hypocatalasemic blood lysates, with antiserum against normal lysate. Lysates were used both in a concentrated form and after fourfold dilution. One of these slides was flooded with H_2O_2 and showed sharp, rapid formation of oxygen bubbles between antiserum and either normal or hypocatalasemic lysate, whether concentrated or dilute. No oxygen formation appeared in front of the well with acatalasemic lysate. When a replicate slide was stained with amido black, faint but definite lines of equal staining intensity appeared in this location in front of all three concentrated lysates, including the acatalasemic. Fourfold dilution of each of the lysates caused total elimination of these lines.

The Cs^a (normal) mice were "immunized" against Cs^b (acatalasemic) red cell lysate, and Cs^b mice were "immunized" against Cs^a cell lysate. Plasma from the recipient mice and nonimmunized control mice was then tested for antibody activity toward both Cs^a and Cs^b antigen by immunoelectrophoresis, Ouchterlony double diffusion, and titration. There was no indication that antibody had been produced.

The work of Tria (*8*) and of Campbell and Fourt (*9*) has shown that catalase-anticatalase complex, while in-

soluble, retains its catalatic activity. This fact has made possible the identification of the catalase-anticatalase line in double-diffusion and immunoelectrophoresis plates.

Our data indicate that erythrocyte catalase proteins produced in five mutant mouse strains are quantitatively and immunologically indistinguishable from that in the normal, wild-type strain. The erythrocyte catalase activity, however, is almost absent in the acatalasemic mutant and is much lower than normal in the four hypocatalasemic mutants. These five mutants, therefore, all represent structural genetic mutations. This conclusion is based on the remarkable similarity in the results obtained in two distinct ways.

1) In all the agar-diffusion tests, the lysates from both the acatalasemic (Cs^b) and the hypocatalasemic (Cs^c) cells produce lines that have immunological identity with and are stained to the same degree as those from normal (Cs^a) lysates. In addition, immunologically identical lines due to catalase and its antibody degrade H_2O_2 if the lysate is from Cs^a or Cs^c, but not if the lysate is from Cs^b mice. After immunoelectrophoresis, lines in the same position and with equal staining show that Cs^a has more catalatic activity than Cs^c, and that Cs^b is acatalatic. Further, if the three antigens are diluted fourfold, all fail to produce precipitin lines. As expected, reciprocal tests of Cs^a and Cs^b lysates with antiserums produced against them in Cs^b and Cs^a mice, respectively, produce no precipitation lines.

2) The antigen titrations demonstrate that red-cell antigen of all four hypocatalasemic mutants attains about 90 percent precipitability, as normal red-cell antigen does, but reaches the region of antigen excess at a catalase concentration well below normal. Because the same antiserum was used in all cases, the implication is that all are behaving in identical immunological fashion, but that an equal number of the mutant catalase molecules, being subnormally active catalatically, reaches the threshold of antigen excess at a lower-than-normal level of catalase activity. Catalase protein in antigen prepared from acatalasemic red-blood cells is detected by its interference with the antigen titration curve of normal erythrocyte lysate. Active but unrelated catalase protein (from rat cells) does not affect the precipitin curve with the antiserum as Cs^b antigen does. Normal rabbit serum has no precipitability, and assorted heterologous proteins have no inhibitory activities.

In view of the foregoing considerations, coupled with the demonstration (*2*) that blood and tissue catalase of the mutant strains differ qualitatively from each other and from the wild-type (normal) strain, we are apparently dealing with a purely structural mutation in which the structural modifications involve the enzyme-activity site but not the antigenic site.

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Nerve Growth Factor of Very High Yield and Specific Activity

Abstract. *Nerve growth factor has been isolated from submaxillary glands of mature male mice at specific activities about a million times, and in yields of biological activity ten million times, greater than best previous results. The major improvement in the isolation is related to the separation of a highly active tosylarginine methyl esterase present in cruder preparations. The new nerve growth factor may be an entity different from the older one, although no gross differences in the qualitative aspects of their actions are apparent on superficial examination of chick ganglia influenced by them. The neurites which develop from a ganglion in the presence of nerve growth factor are of nearly equal length. The amount of nerve growth factor determines the number of neurites but not the extent of individual development. The amount of the new nerve growth factor which evokes the appearance of a hundred neurites from a single ganglion appears to be about ten molecules. Since each neurite seems to arise from a different neuron each molecule of nerve growth factor must affect several cells. This result can be rationalized by a catalytic mechanism or by indirect action of nerve growth factor through a hypothetical cell which produces a neurite evocator on contact with the molecule of nerve growth factor.*

Since very marked overgrowth of nerve fibers from chick embryonic spinal ganglia in the presence of implanted tumors was observed in vivo (*1*), it has become clear that a humoral agent is involved (*2*) and that the agent is protein or closely associated with protein (*3, 4*).

A test (*5*) for activity in vitro evolved into an assay method (*3, 6, 7*). The biological unit (bu) is that amount in one milliliter of medium which produces in 18 hours 3+ growth of neurites from spinal ganglion (of a 7 to 9-day chick embryo) suspended in 0.06 ml of medium. Scoring standards have been described (*6, 7*). The medium is one-third rooster plasma and two-thirds medium 199 containing the material of interest.

The richest known source of nerve growth factor (NGF) is the submaxillary gland of the mature male mouse. Materials having specific activities of 1×10^8 to 1.5×10^8 bu/g have been considered highly purified (*4, 6*). Electrophoresis of such material on paper separates it into three bands that stain with ninhydrin. No one of these is active by itself, but a combination of two is active (*8*). Important aspects of the action of NGF have been reviewed (*9*).

We have found that much more (10^7 times) NGF (in biological units) can be isolated from the crude homogenate than has been demonstrated in it by simple dilution; this purified material has a specific activity of 10^{15} bu/g. An enzyme highly effective in the hydrolysis of tosylarginine methyl ester is also found in the cruder extracts (*10*). The two activities are largely separable, and their separation may