Acta Medica Okayama

Volume 25, *Issue* 2 1971 *Article* 3

APRIL 1971

Properties of catalase protein in immature and mature red cells of acatalasemic and hypocatalasemic mouse mutants

Masana Ogata* Toyoko Fujii†

Shigeo Takahara‡

*Okayama University,

†Okayama University,

‡Okayama University,

Properties of catalase protein in immature and mature red cells of acatalasemic and hypocatalasemic mouse mutants*

Masana Ogata, Toyoko Fujii, and Shigeo Takahara

Abstract

The concentration of catalase protein in anemic blood with enhanced population of reticulocytes and in non-anemic blood was determined immunologically by double diffusion test with anti-mome-liver catalase rabbit serum. The change in catalase protein concentration in anemic blood during incubation at 37°C for 24 hours was also studied. It was indicated that the diminished catalase activity in acatalasemic blood was due to the depletion of the protein and that catalase protein in acatalasemic reticulocytes decreased markedly by in vitro maturation. Furthermore, the possible presence of inactive catalase protein in acatalasemic blood was also suggested. Catalase protein concentration of acatalasemic anemic blood decreased by the incubation at 37°C for 24 hours in parallel with the decrease in reticulocyte count and catalase activity, and the decrease in catalase protein concentration of hemolysate by the same incubation parallel with the decrease in catalase activity. It is hypothesized that the unstable catalase protein with genetical change in structure easily decomposes during acatalasemic reticulocyte maturation is presented.

*PMID: 4258080 [PubMed - indexed for MEDLINE]

Acta Med. Okayama **25**, 101—110 (1971)

PROPERTIES OF CATALASE PROTEIN IN IMMATURE AND MATURE RED CELLS OF ACATALASEMIC AND HYPOCATALASEMIC MOUSE MUTANTS

Masana OGATA, Toyoko FUJII
and Shigeo TAKAHARA*

Department of Public Health, Okayama University Medical School, Okayama, Japan (Director: Prof. M. Ogata)

Received for publication, January 12, 1971

Acatalasemia, apparent absence of catalase activity in blood, was first found in Japan by TAKAHARA in 1948, and considered as a molecular disease due to absence of catalase in blood (1). Subsequently, TAKAHARA demonstrated, however, residual catalase activity in acatalasemic blood (2). AEBI reported in 1960 a case of acatalasemia in Switzerland (3-5) and found that the residual catalase activity in blood was located mainly in reticulocytes (6). FEINSTEIN succeeded in isolating acatalasemic mouse mutant with 2.5% of normal catalase activity by irradiation in 1964 (7-10).

The previous observations in our laboratory indicated that the blood catalase activity of the anemic acatalasemic mouse decreased as the reticulocyte became matured (11-13).

In the present study with the mouse mutant, the catalase protein concentration of acatalasemic anemic blood with enhanced population of reticulocytes and of acatalasemic non-anemic blood with normal population of erythrocytes was determined immunologically by the double diffusion method with catalase antibody. The catalase protein concentration of the anemic acatalasemic blood was also determined similarly before and after the incubation at 37°C for 24 hours, during which concomitant maturation of the population of reticulocytes to red cells was observed.

MATERIALS AND METHODS

Preparation of antibody: Purification of catalase from livers of 30 Cs^d strain mice was carried out by the method of NISHIMURA *et al.* (14) with additional step of gel filtration on Sephadex G-100 column. Mice were killed with ether and

* Department of Oto-Rhino-Laryngology, Okayama University Medical School, Okayama, Japan.

the excised livers were rinsed with 0.85% saline solution. The livers were homogenized in an equal volume of distilled water with a Potter Elvehjem homogenizer. Ethanol and Triton X-100 were added to the homogenate to give a concentration of 0.01M and 0.25% respectively. The homogenate was centrifuged at 1000×g, 4°C. After the supernatant solution was mixed with approximately one-tenth volume of a 5:1 (v/v) ethanol-chloroform, the mixture was recentrifuged immediately. The supernatant solution was obtained as the first extract. The precipitate was re-extracted with the ethanol-chloroform mixture. The first and second extracts were combined. Ethanol (95%) was added slowly to the combined extracts with constant mixing to give a final concentration of 70%. The mixture was allowed to stand for 40 minutes and centrifuged. The precipitated catalase was dissolved in aquous solution and dialyzed against distilled water at 3-5°C for 24 hours. The dialyzed solution was adjusted to pH 4.0 with dilute acetic acid to precipitate the ferritin in the mixture.

The latter was removed by centrifugation. The supernatant solution was subjected to ultrafiltration in a collodion bag under reduced pressure. The concentrated solution (1 ml) thus obtained was applied on a column (1×150 cm) of Sephadex G-100. The gel filtration was performed with 0.1% NaCl containing 0.75% of chloroform. The fractions comprising the first absorbance at 280 mμ was to emerge containing catalase. The fractions were pooled and condensed by ultrafiltration. The concentrated solution was subjected to the fractionation through a diethylaminoethyl cellulose column (1.5×21 cm) by the stepwise elution with 100 ml of 1 mM sodium phosphate, 50 ml of 3 mM sodium phosphate (pH 6.8) and with 100 mM sodium phosphate (pH 6.8). Catalase was eluted with the latter buffer. The fractions containing catalase were pooled and concentrated as described above. The concentrated catalase solution was used as antigen to induce rabbit antibody. The ratio of spectrophotometric absorptions at 405 mμ/280 mμ was approximately 0.323.

An aliquot of the solution containg 5 mg of the antigen was emulsified with Freund's adjuvant and was injected first to palm of rabbit. The four following injections (8 mg of antigen for each) were done in muscle. The blood was drawn by cardiac puncture 3 months after the first injection and used as experimental materials.

Preparation of antigen solution for double diffusion tests: Acatalasemic mice (Cs^b), hypocatalasemic mice (Cs^d) and normal mice (Cs^a) were bled from orbital vein, about 0.5 ml at one time, and 8 times during 8 days, with a glass capillary, (1.2 mm in diameter) coated with heparin (15). At the end of the bleeding, blood was sampled and catalase protein was determined by the double diffusion method described below. These anemic bloods were also used for the incubation experiments. The reticulocytes were counted and catalase activity was measured daily with the depleted blood. Reticulocyte counts were made on the smear of the blood supravitally stained with Nile blue (16). Catalase activity was measured by the method of MATSUBARA, SUTER and AEBI (17). The non-anemic and anemic bloods were centrifuged and collected cells were hemolyzed 3 times with distilled water. Hemolysates thus obtained were used as antigen after the saline

concentration was adjusted to 0.85%.

In the incubation experiment, cells were suspended in 5 volumes of Alserver solution and incubated at 37°C for 24 hours in a water bath and at 0°C in a cold room for 24 hours. After the incubation, the cell suspension was centrifuged. The red cells obtained were lysed by adding distilled water, (two volumes of red cell sediment). Double diffusion tests of hemolysates were performed as described below.

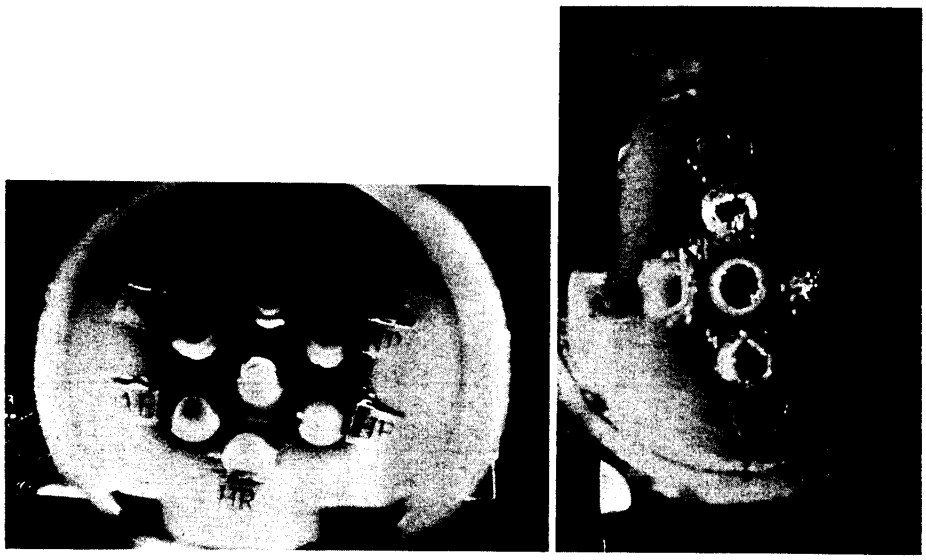
Double diffusion test: One per cent agar was used. Immunodiffusion was carried out by the method of OUCHTERLONY (18) with a disk (4cm in diameter) made of 1% agar (specific noble agar-Difco) in saline containing 0.01% mertionate. A central well and six circumferential wells were punched apart so that the distance from the edge of the center well to that of each circumferential well was about 3mm. Diameters of central well and circumferential wells were 5mm. Antibody and antigens (0.04ml each) were placed in the central well and circumferential wells respectively. Four circumferential wells were punched instead of six for the examination of catalase activity in the precipitin ring.

RESULTS

On agar diffusion, hemolysates of anemic blood with increased population of reticulocytes and non-anemic blood with normal population of erythrocytes from non-acatalasemic, hypocatalasemic and acetalasemic mice produced two precipitin lines against anti-mouse liver catalase rabbit serum as shown in Fig. 1 (A).

The outer precipitin line is considered to be derived from catalase itself, since bubbles were formed when 5 per cent hydrogen peroxide was added to Ouchterlony plate after washing with saline as shown in Fig. 1 (B). The inner precipitin line was probably C reactive protein as described by NISHIMURA, *et al.* (19). Both the outer and inner precipitin lines fused with each other. The outer precipitin line formed by hemolysate of non-acatalasemic non-anemic blood was slightly paler than that by hemolysate of non-acatalasemic anemic blood. The outer precipitin line formed by hypocatalasemic non-anemic blood was considerably paler than that of hypocatalasemic anemic blood. The outer precipitin line of acatalasemic non-anemic blood is markedly paler than that of acatalasemic anemic blood.

The average value of the density of outer precipitin line was obtained by measurement with three samples, wild type anemic blood gave the highest value and hypocatalasemic anemic blood and acatalasemic anemic blood yielded decreased values in that order. The value of the density of precipitin line averaged by the same measurement with non-anemic blood



(A) (B)

Fig. 1 (A) Photograph of double diffusion test between hemolysates and catalase antibody. The center well contained 0.04ml anti-mouse liver catalase rabbit serum and the circumferential wells contained 0.04ml of hemolysates diluted three times (aqua : blood sediment=2 : 1) from acatalasemic, hypocatalasemic and non-acatalasemic mouse bloods at anemic and non-anemic condition.

(B) Photograph of double diffusion test. Note ; the bubble formation indicating catalase activity remains in the outer lines, after reacting, washing with saline, and pouring 5 per cent of hydrogen peroxide on the plate at 4°C.

NE (non-acatalasemic mice, non-anemic hemolysate rich in erythrocyte)
NR (non-acatalasemic mice, anemic hemolysate enhanced reticulocyte)
HE (hypocatalasemic mice, non-anemic hemolysate)
HR (hypocatalasemic mice, anemic hemolysate)
AE (acatalasemic mice, non-anemic hemolysate)
AR (acatalasemic mice, anemic hemolysate)

Reticulocyte count and catalase activity in original blood, and catalase protein in hemolysate used in this experiment are shown in Table 1.

also markedly decreased in the order of wild type, hypocatalasemic and acatalasemic bloods. The density of precipitin line from acatalasemic non-anemic blood was palest.

The amount of catalase protein in hemolysates was also determined semi-quantitatively by double diffusion tests. Anemic and non-anemic bloods were taken from acatalasemic, hypocatalasemic and non-acatalasemic mice. Blood was centrifuged and the collected cells were hemolysed with 2 volumes of distilled water. Catalase activity and hemoglobin concentration of hemolysates were measured. Saline was added to the

hemolysates to yield series of dilution as 1 : 1.4, 1 : 2.0, 1 : 2.8 and 1 : 4.0. The 0.04 ml of original hemolysates and the diluted solutions were put in circumferential wells and 0.04 ml of anti-mouse liver catalase rabbit serum was in a central well. After twenty-four hours' incubation, precipitin lines were examined. The dilution factor of hemolysate showing the palest visible outer precipitin was taken as titer of catalase protein.

Relationship between catalase activity and titer of catalase protein by double diffusion method determined semi-quantitatively is shown in Table 1 and Fig. 2. The catalase activity of non-acatalasemic anemic blood was 67.5 PU/ml (1824.3 PU/gHb) and that of non-anemic blood was 67.1 PU/ml (958.5 PU/gHb). The titers of catlase protein-antigen of the former and the latter were 4.0 and, 4.0 respectively, with 0.04 ml of the antigens. Catalase activity of hypocatalasemic anemic blood was 34.5 PU/ml (718.8 PU/gHb) and that of non-anemic blood was 13.9 PU/ml (185.3 PU/gHb), and the titers of catalase protein-antigen were 2.8 and 1.4 respectively. Catalase activity of acatalasemic anemic blood was 12.8 PU/ml (185.0 PU/gHb) and that of non-anemic blood was 2.3 PU/ml (30.1 PU/gHb), and the titers of catalase protein were 2.8 and 1.0 with the former and the latter respectively.

These results indicate that both the concentration of the catalase protein and the catalase activity of hypocatalasemic and acatalasemic anemic blood decreased as red cells became maturated. The ratio of catalase activity to catalase protein in acetalasemic anemic blood was lower than that in hemolysate from non-acatalasemic anemic blood, and the ratio in acatalasemic non-anemic blood was also lower than that in non-acatalase-

TABLE 1. RETICULOCYTE COUNT AND CATALASE AGTIVITY (PU/gHb) IN ANEMIC AND NON-ANEMIC BLOOD, CATALASE ACTIVITY (PU/ml) AND CONCENTRATION OF CATALASE PROTEIN BY IMMUNE ASSAY IN ANEMIC AND NON-ANEMIC HEMOLYSATE (AQUA : BLOOD SEDIMENT = 2 : 1) USED TO DOUBLE DIFFUSION TESTS AS ANTIGEN FROM NON-ACATALASEMIC, HYPOCATALASEMIC AND ACATALASEMIC MICE

Sample	examination	non-acatalasemic mice		hypocatalasemic mice		acatalasemic mice	
		non-anemic	anemic	non-anemic	anemic	non-anemic	anemic
blood	reticulocyte count (%)	1.6	51.2	1.1	57.7	0.8	45.2
	catalase activity (PU/gHb)	958.9	1824.3	185.3	718.8	30.1	185.0
hemolysate	catalase activity (PU/ml)	67.1	67.5	13.9	34.5	2.3	12.8
	protein conc. by immun. assay (Titer/0.04 ml)	4.0	4.0	1.4	2.8	1.0	2.8
	protein conc. by immun. assay (Titer/ml)	100.0	100.0	35.0	70.0	25.0	70.0
	cat. act./prot. conc.	0.7	0.7	0.4	0.5	0.1	0.2

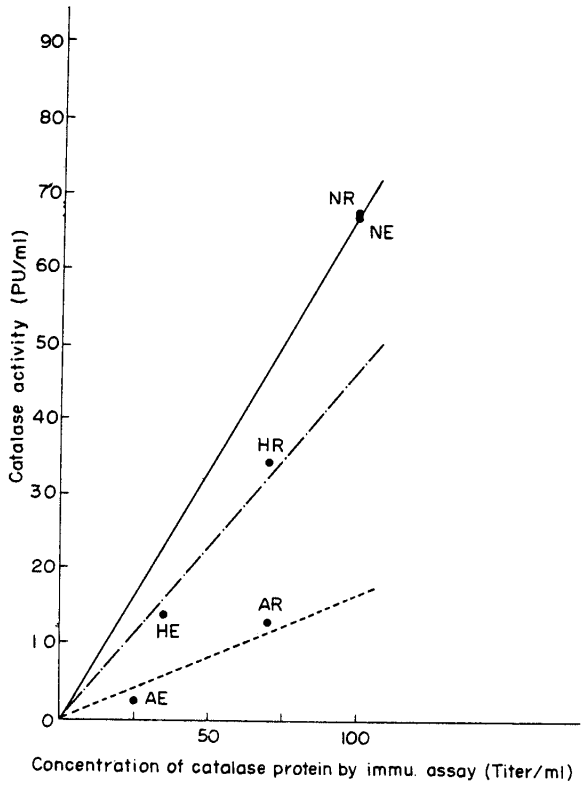


Fig. 2 Correlation between titer of catalase protein measured by double diffusion test, and catalase activity in anemic and non-anemic hemolysates from non-acatalasemic, hypocatalasemic and acatalasemic mouse hemolysates. Symbol of NE, NR, HE, HR, AE and AR is same as described in Fig. 1 respectively.

mic non-anemic blood (Table 1). The observation suggested that inactive catalase protein was present in both acatalasemic reticulocytes and erythrocytes.

Change in the level of catalase protein by the incubation was followed by the double diffusion test described above. The titer of catalase protein in anemic acatalasemic blood decreased from 1 : 4.0 to 1 : 2.0 after the incubation at 37°C, 24 hours. No change of the titer was observed with the blood incubated at 0°C. The observations indicated that catalase protein concentration in acatalasemic reticulocytes decreased by the incubation at 37°C in parallel with decrease of reticulocyte count and catalase activity (in Table 2). In contrast, catalase activity in anemic non-acatalasemic blood slightly decreased by the incubation at 37°C. Hemoglobin levels of bloods in this experiment did not change the incuba-

TABLE 2. CHANGES IN RETICULOCYTE COUNT, HEMOGLOBIN CONCENTRATION, CATALASE ACTIVITY AND PROTEIN CONCENTRATION OF CATALASE IN NON-ACATALASEMIC AND ACATALASEMIC MOUSE *BLOODS* AT ANEMIC CONDITION BY INCUBATION AT 37°C OR 0°C FOR 24 HOURS

mice / condition / examination	non-acatalasemic mice			acatalasemic mice		
	before incubation	after incubation 37°C	after incubation 0°C	before incubation	after incubation 37°C	after incubation 0°C
reticulocyte count* (%)	38.3	11.4	33.3	32.0	9.5	32.4
Hb conc.** (g/dl)	3.7	4.0	3.4	3.8	4.6	3.5
catalase activity**(PU/gHb)	1404.7	1146.3	1441.2	163.8	80.5	163.7
catalase activity**(PU/ml)	52.0	45.9	49.0	6.2	3.7	5.7
cat. prot. conc. by immun. assay** (Titer/0.04ml)	5.6	5.6	5.6	4.0	2.0	4.0

* blood

** hemolysate (aqua : blood sediment = 2 : 1) used to double diffusion test as antigen

TABLE 3. CHANGES IN HEMOGLOBIN CONCENTRATION, CATALASE ACTIVITY AND PROTEIN CONCENTRATION OF CATALASE IN NON-ACATALASEMIC AND ACATALASEMIC MOUSE *HEMOLYSATE* AT ANEMIG CONDITION BY INCUBATION AT 37°C OR 0°C FOR 24 HOURS

mice / condition / examination	non-acatalasemic mice			acatalasemic mice		
	before incubation	after incubation 37°C	after incubation 0°C	before incubation	after incubation 37°C	after incubation 0°C
reticulocyte count* (%)	41.9			52.5		
Hb conc.** (g/dl)	3.8	4.2	3.8	3.8	3.8	3.7
catalase activity**(PU/gHb)	1513.2	1095.2	1539.5	176.2	47.4	214.2
catalase activity** (PU/ml)	57.5	46.0	58.5	6.7	1.8	7.9
cat. prot. conc. by immun. assay** (Titer/0.04ml)	4.0	4.0	4.0	2.0	1.0	2.0

* blood

** hemolysate (aqua : blood sediment = 2 : 1) used to double diffusion test as antigen

Note; titer of catalase antibody used in this experiment is a little weaker than that used in the experiment of Table 2.

tion at 37°C.

The titer of catalase protein in anemic acatalasemic hemolysate decreased from 1 : 2.0 to below 1 : 1.0, by the incubation at 37°C, but remained at the same level after the incubation at 0°C (Table 3). Table 3 also shows the decreases in protein in hemolysates of anemic bloods in parallel with decrease of catalase activity by the incubation at 37°C. Anemic non-acatalasemic hemolysate showed catalase protein concentration and activity of normal level unchanged by incubation procedure at 37°C.

DISCUSSION

The results described above indicate that decrease in catalase activity in hypocatalasemic blood is due to decrease in catalase protein, and that the lowest catalase activity observed with acatalasemic blood corresponds to the lowest concentration of catalase protein.

The results also indicate that the loss of blood catalase activity in hypocatalasemic and acatalasemic mice by maturation is due to the disappearance of catalase protein. Thus decrease in the concentration of catalase protein by maturation explains for the diminishing catalase activity observed with acatalasemic blood and the lower catalase activity of hypocatalasemic blood. Catalase of acatalasemic blood is more unstable against heat denaturation than that of non-acatalasemic blood as measured by the change of the enzymic activity as a function of temperature (9), in either anemic or non-anemic conditions. Therefore, the structure of catalase molecule of the former may be less stable than that of the latter. The protein with unstable structure would likely be affected by maturation process, resulting in the loss of catalase activity. In many inborn errors of metabolism, Swiss and mouse acatalasemia, and glucose-6-P dehydrogenase deficiency (20) are the case showing that the enzymic activity in reticulocytes is higher than that in erythrocytes. However, this report presents the first observation that the loss of enzyme protein in maturated cells, accounts for the decrease in the enzymic activity.

The results with the incubation experiment were also consistent with the parallel change in the protein concentration and the enzymic activity. Therefore, disappearance of catalase protein should be related to the reticulocyte maturation. The mechanism of disappearance of catalase protein with unstable structure during maturation process of reticulocytes remains to be elucidated.

The incubation experiment of hemolysates of anemic acatalasemic blood, also indicated that both catalase protein and catalase activity decreased. This observation is also consistent with instability of catalase protein of acatalasemic blood.

NISIMURA (19) reported that on the agar diffusion between several erythrocyte preparations of normal human individuals and acatalasemic ones with antihuman hepatic catalase rabbit serum. There is a notable and striking absence of precipitin reaction between acatalasemic red blood cells and the antiserum, while normal erythrocytes all show a reaction of complete precipitin ring. These results are similar to those in this experiment, using the agar diffusion between erythrocytes preparations of normal

mice and acatalasemic ones with antimouse hepatic catalase. The experiment using antimouse blood catalase rabbit serum is now being conducted.

CONCLUSION

The concentration of catalase protein in anemic blood with enhanced population of reticulocytes and in non-anemic blood was determined immunologically by double diffusion test with anti-mouse-liver catalase rabbit serum. The change in catalase protein concentration in anemic blood during incubation at 37°C for 24 hours was also studied. It was indicated that the diminished catalase activity in acatalasemic blood was due to the depletion of the protein and that catalase protein in acatalasemic reticulocytes decreased markedly by *in vitro* maturation. Furthermore, the possible presence of inactive catalase protein in acatalasemic blood was also suggested. Catalase protein concentration of acatalasemic anemic blood decreased by the incubation at 37°C for 24 hours in parallel with the decrease in reticulocyte count and catalase activity, and the decrease in catalase protein concentration of hemolysate by the same incubation parallel with the decrease in catalase activity. It is hypothesized that the unstable catalase protein with genetical change in structure easily decomposes during acatalasemic reticulocyte maturation is presented.

ACKNOWLEDGEMENT

We thank Dr. Hiroshi Taniuchi, National Institute of Health, for discussion and criticism of this manuscript.

REFERENCES

1. Takahara, S. and Miyamoto, H.: Clinical and experimental studies on the odontogenous progressive necrotic ostitis due to lack of blood catalase. *J. Otorhinolaryng. Soc. Jap.* **51**, 163, 1948
2. Takahara, S.: Progressive oral gangrene probably due to lack of catalase in the blood (Acatalasemia). *Lancet* **6**, 1101, 1952
3. Aebi, H., Baggilini, M., Dewald, B., Lauber, E., Suter, H., Migheli, A. and Frei, J.: Observations in two Swiss families with acatalasemia. *Enzym. Biol. Clin.* (Basel) **4**, 121, 1964
4. Aebi, H., Heiniger, J. P., Butler, R. and Hassig, A.: Two cases of acatalasemia in Switzerland. *Experientia* **17**, 466, 1961
5. Aebi, H., Jeuner, F., Richterich, R., Suter, H., Butler, R., Frei, J. and Marti, H. R.: Observations in two Swiss families with acatalasemia. *Enzym. Biol. Clin.* **2**, 1, 1962/63
6. Aebi, H. and Cantz, M.: Uber die cellulare Verteilung der Katalase im Blut homozygoter und heterozygoter Defekttrager (Akatalasia), *Humangenetik* **3**, 50, 1966

7. FEINSTEIN, R. N., SEAHOLM, J. E., HOWARD, J. B. and RUSSELE, W. L.: Acatalasemic mice. *Proc. Nat. Acad. Sci. U. S. A.* **52** (3), 661, 1954
8. FEINSTEIN, R. N., HOWARD, J. B., BRAUN, J. T. and SEAHOLM, J. E.: Acatalasemic and hypocatalasemic mouse mutants. *Genetics* **53**, 923, 1966
9. FEINSTEIN, R. N., BRAUN, J. T. and HOWARD, J. B.: Acatalasemic and hypocatalasemic mouse mutants II, Mutational variations in blood and solid tissue catalases. *Arch. Biochem.* **120**, 165, 1967
10. FEINSTEIN, R. N., BRAUN, J. T. and HOWARD, J. B.: Nature of the heterozygote blood catalase in a hypocatalasemic mouse mutants. *Biochem. Genetics.* **1** (3), 277, 1968
11. OGATA, M. and TAKAHARA, S.: On minimal catalatic activity of Japanese acatalasemia and acatalasemic mice. Froceedings of the XII International Congress of Genetics **1**, 151, 1968 (Abstracts)
12. OGATA, M., TOMOKUNI, K. and TAKAHARA, S.: Catalatic activity of immature and mature red blood cells in Japanese acatalasemia (Difference between Japanese and Swiss acatalasemia). *Acta Med. Okayama* **23**, 421, 1969
13. OGATA, M., INOUE, T., TOMOKUNI, K. and TAKAHARA, S.: Catalase activity of immature and mature red cells from acatalasemic mouse mutant. *Acta Haematologica* **44**, 11, 1970
14. NISHIMURA, E. T., KOBARA, T. Y., KALTENBACH, J. P. and WARTMAN, W. E.: Immunological evidence of repressed catalase synthesis in livers of Tumor-Bearing mice. *Archs. Biochem. Biophys.* **97**, 589, 1962
15. RILEY, V.: Adaption of orbital bleeding technic to rapid serial blood studies. *Proc. Soc. Exp. Biol. Med.* **751**, 104, 1950
16. AWAI, M., OKADA, S., TAKEBAYASHI, J., KUBO, T., INOUE, M. and SENO, S.: Studies on the mechanism of denucleation of the erythroblast. *Acta Haemat.* **39**, 141, 1968
17. MATSUBARA, S., SUTER, H. and AEBI, H.: Fractionation of eruthrocyte catalase from normal, hypocatalasemic and acatalatic humans. *Humangenetik* **4**, 29. 1967
18. KABAT, E. A. and MAYER, M. M.: Double diffusion in two dimensions (Ouchterlony method). Experimental immunochemistry, Second Edition. 85 Charles C Thomas Publisher: Springfield, Illinois, U. S. A. (1961)
19. NISHIMURA, E. T., TAKAHARA, S. and HOKAMA, Y.: Interrelationship of catalase and C-reactive protein in acatalasemic erythrocytes, *Arch. Biochem. Biophys.* **126**, 121, 1968