Acta Medica Okayama

Volume 23, *Issue* 5 1969 *Article* 6

OCTOBER 1969

Catalase activity in the red cell ghost of hypocatalasemia and normal subject. I. Catalase in the red cell ghost of hypocatalasemia and normal subject

Keiko Nishioka*

*Okayama University,

Catalase activity in the red cell ghost of hypocatalasemia and normal subject. I. Catalase in the red cell ghost of hypocatalasemia and normal subject*

Keiko Nishioka

Abstract

An attempt was made to find out the nature of catalase coritained in the red cell, especially in the ghost, For this the red cell ghost isolated were washed several times with CO2-saturated water or deionized water and the catalase activity per gram protein of the ghost was estimated. It was found that despite several washings, the catalase activity/gram protein of the ghost do not decrease as compared with the activity of the original red cell solution, indicating the presence of catalase in the ghost. In the case of hypocatalasemic blood the catalase activity in the ghost shows similar behaviors as with normal blood cells. It is assumed theoretically that there are two kinds of catalase having different affinity to the red cell ghost. Namely, one that is readily released from the ghost and the other that has a strong affinity. The affinity of hypocatalasemic blood to the ghost seems to be somewhat weaker.

*PMID: 4244049 [PubMed - indexed for MEDLINE]

Acta Med. Okayama 23, 413—420 (1969)

CATALASE ACTIVITY IN THE RED CELL GHOST OF HYPOCATALASEMIA AND NORMAL SUBJECT

I. CATALASE IN THE RED CELL GHOST OF HYPOCATALASEMIA AND NORMAL SUBJECT

Keiko NISIOKA

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

Received for publication, July 26, 1969

Catalase is known to exist in various animals, especially in all the organs of human being, and its activity has been estimated. Since the discovery of acatalasemia by TAKAHARA (1) the catalase activity and the enzymological characteristics of the red cell have been extensively studied.

Recently, various hypotheses (3, 4, 5) have been presented about red cell ghost (or stroma), and particularly about ultrastructure of this substance as well as some new findings by electron microscopy. In addition, various reports have appeared as regards its chemical composition. Especially noteworthy is the fact that various enzymes are said to be contained in the red cell ghost. For example, ATPase (7, 8), adenosine deaminase (9), DPNase, adenylsulfate kinase, and nucleoside phosphorylase are said to compose this substance, and the presence of cholinesterase (6) has been demonstrated by the use of its enzymatic reaction under electron microscope.

Now, there is a question whether blood catalase is present in the red cell ghost or not. As to this problem there is as yet no report. If it is present, then in what manner would it be bound in the red cell ghost? There arises another question that it would probably appear as a stroma-bound catalase, like the residual hemoglobin of the red cell similar to the stroma-bound hemolobin (11). For the purpose to elucidate the problem still further, the author isolated the red cell ghost by centrifugation, and repeatedly washed the stroma fraction and estimated the catalase activities after each washing. As a result it has been demonstrated that catalase is contained in the red cell ghost as well. On the theoretical basis it is suggested that the physico-chemical affinity to the red cell ghost of blood catalase in the red cell ghost differs from that of free catalase in erythrocyte.

MATERIALS AND METHODS

Materials:

Fresh normal blood used was obtained from healthy normal persons. Hypocatalasemic blood was obtained from one hypocatalasemic individual of the NAK family (12) and another from the GIO family (13), aspirated from the elbow vein in each instance.

Methods;

A. Isolation of red cell ghost

The suspension of the red cell ghost used for the quantitative analysis of catalase activity was isolated and prepared by the following two methods.

a) The method by which the catalase activity per gram protein content of the red cell ghost is measured after each washing.

b) Calculate the percentage of the residual catalase activity of the red cell ghost at each washing, taking the catalase activity of the whole blood prepared at the beginning as 100 %.

In other words, in the experiment a), the blood cells washed 3 times with cold physiological saline solution by PARPART's method (14) are hemolyzed against distilled water, the red cell ghost is repeatedly washed with cold CO_2-saturated water (pH 3.55), and the red cell ghost is separated; and this is further homogenized with the glass homogenizer of Potter-Elvehjem type, and the catalase activity is determined with each homogenate thus prepared.

In the experiment b), 10 ml blood is washed 3 times with about 50 volumes of cold physiological saline solution, physiological saline solution is again added to make the volume 10 ml, to this saline mixture 30 volumes of deionized water at the temperature 4°C is added, left standing at 4°C for 30 minutes to hemolyze, then centrifuged at 3,000 rpm for 20 minutes, after removing the supernatant, to the sediment cold deionized water is added to make the volume 300 ml, stirred thoroughly to have a uniform suspension, then centrifuged (3,000 rpm, 20 min) repeatedly, the stroma fraction (sediment) is dialyzed against 40 % urea, and catalase activity is estimated. Decrease in the catalase activity due to urea treatment proves to be about 2 % within five minutes, indicating it to be within the range of experimental error.

B. Estimation of catalase activity

Catalase activities were all estimated by a modificaton of Euler-Josephson's potassium permanganate drip method. In this estimation the CatK was calculated by the following formula:

$$\text{Cat } K = \frac{D}{t} \log \frac{x_0}{x}$$

In the formula, D stands for the number of dilutions of the samples, t for the reaction time (all the reaction time in the present experiment was expressed in seconds). x_0 stands for the concentration of the H_2O_2-aqueous solution before the estimation, represented by ml units of potassium permanganate solution.

x for ml of the potassium permanganate solution remaining after the reaction (the concentration of the H_2O_2-aqueous solution "t" sec afterwards).

C. Quantitative analyses of protein contents of red cell ghost.

The catalase activity per gram protein of the ghost was calculated by means of the biueret reaction.

D. Quantitative assay of hemoglobin in supernatant at the time of washing the ghost.

By the spectrophotometry (with Hitachi GPO-2 u spectrophotometer) set at the wave length of 540 mμ cyanomethemoglobin formed by sodium cyanide and potassium ferricyanide was estimated.

E. The affinity of blood catalase to the ghost.

Fresh blood collected from normal and hypocatalasemic individuals (13) is washed 3 times with 10 volumes of cold physiological saline solution, the last wash is centrifuged at 10,000 rpm for 15 minutes, and the sediment is put in an equal volume of physiological saline solution to prepare the red cell suspenstion. To 1 ml of the red cell suspension is added 5 volumes of deionized water to hemolyze, and the separation is conducted with Hitachi 20-p type ultracentrifuge with a cooler attachment, and 5 ml CO_2-saturated water (pH 3.55) is added each time, stirring well to obtain a uniform suspension, again centrifuged at 10,000 rpm for 15 minutes, and the supernatant obtained serves as the material for the estimation of catalase activity and the quantitative assay of hemoglobin.

RESULTS

1. Estimation of catalase activity/gram protein content of the ghost (Table 1).

Table 1. Content (CatK/g) of residual catalase in the red cell ghost after washings

Sample No	Blood	Cat K of blood	Hb/g of blood	Cat K/g of ghost				
				No. of washing				
				1.	2.	3.	4.	5.
No. 1	Stored blood			1.51	1.67	1.54	1.12	1.52
No. 2	Fresh normal blood	453	14.5			2.20	2.00	
No. 3	Fresh Hypo. blood	555	12.0			1.83	2.10	
No. 4	Fresh Hypo blood (NAK)	212	8.3			1.82	2.20	
No. 5	Fresh Hypo blood (GIO)	213	13.2			1.25	1.00	

As is obvious from the table, even with stored blood (No. 1) there can be seen no appreciable difference from one washing to 5 washings after hemolysis, likewise with fresh bloods (Nos. 2, 3), especially after the third washing, Cat K values of the sediment do not show any marked change and all the values fall within the range of 1.0 to 2.2 Cat K/g, indicating that the number of washings has no conspicuous influence on the catalase

activity.

In the case of hypocatalasemic blood, one each from the NAK and GIO families (Nos. 4, 5) similarly the catalase activity of the materials after 3 to 4 washings remain within the range of experimental error, showing no decrease.

2. The ratio (%) of catalase activity of the ghost after each washing as against the activity (100 %) of the original red cell solution.

The ratio of the catalase activity of the red cell ghost up to 7 washings changes only by 1—2 % (Table 2), proving that the washing has hardly any remarkable effect on the ratio of the activity of the materials, the value being maintained practically constant.

Table 2. Changes in the catalase activity of the ret cell ghost by several washings

No. of washing	1	2	3	4	5	6	7
Catalase activity (%)	1.09	1.24	1.25	1.31	1.47	1.77	1.56

3. Catalase activities of supernatants as calculated in precentage against the original materials

The results obtained in 1) and 2) were further studied in order to find out the affinity of blood catalase to the ghost. After adding a given quantity of CO_2-saturated water to the ghost, and repeated washings and centrifugation, the supernatant so prepared showed some minute quantity of cataiase being released from the ghost (Fig. 1). For the purpose to see the percentage of catalase activity in the supernatant as against the catalase activity of the original red cell solution, the percentage is plotted on the ordinate as logarithm and the number of washing on the abscissa. The straight line curve begins to bend itself around the fourth wathing and transforms into a straight line with less slope. The curve of the estimated hemoglobin contents (Hb) also begins to band at the fourth washing, making a similar curve. The curve drawn for the hypocatalasemic blood (Fig. 1) likewise bend, its straight line at the third washing. Some discussion will be made on each curve as regards the point where it deflects.

DISCUSSION

I. *Estimation of catalase activity in the red cell ghost*

In attempting to determine the presence or absence of catalase activity in the red cell ghost an important problem will be the procedures involved in the isolation of red cell ghost and methods for estimation. Regarding

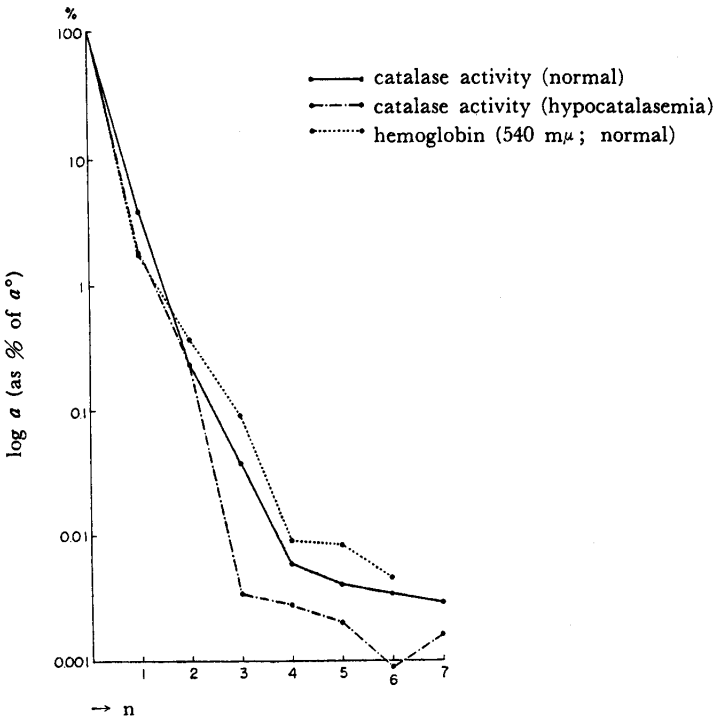

Fig. 1 Changes in the catalase activity of the supernatant by several washings

the isolation of the ghost, there are reports such as by BERNSTEIN (16) and PARPAT, among others.

SINAGAWA and coworders (17) have recently found by electron microscopy that the red blood cell membrane is consisted of three distinct layers. It is to be noted here that nethods of blood dilution and procedure of washing after the dilution are apt to change the structure of red cell membrane. For example, when the blood cells are washed several times with CO_2-saturated water, the inner layer is often completely lost. In view of this, the red cell ghost was isolated by PARPART's method, and the catalase activity per gram protein of the ghost was estimated. Simultaneously, by washing the ghost several times only with deionized water of a given volume and by measuring the catalase activity in the ghost, the percentage of the catalase activity in the ghost to the activity of the orginal red cell solution before washing procedure was obtained. In this instance of quantitative determination, the catalase activity of the primary reaction as represented by Cat K was set at 100 % and Cat K values of the sediment fractions so measured were high enough to estimate in various experiments. Therefore, it is assumed that catalase is contained in the ghost. As

is clear from Tables 1 and 2, the residual catalase activity in the ghost hardly undergoes any change after several washings. In other words, the residual catalase has a quite strong affinity to the ghost. However, in view of the fact that supernatants of 2—7 washings do show slight catalase activity, some of catalase in the ghost also seems to have affinity to water.

II. *The affinity of catalase to the red cell ghost*

As the red cell contains catalase abundantly, unless washing is done thoroughly, catalase to be released will still be adhered to the ghost or it will remain within the red cell. As is evident from heated discussions regarding the presence of hemoglobin, it might be dismissed simply as a contaminant (18). TOMITA (2) reported about physical differences in the affinity of red cell hemoglobin on theoretical basis and stated that under given conditions he could get satisfactory results. In other words, in the case where a substance A has affinity to a substance B, if a fixed amount of solution is added to the substance A of a given concentration and washed repeatedly, the concentration (a) of the supernatant will be:

$$\frac{da}{dn} = -ka$$

n: number of washings
k: constant

Supposing the concentration of $n=0$ time $=a^\circ$

we will have $a=a^\circ e^{-kn}$

$$Ina = Ina^\circ - kn$$

Further, in the case where the substance A is consisted of two components each of which has different affinity to the substance B, then setting the concentrations of each of these two components as a_1 and a_2,

we will have $a=a_1+a_2$ and

the concentration at $n=0$ time to be a_1°, a_2°

$$a^\circ = a_1^\circ + a_2^\circ$$

$$a_1 = a_1^\circ e^{-k_1 n} \qquad a_2 = a_2^\circ e^{-k_2 n}$$

$$\left.\begin{matrix} k_1 \\ k_2 \end{matrix}\right\rangle \text{ constant}$$

$$Ina_1 + Ina_2 = Ina_1^\circ + Ina_2^\circ - k_1 n - k_2 n$$

Supposing that k_1 is sufficiciently large as compared with k_2, this straight line will bend at a certain point on its way, and the angles (k_1, k_2) of inclination of each curve will represent the intensity of affinity.

Now, supposing that catalase is present in the ghost and there is a considerable difference in the affinity between the catalase in the ghost and free catalase in the red cell, then would not the estimation of catalase

activity in the supernatant fraction presents itself as a bend on the straight line in the graph drawn ?

The experimental results (Fig. 1) of normal red cells after 7 times washings show the concentration of hemoglobin and catalase activity at each washing, represented by Log scale, comparing the affinity of Hb and catalase to the ghost and water. The amounts of hemoglobin and catalase released into the supernatant by washing reveal a similar decreasing tendency with each additional washing. However, on investigating still more carefully, the Hb concentration in the supernatant after 4 washings is 0.09 % whereas that of catalase is 0.006 %, the latter being slightly lower. The amount of decrease by the fourth washing to the seventh is somewhat higher with hemoglobin. Therefore, it may be reasonable to say that, as far as the ratios of residual catalase and hemoglobin in the ghost are concerned, residual catalase is equal to hemoglobin or slightly less, having a strong affinity to the ghost.

The percentage of residual catalase after several washings in the case of hypocatalasemic blood tends to be rather small.

CONCLUSION

An attempt was made to find out the nature of catalase contained in the red cell, especially in the ghost. For this the red cell ghost isolated were washed several times with CO_2-saturated water or deionized water and the catalase activity per gram protein of the ghost was estimated.

It was found that despite several washings, the catalase activity/gram protein of the ghost do not decrease as compared with the activity of the original red cell solution, indicating the presence of catalase in the ghost. In the case of hypocatalasemic blood the catalase activity in the ghost shows similar behaviors as with normal blood cells.

It is assumed theoretically that there are two kinds of catalase having different affinity to the red cell ghost. Namely, one that is readily released from the ghost and the other that has a strong affinity. The affinity of hypocatalasemic blood to the ghost seems to be somewhat weaker.

REFERENCES

1. TAKAHARA, S.: Progressive oral gangrene probably due to lack of catalase in the blood. (Acatalasemia). *Lancet* **6**, 1101, 1952
2. TOMITA, S.: Spectrophotometric studies on the erythrocyte and its constituents. *Nara-Igakkai-shi* **12**, 1019, 1961
3. PICKEN, L.E.R.: The fine structure of biological systems. *Biol. Review Chambridge Philo. Soc.* **15**, 133, 1940

4. PONDER, E.: Hemolysis and related phenomena. Grune & Stratton, Inc. New York, 1948
5. MITCHISON, J. M.: Thickness and structure of membrane of human red cell ghost. *Nature*, **166**, 347, 1950
6. SHINAGAWA, Y. and OGURA, M.: Cholinesterase in the red cell membrane. *Kagaku* **30**, 367, 1960 (in Japanese)
7. CLARKSON, E. M., and MAIZELS, M.: Distribution of phosphatases in human erythrocytes. *J. Physiol*, **116**, 112, 1952
8. POST, R. C. *et al.*: Membrane adenosine triphosphatase as a participant in the human erythrocyte. *J. Biol. Chem.* **235**, 1796, 1960
9. LIONETTI, F. J.: The effects of nucleosides on phosphate and pentose metabolism in erythrocyte ghosts. *J. Biol. Chem.* **229**, 817, 1957
10. PONDER, E.: On properties of the red cell ghost. *J. Exp. Biol.* **18**, 257, 1942
11. PONDER, E.: Red cell structure and its breakdown. *Protoplasmotologia*, **10**, 1, 1955
12. TAKAHARA, S. *et al.*: Hypocatalasemia.; A new genetic carrier state. *J. Clin. Invest.* **39**, 610, 1960
13. OGURA, Y. *et al.*: Two families of acatalasemia discovered at dental treatment. *Kohkubyo-Gakki-shi* **32**, 313, 1965 (in Japanese)
14. PARPART, A. K.: The preparation of red cell membranes. *J. Cell and Comp. Physiol.* **19**, 248, 1942
15. EULER, H. and JOSEPHSON, K,: Über katalase *I. Just. Lieb. Amn. Chem.* **452**, 158, 1927
16. BERNSTEIN, S. S., *et al.*: A method for the preparation of posthemolytic residure or stroma of erythrocytes. *J. Biol. Chem* **122**, 507, 1938
17. SHINAGAWA, Y. and OGURA, M.: Study of red cell membrane by the ultra-thin sections. *Nihon Seiri-shi* **22**, 654, 1960 (in Japanese)
18. TISHKOFF, G. H. *et al.*: Red cell stroma in dogs. *Blood J. Haematol.* **8**, 459, 1953