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Abstract

We fractionated the red cells of Japanese acatalasemic individuals (four individuals in two families) by SAss's method and counted the number of reticulocytes and determined the catalatic activity by manometric and perborate methods on each fraction containing reticulocytes in the descending order from the upper to the lower layers. We also incubated each of these fractions at 37°C for 4, 10, 24 and 48 hours, to see the catalatic activity along with the maturation of reticulocytes. Heat stability of catalatic fraction separated by DEAE column was also examined. The results of the study may briefly be summarized as follows. 1. a. There was no parallel relationship between the number of reticulocytes and the catalatic activity in Japanese acatalasemic red cells. b. There could be seen no decrease in catalatic activity while the number of reticulocytes did decrease by the incubation. 2. Heat stability of crude catalatic fraction from acatalasemic blood is approximately the same as that of crude catalase fraction from normal blood.

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**CATALATIC ACTIVITY OF IMMATURE AND MATURE
RED BLOOD CELLS IN JAPANESE ACATALASEMIA
(DIFFERENCE BETWEEN JAPANESE AND SWISS
ACATALASEMIA)**

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It is known that catalase activity per hemoglobin in normal reticulocytes is about 4 times greater than that of normal mature red cells (1). AEBI (2) reported that there could be found a few red cells rich in catalase in Swiss acatalasemic blood and he considered them to be reticulocytes. Further, he (3) divided acatalasemic red cells into several fractions containing different numbers of reticulocytes by a modified method (2) of SASS's centrifugation (4, 5), and estimated catalatic activity of each fraction. As the results he discovered that the catalatic activity of the top fraction containing a greater number of reticulocytes is 8- to 12-fold that of the bottom fraction, and by calculating from the number of reticulocytes he assumed that the catalatic activity per reticulocyte is about 300-fold that of a mature red cells in acatalasemia and is almost the same as that of a normal reticulocyte.

This means the life-span of catalatic substance in the Swiss acatalasemic reticulocytes is shorter than that of catalase molecule in normal reticulocytes. In his further study (6) on the top fraction, he could prove definitely that the above assumption is not incorrect from the following observations, namely when this top fraction containing much reticulocytes was incubated for 1—2 days at 37°C, the catalatic activity of the top fraction decreased with the lapse of time. Also he (7) proved that catalatic substance in the Swiss acatalasemic blood was less heat resistant than that of normal blood catalase. From these results, he deduced that the catalatic substance in acatalasemic reticulocytes must be structurally different from the normal blood catalase.

For the purpose of clarifying whether or not there might be any difference between the catalatic activity of reticulocytes and mature red cells in Japanese acatalasemia, we fractionated the Japanese acatalasemic blood by the modified method of SASS and compared the catalatic activities of

each fraction from the upper to the lower layers. In addition to this, we tested heat stability of acatalasemic catalatic substance separated by DEAE column.

This report describes briefly the results of our study.

MATERIALS AND METHODS

Blood used in this experiment was obtained from the elbow vein of Japanese acatalasemic individuals and heparinized.

Centrifugation: Method 1; 5 ml of acatalasemic blood was put into a plastic centrifuge tube, and centrifuged in a swinging basket at $2000 \times g$ for 2 hours (4, 5). After the centrifugation the sediment of acatalasemic red cells thus obtained was cut into 2 mm-thick layers from the top downward, and the catalatic activity of each layer was estimated.

Method 2: A centrifugation was conducted by the method of AEBI and CANTZ (2) where 2.5 ml of washed red cell sediment was suspended in an equal volume of 26.6 per cent albumin solution in saline and was centrifuged in the swinging basket at $2500 \times g$ for 2 hours.

In vitro incubation: The sediment layer of each tube was separated into 3 layers, i.e. the upper layer containing much reticulocytes, the middle with lesser reticulocytes, and the bottom layer with the least reticulocytes, and 0.05 ml of each layer was poured into 7 ml Alserver solution and incubated in water bath at 37°C for 4, 10, 24 and 48 hours. After incubation, this Alserver solution was centrifuged. To the sediment obtained by centrifugation, 0.45 ml of distilled water was added, and the catalatic activity and hemoglobin concentration were determined.

DEAE column (8): Four ml of washed red cells was hemolysed by adding 8 ml distilled water and hemolysate was dialysed against 0.001 M phosphate-buffer (pH 6.8) overnight. After centrifugation at $20,000 \times g$ for 40 min., 8 ml hemolysate was poured into DEAE column (1.8×15 cm, 18.5 gr). The elution of hemoglobin was carried out with 100 ml of 0.001 M phosphate buffer (pH 6.8) and then traces of hemoglobin was eluted by 100 ml of 0.003 M phosphate buffer. Elution of catalase fraction in normal hemolysate or catalatic fraction in acatalasemic hemolysate was carried out with 150 ml of 0.1 M phosphate buffer. All procedures were conducted in the cold room controlled at 4°C .

Reticulocyte count (9, 10): One per cent Nile-blue chloride in ethanol was prepared. One drop of Nile-blue solution was placed on the glass slide and dried. A drop of each layer was put on the glass slide and mixed with dried Nile-blue, then the reticulocytes were stained supravitaly. The stained layer was smeared on the glass slide and dried. Thereafter the smear was fixed with methanol solution and washed with water. After drying, the smear was restained with Giemsa, washed with water and dried. The smear thus obtained was examined under microscope and number of reticulocytes was counted.

Heat stability test: Normal catalase fraction or acatalasemic catalatic fraction

prepared by DEAE column was incubated at 44°C for ten minutes in water bath.

Determination of catalase activity:

Perborate method: AEBI's method (7), modified FEINSTEIN's method (11), was used.

Manometric method: FUJITA and KODAMA's method (12) was used.

Hemoglobin concentration: Cyanmethemoglobin method was used.

RESULTS

Fig. 1 illustrates differences in the catalatic activities of each layer after the centrifugation of the blood donated from a man of acatalasemic N family. The abscissa shows the distance from the top surface of sediment to the surface of each fraction, and ordinate shows the reticulocyte

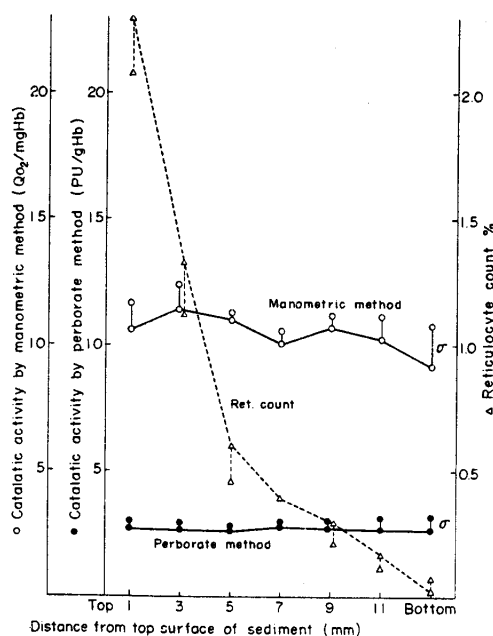


Fig. 1 Reticulocyte count and catalatic activity in the fractions from blood of Japanese acatalasemia (SN) separated by modified Sass's centrifugal method (method 2). Sigma (σ) represents standard deviation.

counts and catalatic activity as represented by $QO_2/mgHb$ (manometric method) and PU/gHb (perborate method). The number of reticulocytes is greater in the upper layer, and decreases to lower layers. The catalatic activities of each layer in our case did not show any significant differences by the manometric and perborate methods. Table 1 demonstrates results

Table 1 Reticulocyte count and catalatic activity of the fractions from Japanese acatalasemic blood, separated by modified Sass's centrifugation method

Family	Case	Number of exp.	Fraction Exp.	Distance from the upper surface of sediments						
				0—1.9mm	2.0—3.9mm	4.0—5.9mm	6.0—7.9mm	8.0—9.9mm	10.0—11.9mm	Bottom
N	T. (♀) ¹⁾	1	QO ₂ /mgHb	12.6	13.5	11.5	11.5			11.0
			Reticulocytes ‰	15.0	9.0	4.0	2.0			2.0
	I. (♀) ¹⁾	1	QO ₂ /mgHb	9.0	8.3	9.7	8.7	9.8	10.2	9.3
			Reticulocytes ‰	44.0	26.0	10.0	1.0	1.0	0	0
	S. (♂) ²⁾	3	QO ₂ /mgHb mean±σ ³⁾	10.6±1.00	11.4±0.92	11.1±0.15	10.1±0.46	10.7±0.46	10.2±0.91	9.1±1.66
			Pu/gHb mean±σ	2.7±0.25	2.7±0.21	2.6±0.11	2.8±0.16	2.7±0.32	2.7±0.40	2.7±0.51
			Reticulocytes ‰ mean±σ ³⁾	23.0±2.16	13.3±2.05	6.0±1.41	4.0±0.00	3.0±0.81	1.7±0.47	0.3±0.47
Y	M. (♂) ²⁾	2	QO ₂ /mgHb mean±d ⁴⁾	9.9±0.51	10.1±0.61	11.1±0.26	10.1±0.42	10.6±0.02	9.5±0.43	9.8±0.39
			Pu/gHb mean±d ⁴⁾	2.0±0.84	2.4±0.60	3.3±1.17	2.8±0.03	2.4±0.09	1.9±0.01	2.6±0.65
			Reticulocytes ‰ mean±d ⁴⁾	24.5±2.50	11.5±1.50	8.5±2.50	2.0±1.00	1.5±0.50	0	0.5±0.50

1) Fractionation by method 1

2) Fractionation by method 2

3) σ=standard deviation

4) d= difference between mean and maximum or minimum value

of our experiments carried out on three acatalasemias in one family and also one acatalasemia of another family. From the above results it can be said there are no significant differences in catalatic activity between reticulocytes and mature red cells of the Japanese acatalasemia.

In Fig. 2 are shown the results of *in vitro* incubation of Japanese acatalasemic top fraction containing reticulocytes in 0.8 per cent and acatalasemic bottom fraction containing reticulocytes in 0.15 per cent. Namely, reticulocyte count in the top fraction decreased markedly by incubation procedure, while there was no decrease in catalatic activity in the top. This also suggests that minimal catalatic activity in Japanese acatalasemic blood comes from both reticulocytes and mature red cells.

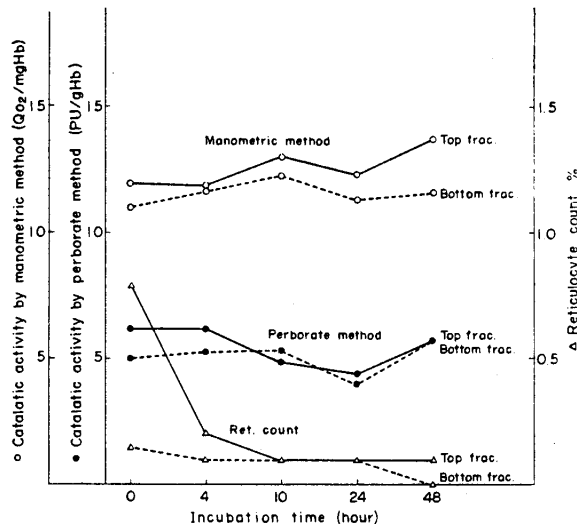


Fig. 2 Maturation and catalatic activity of red blood cells in top and bottom fractions separated from Japanese acatalasemic blood by modified Sass's centrifugation method, and temperature of incubation was kept at 37°C.

Fig. 3 shows the heat stability of the normal blood catalase fraction or acatalasemic catalatic fraction separated by DEAE column chromatography. We find no marked difference in the heat stability of enzyme fraction between normal and acatalasemic blood of Japanese as is evident from this figure. In the Swiss case reported by AEBI, the crude catalatic fraction of acatalasemic blood shows less stability than the normal blood catalase fraction; that is, the catalatic activity of the Swiss acatalasemic blood fraction decreases to the level of 25 per cent of the control, while catalase activity of the normal fraction decreases to 83 per cent, when

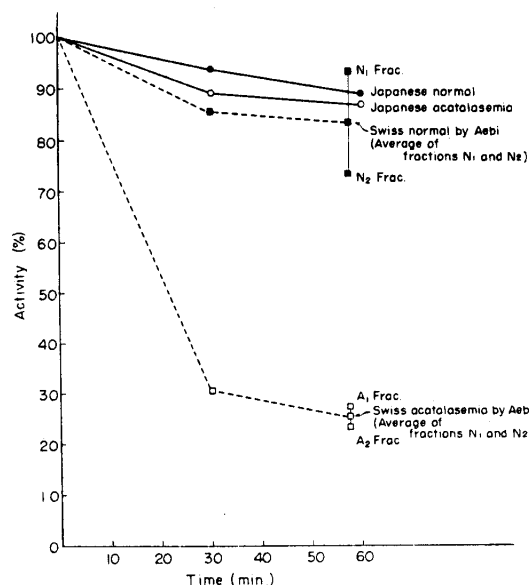


Fig. 3 Heat inactivation of normal catalase fractions and acatalasemic catalatic fractions from Japanese acatalasemia by DEAE column chromatography after incubation at 55°C. Catalatic activity is measured by perborate method.

heat is applied at 55°C for 60 minutes in the water bath as shown in Fig. 3.

DISCUSSION

AEBI (2) reported that residual catalatic activity in acatalasemic blood comes mostly from the reticulocytes (its activity, $300 \times$ mature red cells) and it decreases more than the normal blood when heat is applied in Swiss acatalasemic blood. On the other hand, we (13) reported that the catalatic activity in radiation induced acatalasemic mouse blood (14, 15) is found mostly in reticulocytes (its activity, 30 times of the activity in mature red cells) and heat stability of catalatic substance in reticulocytes has a shorter life-span than that of the normal.

In our study of Japanese acatalasemic blood, the catalatic activity in reticulocytes is not greater than that of immature red cells, as shown in Fig. 1 and Table 1, and this catalatic activity in reticulocytes does not decrease along with the maturation *in vitro* as in Fig. 2. It can be said that heat stability of catalatic substance in acatalasemic blood is almost the same as that of normal blood catalase. This is described in Fig. 3 in comparison with Swiss case.

Comparison among Japanese, Swiss and mouse acatalasemic bloods is summarized in Table 2. From these results, it is considered that residual

Table 2. Difference among Japanese-, and Swiss- radiation induced mouse acatalasemia

	Distribution of cata. activ.	Cata. activ. after <i>in vitro</i> incubation	Heat stability compared with normal
Japanese acatalasemia	Almost equal in reticulocytes and matured erythrocytes	Slight decrease	Stable
Swiss acatalasemia by AEBI	A greater part in reticulocytes	Decrease	Less stable
Radiation induced mouse acatalasemia*	Mostly in reticulocytes	Decrease	Less stable

* Donated by Feinstein

catalatic substances in Swiss acatalasemia as well as radiation induced mouse acatalasemia (Cs^b) are less stable against heat than normal blood catalase, and catalatic substance in reticulocytes has a shorter life-span than that of the normal*. In contrast to this, heat stability of the minimal catalatic substance in Japanese acatalasemic blood is similar to that of the normal blood catalase and the life-span of the catalatic substance in acatalasemic reticulocytes is about the same as that of normal reticulocyte catalase.

CONCLUSION

We fractionated the red cells of Japanese acatalasemic individuals (four individuals in two families) by Sass's method and counted the number of reticulocytes and determined the catalatic activity by manometric and perborate methods on each fraction containing reticulocytes in the descending order from the upper to the lower layers. We also incubated each of these fractions at 37°C for 4, 10, 24 and 48 hours, to see the catalatic activity along with the maturation of reticulocytes. Heat stability of catalatic fraction separated by DEAE column was also examined. The

* Note ; In acatalasemic mice (Cs^b), minimal blood catalatic activity was due to considerable lower activity in reticulocytes than that in normal ones and higher degradation rate than that of normal catalase (13, 16). And in hypocatalasemic mice (homozygous mutant ; Cs^d), the lower activity was due to slightly lower activity in reticulocytes and higher degradation rate which was almost same as the acatalasemic cases than that of normal catalase (13, 16).

Therefore the lower activity of blood catalase in hypocatalasemic mouse mutant is mainly due to higher degradation rate according to maturation and it is similar to that in Swiss acatalasemia, in which lower catalatic activity is mainly due to decrease of catalatic activity according to maturation (2).

results of the study may briefly be summarized as follows.

1. a. There was no parallel relationship between the number of reticulocytes and the catalatic activity in Japanese acatalasemic red cells.

b. There could be seen no decrease in catalatic activity while the number of reticulocytes did decrease by the incubation.

2. Heat stability of crude catalatic fraction from acatalasemic blood is approximately the same as that of crude catalase fraction from normal blood.

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