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# Change of 3'-nucleotidase activities in experimental reticulocytosis of rats*

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## INTRODUCTION

The specific 3'-nucleotidase was first described by Shuster and Kaplan[1].

They studied on the distribution of this enzyme in plant kingdom. The 3'-nucleotidase from rye grass and barley attaks phosphomonoester groups only in the 3' but not in the 5'-nucleotides[2].

The specific 3'-nucleotidase may be of some value in the elucidation of the structure of naturally occuring nucleotides and of nucleic acids and their fragments. Thus, by means of this enzyme, it has been shown that the monoester phosphate on the adenylic acid portion of coenzyme A is in the 3'-position[3].

This enzyme also provides a convenient means for the acid-catalysed interconversion of 2'-3'-nucleotides from both directions[4-5].

3'-Nucleotidase has not been studied in detail on animal tissues, while 5'-nucletidase was described by many investigators from early times[6-9].

The present study was undertaken to observe the changes in the activity of 3'-nucleotidase during the maturation of reticulocytes in rat induced by phenylhydrazine(PH) treatment. It was revealed that the activity of 3'-nucleotidases in both cytosolic and mitochondrial isozymes increased about two-fold above its control level on the 4th day.

## MATERIALS AND METHODS

Reagents; Tris-HCl, EDTA, heparin and 3'-adenylic acid were purchased from Sigma Chemical Co., St. Loius, U.S.A., Giemsa stain, brilliant cresyl blue, and cedar oil were obtained from E. Merck, Darmstadt, West Germany. Phenylhydrazine-HCl and all other chemicals used were of analytical grade.

Induction of rabbit reticulocytosis:

Sprague-Dawley rats, weighing 180-200g were fed a diet of protein-rich Purina Laboratory Chow ad libitum for a week. Reticulocytosis was induced by daily subcutaneous injection of neutralized phenylhydrazine (85.8 mg per Kg body weight) for 3 days[10].

At timed intervals, blood was drawn from cervical vein into heparin-containing vessel. Blood cells were washed with cold physiological saline solution containing 0.1M phosphate, pH 7.2 and centrifuged in a Beckman J-21 B centrifuge at 1,000 x g for 10 min. The procedure was repeated three times.

Hemolysis of separated red blood cells:

The packed red blood cells were lysed in an equal volume of chilled distilled water, and the mixture was kept in a deep freezer. The frozen sample was subjected to repeated freezing and thawing.

Hemolysate was centrifuged for 10 min. at 700 x g, and the supernatant solution was centrifuged at 20,000 x g for 15 min. and separated into two fractions, i. e., cytosol and mitochondria. The mi-

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tochondrial fraction was washed by suspending in the same buffer and the precedure was repeated three times. The samples were stored in a deep freezer for further experiments[11]

Hematocrit was determined by Wintrobe's micromethod by centrifugation in a microhematocrit centrifuge at 12,000 rpm for 3 min. Reticulocyte count was carried out by the New methylene blue method[12-13].

Assay of 3'-nucleotidase activity:

The release of inorganic phosphate from 3'-adenylic acid was measured by the method of Fiske and SubbaRow[1, 14].

The following mixture was incubated for 15 min. at 37°C 0.1 ml of 0.04 M 3'-adenylic acid, 0.1 M Tris buffer, pH 7.5 and enzyme in a total volume of 2.0 ml. The reaction was stopped by the addition of 1.0 ml of 20% trichloroacetic acid (TCA). The control consists of the same mixture with TCA added prior to the addition of enzyme.

If a protein precipitate forms, it should be removed by filtration or centrifugation.

Definition of enzyme unit:

One unit of enzyme was defined as that amount of enzyme which causes the initial release of 1 μmole of inorganic phosphate per hour under the condition of the assay.

Determination of rate constants for enzyme synthesis and degradation:

The rate constants for synthesis(Ks) and degradation(Kd) were determined as described by Schimke and Doyle[15-16]. The rate of change in the enzyme level was expressed as the algebraic sum of zero-order rate of synthesis and the first-order rate of breakdown, $d(E)/dt = Ks - Kd(E)$, where (E) is the enzyme level at any time, t, this equation when integrated and rearranged yields

$In(E-Eo) = In(E'o-Eo) - Kdt$ for the phase of decline in enzyme activity, where Eo denotes the steady-state level of the enzyme under basal conditions, and E'o denotes the elevated steady-state level of the enzyme following phenylhydrazine treatment. The value of Kd were calculated from the slopes of plots of In(E-Eo) against time using the leastsquare method. Values of Ks were calculated by substituting the experimentally determinded values for Kd and Eo into the equation $Ks = Kd(Eo)$ where $d(E)/dt = 0$, The half-life for enzyme degradation was calculated from the following expression: $t\frac{1}{2} = 0.693/Kd$.

The various assumption involved in the use of the methods described herein were delineated by Schimke and Doyle[15].

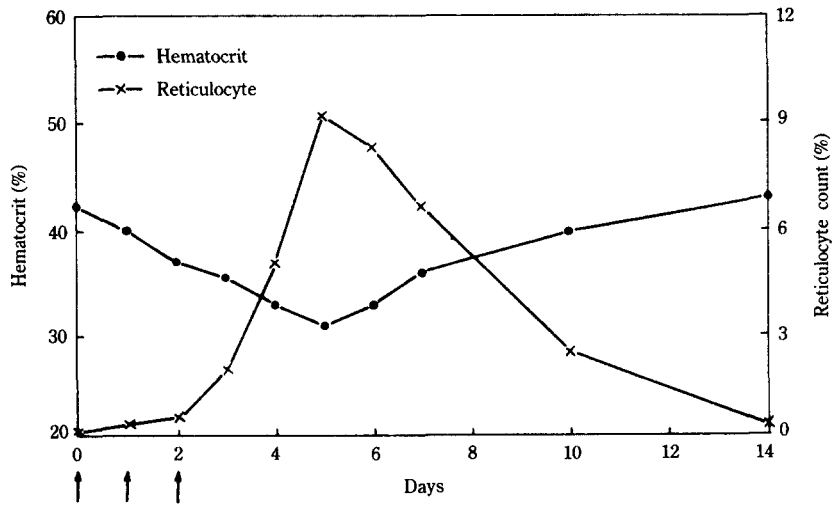


Fig. 1. Sequential change of the hematocrit and reticuocyte count in the peripheral blood of phenylhydrazine-treated rat. Arrows denote the day on which phenyhydrazine was administered.

## RESULTS

As can be seen in Fig 1, the number of reticulocyte was 9 per 1,000 red cells in normal rabbit, and hematocrit was 42%.

Administration of phenylhydrazine caused a steady and gradual decrease in the hematocrit and reached approximately 50% of the normal value on day 5 after drug administation. Thereafter, the value began to increase steadily and attained the normal value on day 10-12. The reticulocyte count increased gradually during 3 days after phenylhydrazine treatment, then showed a drastic increment and reached a peak (96 per 1,000 red blood cells) around day 6. Then, it was followed by a sharp decrease and finally attained normal value on Day 10-12.

The normal red blood cells of rat contained about 64.22 units of 3'-nucleotidase per ml of RBC.

Table 1 shows the time course changes in the 3'-nucleotidase activity in rat red blood cells after phenylhydrazine treatment.

The 3'-nucleotidase in cytosolic fraction increased gradually and reached a peak at day 4, which is about two-fold increment as compared with the initial value.

The mitochondrial 3'-nucleotidase activity in normal red blood cells of rat is very low, because it contains only 2−3% of reticulocytes.

Within about 24 hours after phenylhydrazine treatment, the activity of isozyme in mitochondrial fractions showed a steady and gradual increases and reached a peak, about 2 times over normal value, at day 3, and then the level decreased gradually and attained the initial value at day 10 (Fig.2).

The rate of synthesis (Ks) and degradation (Kd)

Table 1. Changes of 3'-nucleotidase activity in rat erythrocytes during induction of reticulocytosis and recovery from it

| Days | 3'-nucleotidase activities (unit/ml of RBC) | | |
|---|---|---|---|
| after PH | Cytosolic | Mitochondial | Total |
| 0 | 63.24 | 0.99 | 64.22 |
| 1 | 68.09 | 1.47 | 69.56 |
| 2 | 75.34 | 1.72 | 77.06 |
| 3 | 99.38 | 1.97 | 101.35 |
| 4 | 133.41 | 1.72 | 135.14 |
| 5 | 78.12 | 1.58 | 29.70 |
| 6 | 70.42 | 1.23 | 71.65 |
| 7 | 68.05 | 1.02 | 69.07 |
| 10 | 64.23 | 0.99 | 65.22 |
| 14 | 63.23 | 0.98 | 64.21 |

Table 2. The rate of synthesis and degradation of 3'-nucleotidase in rat red blood cells during induction and recovery from reticulocytosis

| Enzyme | Periods | Eo or E'o (units) | Kd (hr) | $t_{\frac{1}{2}}$ (hr) | Calculated. Ks (units/hr) |
|---|---|---|---|---|---|
| Cytosolic | Induction | 133.41 | 0.0095 | 72.9 | 1.2674 |
| | Recovery | 63.24 | 0.0265 | 26.2 | 1.6756 |
| Mitosolic | Induction | 1.97 | 0.0288 | 24.1 | 0.0567 |
| | Recovery | 0.98 | 0.0324 | 21.4 | 0.0318 |

Eo or E'o: Basal level of enzyme(E'o) or peak value(E'o)
Kd : Experimental values derived from plots of In(E-Eo) or In(E'o-E) in against time
$t_{\frac{1}{2}}$ : 0.693/Kd

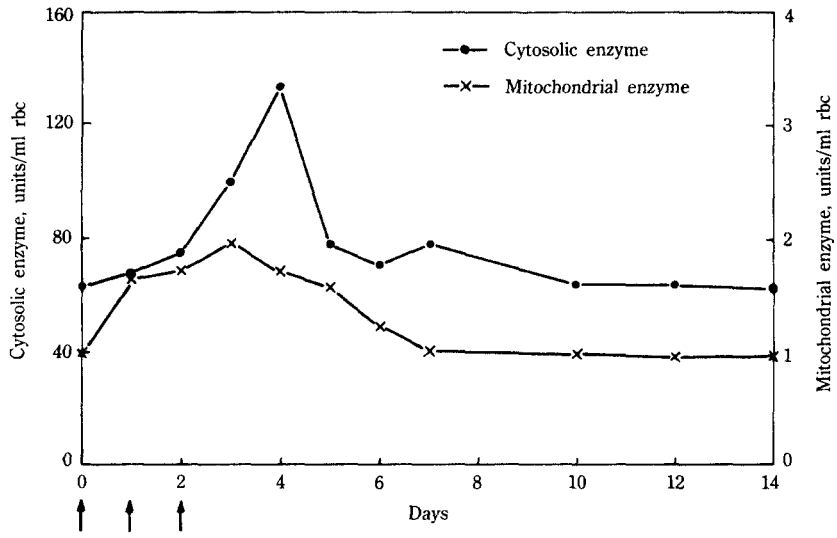


Fig. 2. Changes of 3'-nucleotidase activity in rat erythrocytes during induction of reticulocytosis and recovery from it. Arrows denote day on which phenylhydrazine was administered.

of the enzyme in the cytosolic and mitochondrial compartments during induction and recovery from reticulocytosis were summarized in Table 2. Also included were the values of half-lives of the isozyme in synthesis and degradation periods in the same Table.

The increase in the activity of cytosolic isozyme during the induction of reticulocytosis was remarkable, and showed about 2-fold as great as the normal value (Ks=1.2674; Kd=0.0095; $t\frac{1}{2}$=72.9 hours).

The mitochondrial isozyme also showed an increase as early as 24 hours after phenylhydrazine treatment, and attained its peak value on day 4. This value corresponds to about 2 times initial value (Ks=0.567; Kd=0.0288; $t\frac{1}{2}$=24.1 hours).

The rate constant during the recovery from phenylhydrazine treatment were; Ks=1.6756; Kd=0.0265; $t\frac{1}{2}$=26.2 hours for the cytosolic isozyme and Ks=0.0318; Kd=0.0324; $t\frac{1}{2}$=21.4 hours for the mitochondrial isozyme (Table 2).

Logarithmic plot of In(E-Eo) versus time during induction of reticulocytosis yielded straight line with a slope equal to 0.0095 for cytosolic Kd and 0.0288 for mitochondrial Kd respectively (Fig 3).

In the same manner, a logarithmic plot of In (Eo-E) vs hours during recovery period yielded also straight lines the slopes of which were equal to 0.0265 for cytosolic Kd and 0.0324 for mitochondrial Kd, respectively (Fig. 4).

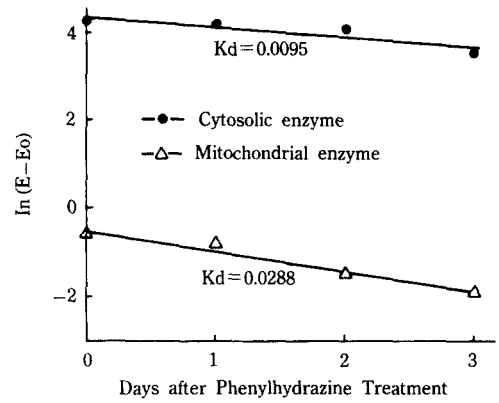


Fig. 3. Logarithmic plot of the decrease of 3'-nucleotidase during induction of reticulocytosis.

As shown in Table 2, during induction period of reticulocytosis, the value of cytosolic isozyme Ks is about 22-fold higher than that of mitochondrial isozyme and the value of mitochondrial Kd is about 3 times higher than that of cytosolic Kd.

At this period, the half-life ($t\frac{1}{2}$)shows that the mitochondrial isozyme is about one-third less stable than cytosolic isozyme. This should mean its rapid rate of degradation.

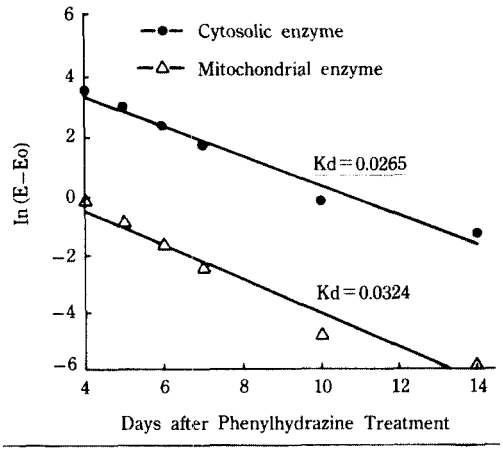


Fig. 4. Logarithmic plot of the decrease of 3'-nucleotidase during the period of recovery from reticulocytosis.

## DISCUSSION

Both the mitochondrial and cytosolic isozymes in phenylhydrazine-induced reticulocytes after phenylhydrazine administration reached their maximal activities on day 3 and 4, respectively. The mitochondrial isozyme increased its activity by only 1 unit, i, e, from 0.97 unit/ml RBC to 1.97 unit/ml RBC. However, the cytosolic isozyme increased its activity by about 70 units, i. e., from 63.24 units/ml RBC to 133.41 units/ml RBC.

These circumstances were fully reflected in Tab le 2.

The rate constants of degradation of mitochondrial isozyme in reticulocytes during induction and recovery periods were shown to be almost the same value (Kd=0.0288 for induction period and Kd=0.0324 for recovery period). The half-lives of the enzyme in each period also exhibited almost the same patterns, i. e., 24.1 hour for induction period and 21.4 hour for recovery period, respectively.

However, the rate constant of synthesis of this isozyme was Ks=0.0567 for induction period, and Ks=0.0318 for recovery period. Thus, the two-fold increase in the activity of the mitochondrial isozyme during reticulocytosis was the result of an increased Ks in induction period.

Although the rate constant of synthesis of cytosolic isozyme during induction period after phenylhydrazine treatment is not very different from that during recovery period (Ks=1.2674, as compared with that of Ks=1.6756), the extremely low rate of degradation, Kd=0.0095, and the longer half-life, $t\frac{1}{2}$=72.90 hours, during induction period appeared to have contributed to the steady increase in the activity of this enzyme during induction period.

The rate constant of synthesis of cytosolic isozyme during recovery period, Ks=1.6756, was about 1.3-fold higher than that of this enzyme during induction, Ks=1.2674. However, the concomitant increase in the rate constant of degradation of this enzyme during recovery period, Kd=0.0265, was about 2.8-fold higher than that of Kd=0.0095 for induction period. Moreover, this enzyme was about 2.8-fold less stable during this period (from $t\frac{1}{2}$= 72.90 hours to $t\frac{1}{2}$=26.2 hours).

It could be concluded that the above mentioned three components accelerated the degradation of cytosolic isozyme during recovery period.

Recently, some observations have been done in our laboratory on the synthesis and degradation of experimental reticulocytosis induced by phenylhydrazine treatment in rabbits, rats and mice.

Studies on the turnover rate of oxidoreductases, i. e., sorbitol[17], galactose[18], and glucose-6-phos phate[22], lactate[19] and malate[20] dehydrogenases have revealed that there are presumably two categories in the half-life of these enzymes.

We have observed that mitochondrial isozymes of malate and lactate dehydrogenases during induction period of reticulocytosis exhibited two or three-fold longer half-lives than that of cytosolic isozymes during same period.

However, sorbitol, glucoss-6-phosphate and galactose dehydrogenases showed a reversed patterns, that is, the half-lives of the above mentioned mitochondrial isozymes had only one-third values than that of cytosolic isozymes.

Malate dehydrogenase plays not only an important role on the respiration in mitochondria, but also transports reduction equivalent from mitochondria to cytosol through malate shuttle. According to Kim

et al[19], lactate dehydrogenase activity appears at 4th day of reticulocytosis, and the subunit type of this enzyme belongs to H which is stable for heat and found in mitochodrial fraction of actively respirating cells[21]. These two enzymes are closely associated within the mitochondria and involved in fulfillment of the energy requirement during the red blood cell morphogenesis.

Above mentioned three substrates for sugar metabolizing dehydrogenases, i. e., sorbitol, galactose and glucose-6-phosphate, are closely related each other in the metabolic pathway, and eventually generate NADPH in cytosolic fraction. NADPH is utilized in reductive biosynthesis, where as ribose-5-phosphate is used in the synthesis of DNA, RNA and nucleotide coenzyme, essential as cell structure in morphogenesis and cell differentiation.

## SUMMARY

The activity of 3'-nucleotidase (3'-ribonucleotide phosphohydrolase, EC 3. 1. 3. 6) isozymes in red blood cells were studied during induction and recovery from phenlhydrazine-induced reticulocytosis in rat.

The results obtained were as follows:

Red blood cells of the normal rat contained about 0.9% of reticulocytes.

The reticulocyte count in phenylhydrazine-treated rats increased to about 10 times on day 4 through day 5. Total 3'-nucleotidase activity was maximal on day 4, about 133.41 units which is about 2-fold higher than normal value. The relative activities of cytosolic and mitochondrial isozymes were 98.7% and 1.3%, respectively. The increase of the total 3'nucleotidase activity during induction of reticulocytosis was due mostly to the increase in the activity of cytosolic isozyme (Ks=1.2674: Kd=0.0095; $t_{\frac{1}{2}}$=72.90 hours), whereas the participation of the mitochondrial isozyme was shown to be insignificant (Ks=0.0567: Kd=0.0288: $t_{\frac{1}{2}}$=24.1 hours). The rate constants of the synthesis and degradation following recovery from phenylhydrazine treatment were: Ks=1.6756: Kd=0.0265: $t_{\frac{1}{2}}$=26.2 hours for cytosolic isozyme and Ks=0.0318: Kd=0.0323: $t_{\frac{1}{2}}$ 21.4 hours for mitochondrial isozyme.

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=국문초록=

# 실험적 흰쥐 망상적혈구 증다증에 있어서 3'-nucleotidase 활성의 변동

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정상 흰쥐에 phenylhydrazine을 주사한 후 시간이 경과하는 동안에 채취한 적혈구 및 망상적혈구에서 3'-nucleotidase: (3'-ribonucleotide phosphohydrolase, EC 3, 1, 3, 6)의 총활성 및 세포질과 mitochondria에서 합성 및 분해과정을 관찰하여 다음과 같은 결론을 얻었다.

정상 흰쥐의 적혈구 중 망상적혈구의 수는 약 0.9%였으며 phenylhydrazine을 주사한 후 심한 망상적혈구 증다증이 유발된 6일경에는 약 9.7%에 달하였다.

정상 적혈구에서 3'-nucleotidase의 총활성은 64.22unit였으며 세포질과 mitochondria에는 각각 63.24unit 및 0.99 unit의 활성을 나타냈다.

망상적혈구 증다증에 걸린 흰쥐 세포질의 3'-nucleotidase 활성은 약물주사 후 4일에 최고에 달하였으며 약 2배의 증가를 보여 133.41unit였다. Mitochondria 분획 내의 이 효소 활성은 0.99unit에서 3일에 최고치에 달했으며 1.97unit였다.

즉 세포질이 98.7%를, mitochondria가 1.3%를 차지하였다.

망상적혈구 증다증이 유발되는 동안 이 효소 활성의 증가와 감소의 양상은 mitochondria에 있어서는 Ks=0.0567: Kd=0.0288: $t\frac{1}{2}$=24.1 hours이며 세포질에 있어서는 Ks=1.2674: Kd=0.0095: $t\frac{1}{2}$=72.90 hours이었다.

Phenylhydrazine으로 처리한 후 회복기에 있어서 3'-nucleotidase의 합성 및 분해속도는 다음과 같았다. 즉 mitochondria에 있어서는 Ks=0.0318: Kd=0.0324: $t\frac{1}{2}$=21.4 hours, 세포질에 있어서는 Ks=1.6756: Kd=0.0265: $t\frac{1}{2}$=26.2 hours이었다.

Key Words: 3'-Nucleotidase, Reticulocytosis