Age-Induced Changes in Adenosine Monophosphate: Pyrophosphate Phosphoribosyltransferase and Inosine Monophosphate: Pyrophosphate Phosphoribosyltransferase from Normal and Lesch-Nyhan Erythrocytes†

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ABSTRACT: The loss of catalytic and immunologic activity of IMP:pyrophosphate phosphoribosyltransferase (EC 2.4.2.8 HPRTase) and AMP:pyrophosphate phosphoribosyltransferase (EC 2.4.2.7 APRTase) was measured in erythrocytes fractionated according to age. HPRTase antigenicity was constant despite the decrease in catalytic activity seen in older cells. In cells obtained from patients with Lesch-Nyhan disease no catalytic activity was seen, but immunologic cross-reacting material was found in all fractions with the same capability as in normal cells. APRTase, on the other hand, decreased with age in catalytic and antigenic activity at the same rate. This is interpreted as evidence that HPRTase is only partially denatured with aging, but that the APRTase molecule is extensively changed in conformation or is drastically degraded under these conditions. Phosphoribosyl pyrophosphate, which is a substrate for the reaction catalyzed by both of these enzymes and also can stabilize the enzymes *in vivo* and *in vitro*, increases greatly in concentration a short time after cells are in the circulation. This can explain the previously reported low thermal stability of the APRTase in the lysates of very young cells.

Normal erythrocytes in man are known to have a life span of about 120 days. During this time there is no *de novo* synthesis of enzymes. As the cells age their composition changes. Several enzyme activities are found to decrease as the cell matures (Sass *et al.*, 1964; Chapman and Schaumbum, 1967). The mechanism underlying the loss of enzyme activity upon aging may be either total destruction of specific proteins, or alterations of the molecule that lead to an inactivation. During the aging of erythrocytes the catalytic activity of IMP:pyrophosphate phosphoribosyltransferase (HPRTase, EC 2.4.2.8.)[1] and AMP:pyrophosphate phosphoribosyltransferase (APRTase, EC 2.4.2.7.) decreases (Rubin *et al.*, 1969).

In the erythrocytes of patients with Lesch-Nyhan disease (L-N) there is essential absence of HPRTase activity which is accompanied by an elevation in APRTase (Seegmiller *et al.*, 1967). The half-life of the L-N erythrocyte APRTase is about 2.5 times longer than that found in normal erythrocytes (Rubin *et al.*, 1969); nevertheless the enzymes from these two sources are immunologically indistinguishable from each other (Yip *et al.*, 1973). These two enzymes, HPRTase and APRTase, because of the facts outlined above permit measurement of the rates of destruction of functional and nonfunctional enzymes, and it further allows us to follow the fate of antigenic as well as catalytic sites. It is hoped that such information will help in evaluating the mechanism by which enzymes are inactivated in normal erythrocyte aging.

Since the stability *in vivo* and *in vitro* of APRTase has been shown to be related to PRPP concentrations (Hori and Henderson, 1966; Murray and Wong, 1967; Gadd and Henderson, 1970; Greene and Seegmiller, 1969; Greene *et al.*, 1970; Sorenson, 1970), it was thought the knowledge of changes in PRPP concentrations would be of significance in understanding the decay of this enzyme. For this reason we assayed the PRPP in erythrocytes of the various ages.

Materials and Methods

Enzyme Assay. The assay of enzyme activity was carried out as described previously based on the retention of [^{14}C]nucleotides on DEAE-cellulose (Yip *et al.*, 1973; Rubin *et al.*, 1971).

Determination of Protein Concentration. Hemoglobin concentration was determined as methoglobin cyanide (Van Kampen and Zijlstra, 1961). Total protein was determined by the method of Lowry *et al.* (1951).

Preparation of Antisera. Both APRTase and HPRTase were purified and antisera prepared according to the procedures previously described (Yip *et al.*, 1973; Rubin *et al.*, 1971). The specificity of the antibody had been demonstrated by the fact that on immunoelectrophoresis the precipitin line coincided with the place to which enzyme had migrated, and by the fact that absorption with purified enzyme removed all neutralizing activity.

Enzyme Neutralization. An appropriately diluted rat or rabbit antiserum (20 μl) was mixed with varying amounts of hemolysate. Phosphate-buffered saline was added to a final volume of 200 μl. Nonimmunized animal serum or a bovine serum albumin solution was used as control. The mixture was incubated at 4° for 16 hr, and then centrifuged at 2000*g* for 30 min. The supernatant (50 μl) was used for assay of enzyme activity.

Absorption of Antibody. Aliquots (10 μl) of properly diluted antiserum or anti-γ-globulin were mixed with varying amounts

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[1] Abbreviations used are: HPRTase, IMP:pyrophosphate phosphoribosyltransferase; APRTase, AMP:pyrophosphate phosphoribosyltransferase; PRPP, phosphoribosyl pyrophosphate.

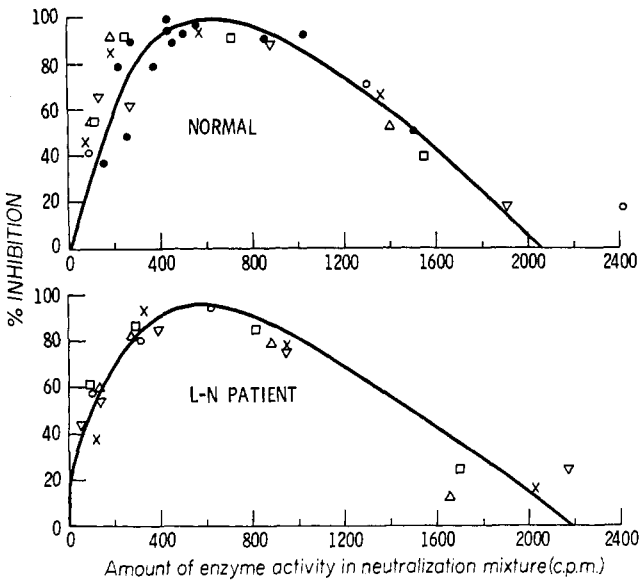

FIGURE 1: Inhibition of adenine phosphoribosyltransferase (APRTase) by rabbit anti-APRTase serum. A fixed amount of antiserum (10 μl undiluted) was used to neutralize a varying volume of 1:10 diluted hemolysate of fractionated red cells from normal and Lesch-Nyhan subjects: fraction 0, whole blood; fraction 1–5, red cells increasing in age; (●) fraction 0; (×) fraction 1; (○) fraction 2; (△) fraction 3; (▽) fraction 4; (□) fraction 5. Experimental procedure is described in text. The results are given as the catalytic activity inhibited after the antibody neutralization relative to the control value in the absence of antibody. There is no detectable difference in the inhibition curves. At maximum inhibition 6.53×10^{-5} IU was neutralized. This represented 272, 336, 405, 405, and 593 μg of protein in fractions 1–5, respectively, of normal and 118, 155, 166, 170, and 178 in fractions 1–5, respectively, of L-N patient.

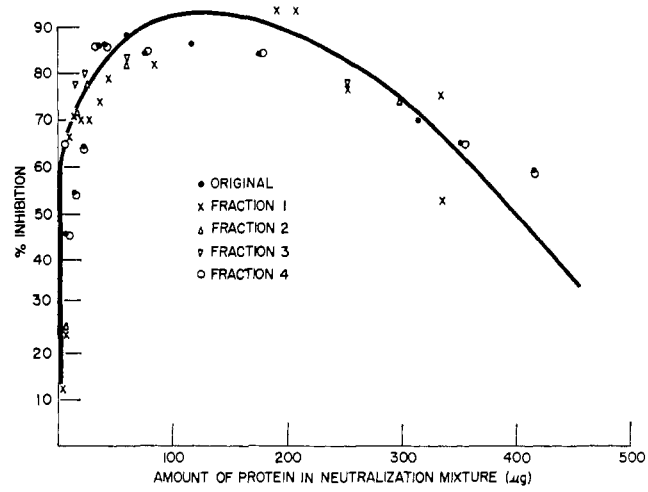

FIGURE 2: Inhibition of IMP:phosphoribosyltransferase (HPRTase) by rat anti-HPRTase γ-globulin. Rat anti-HPRTase γ-globulin (15 μl; 315 μg) were used to neutralize a varying amount of diluted hemolysate (protein content 10–400 μg) of fractionated red cells from a normal subject: fraction 0, unfractionated blood; fraction 1–4, red cells increasing in age. Experimental procedure is described in text. The results are given as the activity inhibited by antibody neutralization relative to the amount of antigen protein used in the neutralization assay. At maximum inhibition the enzyme found in 120 μg of erythrocyte lysate protein was neutralized. This represented 19.8, 28.9, 24.3, 23.3, 20.2 $\times 10^{-5}$ IU in unfractionated material and fractions 1–4, respectively.

of hemolysate from normal or L-N erythrocytes. The absorption mixture was allowed to stand at 4° for 16 hr. The resulting precipitates were removed by centrifugation. The amount of free antibody which remained in the supernatant was determined by a second neutralization assay. In the second neutralization, a known amount of normal hemolysate (50 μl, 1:50 dilution) was incubated with 100 μl of the above supernatant at 4° for 16 hr. The remaining enzyme activity was determined at the end of the neutralization.

Fractionation of Erythrocytes. Red blood cells were fractionated according to age by ultracentrifugation in an isosmotic discontinuous gradient of bovine serum albumin, according to the precedure of Piomelli *et al.* (1967).

Endogeneous PRPP Determination. A modification of the method of Sperling *et al.* (1972) was used. Fractionated red blood cells were lysed by freezing and thawing three times. The reaction mixture contained 100 μl of the lysed red cells, 10 μmol of Tris-HCl (pH 8.0) buffer, 1 μmol of Mg^{2+}, 5 μl of purified APRTase (specific activity 0.4 IU/mg), and 0.25 mmol of [8-^{14}C]adenine (specific activity 3.3 Ci/mol). The reaction mixture was incubated at 37° for 1 hr and the incubation was terminated by immersion of the reaction mixture in an ethanol–Dry Ice bath. Nucleotide formation was measured as described above and the amount of PRPP present was calculated from the amount of adenosine phosphate formed.

Results

When a fixed amount of rabbit anti-APRTase serum was mixed with serial dilutions of lysates of erythrocytes fractionated according to age from normal and Lesch-Nyhan patients, a typical parabolic inhibition curve (Horowitz and Scharff, 1969) was obtained (Figure 1). Despite the differences in catalytic activity per mg protein and heat stability of APRTase among cells of different age and between cells of normal and L-N subjects (Rubin *et al.*, 1969), no significant difference in immunological behavior among any blood lysates was found. A constant amount of APRTase catalytic activity was neutralized by a fixed amount of antiserum in all cases. For example, at one point in the equilibrium portion of the neutralization curve, 10 μl of rabbit antiserum neutralized 6.53×10^{-5} IU of APRTase activity of all fractions from normal and L-N erythrocytes, while the amount of the erythrocyte protein being neutralized in each fraction was different, depending on the specific activity of the fraction. This confirms our previous observation (Yip *et al.*, 1973) that the ratio of the APRTase catalytic activity to antigenicity remains constant. It further indicates that the decay of APRTase during erythrocyte aging process affects both catalytic and antigenic sites at the same rate.

A different neutralization result was obtained with fractionated HPRTase (Figure 2). A single parabolic response curve was observed for erythrocytes of all ages when the relative inhibition of the catalytic activity was plotted against the amount of erythrocyte protein in the neutralization mixture. The fact that a fixed amount of antibody neutralized the same amount of different age erythrocyte protein, with different specific enzyme activity, indicates that the aging process has altered only the catalytic activity of the protein molecule. In the equilibrium portion of the neutralization curve, while the same amount of erythrocyte protein (120 μg) was neutralized in all fractions, the catalytic activity of HPRTase being neutralized decreased with age (Figure 2).

Using antibody absorption followed by neutralization, we were able to demonstrate that the amount of inactive HPRTase protein (cross-reacting material) in fractions of erythrocytes of different ages from Lesch-Nyhan patients was about the same as that of the amount of active HPRTase in

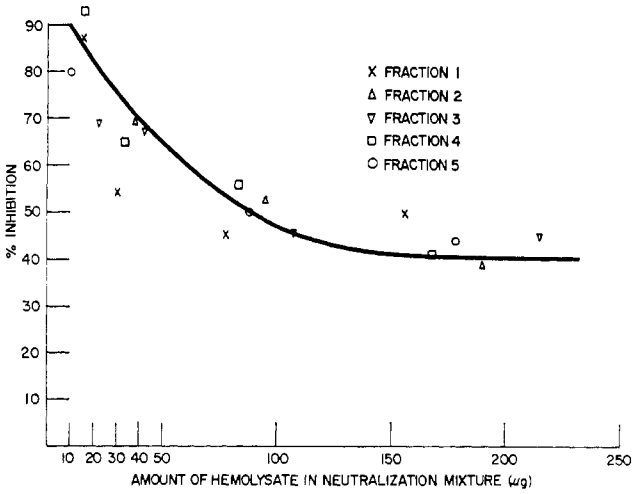

FIGURE 3: Inhibition of IMP:phosphoribosyltransferase (HPRTase) from fractionated Lesch-Nyhan (L-N) erythrocytes by rat anti-HPRTase γ-globulin. Rat anti-HPRTase γ-globulin (15 μl; 315 μg) were used to neutralize a varying amount of diluted L-N hemolysate (protein content 10–400 μg; activity = 0) of fractionated blood; F1–F5, red cells increasing in age. The free antibody after first antibody absorption was determined by a second neutralization assay (see text). The results are given as the activity inhibited at the end of the neutralization (compared to the total catalytic activity added) relative to the amount of antigen protein present in the first neutralization step.

normal control subjects (Figure 3). In addition, as was seen with cells from control subjects, the amount of antigenically identifiable HPRTase was independent of the age of the cells.

In Table I is shown the amount of endogeneous PRPP present in erythrocytes fractionated according to age. A 6-fold increase between the very young (fraction 1) and matured erythrocytes was observed. The mean amount of PRPP in the four fractions is the same as that in the unfractionated within experimental uncertainty.

Discussion

It has been clearly demonstrated by Cinader (1967), that the inhibition of enzyme activity subsequent to binding of antibody is caused by either the steric hindrance of binding of substrate or product at the catalytic site, or by a conformational change of the enzyme molecule induced by the binding of antibody. In either case, the inhibition thus produced is constant, and the magnitude depends on the nature of the antigenic and catalytic sites on the enzyme molecule. A change in either the catalytic or the antigenic capacity of the enzyme

TABLE I: Comparison of the Concentration of PRPP in Erythrocyte of Different Ages.

Samples[a]	pmol/mg of Erythrocyte
Unfractionated	131
Fraction 1	31
Fraction 2	156
Fraction 3	193
Fraction 4	172
Mean of the fractions	137

[a] Samples increase in age from fraction 1 to fraction 4.

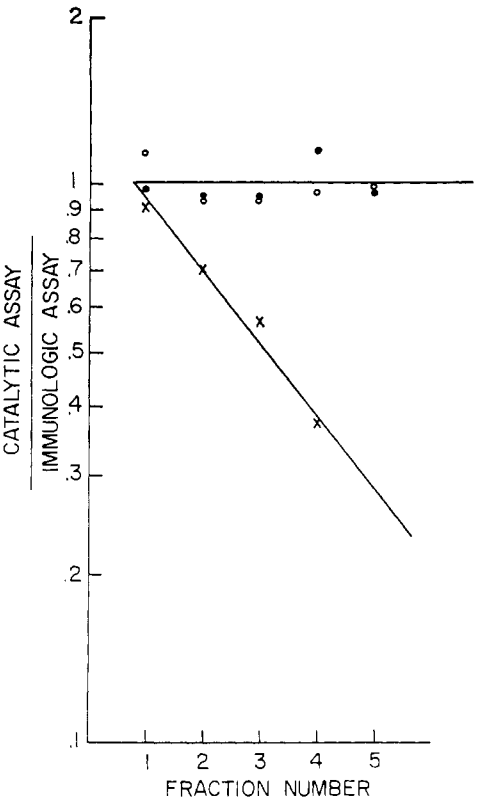

FIGURE 4: Ratio of catalytic to antigenic activity of APRTase and HPRTase in red cell fractions of increasing age. Results were obtained from red cells increasing in age from fraction 1 to 5. The ratio of catalytic to antigenic activity of HPRTase (×), L-N APRTase (○), and normal APRTase (●) are shown.

molecule would be shown by a change of the amount of catalytic inhibition by antibody binding under the specific experimental conditions.

The results of the immunochemical studies on the adenine phosphoribosyltransferase of erythrocytes, fractionated according to age, have shown that at a given enzyme concentration (shown by catalytic activity) the relative amount of catalytic inhibition by antibody binding remained a constant regardless of the age and the source of the erythrocytes (Figure 1). No age-induced immunological characteristic change on APRTase was detectable. This is shown graphically in Figure 4 in which the ratio of catalytic to antigenic activity is plotted for APRTase of a normal subject and a Lesch-Nyhan patient. Despite the altered half-life in the latter subject the ratio is essentially one. In contrast, the decay in catalytic activity of HPRTase is not paralleled by a decrease in antigenic activity and hence the ratio decreases. Therefore, we conclude that degradation of the entire APRTase molecule during the erythrocyte aging process is the underlying mechanism for the decrease of APRTase activity observed.

Recently, Sachs *et al.* (1972) have identified an antigenic determinant region on staphylococcal nuclease molecule. They have also shown that a catalytically inactive fragment of the staphylococcal nuclease molecule can bind with the antibody prepared against the nuclease antigenic determinant fragment. This observation indicates independence of the catalytic region and the antigenic region on the same protein molecule. Our immunological studies on the HPRTase of L-N and gouty subjects (Rubin *et al.*, 1971; Balis *et al.*, 1974) have shown that the mutation of the catalytic site of this enzyme does not affect its antigenicity. The age-induced disintegration of HPRTase in erythrocytes is also found to be a

partial modification of one site but not the other. Figure 2 shows that the relative catalytic inhibition of HPRTase by antibody binding remains a constant for all fractions with respect to the amount of protein present in the neutralization mixture. The HPRTase specific activity decreases with age (Rubin *et al.*, 1969). In other words, the HPRTase of older fractions, though lower in catalytic activity, can bind as effectively with the antibody as that of the younger cells. An age-induced modification of the HPRTase molecule seems to be the mechanism for the decrease of HPRTase activity during erythrocyte aging process. The result on the immunoassay on HPRTase of fractionated erythrocytes from a Lesch-Nyhan subject (Figure 3) further confirmed our previous observation (Rubin *et al.*, 1971); a loss of HPRTase catalytic activity is not accompanied by a concomitant loss of enzyme antigenicity.

The change that occurs in HPRTase during erythrocyte aging process can result from either a modification of the enzyme molecule to a less active enzyme, similar to the age-induced modification of glucose-6-phosphate dehydrogenase (Piomelli *et al.*, 1968), or a complete inactivation of part of the enzyme population like that of fructose-1,6-bisphosphate aldolase in the liver of senescent mice (Gershon and Gershon, 1973). Identification of HPRTase isozymes in normal and gout erythrocytes (Rubin *et al.*, 1971; Balis *et al.*, 1974; Dancis *et al.*, 1973; Arnold and Kelley, 1971) is evidence of the existence of a less active HPRTase component in the circulating erythrocyte system and supports the former explanation.

Since no *de novo* protein biosynthesis occurs in erythrocytes, the modification of HPRTase during the aging process has to be a posttranscriptional alteration of the molecule. A like observation of this enzyme has been reported (Arnold and Kelley, 1971). Evidence of similar modification is seen with erythrocyte lactic dehydrogenase (Rosa and Schapira, 1964; Schapira and Rosa, 1967; Starkweath *et al.*, 1965) and hexokinase (Eaton *et al.*, 1966).

The intracellular PRPP level in very young cells (fraction I, in Table I) is only one-sixth of that of the more mature cells. It has previously been reported that APRTase in lysates of younger erythrocytes is more susceptible to heat denaturation than is that from older cells (Rubin *et al.*, 1969). These results explain that observation.

The intracellular PRPP concentration is influenced by many factors (Meyskens and Williams, 1971; Fox and Kelley, 1971, 1972). The observed 6-fold increase in intracellular PRPP concentration in older cells is more likely to be the result of a slower rate of utilization rather than a faster rate of generation of this molecule in the aging erythrocyte. Because of the several reactions in which PRPP is a substrate it is not possible at this time to identify the significant biochemical sites involved.

Acknowledgments

The authors express their sincere thanks to Dr. S. Piomelli for his gift of the most valuable age-fractionated blood cells. The authors wish to acknowledge the excellent technical assistance of Mrs. Carol Gryte. Thanks is also extended to the New York Blood Bank for their gift of outdated erythrocytes.

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