Purine Nucleoside Phosphorylase. Microheterogeneity and Comparison of Kinetic Behavior of the Enzyme from Several Tissues and Species†

Kailash C. Agarwal, Ram P. Agarwal, Johanna D. Stoeckler, and Robert E. Parks, Jr.*

ABSTRACT: The purine nucleoside phosphorylases (PNPases) from human and rat erythrocytes and bovine spleen have been subjected to isoelectric focusing. The crystalline bovine spleen PNPase emerged as a single peak of pI = 5.4 whereas the rat erythrocytic PNPase was distributed into two variants of pI = 5.6 and 5.7 and the crystalline human erythrocytic enzyme produced six variants ranging from pI = 5.85 to 6.25. Treatment of human erythrocytic PNPase with dithiobisnitrobenzoate changed the enzyme to a more acidic form (pI = 5.05). The kinetic behaviors of these electrophoretic variants were studied and compared with the unresolved bovine erythrocytic PNPase. All six variants of the human erythrocytic PNPase and the two variants of rat erythrocytic PNPase displayed substrate activation at high concentrations of inosine and deoxyinosine. Bovine erythrocytic PNPase did not show activation with any of the nucleosides whereas with the bovine spleen enzyme activation occurred only with the deoxynucleosides, deoxyinosine and deoxyguanosine. The K_m values for inosine, deoxyinosine, guanosine, deoxyguanosine, guanine, and hypoxanthine, where determined, ranged from 1.3×10^{-5} to 3.0×10^{-5} M for all the enzymes except the rat erythrocytic PNPase variants which have higher K_m values for inosine (5.9×10^{-5} M, 8.3×10^{-5} M) and deoxyinosine (13×10^{-5} M, 20×10^{-5} M).

A still unexplained phenomenon observed with human erythrocytic purine nucleoside phosphorylase (PNPase)[1] is the enzymic activation that occurs at high concentrations of inosine (Agarwal and Parks, 1969; Kim *et al.*, 1968a,b). Possible explanations proposed were cooperativity among the subunits of the enzyme and/or the occurrence of isozymes with different kinetic properties. PNPases from human erythrocytes and bovine spleen have been shown to be trimeric in structure by substrate-binding studies, genetic analysis, and by electrophoretic and sedimentation methods (Agarwal *et al.*, 1973; Agarwal and Parks, 1969; Edwards *et al.*, 1971, 1973) with a monomeric molecular weight of about 30,000 (Agarwal *et al.*, 1973; Edwards *et al.*, 1973). In a study of the behavior of sulfhydryl groups with the human erythrocytic enzyme it was shown that only 2–4 sulfhydryl groups of a total of 12 react with DTNB with a concomitant loss of about 60% of the enzymic activity. However, kinetic analysis of the residual (40%) activity revealed a loss of the phenomenon of substrate activation and an increase of about fivefold in the K_m for inosine. When this partially inactivated enzyme was treated with dithiothreitol (DTT), the activity was fully restored, the phenomenon of substrate activation returned, and the K_m for inosine decreased about fivefold to its original value (Agarwal and Parks, 1971). These observations *per se*, however, do not permit a choice among the possible explanations for the activation by inosine. Titration with DTNB could have abolished cooperativity among the subunits or could have specifically titrated one or more isozymes with low K_m values leaving unaffected an isozyme with a high K_m value. These observations, and others, such as the occasional appearance of aberrant peaks in column chromatography during purification, and a relatively broad band seen during gel electrophoresis of the crystalline enzyme, led us to subject human erythrocytic PNPase to isoelectric focusing. This study, which was performed several years ago and reported in preliminary form, revealed that the crystalline human erythrocytic enzyme displays a marked degree of microheterogeneity with variation from preparation to preparation (Agarwal *et al.*, 1973). Independently, by the use of starch gel electrophoretic methods, Edwards *et al.* (1971) also showed the marked microheterogeneity of human erythrocytic PNPase and, furthermore, made the intriguing observation that the pattern of enzymic heterogeneity changes as the erythrocyte ages from the reticulocyte to the dense senescent cell (Turner *et al.*, 1971). The proportion of more acidic PNPase variants increased as the erythrocyte aged which suggests that the enzyme is subject to progressive degradation, possibly deamination reactions, during the lifetime of the cell. In the present study an attempt was made to learn whether the microheterogeneity of human erythrocytic PNPase can explain the unusual kinetic behavior and the reactivity with sulfhydryl reagents. The behavior of the human erythrocytic enzyme was compared with partially purified preparations of rat and bovine erythrocytic PNPases and with commercially available crystalline bovine spleen PNPase.

Materials and Methods

Inosine, deoxyinosine, hypoxanthine, guanine, guanosine, and α-D-ribose 1-phosphate were purchased from P-L Biochemicals (Milwaukee, Wis.); dithiothreitol and 2′-deoxy-

† From the Section of Biochemical Pharmacology, Division of Biological and Medical Sciences, Brown University, Providence, Rhode Island 02912. *Received August 26, 1974.* This paper is the sixth in a series from this laboratory on Purine Nucleoside Phosphorylase. The preceding paper in this series is Agarwal and Parks (1971). Supported by Grant IC 62-0 from the American Cancer Society.

[1] Abbreviations used are: PNPase, purine nucleoside phosphorylase (purine nucleoside:orthophosphate ribosyltransferase, EC. 2.4.2.1); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; PMS, phenazine methosulfate, Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

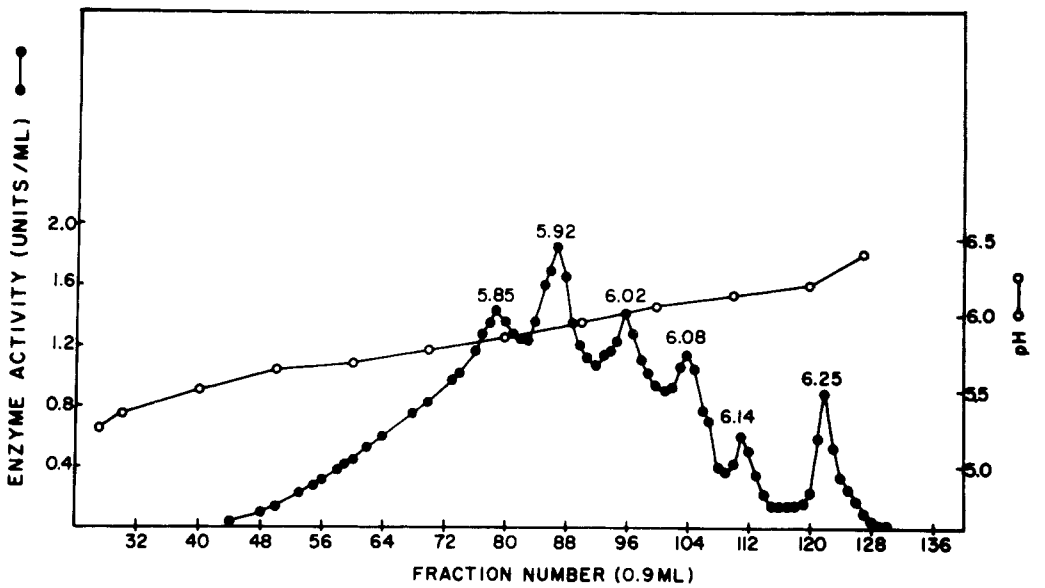

FIGURE 1: Electrofocusing profile of crystalline human erythrocytic purine nucleoside phosphorylase. About 20 units of crystalline PNPase were electrofocused in the Ampholine mixture (pH 5.1–6.3) at 400 V for 24 hr followed by 600 V for 40 hr; 1-ml fractions were collected in tubes containing 0.1 mg of bovine plasma albumin dissolved in 10 μl of water. Enzymic activity and pH were determined in each fraction.

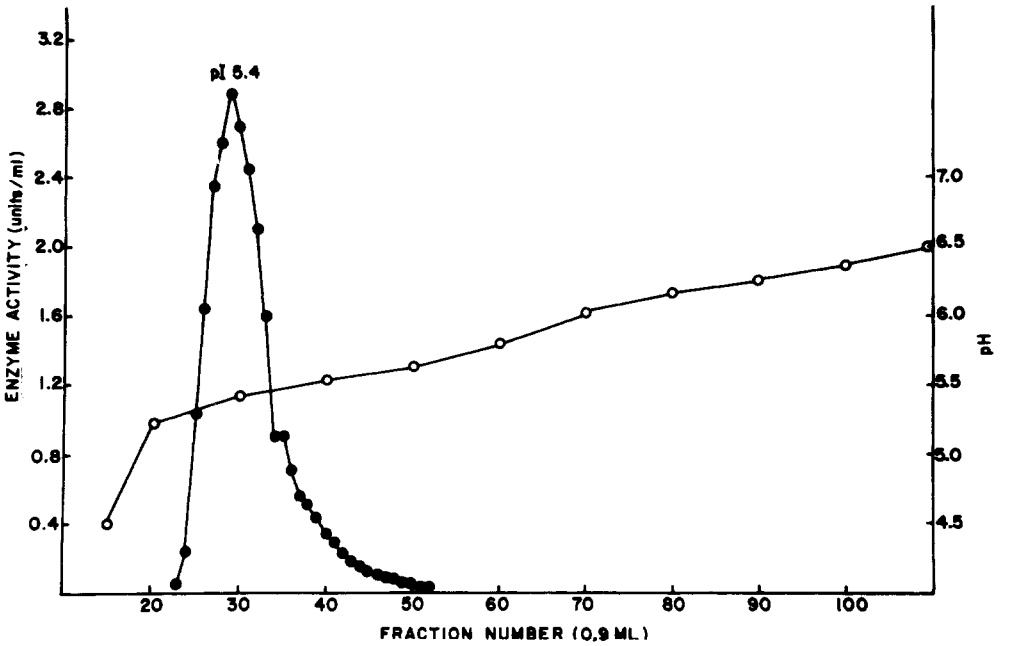

FIGURE 2: Electrofocusing profile of crystalline bovine spleen purine nucleoside phosphorylase. About 40 units of crystalline bovine spleen PNPase were electrofocused in 1% Ampholine (pH 5–7) as described in Figure 1.

guanosine were obtained from Calbiochem (La Jolla, Calif.); DTNB was a product of Aldrich Chemical Co. (Milwaukee, Wis.); agarose, PMS, and xanthine oxidase (Grade I, milk) were obtained from Sigma Chemical Co. (St. Louis, Mo.); MTT was from Nutritional Biochemicals Corp. (Cleveland, Ohio); Ampholine carriers (40% solution) were from LKB Instruments Inc. (Rockville, Md.); and PNPase (crystalline, bovine spleen; specific activity 40 units/mg of protein) was a product of Boehringer Mannheim (New York, N.Y.) whereas human erythrocytic PNPase was crystallized as described earlier (Agarwal and Parks, 1969).

Rat erythrocytic PNPase (present at 0.9 unit/ml of packed erythrocytes) was partially purified to a specific activity of 0.09 unit/mg of protein by adsorption on calcium phosphate gel and elution with and precipitation by ammonium sulfate as described earlier (Agarwal *et al.*, 1971). The enzyme was dialyzed against 1% glycine solution prior to electrofocusing.

Bovine erythrocytic PNPase was partially purified from freshly drawn bovine erythrocytes (present at 0.05 unit/ml of packed cells) to a specific activity of 7.0×10^{-4} units/mg of protein by passing through a column of calcium-phosphate gel-cellulose. The column was washed with 0.03 M Tris-acetate (pH 7.5), and the enzyme was eluted with 0.2 M potassium phosphate buffer (pH 7.5). This preparation was used for the kinetic studies.

Determination of Enzyme Activities. The enzyme was assayed spectrophotometrically by the coupled xanthine oxidase method of Kalckar (1947) essentially as described by

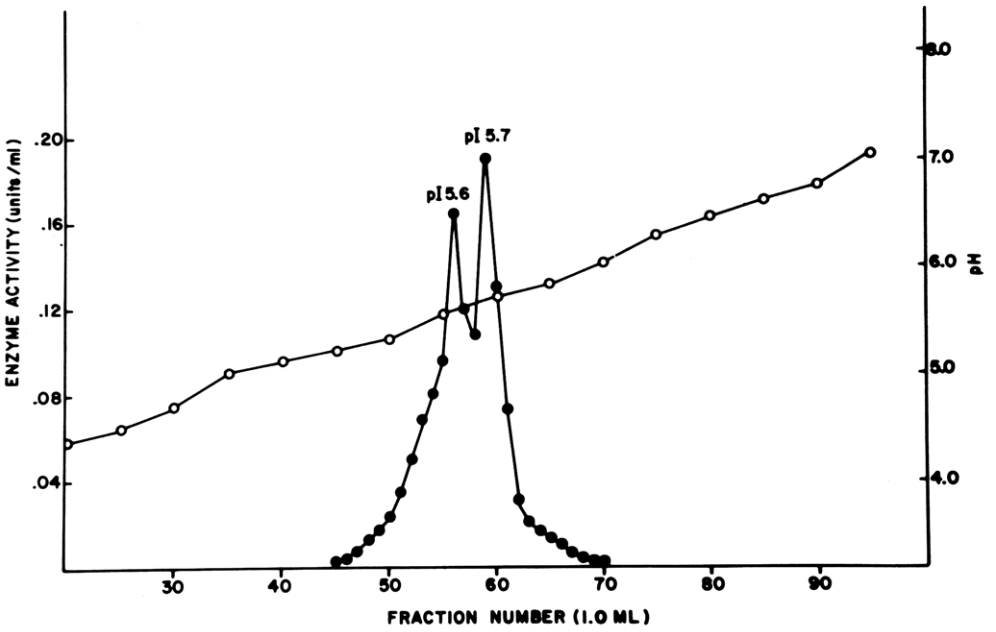

FIGURE 3: Electrofocusing profile of partially purified rat erythrocytic purine nucleoside phosphorylase. About 1 unit of rat erythrocytic PNPase was electrofocused in 1% Ampholine (pH 4–8) as described in Figure 1.

Kim *et al.* (1968a). The standard assay reaction mixture contained: inosine, 0.5 mM; potassium phosphate buffer (pH 7.5), 50 mM; xanthine oxidase, 0.02 unit, and an appropriate amount of PNPase in a final volume of 1 ml. Increase in absorbance at 30° was followed at 293 nm, using a cuvet of 1 cm light path in a Gilford Model 2000 spectrophotometer.

One unit of PNPase is the amount of enzyme that catalyzes the phosphorolysis of 1 μmol of inosine under standard assay conditions. Protein concentrations were determined by the method of Warburg and Christian (1941).

The K_m values for guanine and hypoxanthine were determined as described by Ross *et al.* (1973). Reactions were carried out at 30° in Hepes–NaOH buffer (pH 6.5), 50 mM; ribose 1-phosphate, 1 mM; varying amounts of substrate; and about 0.003 unit of PNPase. The molar absorbancy changes of guanine and hypoxanthine for their conversion to the respective ribonucleosides at pH 6.5 were 5100 at 252 nm and 1400 at 248 nm, respectively (Ross *et al.*, 1973). The phosphorolysis of guanosine or deoxyguanosine was followed at 252 nm in potassium phosphate buffer, 50 mM, pH 7.5.

Isoelectric Focusing. Electrofocusing was carried out in an LKB electrofocusing column (110 ml) in a sucrose gradient with 1 or 2% Ampholine for 20–24 hr at 400 V, followed by an increase in voltage to 600 V for 40 hr. Ampholine of the narrow pH range (5.1–6.3) was prepared according to the method described in the LKB 8100 Ampholine® Electrofocusing Equipment Instruction Manual (LKB-Produkter AB, S-161 25 Bromma 1. Sweden).

Agarose Gel Electrophoresis. Electrophoresis was carried out on 1% agarose gel plates using Tris-maleate buffer (pH 7.2) containing EDTA and $MgCl_2$ as described earlier (Agarwal and Parks, 1972). 5–10 μl of enzyme samples were employed and electrophoresed for about 30 min at 180 V and 100 mA current.

After the run, the enzyme bands were identified by an overlay technique similar to that of Edwards *et al.* (1971) as described below. Sufficient agarose to yield a final concentration of 1.5% was added to 20 ml of 0.05 M Tris-chloride buffer (pH 8.0) and dissolved by gentle boiling. This solution was allowed to cool to about 42°, mixed rapidly with the reaction mixture containing: inosine, 20 μmol; xanthine oxidase, 0.5 unit; potassium phosphate, 1.0 mmol (pH 8.0); PMS, trace amount; and MTT, 0.5 mg, and poured into a mold formed by two glass plates separated by a 1-mm thick plastic gasket and allowed to solidify at 4°. The overlay gel was then placed carefully over the electrophoretic gel and incubated at 37° for 30–45 min. The overlay and running gels were separated and the excess dye was removed by washing with cold running tap water in the dark. The gels were allowed to dry on a glass plate to form a permanent preparation.

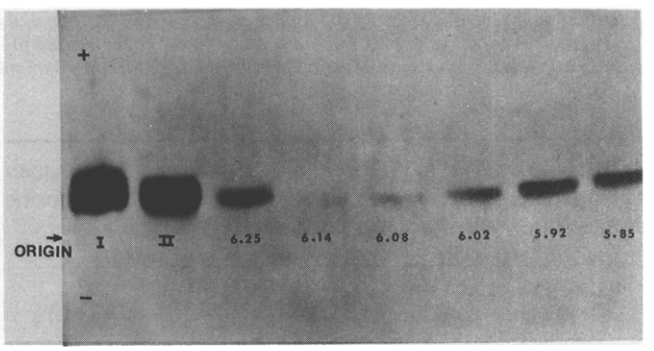

FIGURE 4: Agarose gel electrophoretogram of crystalline human erythrocytic purine nucleoside phosphorylase and its variants; 5–10 μl of PNPase from each electrofocusing peak (Figure 1) and unresolved enzyme (I, II) were electrophoresed for 30 min at 180 V. The enzymic bands were detected by the overlay technique described under Materials and Methods.

Results

Electrofocusing Profiles of PNPases. A typical electrofocusing profile of crystalline human erythrocytic PNPase is presented in Figure 1. On electrofocusing over a narrow range of pH (5.1–6.3), the enzymic activity was distributed among six peaks with p*I* values ranging from 5.85 to 6.25. In contrast, crystalline bovine spleen PNPase electrofocused into a single peak of p*I* = 5.4 (Figure 2) and partially

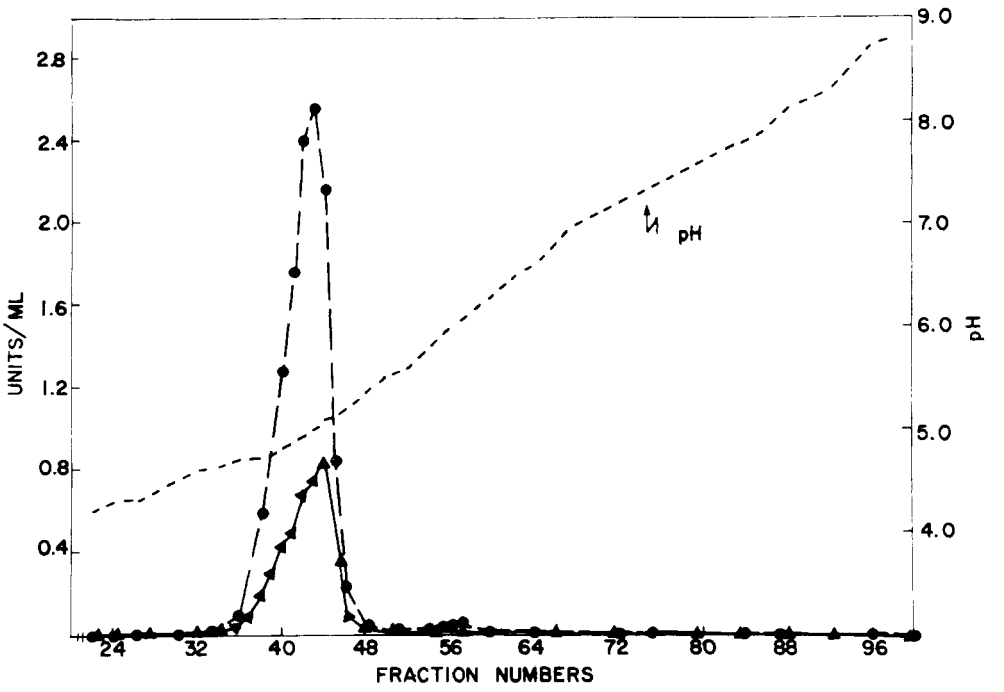

FIGURE 5: Electrofocusing profile of human erythrocytic nucleoside phosphorylase after DTNB treatment. Crystalline human erythrocytic PNPase (about 66 units) was incubated in 1.2 ml of 0.05 M Tris-acetate (pH 7.5) containing 2 mM DTNB, at room temperature for 20 min and then dialyzed overnight against 0.05 M Tris-acetate (pH 7.5). The dialyzed sample was electrofocused in a sucrose solution containing 1% Ampholine (pH 3-10) for 60 hr at 600 V; 1-ml fractions were collected and pH and enzymic activity (▲—▲) were measured in each fraction. The enzymic activity was reassayed after treating 0.2 ml of each fraction with 0.05 ml of 100 mM dithiothreitol for at least 2 hr (●—●).

TABLE 1: Michaelis Constants of the Variants of Human Erythrocytic Purine Nucleoside Phosphorylase Using Inosine as Substrate.

p*I*	K_m (M × 10^5)	p*I*	K_m (M × 10^5)
5.85	2.2	6.08	3.2
5.92	2.9	6.14	4.0
6.02	2.8	6.25	5.3

purified rat erythrocytic PNPase was distributed into two peaks of p*I* = 5.6 and 5.7 (Figure 3).

The individual peaks of human erythrocytic PNPase were subjected to agarose gel electrophoresis. As shown in Figure 4, the enzymic variants moved in sharp and distinct bands in accordance with their p*I* values whereas unresolved enzyme produced diffuse broad bands.

Electrofocusing after DTNB Treatment. The partial inactivation and change in the kinetic behavior on treatment of crystalline human erythrocytic PNPase with DTNB, reported earlier (Agarwal and Parks, 1971), suggested the possibility of differential inactivation of the enzymic variants by DTNB. To test this hypothesis, the enzyme was electrofocused after treatment with DTNB. As shown in Figure 5, all of the enzymic activity emerged in a single peak shifted markedly to the acidic directions (p*I* = 5.05). No change in the molecular weight as determined by a molecular sieving method was detected after partial inactivation of the enzyme by DTNB as described previously (Agarwal and Parks, 1971).

Kinetic Studies. As shown in Table I no marked differences in the K_m values were observed among the different variants of human erythrocytic PNPase. The K_m values

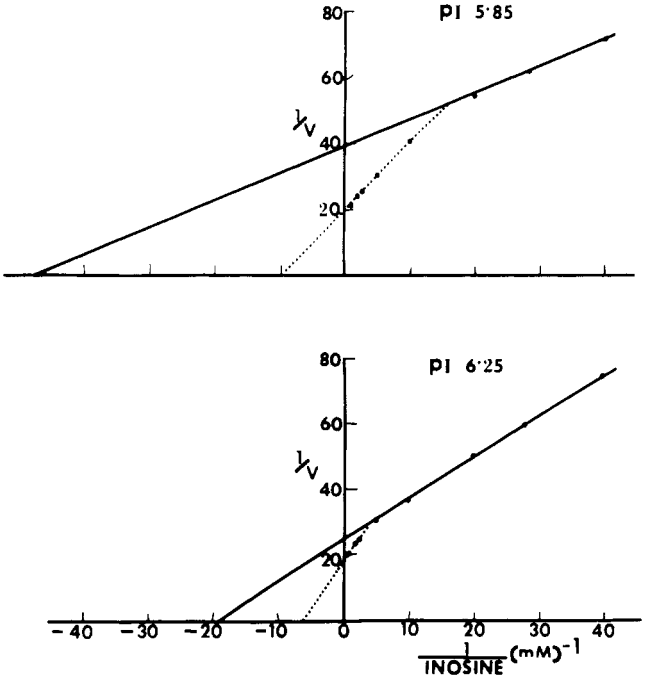

FIGURE 6: Double reciprocal plots of initial velocities and concentrations of inosine with the two variants of the human erythrocytic purine nucleoside phosphorylase. Reaction mixtures contained in 1.0 ml: potassium phosphate, 50 mM, pH 7.5; xanthine oxidase, 0.02 unit; PNPases, 0.002 unit, and inosine, varying amounts. An increase in absorbance was followed at 293 nm at 30°.

ranged from 2.2 × 10^{-5} to 5.3 × 10^{-5} M which are in good agreement with the values reported for the unresolved crystalline enzyme (Agarwal and Parks, 1969). Substrate activation occurred with all six electrophoretic peaks. However, the substrate activation was much more pronounced in variants with more acidic p*I* values. Figure 6 presents double-

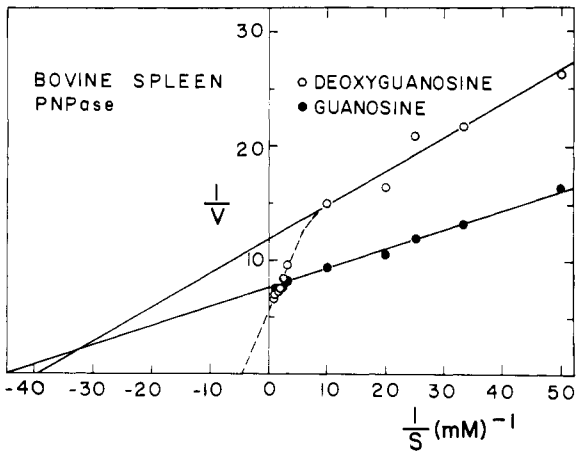

FIGURE 7: Double reciprocal plots of initial velocities and concentrations of guanosine and deoxyguanosine with bovine nucleoside phosphorylase. Reaction mixtures contained in 1.0 ml: potassium phosphate, 50 mM, pH 7.5; bovine spleen PNPase, 0.002 unit, and varying amounts of the substrates. A decrease in absorbance was followed at 252 nm at 30° in a cuvet of 1-cm light path. For the higher concentrations of the substrates (0.3–1.0 mM) a cuvet of 1-mm light path and 0.005 unit of the enzyme were used. The velocities were normalized to correspond to those obtained with the 1-cm cuvet.

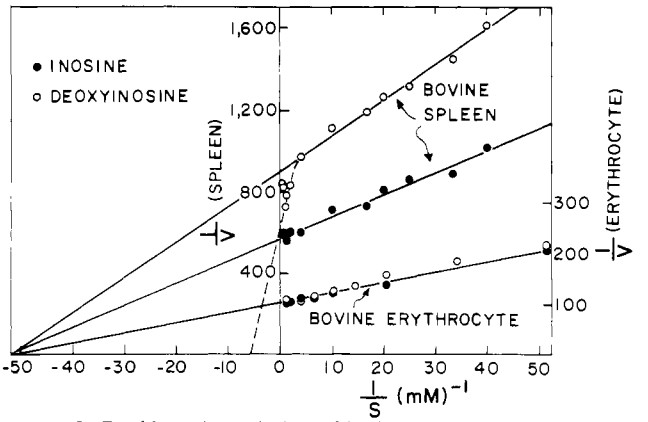

FIGURE 8: Double reciprocal plots of initial velocities and concentrations of inosine and deoxyinosine with bovine spleen and erythrocytic purine nucleoside phosphorylases. Reaction mixtures contained in 1.0 ml: Tris-acetate, 50 mM, pH 7.5; potassium phosphate, 50 mM, pH 7.5; xanthine oxidase, 0.2 unit; bovine spleen PNPase, 0.002 unit, and varying amounts of the substrates. An increase in absorbance was followed at 293 nm at 30°. In the case of the bovine erythrocytic PNPase, 100 mM potassium phosphate, pH 7.5, and 0.001 unit of the enzyme was used.

reciprocal plots of initial velocity *vs.* concentration with inosine as the varying substrate for the variants of p*I* = 6.25 and 5.85. With the p*I* = 5.85 enzyme the phenomenon of substrate activation becomes apparent at low substrate concentrations.

Substrate activation at high concentrations of inosine or deoxyinosine occurred with both electrophoretic variants of rat erythrocytic PNPase. It was surprising, however, to note that no substrate activation occurred with bovine spleen PNPase with inosine or guanosine but that activation was seen with the deoxyribonucleosides, deoxyinosine and deoxyguanosine (Figures 7 and 8). In view of earlier observations with human erythrocytic PNPase (Agarwal and Parks, 1971), in which DTNB caused partial inactivation and elimination of substrate activation by inosine, bovine spleen PNPase was similarly treated with DTNB. In contrast to the observations with the human erythrocytic enzyme the bovine spleen enzyme was totally inactivated with concomitant loss of both deoxyribonucleoside and ribonucleoside activity. To examine whether this kinetic behavior was due to a species or tissue difference, bovine erythrocytic PNPase was studied. In contrast to the spleen enzyme, bovine erythrocytic PNPase did not exhibit activation with either inosine or deoxyinosine (Figure 8). Also, the V_{max} values for inosine and deoxyinosine were similar, whereas with all other PNPases examined in this study deoxyinosine was phosphorolyzed at a lower rate (60–70%). These results indicate that PNPases vary not only from one species to the next but also among tissues from a single species.

Apparent K_m values for several substrates with PNPases from bovine spleen, bovine erythrocytes, and rat erythrocytes are presented in Table II. The K_m value for inosine (1.9×10^{-5} M) with the bovine spleen enzyme is similar to that reported earlier (Agarwal and Parks, 1969), and there is no significant difference in the K_m values for the different substrates. While the two peaks of rat erythrocytic PNPase have K_m values for hypoxanthine and guanine similar to those of the enzyme from other species, the values are 3–15 times higher for inosine and deoxyinosine; however, the very high K_m (2×10^{-2} M) for inosine reported by Oski *et al.* (1972) has not been confirmed.

TABLE II: Apparent K_m Values of Substrates for Purine Nucleoside Phosphorylases from Various Sources.

	Bovine Spleen (5.4)[a] (M × 10⁵)	Bovine Erythrocytes (M × 10⁵)	Rat Erythrocytes (5.6)[a] (M × 10⁵)	Rat Erythrocytes (5.7)[a] (M × 10⁵)
Inosine	1.9	2.1	8.3[b]	5.9[b]
Deoxyinosine	1.9[b]	2.4	20.0[b]	13.0[b]
Guanosine	2.3			
Deoxyguanosine	2.6[b]			
Hypoxanthine	2.6		1.3	1.5
Guanine	3.0		2.4	2.1

[a] Numbers in parentheses are the *pI* values. [b] The values are from the linear portion of the double reciprocal plots at low concentrations of the substrates. Plots were nonlinear at high concentrations of the substrate because of substrate activation.

Discussion

In comparison with most other mammalian cells, the erythrocyte is unique in that it is anucleate and devoid of many biochemical pathways including the synthesis of protein. Therefore, this cell is incapable of renewing its enzymes and the original enzymic complement must function throughout the life of the cell, *i.e.*, about 120 days. In most other tissues, the various enzymes have relatively constant half-lives unique for each enzyme which are the resultant of the relative rates of synthesis and degradation of the enzymes. Therefore, a special opportunity exists to examine progressive changes in protein structure as the erythrocytes age. Through the use of gradient density centrifugation techniques, Turner *et al.* (1971) have demonstrated progressive modifications in the electrophoretic pattern of human erythrocytic PNPase as the erythrocyte proceeds through various stages from the reticulocyte to the senescent cell. With aging, the electrophoretic profile shifts progressively to more acidic forms suggesting the possibility that deamination of glutamine and asparagine residues of

the enzymic proteins may occur. Of considerable interest is the observation that microheterogeneity does not occur with PNPase from human lymphocytes which have active protein synthesis. The electrophoretic characteristics of this enzyme resemble those of the predominant electrophoretic variant of the human reticulocyte (Turner *et al.*, 1971). It may be noted that the electrophoretic profile of PNPase from rat erythrocytes consists of only two peaks with p*I* values of 5.6 and 5.7 in contrast to the much more marked heterogeneity of the human erythrocytic enzyme. A possible explanation might be that the average survival time of rat erythrocytes (45–50 days; Burwell *et al.*, 1953) is much shorter than that of human erythrocytes which may result in a lesser degree of modification of the enzyme as the rat erythrocyte becomes senescent.

Although PNPases from various mammalian sources resemble each other in their substrate activities and in many of their molecular properties, such as molecular weight and subunit structure (Agarwal *et al.*, 1973; Parks and Agarwal, 1972; Edwards *et al.*, 1973), significant differences may be demonstrated by examination of parameters such as electrophoretic characteristics, response to sulfhydryl reagents, and kinetic behavior, especially with regard to the phenomenon of substrate activation. For example, on electrofocusing, the bovine spleen enzyme yields one peak of p*I* = 5.4, the rat erythrocytic enzyme two peaks of p*I* = 5.6 and 5.7, whereas the human erythrocytic enzyme yields six peaks of activity ranging from p*I* = 5.85 to 6.25. Since the crystalline PNPase was obtained from the pooled erythrocytes of more than 100 persons, it might be argued that a part of the heterogeneity may be due to genetic variance in this population. However, the low frequency of genetic variants of PNPase (Edwards *et al.*, 1971) suggests that genetic contribution to the observed heterogeneity is negligible. The phenomenon of substrate activation with both inosine and deoxyinosine was observed with all six electrophoretic variants of the human erythrocytic enzyme and the two variants of the rat erythrocytic enzyme. On the other hand, no such activation was apparent with the bovine erythrocytic enzyme. Furthermore, the kinetic parameters, *i.e.*, K_m and V_{max} values, for inosine and deoxyinosine were virtually identical with this enzyme, in contrast to other PNPases where the V_{max} value for the deoxyinosine is significantly lower than that for inosine. An unusual situation exists with the bovine spleen enzyme where no substrate activation is observed with the ribonucleosides, inosine and guanosine, while striking activation occurs at high concentrations of the deoxyribonucleosides, deoxyinosine and deoxyguanosine. Further significant differences exist in the response of different PNPases to the sulfhydryl reagent, DTNB. With the human erythrocytic enzyme DTNB causes inhibition of about 60% of the activity and eliminates the phenomenon of substrate activation. Both activity and substrate activation are restored by subsequent treatment with dithiothreitol. With the crystalline bovine spleen enzyme, however, DTNB causes a complete loss of enzymic activity with no discrimination between the substrates inosine and deoxyinosine. At present no obvious explanation of these findings is apparent.

In earlier studies it was suggested that the phenomenon of substrate activation of human erythrocytic PNPase might be due to cooperativity between the subunits or to presence in the crystalline enzyme of a mixture of isozymes with different kinetic parameters for the nucleoside substrates. Unfortunately, the present studies have not significantly clarified this issue. For example, an attractive explanation of our earlier studies, in which the sulfhydryl reagent caused about 60% decrease in the activity of human erythrocytic PNPase but a loss of substrate activation, was that this sulfhydryl reagent selectively inactivated an isozyme with a low K_m value for inosine. Although the six peaks of PNPase activity separated by isoelectric focusing displayed substrate activation to different degrees, no selective inactivation was caused by DTNB. In fact, the reaction with DTNB caused a marked alteration in the p*I* values of all isoelectric focusing variants to a much more acidic pH value (5.05) as seen in Figure 5. The possibility that this unusual response to treatment with DTNB might have been the result of dissociation of the enzyme into subunits was ruled out by the failure to observe any alteration in the molecular weight as determined by gel filtration. This interesting phenomenon is being subjected to further study. Also when greater amounts of the purified electrophoretic variants become available, the nature of the differences among them should be examined by such techniques as amino acid analysis.

References

Agarwal, K. C., Agarwal, R. P., and Parks, R. E., Jr. (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32,* 581 Abs.

Agarwal, K. C., and Parks, R. E., Jr. (1972), *Mol. Pharmacol. 8,* 128.

Agarwal, R. P., and Parks, R. E., Jr. (1969), *J. Biol. Chem. 244,* 644.

Agarwal, R. P., and Parks, R. E., Jr., (1971), *J. Biol. Chem. 246,* 3763.

Agarwal, R. P., Scholar, E. M., Agarwal, K. C., and Parks, R. E., Jr. (1971), *Biochem. Pharmacol. 20,* 1341.

Burwell, E. L., Brickley, B. A., and Finch, C. A. (1953), *Amer. J. Physiol. 172,* 718.

Edwards, Y. H., Edwards, P. A., and Hopkinson, D. A. (1973), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 32,* 235.

Edwards, Y. H., Hopkinson, D. A., and Harris, H. (1971), *Ann. Hum. Genet. 34,* 395.

Kalckar, H. M. (1947), *J. Biol. Chem. 167,* 429.

Kim, B. K., Cha, S., and Parks, R. E., Jr. (1968a), *J. Biol. Chem. 243,* 1763.

Kim, B. K., Cha, S., and Parks, R. E., Jr. (1968b), *J. Biol. Chem. 243,* 1771.

Oski, F. A., Sugarman, H. J., and Miller, L. D. (1972), *Blood 39,* 522.

Parks, R. E., Jr., and Agarwal, R. P. (1972), *Enzymes, 3rd Ed. 7,* 483.

Ross, A. F., Agarwal, K. C., Chu, S. H., and Parks, R. E., Jr. (1973), *Biochem. Pharmacol. 22,* 141.

Turner, B. M., Fisher, R. A., and Harris, H. (1971), *Eur. J. Biochem. 24,* 288.

Warburg, O., and Christian, W. (1941), *Biochem. Z. 310,* 384.