Trotta, P. P., Ahland, M. P., Brown, G. F. and Balis, M. E. (1978), *Mol. Pharmacol.* (in press).
Valentine, W. N., Tartaglia, A. P., and Gilanz, F. (1977), *Science 195,* 783.
Van der Weyden, M. B., and Kelley, W. N. (1976), *J. Biol. Chem. 251,* 5448.
Van Kampen, E. J., and Zijlstra, W. G. (1961), *Clin. Chim. Acta 6,* 538.

Purine Nucleoside Phosphorylase from Human Erythrocytes: Physicochemical Properties of the Crystalline Enzyme†

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

ABSTRACT: The major physicochemical properties of human erythrocytic purine nucleoside phosphorylase (PNPase) have been described. The molecular weight, estimated by ultracentrifugation, molecular sieving and sucrose density gradient centrifugation, ranged from 87 000 to 92 000. Other physical constants of erythrocytic PNPase were: sedimentation coefficient ($s_{20,w}$), 5.4 S obtained by sedimentation analysis and 5.5 S by the sucrose density gradient procedure; Stokes radius, 38 Å; calculated diffusion coefficient ($D_{20,w}$), 5.7×10^{-7} cm^2 s^{-1}; frictional ratio, 1.29; and partial specific volume calculated from amino acid analysis, 0.73 cm^3 g^{-1}. The CD spectra of the human erythrocytic and bovine spleen PNPases were almost identical and indicated a very low α-helical content. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate indicated that the molecular weight of the PNPase subunit is 30 000 ± 500. These results corroborate earlier reports that the native enzyme is a homologous trimer. Comparative studies with crystalline bovine spleen PNPase confirmed that it is also a trimer but is somewhat smaller than the human erythrocytic enzyme with a molecular weight of about 86 000.

The purification, crystallization, and some properties of human erythrocytic purine nucleoside phosphorylase (purine-nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1) have been reported earlier (Kim et al., 1968a,b; Sheen et al., 1968; Agarwal and Parks, 1969, 1971; Agarwal et al., 1975), including a recent review of the literature by Parks and Agarwal (1972). Substrate binding studies (Agarwal and Parks, 1969) and the electrophoretic patterns of rare allelic variants (Edwards et al., 1971) have indicated that the enzyme consists of three catalytically active subunits. A trimeric structure has been demonstrated for the PNPase[1] from bovine spleen (Edwards et al., 1973); Chinese hamster liver, kidney, and V79 tissue culture cells (Milman et al., 1976), and chicken liver (Murakami and Tsushima, 1976), with the last possessing subunits of two different sizes. Hybrids formed from human and mouse liver PNPases suggest that each of these has three electrophoretically identical subunits (Edwards et al., 1971). However, trimers are rare among enzymes (Klotz et al., 1975) and a variety of quaternary structures has been reported for PNPases from other sources. Rabbit liver PNPase is monomeric whereas bovine brain PNPase is dimeric (Lewis and Glantz, 1976a,b). The enzymes from *E. coli* and *S. typhimurium* have six subunits of equal size (Jensen and Nygaard, 1975). The present report offers further evidence for the trimeric structure of human erythrocytic PNPase, describes some of the important physicochemical properties of the enzyme, and compares them with those of the crystalline bovine spleen PNPase. A preliminary report of these studies has appeared earlier (Agarwal et al., 1973).

Materials and Methods

Materials. Peroxidase (horseradish) was purchased from P-L Biochemicals, Milwaukee, Wis., and alcohol dehydrogenase (crystalline, horse liver), creatine kinase (crystalline, rabbit muscle), and cytochrome *c* (horse heart type VI) were obtained from Sigma Chemical Co., St. Louis, Mo. Chymotrypsinogen A, ovalbumin, ribonuclease, blue dextran 2000, and Sephadex were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. Purine nucleoside phosphorylase (crystalline, bovine spleen) and lactate dehydrogenase (crystalline, beef heart) were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind., and crystalline human erythrocytic PNPase was prepared according to the method described earlier (Agarwal and Parks, 1969).

Methods. PNPases were assayed by the coupled xanthine oxidase method of Kalckar (1947) as modified by Kim et al. (1968a). Lactate dehydrogenase, alcohol dehydrogenase, and creatine kinase activities were measured spectrophotometrically by the methods of Kornberg (1955), Bonnichsen and Brink (1955), and Tanzer and Gilvarg (1959), respectively. Peroxidase activity was determined by following the rate of decomposition of hydrogen peroxide in the presence of *o*-dianisidine as hydrogen donor essentially as described in the Manual of Worthington Biochemical Corp., Freehold, N.J. (1968). The increase in color development was followed spectrophotometrically at 460 nm. Cytochrome *c* and chymotrypsinogen were determined by following absorbancy at 410 and 280 nm, respectively.

† From the Section of Biochemical Pharmacology, Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912 (J.D.S., R.P.A., K.C.A., and R.E.P.), and the Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118 (K.S.). *Received February 14, 1977; revised manuscript received September 12, 1977.* This work was supported by American Cancer Society Grant CH-7Q, National Science Foundation Grant No. GB-24813, and U.S. Public Health Service Grant No. GM-10374. This paper is the seventh in a series from this laboratory on Purine Nucleoside Phosphorylase. The preceding paper in this series is Agarwal et al. (1975).

[1] Abbreviations used: DTNB, 5,5′-dithiobis(2-nitrobenzoate); PCMB, *p*-chloromercuribenzoate; PNPase, purine nucleoside phosphorylase; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/78/0417-0278$01.00/0

A Model E Spinco ultracentrifuge equipped with Schlieren optics and a rotor temperature control unit (RITC) was employed for the sedimentation velocity runs. Centrifugation was performed at 20 °C in potassium phosphate, pH 7.0, ionic strength 0.1, at a full speed of 60 000 rpm. Sedimentation coefficients were estimated according to Schachman (1957) at concentrations of approximately 6.8 and 3.9 mg/mL of the erythrocytic and spleen enzymes, respectively. Sedimentation equilibrium experiments using interference optics were carried out as described by Chervenka (1970). The enzymes were dialyzed against 0.01 M potassium phosphate, pH 7.0, containing 0.08 M NaCl. Each run employed 0.04 mL of enzyme solution. Duplicate runs were made: erythrocytic PNPase (1.80 mg/mL) at 14 000 rpm, 20 °C, 7 h, and at 15 000 rpm, 16 °C, 21 h; spleen PNPase (1.86 mg/mL) at 14 000 rpm, 19 °C, 22 h, and at 15 000 rpm, 17 °C, 17 h.

Sucrose density gradient centrifugation was performed by the procedure of Martin and Ames (1961). A linear sucrose gradient was obtained by mixing 5 and 20% (w/v) sucrose solutions buffered with 0.05 M Tris–acetate, pH 7.5, in a Buchler density gradient mixer (Buchler Instrument Division, Fort Lee, N.J.). Either spleen or erythrocytic PNPase was added to marker proteins for which sedimentation coefficients, $s_{20,w}$, have been published: cytochrome *c*, 1.80 S (Stewart and Margoliash, 1965); peroxidase, 3.48 S (Cecil and Ogsten, 1951); creatine kinase, 5.0 S (Noda et al., 1954); alcohol dehydrogenase, 5.11 S (Ehrenberg and Dalziel, 1958); lactate dehydrogenase, 7.0 S (Markert and Appella, 1961). The enzyme mixtures were layered on 5-mL sucrose gradients and centrifuged in a Beckman SW 50 L rotor and Model L-2 ultracentrifuge. Fractions (approximately 0.15 mL) were collected with a Buchler Auto Densi-Flow apparatus.

Stokes radius, *a*, was determined by molecular sieving on Sephadex G-150 (0.9 × 50 cm) and G-100 (2.5 × 76 cm) columns and the data were expressed in terms of K_{av}, a parameter defined by Laurent and Killander (1964) as follows:

$$K_{av} = \frac{V_e - V_0}{V_t - V_0} \tag{1}$$

where V_e is the elution volume, V_0 is the void volume of the column, and V_t is the total volume of the gel bed. The K_{av} values of known proteins were plotted against their Stokes radii according to the equation:

$$(-\log K_{av})^{1/2} = \alpha(\beta + a) \tag{2}$$

where α and β are constants. The values of *a* for standard proteins were calculated from their diffusion coefficients, $D_{20,w}$ [lactate dehydrogenase, 5.10×10^{-7} (Markert and Appella, 1961); alcohol dehydrogenase, 5.95×10^{-7} (Ehrenberg, 1957); peroxidase, 7.05×10^{-7} (Cecil and Ogsten, 1951); chymotrypsinogen A, 9.5×10^{-7} (Schwert, 1951); cytochrome *c*, 12.6×10^{-7} (Stewart and Margoliash, 1965)], by the Stokes–Einstein equation (Gosting, 1956):

$$a = \frac{kT}{6\pi\eta D} \tag{3}$$

where *k* is the Boltzman constant, *T* is the absolute temperature, and η is the viscosity of the system. A temperature of 20 °C and viscosity of water at 20 °C were employed in the calculations.

The molecular weight was calculated from the sedimentation constant, free diffusion coefficient and apparent partial specific volume, $\bar{v}$, according to the following equation:

$$M = \frac{s}{D} \frac{RT}{(1 - \bar{v}\rho)} \tag{4}$$

where *R* is the gas constant and ρ is the density of the solvent, i.e., water. The apparent partial specific volume was computed from the amino acid composition, according to Cohn and Edsall (1943).

The frictional ratio (f/f_{min}) was calculated according to Tanford (1961) by means of the relation:

$$f/f_{min} = D^0_{max}/D^0 = f/f_0 \left(\frac{\bar{v}_2 + \delta_1 \bar{v}_1^0}{\bar{v}_2}\right)^{1/3} \tag{5}$$

where D^0 is the diffusion coefficient calculated from the observed Stokes radius and D^0_{max} is calculated for a hypothetical unhydrated sphere of known molecular weight by the equation:

$$D^0_{max} = kT/6\pi\eta \left(\frac{3M\bar{v}}{4\pi N}\right)^{1/3} \tag{6}$$

where *N* is Avogadro's number. In eq 5, f/f_0 and δ are the contributions of asymmetry and hydration, respectively, to the frictional ratio. These factors could not be evaluated individually by the present methods.

Polyacrylamide gel electrophoresis was performed in 10% polyacrylamide gels containing 0.01 M sodium phosphate buffer, pH 7.0, 0.1% $NaDodSO_4$, and 0.1% β-mercaptoethanol. Samples of protein markers and PNPases were prepared by incubating about 100 μg of each protein in 0.1 mL of 0.01 M phosphate buffer, pH 7.0, containing 1.0% $NaDodSO_4$ and 1.0% β-mercaptoethanol, at 37 °C for 3 h. Aliquots containing 10–20 μg of protein in a drop of glycerol with a trace of bromophenol blue as a tracking dye were applied onto the top of the gel and electrophoresed for 4–5 h at a constant current of 6 mA per gel column. After electrophoresis the protein bands were located by staining with 0.25% Coomassie brilliant blue in 7% acetic acid and 5% methanol. The apparent mobilities of the bands were calculated as described by Weber and Osborn (1969).

The amino acid compositions of crystalline erythrocytic and bovine spleen PNPases were estimated by single column analyses on a Beckman Model 120C amino acid analyzer after hydrolysis for 24, 48, and 72 h in 6 N HCl at 110 °C. The concentrations of threonine, serine, valine, and tyrosine are extrapolated values. The half-cystine concentration of bovine spleen PNPase was estimated by extrapolation to zero time. The cysteine concentration of human erythrocytic PNPase was determined earlier by sulfhydryl group titration employing DTNB and labeled PCMB (Agarwal and Parks, 1971). Tryptophan was determined by the method of Penke et al. (1974).

The circular dichroism spectra were recorded on a Cary 61 circular dichrometer at 20 °C with approximately 0.22 and 0.13 mg/mL of the human erythrocytic and bovine spleen PNPases, respectively. The protein concentrations of both enzymes were calculated by use of $E_{1cm}^{1\%} = 9.64$ at 280 nm, which was based on the nitrogen analysis of the human erythrocytic PNPase by the Kjeldahl method. The CD data were interpreted by method I of Greenfield and Fasman (1969). As a control, the spectrum of lysozyme was also recorded. Its estimated percent α helix, β structure, and random coil agreed with that reported by these authors.

Microzone electrophoretic analysis for glycoproteins by a periodate–Schiff reagent was performed by the method of Köiw and Grönwall (1952).

Results

Ultracentrifugation. Table I summarizes the main physicochemical characteristics of the human erythrocytic and bovine spleen PNPases. The sedimentation equilibrium ex-

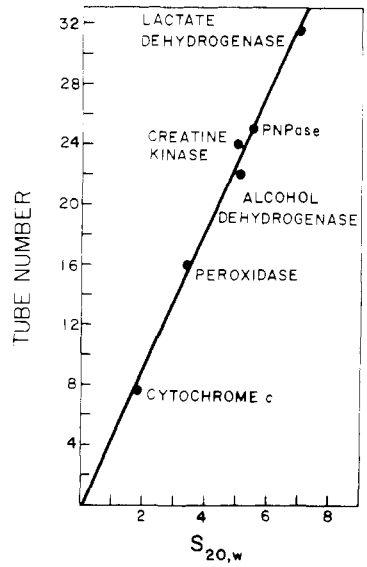

FIGURE 1: Sedimentation coefficient of human erythrocytic PNPase determined by the sucrose density gradient method. The sedimentation behavior of standard proteins and PNPase was studied in 5 to 20% (w/v) sucrose gradients in 0.05 M Tris–acetate, pH 7.5. Cytochrome *c* (250 mg), peroxidase (1.3 μg), lactate dehydrogenase (4 μg), creatine kinase (25 μg), and 30–50 μg of PNPase, dissolved in 0.1 mL of 0.05 M Tris-acetate, pH 7.5, were layered on the gradient. The samples were centrifuged at 38 000 rpm for 23 h at 3–4 °C. The sedimentation coefficients of standard proteins are those reported in the literature (see Methods).

TABLE I: Physicochemical Properties of Human Erythrocytic and Bovine Spleen Purine Nucleoside Phosphorylases.

	Human erythrocytic	Bovine spleen
Sedimentation coefficient, $s_{20,w}$ $\times 10^{-13}$ cm s^{-1}		
Sedimentation velocity in phosphate buffer	5.4	5.3
Sedimentation velocity in 4 M guanidinium chloride	1.97	
Sucrose density gradient centrifugation	5.5[a]	5.5[b]
Stokes radius (Å)	36.4, 39.5 (av = 38.0)	34.2
Diffusion coefficient, $D_{20,w}$ $\times 10^{-7}$ cm^2 s^{-1} (calcd)	5.89, 5.43 (av = 5.66)	
Appar partial spec vol, $\bar{v}_{app}$ mL g^{-1} (calcd)	0.73	0.73
Frictional ratio	1.25, 1.36 (av = 1.29)	
Axial ratio[c]	1:5	
Molecular weight		
Sedimentation equilibrium (M_w)	91 000	86 000
From s and D[d] ($M_{s,D}$)	87 000	
Subunit mol wt	30 000 ± 500	30 000 ± 500
Secondary structure estimated by circular dichroism		
% α helix	Approx. 3.2	Approx. 2.9
% β structure	Approx. 32	Approx. 32
% random coil	Approx. 65	Approx. 65

[a] Average of eight determinations, ranging from 5.4 to 5.8. [b] Average of three determinations, ranging from 5.4 to 5.5. [c] Assumes unhydrated sphere. [d] The average D value was used.

periments showed the erythrocytic PNPase to be homogeneous. M_w values of 91 000 and 92 000 were obtained and are averages across the centrifuge cell. An M_z of 95 000 was also calculated. M_w values of 86 000 and 87 000 were obtained for spleen PNPase from the upper part of the cell. Higher values were calculated closer to the bottom. M_z increased across the entire cell. Significant loss due to precipitation of aggregates was also usually seen with the spleen enzyme during dialysis.

The sedimentation velocity patterns for both PNPases exhibited single peaks. Almost the same $s_{20,w}$ values, 5.3 and 5.4, were calculated from these patterns. In the presence of 4 M guanidine hydrochloride, the erythrocytic PNPase sedimented as a single symmetrical peak with an uncorrected s value of 1.97 S. This treatment was not applied to the spleen enzyme.

Sucrose Density Gradient Centrifugation. Figure 1 presents a typical $s_{20,w}$ determination for erythrocytic PNPase. Table I lists the results from four centrifugation experiments, each of which employed one tube with spleen PNPase and two with the erythrocytic enzyme. By this method the two enzymes had the same sedimentation coefficient (5.5 S).

Determination of Stokes Radius by Gel Filtration. A Stokes radius of 39.5 Å was estimated for erythrocytic PNPase using Sephadex G-150 with peroxidase, chymotrypsinogen, and cytochrome *c* as markers. In a later series of gel filtrations, with lactate dehydrogenase, alcohol dehydrogenase, peroxidase, and cytochrome *c* as markers on Sephadex G-100, Stokes radii of 36.4 and 34.2 Å were determined for the crystalline erythrocytic and spleen enzymes, respectively. Figure 2, the composite of two gel filtrations, shows that the human PNPase eluted before and the spleen enzyme eluted after alcohol dehydrogenase. A sample of human PNPase partially purified from freshly drawn blood behaved identically in a comparable gel filtration study. Also, replacement of the Tris–acetate elution buffer by 0.1 M potassium phosphate, pH 7.5, containing 1.0 mM dithiothreitol, did not alter the rate of elution of erythrocytic PNPase.

The diffusion coefficients calculated for erythrocytic PNPase by eq 3 are 5.89×10^{-7} and 5.43×10^{-7} cm^2 s^{-1} for a = 36.4 and 39.5 Å, respectively. These $D_{20,w}$ values, in conjunction with $s_{20,w}$ = 5.5 from sucrose density gradient centrifugation, yield molecular weights of 83 400 and 91 000, respectively. The average of the two diffusion coefficient values, $D_{20,w} = 5.66 \times 10^{-7}$ cm^2 s^{-1}, which gives a molecular weight of 87 000, was used to determine the frictional ratio. The molecular weight corresponding to the higher Stokes radius coincides with the value determined by equilibrium centrifugation.

Polyacrylamide Disc Gel Electrophoresis. The electrophoretic patterns of marker proteins and human erythrocytic and bovine spleen PNPases in the presence of $NaDodSO_4$ showed no evidence of subunit heterogeneity. From plots of the migration of known proteins vs. their molecular weights (Figure 3), the average molecular weights of the PNPase subunits were estimated to be 30 000 ± 500 (average of ten experiments).

Amino Acid Analysis. Molar ratios of the amino acids were calculated by assuming three identical subunits (Table II). Since histidine is stable to acid hydrolysis and is one of the amino acids occurring in lowest concentration in both enzymes, this amino acid was selected for estimation of the molar ratios of the other residues. For both enzymes, seven histidine residues per subunit yielded molecular weights compatible with those obtained from physical measurements.

In our hands, hydrolysis of lysozyme by mercaptoethanesulfonic acid (Penke et al., 1974) yielded incomplete re-

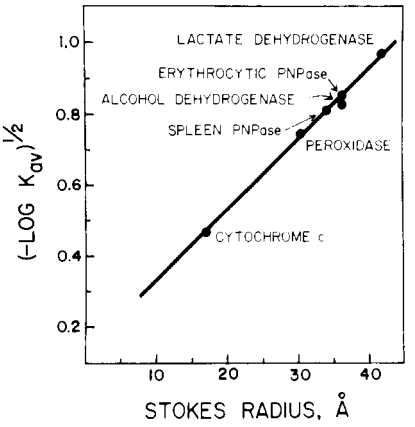

FIGURE 2: Stokes radius of human erythrocytic PNPase determined by gel filtration. Peroxidase (20 μg), cytochrome *c* (3 mg), lactate dehydrogenase (125 μg), alcohol dehydrogenase (3 mg), and erythrocytic (or spleen) PNPase (about 4 units), in 0.5 mL of 0.1 M Tris–acetate, pH 7.5, containing 0.1 M KCl, were chromatographed on a Sephadex G-100 column, at about 8 °C. Aliquots of about 1.0 mL per tube were collected. The void volume of the column was estimated using blue dextran 2000. The peaks of the standard proteins and their elution constant (K_{av}) were determined as described in Methods.

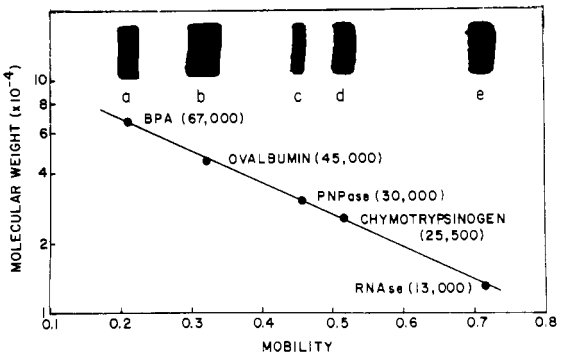

FIGURE 3: Plot of molecular weight and electrophoretic mobility of PNPases and marker proteins. Conditions of electrophoresis are described in Methods. Figures in parentheses show the molecular weight of each protein or its subunits. Inset is a photograph of a polyacrylamide gel enlarged to show the protein bands corresponding to the points on the graph: (a) bovine plasma albumin (BPA); (b) ovalbumin; (c) PNPase; (d) chymotrypsinogen; (e) ribonuclease (RNase). Erythrocytic and spleen PNPases gave identical results.

coveries of tryptophan and other amino acids, excessively high values for proline and total loss of cysteine. The tryptophan content of the PNPases was estimated on the basis of percentage recovery of this amino acid with the lysozyme standard.

Circular Dichroism. Almost identical curves were obtained for the human erythrocytic and bovine spleen enzymes as shown in Figure 4. The α helix content and β structure were estimated to be approximately 3% and 32%, respectively, leaving about 65% random coil.

Microzone Electrophoresis. Analysis for glycoproteins of as much as 70 μg of purified erythrocytic PNPase gave a negative result whereas 15 to 45 μg of a serum protein standard were readily detected.

Discussion

A molecular weight for human erythrocytic PNPase of 87 000 to 92 000 is consistent with the physicochemical parameters presented in Table I. This differs somewhat from the values of 80 000 (Kim et al., 1969a) and 82 470 ± 7700 (Edwards et al., 1971) reported earlier on the basis of the molecular sieving method of Andrews (1964). These earlier estimates, obtained by plotting the elution volumes of marker proteins vs. the logarithms of their molecular weights, assumed the same shape, degree of hydration and partial specific volume for all the proteins used. It has been shown that a better correlation exists between elution volume and the Stokes radius of a given molecule (Laurent and Killander, 1964; Siegel and Monty, 1966). The average Stokes radius of 38 Å and the sedimentation coefficient of 5.5 S obtained from sucrose density gradient centrifugation permitted the calculation of a molecular weight of 87 000 that agrees well with that derived from sedimentation equilibrium.

Based on a Stokes radius of 38 Å, the frictional ratio (f/f_{min}) of erythrocytic PNPase is 1.29. If one were to assume that PNPase is a nonhydrated prolate ellipsoid, this frictional ratio would indicate an axial ratio of about 5:1, an unlikely value for a homologous trimeric protein. It seems probable that the deviation of the frictional ratio from unity is mainly due to hydration. Additional studies, such as x-ray crystallography, are required to clarify this question.

TABLE II: Amino Acid Analyses of Crystalline PNPases from Human Erythrocytes and Bovine Spleen.

Amino acid	Residue wt %		Molar ratio to nearest integer	
	Human	Bovine	Human	Bovine
Trp	1.9	1.9	9	9
Lys	4.8	5.0	33	36
His	3.3	3.3	21	21
Arg	8.1	7.7	45	42
Asp	9.7	9.6	75	72
Thr	5.3	4.9	48	42
Ser	3.7	4.6	39	48
Glu	13.6	15.4	93	105
Pro	4.2	4.0	39	36
Gly	5.3	5.2	81	78
Ala	5.6	5.1	69	63
Cys	1.4	1.5	12	12
Val	6.9	7.1	63	63
Met	3.6	2.8	24	18
Ile	3.0	2.8	24	21
Leu	8.9	8.2	69	63
Tyr	4.3	4.9	24	27
Phe	6.4	5.8	39	36
Totals	100.0	99.8	807	792

The PNPase from spores of *Bacillus cereus* showed an increase in Stokes radius from 39 to 57 Å that was attributed to an increase in phosphate concentration from 1 to 50 mM in the presence of 50 mM Tris (Englebrecht and Sadoff, 1969). It is not clear whether this effect was due to the phosphate or to increasing ionic strength (from 0.05 to 0.15). No difference was found in the Stokes radius of erythrocytic PNPase in 0.1 M Tris–acetate containing 0.1 M NaCl or in 0.1 M potassium phosphate, with both buffers at pH 7.5.

The CD spectra of both PNPases suggest a very low percentage of α helix and about 65% random coil. It has been shown by Greenfield and Fasman (1969) that good agreement exists in the secondary structures of certain proteins deduced from CD spectra and from x-ray data, whereas a poor correlation is seen for proteins with a large amount of random coil. Since the CD spectra of both PNPases suggest a very low α helical and high (about 65%) random coil content, the present results must be regarded as approximations of the conformations of the PNPases.

Differences are seen in the amino acid compositions reported

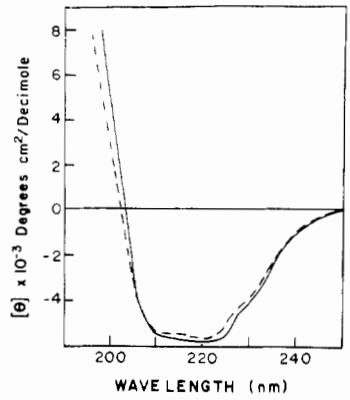

FIGURE 4: Circular dichroism spectra of human erythrocytic (—) and bovine spleen (- - - -) PNPase in potassium phosphate, pH 7.0, ionic strength 0.1 at 20 °C.

earlier for spleen PNPase (Edwards et al., 1973) and erythrocytic PNPase (Parks and Agarwal, 1972) and presented here. These discrepancies may be due to differences in the enzyme preparations and the use of multiple hydrolysis times for the spleen enzyme in the present study. In addition, the molar ratios for spleen PNPase (Edwards et al., 1973) appear to have been based on calculations employing the amino acid molecular weights rather than residue weights, which results in errors ranging from 10 to 25%.

Bovine spleen PNPase was reported by Edwards et al. (1973) to have a subunit size of 28 000 and molecular weight of about 84 000 by $NaDodSO_4$-polyacrylamide gel electrophoresis and gel filtration. The Stokes radius of 34.2 Å (Table I) is probably too low since it leads to a molecular weight of about 79 000, whereas the subunit weight was found to be about 30 000 and a molecular weight of 86 000 was obtained by equilibrium centrifugation. Therefore, $D_{20,w}$ and $M_{s,D}$ for spleen PNPase are not listed in Table I. On the basis of the very similar sedimentation coefficients but distinctly different elution patterns with respect to alcohol dehydrogenase during gel filtration, it may be concluded that the bovine spleen enzyme is similar to but smaller than human erythrocytic PNPase (see Figure 2). The dissimilarities in the amino acid compositions (Table II), isozyme patterns, certain kinetic properties (Agarwal et al., 1975), and the reaction of sulfhydryl groups with DTNB (unpublished results) further attest to the differences between the human erythrocytic and bovine spleen PNPases.

All evidence to date indicates that human erythrocytic PNPase is a homologous trimer. Early studies, in which 3 mol of hypoxanthine bound per mole of enzyme, together with more recent evidence, suggest that each subunit is identical and has a purine binding site (Agarwal and Parks, 1969). Electrophoretic studies of PNPases from individuals heterozygous for a relatively rare allelic variant yielded further evidence for the trimeric structure of the enzyme (Edwards et al., 1971). Our studies on the cross-linking of erythrocytic PNPase with suberimidate have given results consistent with a trimeric structure.[2] The results of gel electrophoresis and equilibrium centrifugation described above and elsewhere (Agarwal et al., 1973) offer definitive evidence of the trimeric structure of human erythrocytic PNPase. Studies with crystalline bovine spleen PNPase described above confirm the report of Edwards et al. (1973) that this enzyme is a trimer. Since the occurrence of trimeric enzymes is rare, originally it was tempting to speculate that this unusual structure was somehow related to the catalytic behavior of the enzyme and might play a role in the phenomenon of substrate activation observed with human erythrocytic and bovine spleen PNPases (Kim et al., 1968a; Agarwal et al., 1975). A complication, however, is that crystalline erythrocytic PNPase has multiple isoelectric variants (p*I* values from 5.8 to 6.3), each showing the phenomenon of substrate activation to a different degree (Edwards et al., 1971; Turner et al., 1971; Parks and Agarwal, 1972; Agarwal et al., 1973, 1975). Another significant finding is that substrate activation can be abolished by titration with DTNB and restored by treatment with dithiothreitol (Agarwal and Parks, 1971). When the first two PNPases examined proved to be trimers, it was suspected that this might be a common feature of all enzymes of this class. More recently, however, a monomeric PNPase from rabbit liver (mol wt 46 000) and a dimeric PNPase from rabbit brain (subunit mol wt 38 000) have been described (Lewis and Glantz, 1976a,b). Furthermore, a heterologous trimeric PNPase (mol wt 90 000) has been isolated from chicken liver (Murakami and Tsushima, 1976). Therefore, the significance of the trimeric quaternary structure of certain PNPases awaits clarification.

To date, limited amounts of the crystalline enzyme have been available. It should be noted, however, that human erythrocytes possess remarkably high activities of PNPase, i.e., about 15 units/mL of packed cells (Parks and Agarwal, 1972) and methods developed for the large scale isolation (Agarwal et al., 1971) have provided more than 50 000 units of the enzyme at 1–5% homogeneity (equivalent to 500 mg of crystalline PNPase). As soon as large quantities of the pure enzyme are made available by more efficient methods such as affinity chromatography (currently under development in our laboratory), further characterization such as x-ray crystallography and amino acid sequence determination will be possible.

Several considerations make human erythrocytic PNPase an important subject for further study. (1) Lack of PNPase associated with a defect in T-lymphocyte function (Giblett et al., 1975) suggests that the enzyme plays a previously unsuspected role in immune mechanisms and may offer an attractive target site for the design of specific immunosuppressive agents. (2) It is one of the few readily available enzymes that is trimeric in structure. (3) Previous studies (Agarwal and Parks, 1971) have shown that it may be possible to selectively label either the thiols associated with catalytic activity or those involved in the phenomenon of substrate activation. The last feature offers a special opportunity for the application of physical techniques such as x-ray crystallography.

Acknowledgments

We are indebted to Dr. C. H. Chervenka of the Spinco Division of Beckman Instruments, Inc., Palo Alto, Calif., for the molecular weight determinations by equilibrium ultracentrifugation and to Mr. Robert Hyde for performing the sedimentation velocity runs at the Woods Hole Marine Biological Laboratories. We also wish to thank Drs. David Yphantis and K. E. VanHolde for their helpful advice. We also thank Mr. Leon Johnston for conducting the single-column amino acid analysis, Mr. William Henry for the microzone electrophoresis, and Mr. Dan Reitenbach for operating the circular dichrometer.

[2] Cross-linking of erythrocytic PNPase with suberimidate has yielded monomers, dimers, and trimers. In addition, faster moving bands appear in each region and are related to the extent of reaction with suberimidate. Spleen PNPase showed no cross-linking with suberimidate. These results will be reported in greater detail elsewhere.

References

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.

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

Agarwal, K. C., Agarwal, R. P., Stoeckler, J. D., and Parks, R. E., Jr. (1975), *Biochemistry 14,* 79.

Andrews, P. (1964), *Biochem. J. 91,* 222.

Bonnichsen, R. K., and Brink, N. G. (1955), *Methods Enzymol. 1,* 495.

Cecil, R., and Ogston, A. G. (1951), *Biochem. J. 49,* 105.

Chervenka, C. H. (1970), *Anal. Biochem. 34,* 24.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids, and Peptides, New York, N.Y., Reinhold Publishing Co., p 370.

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

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

Ehrenberg, A. (1957), *Acta Chem. Scand. 11,* 1257.

Ehrenberg, A., and Dalziel, K. (1958), *Acta Chem. Scand. 12,* 465.

Engelbrecht, H. L., and Sadoff, H. L. (1969), *J. Biol. Chem. 244,* 6228.

Giblett, E. R., Ammann, A. J., Sandman, R., Wara, D. W., and Diamond, L. K. (1975), *Lancet 1,* 1010.

Gosting, L. J. (1956), *Adv. Protein Chem. 11,* 449.

Greenfield, N., and Fasman, G. D. (1969), *Biochemistry 8,* 4108.

Jensen, K. F., and Nygaard, P. (1975), *Eur. J. Biochem. 51,* 253.

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.

Klotz, I. M., Darnall, D. W., and Langerman, N. R. (1975), in The Proteins, Vol. I, Neurath, H., and Hill, R. L., Ed., New York, N.Y., Academic Press, p. 293.

Köiw, E., and Grönwall, A. J. (1952), *Scand. J. Clin. Lab. Invest. 4,* 244.

Kornberg, A. (1955), *Methods Enzymol. 1,* 441.

Laurent, T. C., and Killander, J. (1964), *J. Chromatogr. 14,* 317.

Lewis, A. S., and Glantz, M. D. (1976a), *J. Biol. Chem. 251,* 407.

Lewis, A. S., and Glantz, M. D. (1976b), *Biochemistry 15,* 4451.

Markert, C. L., and Appella, E. (1961), *Ann. N.Y. Acad. Sci. 94,* 678.

Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236,* 1372.

Meuwissen, H. J., Pickering, R. J., Pollara, B., and Porter, I. H., Ed. (1975), Combined Immunodeficiency Disease and Adenosine Deaminase Deficiency, New York, N.Y., Academic Press.

Milman, G., Anton, D. L., and Weber, J. L. (1976), *Biochemistry 15,* 4967.

Murakami, K., and Tsushima, K. (1976), *Biochim. Biophys. Acta 453,* 205.

Noda, F., Kuby, S. A., and Lardy, H. A. (1954), *J. Biol. Chem. 209,* 203.

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

Penke, B., Ferenczi, R., and Kovacs, K. (1974), *Anal. Biochem. 60,* 45.

Schachman, H. K. (1957), *Methods Enzymol. 4,* 32.

Schwert, G. W. (1951), *J. Biol. Chem. 190,* 799.

Sheen, M. R., Kim, B. K., and Parks, R. E., Jr. (1968), *Mol. Pharmacol. 4, 293.*

Siegel, L. M., and Monty, K. J. (1966), *Biochim. Biophys. Acta 112,* 346.

Stewart, J. W., and Margoliash, E. (1965), *Can. J. Biochem. 43,* 1187.

Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N.Y., Wiley, p 356.

Tanzer, M. L., and Gilvarg, C. (1959), *J. Biol. Chem. 234,* 3201.

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

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.