PURINE NUCLEOSIDE PHOSPHORYLASE: ISOLATION AND CHARACTERIZATION

Katarina ŠEDIVÁ, Ivan VOTRUBA, Antonín HOLÝ and Ivan ROSENBERG
Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Science, 166 10 Prague 6

Received March 16, 1990
Accepted May 31, 1990

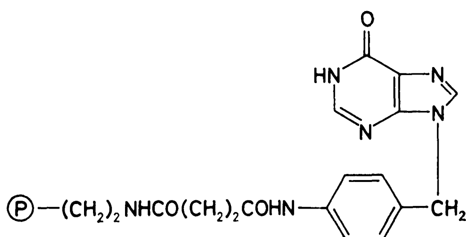
Purine nucleoside phosphorylase (PNP) from mouse leukemia cells L1210 was purified to homogeneity by a combination of ion exchange and affinity chromatography using AE-Sepharose 4B and 9-(*p*-succinylaminobenzyl)hypoxanthine as the matrix and the ligand, respectively. The native enzyme has a molecular weight of 104 000 and consists of three subunits of equal molecular weight of 34 000. The results of isoelectric focusing showed that the enzyme is considerably microheterogeneous over the pI-range 4·0—5·8 and most likely consists of eight isozymes. The temperature and pH-optimum of phosphorolysis, purine nucleoside synthesis and also of transribosylation is identical, namely 55°C and pH 7·4. The transribosylation reaction proceeds in the presence of phosphate only. The following K_m-values (μmol l^{-1}) were determined for phosphorolysis: inosine 40, 2′-deoxyinosine 47, guanosine 27, 2′-deoxyguanosine 32. The K_m-values (μmol l^{-1}) of purine riboside and deoxyriboside synthesis are lower than the values for phosphorolysis (hypoxanthine 18 and 34, resp., guanine 8 and 11, resp.). An affinity lower by one order shows PNP for α-D-ribose-1-phosphate, α-D-2-deoxyribose-1-phosphate (K_m = 200 μmol . . l^{-1} in both cases) and phosphate (K_m = 805 μmol l^{-1}). The substrate specificity of the enzyme was also studied: positions N(1), C(2) and C(8) are decisive for the binding of the substrate (purine nucleoside).

Purine nucleoside phosphorylase (purine nucleoside orthophosphate ribosyltransferase, PNP, EC 2.4.2.1) catalyzes the reversible degradation[1] of purine ribo- and 2′-deoxyribonucleosides, to the purine base hypoxanthine and guanine, and α-D- -ribose- and 2-deoxyribose-1-phosphate, resp. The enzyme has received increased interest after the discovery by Giblett and coworkers[2] of the relationship between purine metabolism and inherited disorders of the immune system. Unlike in adenosine deaminase deficiency patients with PNP deficiency suffer from severely and selectively impaired function of the T-cells yet the humoral immunity is less affected[2–4]. These findings have stimulated the research on new and efficient purine nucleoside analogs, PNP inhibitors, which could serve as potential drugs in the treatment of T-cell type leukemias and in the therapy of autoimmune disorders[5–8]. Since L12010 cells (tumor cells of the lymphoid type) represent a generally recognized model for studies on cytostatic and cytotoxic effects of newly synthesized drugs[9] and since the properties of PNP from this neoplastic tissue have not been described so

far, we decided to isolate the enzyme and determine its characteristics. The characteristics are known of this enzyme isolated so far from erythocytes[35,36], granulocytes[15], liver[25,37,38], spleen[39,40] and kidney[25].

RESULTS AND DISCUSSION

The washed cells were suspended in 50 mM phosphate buffer, pH 7·4 (according to Sørensen), containing 2 mM dithiothreitol (DTT) and disintegrated by sonication. The sonicate was centrifuged off at 30 000 ***g*** and the supernatant was again centrifuged at 100 000 ***g***. Nucleic acids were then precipitated in the supernatant by the addition of 15% streptomycin sulfate. The supernatant was then treated with ammonium sulfate to 35–65% saturation and the precipitated proteins amounting to 41% of the total enzyme activity were isolated. The protein precipitate was dissolved and desalted by passage through a Sephadex G-25 column and transferred to 50 mM Tris–HCl buffer, pH 7·6, containing 2 mM DTT. In the subsequent chromatography on DEAE-Sephadex A-50 PNP was resolved from a considerable excess of adenosine aminohydrolase (EC 3.5.4.4). The separation of the two enzymes was achieved in a medium of increasing ionic strength of KCl; ADA was separated from PNP to 80–90% (Fig. 1). The effluent was a very suitable starting material for the final purification step on the affinity column of AE-Sepharose 4B with 9-(*p*-succinylaminobenzyl)hypoxanthine *I* as ligand (Fig. 2). The final product was immediately purified 1 712 times to a specific activity of 1 543 nkat mg^{-1} in terms of inosine phosphorolysis and of 196 nkat mg^{-1} in terms of inosine synthesis. The yield of the isolation procedure was 20% (Table I).



I

P Sepharose 4B matrix

The purity of the enzyme isolated was checked by electrophoresis in 10% polyacrylamide gel (PAGE) (Fig. 3) and in a gradient gel (4–20% polyacrylamide) (Fig. 4). The enzyme was detected as a single band in both cases and its identity was proved by the enzyme activity assay after the gel slab had been cut to slices. We observed that native PNP of L1210 has a relative molecular weight of 104 200 ± 4 300.

TABLE I

Isolation of PNP from mouse leukemia cells L1210

Isolation step	Volume ml	Protein content mg	Total activity[a] nkat	Specific activity[a] nkat mg^{-1}	Yield %	Purification degree
Crude extract	69	1 099	989·3	0·90	100	—
100 000 *g* supernatant	55	501	885·3	1·77	89·4	2·0
Precipitation by 15% streptomycin sulfate	57	501	668·1	1·33	67·5	—
35—65% Ammonium sulfate[b]	21·8	198	409·3	2·07	41·3	2·3
DEAE-Sephadex A-50	45	47	391·7	8·33	39·5	9·3
Affinity chromatography	88·2	0·13	200·7	1 543·64	20·2	1 712·0

[a] The enzyme activity was determined in terms of inosine phosphorolysis; [b] value subject to error due to the presence of sulfate ions.

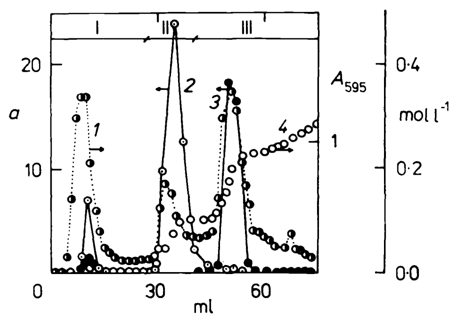


FIG. 1

Separation of ADA and PNP on DEAE-Sephadex A 50. *1* Absorbance at 595 nm, *2* activity of adenosine deaminase, *3* phosphorolytic activity of PNP, *4* KCl concentration. Elution: I 50 mM Tris–HCl, pH 7·6, containing 2 mM DTT; II 50 mM Tris–HCl, pH 7·6, containing 0·1M-KCl and 2 mM DTT; III concentration of KCl in 50 mM Tris–HCl, pH 7·6, containing 2 mM DTT. *a* Activity of PNP and ADA in nkat ml^{-1}, mol l^{-1} KCl concentration, ml elution volume

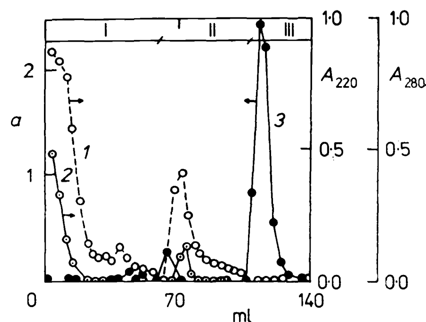


FIG. 2

Affinity chromatography of PNP. *1* Absorbance at 220 nm, 2 absorbance at 280 nm, *3* phosphorolytic activity of PNP. Elution: I 50 mM Tris–HCl, pH 7·6, containing 2 mM DTT; II 0·1M Tris–HCl, pH 8·0, containing 0·5M-NaCl and 2 mM DTT; III 50 mM phosphate buffer, pH 7·5, containing 2 mM inosine and 2 mM DTT. *a* Activity of PNP in nkat . . ml^{-1}, ml elution volume

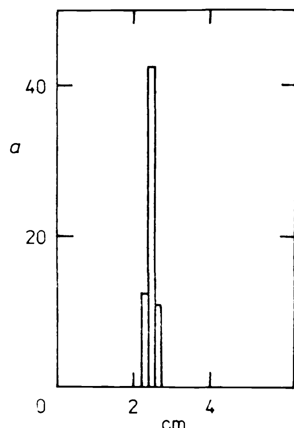


FIG. 3

PAGE of native PNP in 10% gel. cm Mobility, a activity of PNP in pmol min^{-1} of hypoxanthine. The phosphorolytic activity of PNP was determined in 2-mm gel slices immersed in reaction mixture F_2 (cf. Experimental)

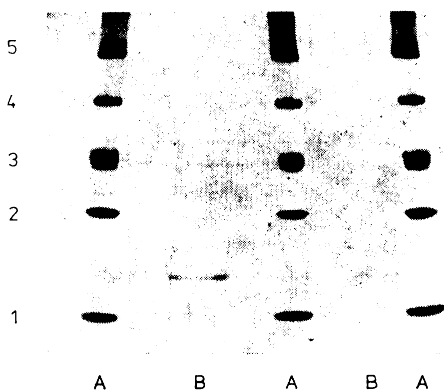


FIG. 4

Determination of relative molecular weight of PNP (4–20% PAGE). A HMW Electrophoresis Calibration Kit: 1 bovine serum albumin (67 000), 2 lactate dehydrogenase (140 000), 3 catalase (232 000), 4 ferritin (440 000), 5 thyreoglobulin (669 000); B and B′ purine nucleoside phosphorylase (50 ng and 20 ng)

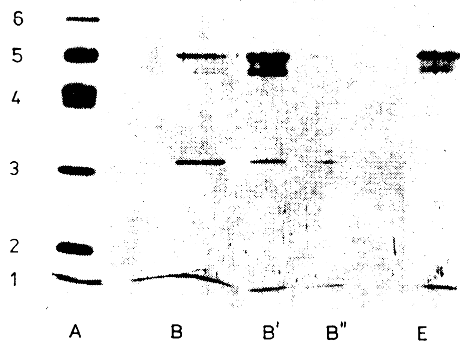


FIG. 5

Determination of relative molecular weight of PNP subunit (12·5% SDS–PAGE). A LMW Electrophoresis Calibration Kit: 1 α-lactalbumin (14 000), 2 soybean trypsin inhibitor (20 000), 3 carbonic anhydrase (33 000), 4 ovalbumin (43 000), 5 albumin, (67 000), 6 phosphorylase b (94 000); B, B′ and B″ purine nucleoside phosphorylase (120 ng, 50 ng and 20 ng); E control sample (the protein fraction was replaced by the buffer during the procedure). The visible bands are due to impurities present as contaminants in the reagents[34]

The relative molecular weight of the enzyme subunit was determined by SDS-PAGE in 12·5% gel (Fig. 5). A comparison of the gradient PAGE pattern (Fig. 4) with the pattern of SDS-PAGE (Fig. 5) showed that the native enzyme consists of three identical subunits of a relative molecular weight of 34 000 ± 970. In contrast, the enzyme isolated showed a considerable microheterogeneity when examined by isoelectric focusing. We demonstrated by this method that PNP is most likely composed of eight isozymes in the pI-range 4·1 – 5·8 (4·00; 4·35; 4·80; 4·95; 5·10; 5·25; 5·40; 5·80). Interest deserves the fact that the distribution of isozymes showing the highest enzymatic activity (pI 4·35 and 4·90, designated 13 and 14 – 15, resp.) in the PNP isolate is the lowest (Fig. 6). A number of papers which have appeared so far contain information on several PNP isozymes purified both from animal and human tissues[10–14]. Likewise Mably and coworkers[15] observed that different tissues of the same mouse strain may contain 1 – 4 isozymes differing in pI. The existence of several isozymes may be expected also in view of the fact that the enzyme was isolated from ascites of different individuals and also because it has been known[16] that mouse PNP is encoded by two alleles. The possibility of posttranscriptional or posttranslational modification[17,18], however, cannot be excluded. Wiginton and co-

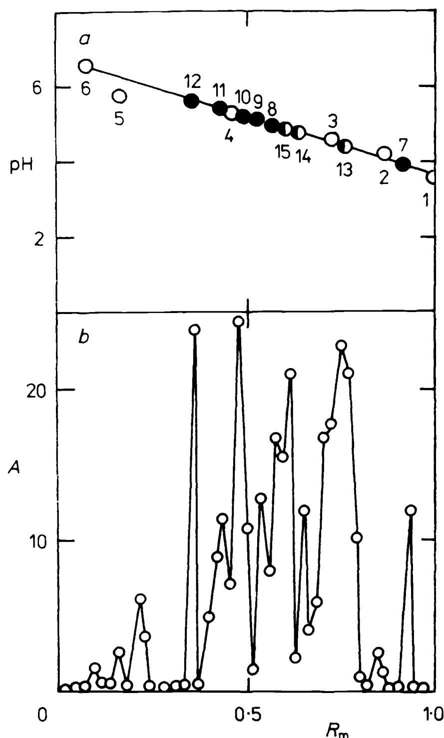


FIG. 6

Isoelectric focusing of PNP (5% gel). *a* Low pI Calibration Kit pH 2·5—6·5; **1** amyloglycosidase (3·50), **2** glucose oxidase (4·15), **3** soybean trypsin inhibitor (4·55), **4** α-lactalbumin (5·20), **5** bovine carbonic anhydrase B (5·85), **6** human carbonic anhydrase B (6·55), **7—15** PNP isozymes; after protein staining (**7—12**) and enzyme activity determination (**13—15**); *b* enzyme activity of PNP determined by inosine phosphorolysis. *A* PNP activity expressed as quantity of hypoxanthine in pmol min^{-1}; R_m relative mobility

workers[19] were able to obtain from a similar source of human leukemic granulocytes a PNP isolate containing three isozymes of pI 6·1, 5·9, and 5·8.

Our knowledge of the properties of PNP represents two groups of data on *a*) the activity of the enzyme at phosphorolysis and reverse reaction, i.e. synthesis of nucleosides from the purine base and α-D-ribose-1-phosphate, and *b*) on the trans-ribosylation reaction, i.e. on D-ribose transfer from a nucleoside to a free base.

Phosphorolysis and Synthesis of Purine Nucleosides

The Michaelis constants K_m and V_{max} were determined from the plot of initial rate of the enzyme reaction versus substrate concentration (Table II). As shown in the table it may be generally stated that the affinity of PNP from L1210 cells for the substrate decreases in the series guanosine > 2′-deoxyguanosine > inosine > 2′-deoxyinosine, both in phosphorolysis and synthesis. The maximal rate of synthesis of guanine nucleosides is comparable to the rate of synthesis of hypoxanthine nucleosides, unlike with phosphorolysis where the rate decreases in the series inosine > guanosine > 2′-deoxyguanosine > 2′-deoxyinosine. It is also apparent from Table II that the ratio of maximal rates of phosphorolysis and synthesis of inosine

TABLE II
Kinetic characteristics of PNP

Substrate	Phosphorolysis		Synthesis	
	K_m μmol l^{-1}	V_{max} μmol l^{-1} mg^{-1}	K_m μmol l^{-1}	V_{max} μmol l^{-1} mg^{-1}
Inosine	40	2·26		
Inosine[a]	40	1·20		
2′-Deoxyinosine	47	1·25		
Guanosine	27	1·63		
2′-Deoxyguanosine	32	1·60		
Hypoxanthine[b]	—	—	18	0·39
Hypoxanthine[c]	—	—	34	0·38
Guanine[d]	—	—	8	0·41
Guanine[e]	—	—	11	0·48
α-D-Ribose-1-phosphate	—	—	200	0·44
α-D-2-Deoxyribose-1-phosphate	—	—	200	0·39
Inorganic phosphate	805	1·26	—	—

[a] Determined at 1 mM phosphate concentration in the reaction mixture; [b] measured in terms of inosine synthesis; [c] measured in terms of 2′-deoxyinosine synthesis; [d] measured in terms of guanosine synthesis; [e] measured in terms of 2′-deoxyguanosine synthesis.

and guanosine is 5·79 and 3·79, resp., and that under the in vitro conditions phosphorolysis is faster than synthesis.

As shown by the results listed in Table III the substrate specificity of PNP isolated from L1210 cells does not differ from that of the other enzymes[1,17,20,21] isolated so far from eukaryotes and cannot cleave either adenosine or 2′-deoxyadenosine by phosphorolysis. Likewise 8-dimethylaminoguanosine, 8-methylthioguanosine, 8--hydroxyguanosine, 8-chloroguanosine, 8-bromoinosine, 8-chloroinosine, 7-deazainosine, 2-aminopurine riboside, 6-hydroxylaminopurine 9-β-D-ribofuranoside, inosine N^1-oxide, uric acid N^3-riboside, 6-hydrazino-2-aminopurine 9-β-D-ribofuranoside, 2,6-diaminopurine 9-β-D-ribofuranoside, and 8-bromoxanthosine do not undergo phosphorolytic cleavage. Several general conclusions can be drawn from the data obtained. Since the modification of N(1) of the purine ring of the nucleoside results in the loss of ability of the latter to act as a PNP substrate, we may assume that this position plays most likely an important role in the binding of the substrate to the enzyme, as documented also by recorded data[21–23]. We have also observed that the introduction of a bulky group into position C(6) of the purine ring results in the loss of substrate activity. In contrast, simultaneous modification of position C(6) and C(2) (such as in, e.g. 6-thioguanosine or isoguanosine) leads to "weaker" substrates compared to natural nucleosides. For this reason and also in view of the fact that we observed a similar increase in the affinity of purine inhibitors for PNP after introduction of the NH_2-group into position C(2) of the purine ring[24], it may be

TABLE III

Phosphorolysis of modified nucleoside substrates of PNP. ND not measured, v_N rate of nucleoside phosphorolysis, v_{Ino} rate of inosine phosphorolysis

Compound	v_N/v_{Ino}	
	in 20 min	in 24 h
Inosine	1	1
Guanosine	0·93	1
8-Mercaptoguanosine	0·18	ND
8-Methylguanosine	0·09	1
8-Aminoguanosine	0·52	ND
Isoguanosine	0	0·12
6-Thioguanosine	0·16	ND
8-Azaguanosine	0	0·13
8-Mercaptoinosine	0·28	ND
8-Aminoinosine	0·37	ND
8-Mercaptoxanthosine	0·34	ND
Xanthosine	0·23	ND

concluded that this position is important for the binding of the purine base of the nucleoside. It follows from this consideration and also from the data listed in Table III that the rate of phosphorolysis of nucleosides modified at the position C(8) decreases in the series: $NH_2 > SH > CH_3 \gg OH$ and that it is on the whole lower than the rate of phosphorolysis of natural substrates. Nucleosides with a bulky group (such as, e.g. dimethylamino-, -Cl, -Br) in position C(8) do not undergo, however, phosphorolytic cleavage by PNP from L1210 cells.

Transribosylation

The transribosylation reaction was studied in detail by the estimation of ribose transfer from inosine to guanine in a reaction mixture not containing exogenously added phosphate. The course of the reaction was evaluated by the detection of the synthesized guanosine and hypoxanthine simultaneously formed by phosphorolysis (Fig. 7). It follows from the data obtained that PNP from L1210 cells catalyzes the above transribosylation reaction yet that the specific activity of the enzyme without the addition of exogenous phosphate represents merely 0·4 to 1·2% of the specific activity of inosine phosphorolysis. As documented in Figs 8 and 7 the reaction primarily in its early stages depends on the concentration of exogenously supplied phosphate or on the concentration of phosphate endogenously bound to the protein molecule (the reaction mixture contained in the latter case only Tris–HCl buffer and the enzyme which had been transferred to the same buffer after affinity chromatography). Since the value of the pH-optimum (7·4) and of the temperature optimum (55°C) are identical to the data on the phosphorolytic activity of PNP and since the reaction depends on the presence of phosphate, it is very likely that the first step in the transribosylation reaction is the phosphorolytic cleavage of inosine to α-D-ribose-1-phosphate as an intermediate; the latter is subsequently transferred to guanine. This assumption is in accordance with the finding that after an increase of guanine concentration above a value of 4 $\mu mol\ l^{-1}$ the transribosylation reaction cannot be detected. A most likely explanation of this phenomenon is competitive inhibition of inosine phosphorolysis by guanine which has a higher affinity for PNP than hypoxanthine formed (Table II). Our finding is in accordance with the data of Ananiev and coworkers[25] that the transribosylation reaction proceeds in the presence of phosphate only.

We also studied the temperature and pH-optima of PNP as regards the phosphorolytic, synthetic, and transribosylation reaction. These values are identical for all these reactions, i.e. 55°C and 7·4. Purified PNP from L1210 cells is stable during preincubation in phosphate buffer, pH 7·4, containing DTT and bovine serum albumin over the temperature range 0–45°C for 3 h. A subsequent temperature increase to 50–60°C leads to a loss of enzyme activity after 30 min already. The value depends on the concentration of phosphate buffer in the preincubation mix-

ture; the pH of the phosphate buffer in the range 6–8 is without effect on the phosphorolytic activity of the enzyme (Fig. 9).

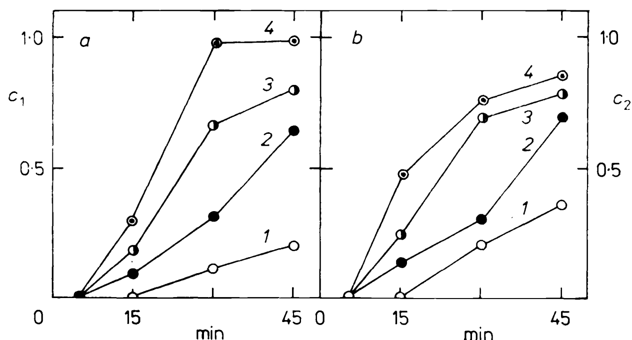


FIG. 7

Time of transribosylation: effect of endogeneous phosphate *a* guanosine synthesis; *b* concurrent inosine phosphorolysis to hypoxanthine; PNP concentration: *1* 0·06 μg ml^{-1}; 2 0·11 μg ml^{-1}, *3* 0·17 μg ml^{-1}, *4* 0·22 μg ml^{-1}. min Time profile of reaction; c_1 the quantity of guanosine in nmol, c_2 the quantity of hypoxanthine in nmol

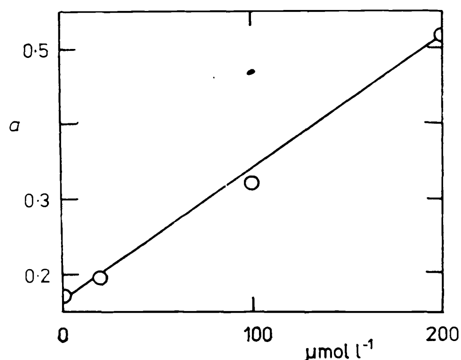


FIG. 8

Effect of exogeneous phosphate on transribosylation. The reaction mixture contained 0·11 μg ml^{-1} of PNP; *a* PNP activity expressed in nmol of guanosine per 20 min, μmol l^{-1} concentration of inorganic phosphate

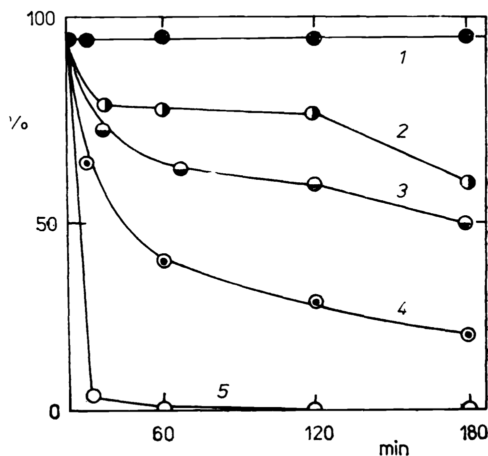


FIG. 9

Temperature stability of PNP. % Residual activity of PNP; *1* at 0–45°C, 2 at 50°C in 41 mM phosphate, *3* at 60°C in 41 mM phosphate, *4* at 50°C in 1 mM phosphate, *5* at 60°C in 1 mM phosphate. min Time profile of reaction

PNP from L1210 cells belongs to SH-enzymes since it is sensitive to *p*-chloromercuribenzoic acid and to 5,5′-dithiobis(2-nitrobenzoic) acid; their inhibitory effect is reversible after the addition of dithiothreitol to the reaction mixture (results not presented).

EXPERIMENTAL

Materials

Mouse leukemia cells L1210 were obtained from DBA/2 female mice on the eighth day after the inoculation of 10^5 cells. The cell suspension was washed twice with 50 mM Tris–HCl buffer, pH 7·4 (sodium–potassium buffer according to Sørensen, phosphate buffer in what follows), containing 0·15M KCl and 5 mM 2-mercaptoethanol. After centrifugation (10 min, 12 000 *g*) the cell sediment was suspended in the same buffer (5 : 1) and was kept at −78°C or was used in the next isolation step. 9-(*p*-Succinylaminobenzyl)hypoxanthine and the modified nucleosides were prepared in this Institute. 2′-Deoxyinosine was prepared according to Votruba and Holý[26]. [U-^{14}C]-Inosine was prepared by enzymatically catalyzed deamination of [U-^{14}C]-adenosine (17 GBq mmol l^{-1}; Prague, Czechoslovakia) using calf intestinal adenosine deaminase purchased from Boehringer (Mannheim, F.R.G.). Adenosine, 2′-deoxyadenosine, guanosine, 2′-deoxyguanosine, α-D-ribose-1-phosphate, 2-deoxy-D-ribose-1-phosphate, hypoxanthine, and guanine were purchased from Calbiochem (Los Angeles, CA, U.S.A.). and inosine from Fluka (Buchs, Switzerland). Acrylamide, N,N′-methylenebisacrylamide, sodium dodecyl sulfate, Coomassie Brilliant Blue G-250, glycine, and Tris were products of Serva (Heidelberg, F.R.G.). Pharmalyte (pH 4−6·5), Low pI Calibration Kit (pH 2·5−6·5), HMW and LMW Electrophoresis Calibration Kit, CNBr-Sepharose 4B, and DEAE-Sephadex A-50 were products of Pharmacia (Uppsala, Sweden).

Reaction Mixtures

The phosphorolytic activity was measured in a reaction mixture (total volume 250 μl) consisting of 41 mM phosphate buffer, pH 7·4 containing 2 mM DTT, 50 μg ml^{-1} of bovine serum albumin and 40 μm inosine (mixture F_1). Inosine was replaced by 20 μM adenosine in the mixture used for adenosine deaminase (mixture A). When a radioactive substrate was used the reaction mixture contained 40 μM [U-^{14}C]-inosine (specific activity 0·30 MBq μmol^{-1}) (mixture F_2). The standard reaction mixture used for specific elution of the affinity support (mixture F_3) did not contain inosine. The reaction mixture used for the determination of K_m-values contained 10 to 80 μM purine nucleosides and 2·8 ng ml^{-1} of PNP. The phosphate concentration varied between 1 and 100 mmol l^{-1}.

The synthesis of nucleosides was measured in a reaction mixture (total volume 250 μl) containing 40 mM Tris–HCl, pH 7·4, 2 mM DTT, 20 μM hypoxanthine (or guanine), 100 μM α-D-ribose-1-phosphate (2-deoxy-α-D-ribose-1-phosphate), 50 μg ml^{-1} of bovine serum albumin and 5·6 ng ml^{-1} of PNP (mixture S). For the determination of the K_m-values for purine bases reaction mixture S_1 contained 4−40 μM hypoxanthine or guanine, resp. The determinations of the K_m-values for ribose-1-phosphate or for 2-deoxyribose-1-phosphate were effected in reaction mixture S_2 which unlike S contained 20 μM hypoxanthine or guanine, resp. and ribose-1-phosphate or 2-deoxyribose-1-phosphate in the range 20−100 μmol l^{-1}. The transribosylation was assayed in a reaction mixture (total volume 250 μl) containing 40 mM Tris–HCl, pH 7·4, 2 mM DTT, 4 μM guanine, 20 μM inosine, 50 μg ml^{-1} of human serum albumin and 0·06 to 0·22 μg ml^{-1} of the enzyme preparation (reaction mixture T).

All reactions were triggered by the addition of the enzyme preparation to the reaction mixture which was subsequently incubated 5—30 min at 37°C.

Analysis

The determination of nonradioactive products of PNP catalyzed reactions was effected by HPLC on Separon SIX C18 5 μm columns (3·2 × 150 mm, Tessek, Prague, Czechoslovakia). The mobile phase was 10 mM potassium dihydrogenphosphate, pH 3·1 or 3·9 in 5 or 10% methanol. The flow rate was 0·50 ml min^{-1} and the effluent was monitored at 254 nm and a sensitivity of 0·02—0·04 AUFS. For the quantitation Model CI 100 Integrator (Laboratorní přístroje, Praha) was used.

The determination of the reaction product in reaction mixtures containing radioactive substrate was carried out by descending paper chromatography on Whatman 3MM paper (16—20 h) in the system 1-butanol–acetic acid–water (10 : 1 : 3). The radioactivity of the spots was determined in toluene based scintillation cocktail in Beckman LS 5801 Spectrometer.

Polyacrylamide gel electrophoresis: Discontinuous electrophoresis (focusing gel[29]: T 4·5%, C 2·5%; separation gel T 10%, C 2·6%) was carried out according to Davis[27]. Discontinuous electrophoresis in the presence of SDS was carried out in the system of Laemli[28] (focusing gel[29]: T 4·5%, C 2·6%; separation gel: T 12·5% and C 2·6%). Both electrophoreses were performed in flat gel slabs (8·3 × 6·2, 15 cm) in 2050 MIDGET Electrophoresis Unit, LKB-Pharmacia; a constant voltage of 120 V (initial current intensity 40 mA/2 gels) was applied for 2—3 h.

Electrophoresis in the gel with linear polyacrylamide gradient (4—20%) was carried out in flat gel slabs (8·3 × 6·2 × 0·075 cm) containing Tris–borate buffer, pH 8·3 (ref.[29]) at 110 V (initial current intensity 25 mA/2 gels) for 10 h. The gels were stained with the silver nitrate solution according to Blum and coworkers[30]. For determination of the molecular weight of the subunit and of native PNP a set of standard proteins was used. Some of the gels were cut after the electrophoresis to 2—3 mm slices which were then placed in standard reaction mixture F_2. The phosphorolysis was allowed to proceed 30 min at 37°C and the reaction products were analyzed by paper chromatography.

Isoelectric focusing: The determination of the isoelectric point of the enzyme was carried out on a glass plate (23 × 11·5 cm) whose surface had been treated with Silan A 174. The isoelectric focusing was performed in Flat Bed Apparatus FBE-3000 in polyacrylamide gel of the following composition[31]: T 5%, C 3%, thickness 1 mm; the pH-gradient was developed by the addition of Pharmalyte 4—6·5 (ref.[31]) 0·04M L-glutamic acid and 0·2M histidine served as electrode solutions. The samples and the standards were applied after prefocusing at 500 V h and a constant output of 25 W. The focusing itself was carried out at a constant output of 15 W and was terminated after 4 000 V h. A part of the gel was fixed in a solution containing 10% TCA and 5% sulfosalicylic acid for 45 min. The gel was washed in the mixture methanol–water–acetic acid (3 : 6 : 1) and the proteins were then stained with the silver nitrate solution according to Blum and coworkers[30].

The pH gradient was determined according to the positions of the standard proteins. The other part of the gel was used for the determination of phosphorolytic activity after slicing (see above).

Ion exchange chromatography: A 7-ml sample containing 72·8 mg of proteins was applied to a column (2 × 1 cm) of DEAE-Sephadex A50 equilibrated in 50 mM Tris–HCl, pH 7·6, containing 2 mM DTT. The flow rate was 12 ml h^{-1}. The column was eluted first with the equilibration buffer (25·5 ml), then with 50 mM Tris–HCl, pH 7·6, containing 2 mM DTT, 0·1M-KCl (15 ml) and finally with the following elution gradient: 0·1M-KCl—0·5M-KCl in the equilibration

buffer. The protein content of the fractions was determined according to Bradford[32]; PNP and ADA activity was assayed in reaction mixtures F_1 and A, resp., as described above. The course of the concentration gradient in the individual fractions was assayed refractometrically. PNP--containing fractions were pooled and dialyzed 16 h against 50 mM Tris–HCl, pH 7·6, containing 2 mM DTT.

Affinity chromatography: Preparation of the affinity support. The gel slurry, prepared from lyophilized CNBr-Sepharose 4B (ref.[33]) was placed in two volumes of 2M 1,2-diaminoethane hydrochloride, pH 8·3. The suspension was shaken overnight. AE-Sepharose 4B was washed with 0·1M Tris–HCl, pH 8·0, water, 1M-NaCl and again with water; it was then filtered off on a glass filter. 9-(*p*-Succinylaminobenzyl)hypoxanthine was dissolved in the minimal volume of water by stepwise adjustment of the pH of the suspension with sodium hydroxide to pH 6 (120 μmol of ligand per ml of support) to which AE-Sepharose 4B had been added. The suspension was then kept at pH 5·5—6 by the addition of HCl and solid CDI (N-cyclohexyl-N′-(3-trimethylammoniumpropyl)carbodiimide *p*-toluenesulfonate; 1 mmol per 1 ml of gel slurry) was added with constant stirring during the first 30 min of the reaction. An equal portion of CDI was added afterwards and the pH of the suspension was adjusted to pH 5·5. On the subsequent day the support was again washed with 0·1M phosphate buffer, 1M-NaCl and water. The saturation of the free amino groups and the final washing of the support were effected according to the instructions of Pharmacia[33]. The affinity support was stored in 0·02% NaN_3 at 0°C.

Affinity purification of PNP. The affinity support (see above) was equilibrated first with 50 mM Tris–HCl, pH 7·6, containing 2 mM DTT. The binding of the enzyme to the support was effected batchwise in an ice bath. To bind the enzyme obtained from 20 ascites 14 ml of the affinity support was used. The latter with the immobilized enzyme was placed in a column (1·6 × 7 cm), which was washed stepwise with the equilibration buffer (120 ml, average flow rate 15 ml h^{-1}), with 0·1M Tris–HCl, pH 8·0, containing 2 mM DTT and with 0·5M-NaCl (90 ml). After the absorbance (at 220 nm) of the effluent had dropped to the level of the blank PNP was specifically eluted with 50 mM phosphate buffer, pH 7·5, containing 2 mM inosine and 2 mM DTT. Inosine was removed from the effluent by gel filtration. The isolate obtained was treated with glycerol and bovine serum albumin to a final concentration of 20% and 50 μg ml^{-1}, resp. In other isolation runs the enzyme was concetrated by ultrafiltration (Amicon apparatus) using YM-10 ultrafilter and again treated with glycerol to a final concentration of 50%. The purification of PNP was allowed to proceed at 4°C. All isolates were stored at −78°C for long periods without loss of enzyme activity.

REFERENCES

1. Parks R. E., Jr., Agarwal R. P. in: *The Enzymes* (P. D. Boyer, Ed.) 3rd ed., Vol. 7, p. 483. Academic Press, New York 1971.
2. Giblett E. R., Anderson J. E., Cohen F., Pollara B., Meuwissen J.: Lancet *2*, 1067 (1972).
3. Giblett E. R.: Lancet *3*, 1010 (1975).
4. Giblett E. R.: Ann. N. Y. Acad. Sci. *451*, 1 (1985).
5. Kazmers I. S., Mitchell B. S., Daddona P. E., Wotring L. L., Townsend L. B., Kelley W. N.: Science *214*, 1137 (1981).
6. Ho A. D., Ganeshaguru K.: Klin. Wochenschr. *66*, 457 (1988).
7. Stoeckler J. D. in: *Developments in Cancer Chemotherapy* (R. J. Glazer, Ed.), p. 35. CRC Press, Inc. Boca Raton 1984.
8. Montgomery J. A.: Med. Res. Reviews *2*, 271 (1982).

9. *Description of Systems Used in Experimental Screening of Anticancer Preparations*, World Health Organization, Geneva 1974.
10. Lukey T., Noete K., Loman J. F., Unger A. E., Biddle F. G., Snyder F. F.: Biochem. Genet. *23*, 347 (1985).
11. Snyder F. F., Biddle F. G., Lukey T., Sparling M. J.: Biochem. Genet. *21*, 323 (1983).
12. Board P. G., Smith J. E.: Biochem. Genet. *15*, 439 (1977).
13. Zannis V., Gudas D. W., Martin D. W. Jr.: Biochem. Genet. *17*, 621 (1979).
14. Edwards Y. H., Hopkinson D. A., Harris H.: Ann. Hum. Genet. *34*, 395 (1971).
15. Mably E. R., Carter-Edwards T., Biddle F. G., Snyder F. F.: Comp. Biochem. Physiol., B *89*, 427 (1988).
16. Lukey T., Neote K., Loman J. F., Unger A. E., Biddle F. G., Snyder F. F.: Biochem. Genet. *23*, 347 (1985).
17. Zannis V., Doyle D., Martin D. W., Jr.: J. Biol. Chem. *253*, 504 (1978).
18. Changas G., Reem G. H.: J. Biol. Chem. *254*, 4233 (1979).
19. Wiginton D. A., Coleman M. S., Hutton J. J.: J. Biol. Chem. *255*, 6663 (1980).
20. Mora M., Bozal J.: Comp. Biochem. Physiol., B *82*, 805 (1985).
21. Bzowska A., Kulikowska E., Darzynkiewicz E., Shugar D.: J. Biol. Chem. *263*, 9212 (1988).
22. Jordan F., Wu A.: J. Med. Chem. *21*, 877 (1978).
23. Krenitsky T. A., Elion G. B., Henderson A. M., Hitchings G. H.: J. Biol. Chem. *243*, 2876 (1968).
24. Šedivá K., Ananiev A. V., Votruba I., Holý A., Rosenberg I.: Int. J. Purine Pyrimidine Res., in press.
25. Ananiev A. V., Bezirdzhyan Kh. O., Akopyan Zh. I.: Biochemistry (U.S.S.R.) *52*, 2022 (1987).
26. Holý A., Votruba I.: Nucleic Acids Res. Symp. Ser. *18*, 69 (1987).
27. Davis B. J.: Ann. N. Y. Acad. Sci. *121*, 404 (1964).
28. Laemmli U. K.: Nature *227*, 680 (1970).
29. *Polyacrylamide Gel Electrophoresis Laboratory Techniques:* Pharmacia Fine Chemicals AB, Box 175, S-75104 Uppsala, Sweden.
30. Blum H., Beier H., Gross H. J.: Electrophoresis *8*, 93 (1987).
31. *Isoelectric Focusing, Principles and Methods:* Pharmacia Fine Chemicals AB, Box 175, S-75104 Uppsala, Sweden.
32. Bradford M.: Anal. Biochem. *72*, 248 (1976).
33. *Affinity Chromatography, Principles and Methods:* Pharmacia Fine Chemicals AB, Box 175, S-75104 Uppsala, Sweden.
34. Dunn M. J. in: *Advances in Electrophoresis* (A. Chrambach, M. J. Dunn and B. J. Radola, Eds), Vol. 1, p. 68. Weinham, New York 1987.
35. Stoeckler J. D., Agarwal R. P., Agarwal K. C., Parks R. E., Jr.: Methods Enzymol. *51*, 530 (1978).
36. Osborne W. R. A.: J. Biol. Chem. *235*, 7089 (1980).
37. Hoffee P., May R., Robertson B. C.: Methods Enzymol. *51*, 517 (1978).
38. Milman G., Anton D. L., Weber J. L.: Biochemistry *15*, 4967 (1976).
39. Edwards Y. H., Edwards P. A., Hopkinson D. A.: FEBS Lett. *32*, 235 (1973).
40. Stoeckler J. D., Agarwal R. P., Agarwal K. C., Schmid K., Parks R. E.: Biochemistry *17*, 278 (1978).

Translated by V. Kostka.