REVISTA ESPAÑOLA DE FISIOLOGIA, 26, 157-166. 1970

Phosphorolytic and Transferase Activities of the Purine Nucleoside Phosphorylase from Human Erythrocytes *

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(Received on October 20, 1969)

B. PINTO, *Phosphorolytic and Transferase Activities of the Purine Nucleoside Phosphorylase from Human Erythrocytes.* R. esp. Fisiol., 26, 157-166, 1970.

Purine nucleoside phosphorylase obtained from human erythrocytes was purified 600 to 800 fold by acid precipitation and filtration through p-cellulose and DEAE-cellulose columns.

The enzyme was found to contain both phosphorylase and transferase activities and the ratio of the two activities remaind constant throughout the purification procedure. Apparently different molecular aggregates were obtained by sucrose gradient centrifugation. No interconversion of the two activities was found.

The ability to catalyze pentosyl exchange reactions in the presence and absence of phosphate has been reported to be associated with the purine nucleoside phosphorylase from calf spleen, Ehrlich ascites tumor cells (1, 11) and human erythrocytes (8). The constant ratios of the two enzymatic activities found after different methods of purification suggest that they are associated with the same enzymatic entity. Suggestive evidence for the separation of the two activities by sucrose centrifugation gradients and their apparent interconversion has been reported for the Ehrlich ascites tumor enzyme (11). Such data suggest the association of the two activities in different molecular aggregates and their interconversión under various conditions. Kinetic data (9) seem to support this hypothesis.

In preliminary experiments, PARKS *et al.* (8) were unable to separate the two activities of purine nucleoside phosphorylase from human erythrocytes by using Sephadex G-200 columns. These authors could find no evidence for the interconversion of phosphorylase and transferase activities in the same source of enzyme. The present paper describes the results obtained with the enzyme in human erythrocytes and some of the basis for these discrepancies.

* This investigation was supported in part by Grant MA-2357 from the Medical Research Council of Canada, and preformed at the Department of Biochemistry Queen's University. Kingston, Ontario (Canada).

Materials and Methods

MATERIALS. Phospho-cellulose, coarse type, 0.9 meq/g capacity was purchased

from Carl Schleicher and Schuell Co., Keene, New Hampshire. DEAE-cellulose, standard type, 0.95 meq/g capacity, and CM-cellulose-Sephadex G-25 type, 4.5 ± CM-cellulose-Sephadex G-25 type, 4.5 ± 0.5 meq/g capacity, were obtained from Sigma Chemical Co., St. Louis, Missouri. Xanthine oxidase (Xanthine: oxygen oxidoreductase E.C. 1.2.3.2.) prepared from milk by a modification of the method of BALL (2) catalase (H_2O_2:H_2O_2 oxidoreductase E.C. 1.11.1.6.) crystallized from beef liver by the method of TAUBER *et al.* (14), alcohol dehydrogenase (alcohol: NAD oxidoreductase E.C. 1.1.1.1.) purified from yeast according to RACKER (13) and from horse liver by the procedure of BONNICHSEN *et al.* (4), carboxypeptidase A (carboxypeptidase A, E.C. 3.4.2.1.) prepared from bovine pancreas according to PUTMAN *et al.* (12), were bought from Worthington Biochemical Corp., Freehold, New Jersey. Unlabeled nucleosides and bases, adenosine-8-^{14}C, xanthosine-8-^{14}C, thymidine-2-^{14}C, cytidine-2-^{14}C, guanosine-8-^{14}C, uridine-2-^{14}C, uracil-2-^{14}C, thymine-2-^{14}C, cytosine-2-^{14}C, xanthine-8-^{14}C, adenine-8-^{14}C, hypoxanthine-8-^{14}C and guanine-8-^{14}C were obtained from Schwartz Biochemicals, Orangeburg, New York. Human erythrocytes were a gift of the Canadian Red Cross Society, Ottawa, Ontario. Osray, DW Blue base x-ray films from Agfa-Gevaert, Belgium were supplied by Philips Electronics, Toronto, Ontario.

ENZYMATIC ASSAYS

Phosphorylase Activity. Phosphorylase activity was measured by determining the rate of transformation of inosine-8-^{14}C to hypoxanthine-8-^{14}C in the presence of phosphate buffer at pH 5.5. Incubation mixtures contained 100 μmoles of potassium phosphate buffer (pH 5.5), 5 μmoles of nucleoside (0.2 μC of nucleoside-8-^{14}C) and enzyme in a total (final) volume of 1 ml. Reactions were run at room temperature for, usually, 5 min. and aliquots (0.2 ml) were removed at 1 min. intervals and deposited in 5 ml glass tubes which were kept standing in a mixture of dry iceacetone. The samples were kept frozen until required when the products of the reaction were separated by paper chromatography.

Transferase Activity. Transferase activity was determined by measuring the rate of formation of labeled nucleoside in the presence of ^{14}C base and unlabeled nucleoside. Assays were performed in 1 ml incubation mixtures containing 2.5 μmoles of unlabeled nucleoside, 0.5 μC in 0.02 μmoles of 8-^{14}C or 2-^{14}C base and 100 μmoles of sodium Tris maleate buffer (pH 6.0). Incubations were carried out in a Warner-Chilcott shaker at 37° for 2 hours. Aliquots of 0.25 ml were taken out every 30 min. The reaction was terminated and the products separated in the same manner as described for the phosphorylase. An enzymatic unit is defined as the ability to transfer 1 μmole of pentose either to phosphate or base, per hour.

Paper Chromatography. Descending paper chromatography was carried out in a steel chromatocabinet at room temperature. From each aliquot, 0.1 ml of sample were spotted on standard size Whatman N.° 3 paper sheets and two of them developed with 180 ml of solvent. Spots from the phosphorylase and transferase assay systems contained about 10.000 and 30.000 c.p.m. respectively. Guanine and hypoxanthine were separated from their nucleosides by developing for 7 hours with 5 % disodium phosphate saturated with amyl alcohol. Other bases and nucleosides were separated with butanol-propionic acid-water (125:62:87) for 16 hours.

Radioactivity Measurements. Radioactive compounds were identified by autoradiography after exposing the papers to film for 8 days. Alternatively the identification was carried out by the position

of added unlabeled standards. Labeled spots were cut out and counted in a model 6725 Nuclear Chicago scintillation counter by the method of HAYES *et al.* (6) using zero time samples as background.

Miscellaneous Determinations. Protein was determined in mercaptoethanol-free samples, according to the technique of LOWRY *et al.* (10), using bovine serum albumin as standard. Other enzymatic determinations were performed as follows: alcohol dehydrogenase by the method of VALLEE *et al.* (16); catalase by the procedure of BEERS *et al.* (3); carboxypeptidase A according to FOLK *et al.* (5); and xanthine-oxidase as determined by KALCKAR (7).

Sucrose Centrifugation Gradients. Linear sucrose centrifugation gradients were carried out in 30 ml nitrocellulose tubes by delivering 27 ml of sucrose solution of density ranging from 1.01 to 1.10 buffered with 2.5 mM sodium Tris maleate (pH 7.4) and containing 10 mM mercaptoethanol. The delivery system consisted of a Buchler mixing device equipped with three outlets connected to tygon tubing (inside diameter 0.078 cm) and connected to a Polystaltic pump, set to deliver 35 ml/hour. The purified enzyme was dissolved in 1 ml of the buffer and layered on the top of the gradient. Tubes containing the enzyme-sucrose solution were centrifuged for 52 hours in a model L Spinco ultracentrifuge set at 24.000 r.p.m. and 4° using the SW 25.1 rotor. After centrifugation the bottom of the tubes were pierced and 55 to 59 fractions (13 drops each) collected. Each fraction was dialyzed for a total of 16 hours against 3 changes of 8 l of 2.5 mM sodium Tris maleate buffer (pH 7.4) and 10 mM mercaptoethanol. After dialysis, phosphorylase and transferase activities were determined in alternate fractions, using 0.5 ml samples. Estimation of molecular weights was performed by standardizing the gradients with horse liver and yeast alcohol dehydrogenases, catalase, carboxypeptidase A and xanthine oxidase.

Enzyme Purification. All operations were performed at 4° unless otherwise indicated.

First Stage Separation of Cells. Erythrocytes from apparently normal healthy individuals were separated from the plasma by centrifugation at 2.000 r.p.m. for 15 min. a Sorvall RC-2B centrifuge and SGA Sorvall rotor. The precipitated red cells collected and kept frozen at —19° (for usually 3-4 months), until they were used.

Second Stage Acid Precipitation. After thawing, the erythrocyte suspension was adjusted to pH 5.0 with 1 M acetic acid and the resulting precipitate was removed by centrifugation at 15.000 r.p.m. for one hour. The supernatant was brought to pH 6.5 with 0.1 N NaOH.

Third Stage Filtration Through Phospho-cellulose. Further purification was accomplished by removing hemoglobin from the supernatant on a phospho-cellulose column. For the preparation of the column, 180 g of phospho-cellulose were successively washed with 8 liters of 0.1 N NaOH, 8 liters of 0.1 N HCl followed by water until the eluate was free of acid. The cellulose was then suspended in 2.5 mM sodium Tris maleate buffer (pH 6.5) and 10 mM mercaptoethanol. After the pH of the suspension had been adjusted to 6.5 with 1 N NaOH, it was poured into a 100×5 cm colum and washed with 5 liters of the same buffered Tris maleate mercaptoethanol solution at a flow rate of 6-8 ml/min. Samples from the second stage supernatant containing 10-15 g of protein in 60-70 ml were dialyzed overnight against the buffer and layered on the top of the column with a Sephadex applicattor. The column was wa-

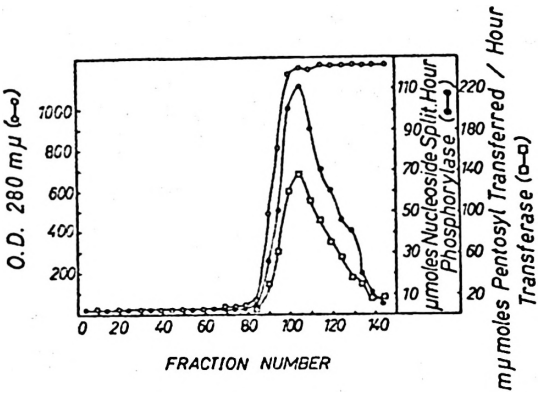

Fig. 1. *Purification of purine nucleoside phosphorylase on P-cellulose columns.* Enzyme from «acid precipitation» fraction was purified on P-cellulose columns as described in «Methods». Phosphorylase and transferase activities were determined as described in the text. Protein was determined by 280 mμ absorption.

shed at the rate of 1.0-1.5 ml/minute with 2.5 mM sodium Tris maleate buffer (pH 6.5) containing 10 mM mercaptoethanol. Fractions (15 ml) of the eluate were then collected. The enzyme appeared in the void volume mixed with several red and green colored components. Fractions containing enzyme activity were pooled and further purified. The results of a typical experiment are shown in figure 1.

Fourth and Fifth Stages Filtration Through DEAE-cellulose. Two additional purification stages were carried out on DEAE-cellulose at pH 5.2. The DEAE-cellulose was washed with NaOH and HCl solutions, and then suspended in 2.5 mM sodium Tris maleate buffer (pH 5.2) and 10 mM mercaptoethanol and the pH of the suspension adjusted to 5.2 with 1 N HCl. The first-DEAE-cellulose colum was prepared by suspending 30 g of DEAE-cellulose in the above mentioned buffer and the suspension was poured into a 105-110 × 2.5 cm column. Equilibration was effected by washing the column with the same 2.5 mM sodium Tris maleate buffer (pH 5.2) and 10 mM mercaptoethanol until the eluate no longer absorbed at 280 mμ. The pooled enzyme from the previous step was adjusted to pH 5.2 with 0.1 N acetic acid and poured on the top of the DEAE-cellulose column. The flow rate was adjusted to 0.8-1.0 ml/minute and elution begun by washing the column with 2.5 mM sodium Tris maleate buffer (pH 5.2) and 10 mM mercaptoethanol. Fractions (10 ml) were collected and enzymically active fractions were pooled together. At this stage, small amounts of a reddish colored component were still present, but this was removed by the following stage (Fig. 2). An identical second-DEAE-cellulose column was prepared by the same procedure as described for the first-DEAE-cellulose column. The enzyme solution obtained from the previous column (first-DEAE-cellulose) was poured on top of the column and collected under the same conditions, upon elution of the column with the 2.5 mM sodium Tris ma-

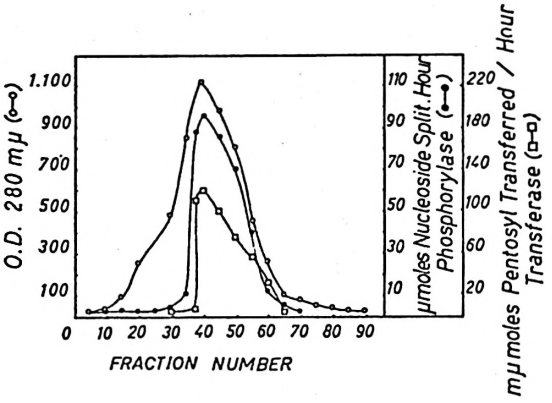

Fig. 2. *Purification of purine nucleoside phosphorylase on DEAE-cellulose colums.* Enzyme from P-cellulose column was deposited on a DEAE-cellulose column. Assays and purification were performed as described in «Methods». Active enzyme fraction from this column was again purified on a second similar DEAE-cellulose colum. Protein was determined by the 280 mμ absorption.

Table I. *Purification Procedure for the Purine Nucleoside Phosphorylase.*

Phosphorylase activity was determined by the rate of hypoxanthine-8-^{14}C formation in 1.0 ml solutions containing 100 μmoles of potassium phosphate buffer (pH 5.5), 5 μmoles of inosine having 0.2 μC of nucleoside-8-^{14}C, and enzyme in not greater concentration than 0.6 mg of protein. Transferase reactions were assayed in the presence of 100 μmoles of sodium Tris maleate buffer (pH 6.0); 2.5 μmoles of guanosine, 0.02 μmoles of guanine-8-^{14}C and enzyme. Incubation, removal of aliquots and separation of products were performed as described in Materials and Methods.

	VOLUME ml	PROTEIN mg/ml	SPECIFIC ACTIVITY units/mg		TOTAL ACTIVITY UNITS		YIELD %		RATIO
			Phospho-rylase	Trans-ferase	Phospho-rylase	Trans-ferase	Phospho-rylase	Trans-ferase	Phosphorylase/ Transferase
HEMOLYSATE	68.0	390.00	0.046	0.00006	1219.0	1.591	100.0	100.00	766
ACID SUPERNATANT SOLUTION (pH 5.0)	60.0	250.00	0.072	0.00009	1080.0	1.350	88.5	84.80	800
P-CELLULOSE FRACTION	850.0	0.60	6.000	0.00840	3060.0	4.284	251.0	269.00	714
FIRST-DEAE-CELLULOSE	288.0	0.25	19.000	0.02300	1368.0	1.656	112.2	103.90	826
SECOND-DEAE-CELLULOSE	120.0	0.12	29.300	0.04000	421.9	0.576	34.6	36.17	732
DEAE-CELLULOSE CONC.	7.4	1.00	25.000	0.03300	185.0	0.244	15.2	15.23	757

leate buffer (pH 5.2) and 10 mM mercaptoethanol. Fractions (10 ml) were collected and those containing the enzyme were pooled.

Sixth Stage Concentration on DEAE-cellulose. A DEAE-cellulose column (1.5 × 15 cm) was prepared as previously described and equilibrated by washing with 1 liter of 2.5 mM sodium Tris maleate buffer (pH 7.4) and 10 mM mercaptoethanol. Pooled fractions from previous stage (second-DEAE-cellulose column) were adjusted to pH 7.4 with 0.1 N NaOH and added to the colum without interruption at a speed of 1 ml/minute. The column was then eluted with 2 M KCl in 2.5 mM sodium Tris maleate buffer (pH 7.4) and 10 mM mercaptoethanol. Fractions (1 ml) containing the peak of activity were pooled together and KCl removed by dialysis against 6 changes of 8 liters each of 2.5 mM sodium Tris maleate buffer (pH 7.4) and 10 mM mercaptoethanol. By this procedure a 600-800 fold purification was obtained with an overall recovery of about 10 %. Similar ratios of the phosphorylase-transferase activities were obtained throughout the different purification steps. The results of a typical experiment are shown in Table I. Several attempts to purify the enzyme by using the procedure of VERDIER *et al.* (17) were unsuccessful. Traces of phosphate (method by VERDIER) were always present in the solutions, in spite of thorough dialysis against 12 changes of 2.5 mM sodium Tris maleate buffer (pH 7.4) and 10 mM mercapthoethanol. Similar disappointing results were obtained in attemps to remove phosphate by passing enzyme preparations through Sephadex-G-25 columns equilibrated with the same Tris maleate buffered solution. Furthermore, most of the enzyme activity (99-99.5 %) was lost on the step where the enzyme is purified by using CM-Sephadex C-25 at pH 5.5 in acetate buffer (17).

Results

pH Optimum. pH optima for the phosphorylase and transferase activities were obtained at 5.5 and 6.0 respectively by using the assay conditions previously described (Fig. 3).

Stability. Concentrated solutions containing up to 4 mg of protein/ml were stable for two months. Diluted solutions (<1.5 mg/ml) in the absence of reagents containing SH groups lost activity very rapidly. Similar results were obtained by TSUBOI *et al.* (15). No indications of different behaviour with either transferase and phosphorolytic activities were found. In fact, the loss of phosphorolytic activity

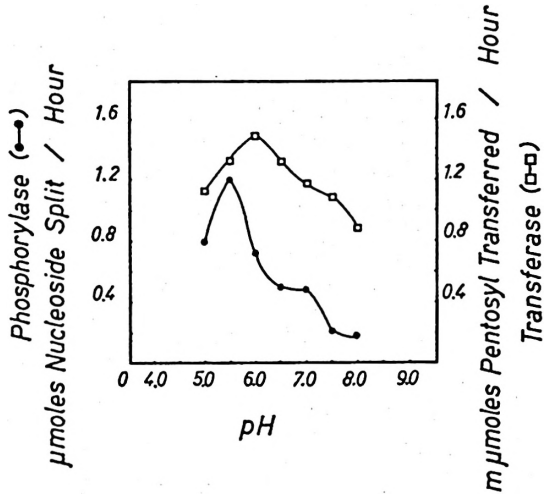

Fig. 3. *pH effect on the transferase and phosphorylase activities.*
Purified enzyme was tested under different pH conditions. Phosphorylase assays contained in 1 ml at room temperature, 100 μmoles of potassium phosphate buffer at different pHs, 5 μmoles of inosine containing 0.2 μC of inosine-8-^{14}C and enzyme. Transferase activity was determined in incubation mixtures a 37° in a volume of 1 ml in the presence of 100 μmoles of sodium Tris maleate buffer at different pHs, 2.5 μmoles of guanosine and 0.02 μmoles of guanosine and 0.02 μmoles of guanine-8-^{14}C in 0.5 μC. Incubation, removal of aliquots, separation and counting of the products were performed as described in «Methods».

Table II. *Effect of Mercaptoethanol Removal on Phosphorylase and Transferase Activities.* Purified enzyme containing 0.6 mg of protein per ml was layered on a sucrose centrifugation gradient as described in Materials and Methods. Fractions of intermediate rate of sedimentation were pooled and sucrose dialyzed against 2.5 mM sodium Tris maleate buffer (pH 7.4) with and without 10 mM mercaptoethanol for fractions A and B respectively. Fraction C was obtained by incubating and aliquot from fraction B with 10 mM mercaptoethanol for 15 minutes at 4°. Phosphorylase and transferase activities were determined on 0.3 ml samples as described in Materials and Methods.

FRACTION	Phosphorylase Units/0.3 ml	Transferase Units/0.3 ml
A	3.06	0.00370
B	0.90	0.00038
C	0.87	0.00025

was accompanied by a similar loss of the transferase activity (Table II). Both activities were protected by reagents containing SH groups. However, no restoration of lost activity was found.

Specificity. The enzyme was able to split inosine, guanosine, deoxyinosine and deoxyguanosine in the presence of inorganic phosphate as has already been reported (17). In order to measure the deoxynucleosides split, the liberated bases and unreacted nucleosides were eluted with water from the paper, after chromatographic separation and the U.V. absorption of the eluates was measured at the appropriate wave length and the concentrations calculated by using the respective extinction coefficient (7). The enzyme transferred pentosyl moities from the same four purine nucleosides, namely inosine, guanosine, deoxyinosine, and deoxyguanosine when they were used as donors and guanine as acceptor. No interchange was observed when other acceptors and donors were used (Table III).

Study of the Enzyme on Sucrose Gradient Centrifugation. After layering and centrifuging the enzyme solution on sucrose gradient, fractions were tested for transferase and phosphorolytic activities. Depending upon protein concentration used, different activity profiles were obtained. When the amount of protein containing the purified enzyme was 0.6 mg, three peaks of activity were evident. The first and second peaks showed transferase and phosphorolytic activities. The last peak, however, possessed transferase activity and negligible phosphorolytic activity. The approximate molecular weight of these three peaks were 160.000, 80.000 and 40.000 respectively. When 0.1 mg of protein per ml was layered on the gradient

Table III. *Phosphorylase and Transferase Specificity.* Purified enzyme from DEAE-cellulose concentrated fractions, possessing a specific activity of 22.2 phosphorylase units, was tested for phosphorylase and transferase activities by addition of 0.22 phosphorylase units to each incubation mixture. Assays for both activities were performed as described in Materials and Methods. Data is expressed in percentage of pentosyl exchange by using inosine as donor and inorganic phosphate (Pi) and guanine-8-^{14}C (G-8-^{14}C) as acceptors. Hypoxanthine, xanthine, adenine, uracyl, cytosine and thymine were also tested as pentosyl acceptors.

Pentosyl donor	Pentosyl acceptor	
	Pi (Phosphorylase)	G-8^{14}C (Transferase)
Inosine	100	100
Deoxyinosine	46	20
Guanosine	148	214
Deoxyguanosine	72	62
Adenosine	0	0
Deoxyadenosine	0	0
Uridine	0	0
Cytidine	2	0
Thymidine	0	0

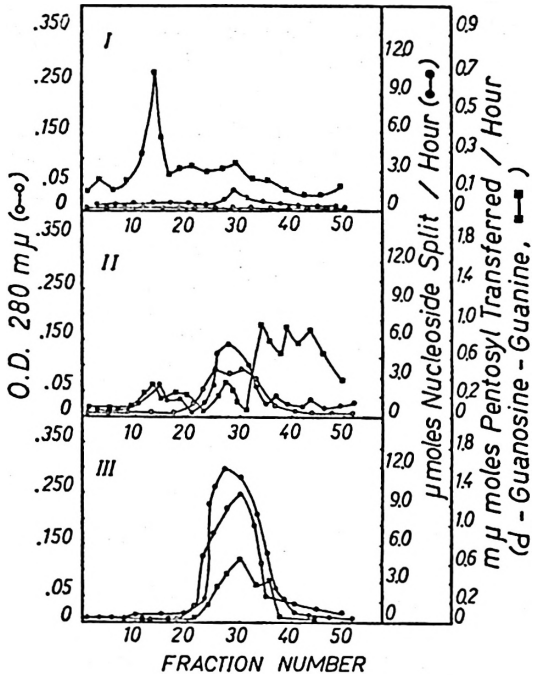

Fig. 4. *Phosphorylase and transferase profiles on sucrose density centrifugation gradients.*
By dissolving the purified enzyme from DEAE-cellulose concentrate in 2.5 mM sodium Tris maleate buffer (pH 7.4) and 10 mM mercaptoethanol, three protein solutions containing 0.1 mg/ml, 0.6 mg/ml and 1mg/ml were made up. One ml of each one of these three respectively solutions were layered on gradients I (0.1 mg/ml), II (0.6 mg/ml) and III (1 mg/ml). Centrifugation run, fractionation, dialysis and enzymatic determinations were performed as described in «Methods». Protein was determined by the 280 mμ absorption.

the slowest peak, showing mainly transferase activity, was smaller and even disappeared on some occasions. Experiments in which 1.0 mg of protein was layered, showed only one single peak having both activities and a molecular weight of 80.000 (Fig. 4). The transferase peaks showed slight differences in specificity towards the transfer of the pentosyl moiety (Table IV). The presence of mercaptoethanol was needed to recover the two activities from the gradients containing diluted solutions of the enzyme. Removal of mercaptoethanol was associated with an irreversible loss of both activities.

Table IV. *Transferase Specificity from Sucrose Centrifugation Fractions.*
Purified enzyme containing 0.6 mg/ml of protein was layered, centrifuged and fractioned as described in Materials and Methods. Fractions corresponding to each separated peak were pooled and each pool assayed for transferase activity in 0.3 ml samples. Results are expressed for each peak in relative percentage of pentosyl exchange by using inosine as donor an guanine-8-^{14}C as acceptor.

DONOR	GUANINE-8-^{14}C ACCEPTOR		
Moving:	Fastest	Intermed.	Slowest
Inosine	100	100	100
Deoxyinosine	91	42	89
Guanosine	99	121	77
Deoxyguanosine	142	51	90

Discussion

The purine nucleoside phosphorylase from human erythrocytes purified by the procedure described in this paper possesses both transferase and phosphorylase activities depending upon the absence or presence of phosphate in the testing procedures. Phosphorolytic specificity was similar to previously reported results (15), however, transferase reactions only showed interchange ability when guanine was used as acceptor and guanosine, inosine, deoxyguanosine and deoxyinosine as donors. No apparent inter-conversion of both activities was found when mercaptoethanol was removed or added to diluted solutions of the enzyme, and this is a major difference with the results reported for the Ehrlich tumor (11). Discrepancies between the results herein and those reported by PARKS *et al.* (8) can be explained by the different pH of the enzymatic assays (pH 7.2). Fur-

thermore transferase activity was tested spectrophotometrically by measuring in the absence of phosphate the rate of hypoxanthine liberation from inosine in the presence of guanine. Hypoxanthine is then determined by xanthine oxidase. Under these conditions the xanthine oxidase is inhibited by the guanine. Different pH and inhibition of the xanthine oxidase by guanine certainly have to influence the rate of the reaction.

The separation of different peaks with apparently different molecular weights and also slightly differing in the type of activity implies the following: 1) the aggregate sub unit nature of the enzyme, 2) the separation of these aggregates under very diluted conditions and 3) the presence ot both activities in the same enzyme. Although it is shown herein that the ratio of both activities in the light and heavy peaks can change after sucrose gradient centrifugation, no evidence of mayor specificity difference associated to distinct molecular aggregates was found. Such results may indicate the association of both activities with each molecular aggregate.

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