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PURIFICATION AND PROPERTIES OF RAT LIVER ADENINE PHOSPHORIBOSYLTRANSFERASE

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Summary

The adenine phosphoribosyltransferase (AMP : pyrophosphate phosphoribosyltransferase, EC 2.4.2.7) of rat liver was purified to a specific activity of 1.1 μmol of AMP formed per min per mg. The enzyme activity is associated with an apparently homogeneous protein as shown by isoelectrofocusing, acrylamide gel electrophoresis, and N-terminal amino acid analysis (phenylalanine). The molecular weight of the enzyme was estimated to be approx. 20 000 by acrylamide gel electrophoresis in the presence of sodium dodecylsulfate and by sucrose density gradient zone sedimentation. The rat liver enzyme exhibited initial burst synthesis of AMP when 1-pyrophosphorylribose 5-phosphate was added. The 1-pyrophosphorylribose 5-phosphate initial-burst activity copurifies with the adenine phosphoribosyltransferase activity. A pH optimum of 10.0 was demonstrable for the adenine phosphoribosyltransferase.

The initial-burst and steady-state phases of AMP synthesis catalyzed by highly purified rat liver adenine phosphoribosyltransferase have been partially characterized by the use of ligands which bind to sulfhydryl groups. Studies utilizing *p*-chloromercuribenzoate and $HgCl_2$ as inhibitors of AMP synthesis during the initial-burst and steady-state phases have revealed that sulfhydryl groups with different rates of ligand binding are present in the enzyme. The initial-burst phase was thereby delineated from the steady-state phase by use of these mercurial ligands. This delineation was also accomplished by titration with the Mg^{2+} chelator, EDTA. The inhibitory effects of mercurials and EDTA were reversed by β-mercaptoethanol and excess Mg^{2+}, respectively.

Quantitative binding studies with 5,5′-dithiobis(2-nitrobenzoic acid) and *p*-chloromercuribenzoate yielded values of 3.65 and 3.6 mol of sulfhydryl per

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mol of enzyme, respectively. 3.3 mol of cysteic acid per mol of performic acid-oxidized enzyme were found by amino acid analysis.

Introduction

It was observed in our laboratory that the addition of 1-pyrophosphoryl-ribose 5-phosphate (*PP*-ribose-*P*) and adenine to a dialyzed soluble fraction of rat liver homogenate resulted in AMP synthesis during two distinct reaction phases. An extremely rapid limited "initial-burst" synthesis of AMP was accompanied by a sustained steady-state synthesis of AMP [1,2]. Although several alternatives were considered, preliminary fractionation studies indicated that adenine phosphoribosyltransferase (AMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.7) was the catalyst for both reaction phases. This enzyme has been purified previously from several mammalian tissues [3—6] and microorganisms [7—9]. Murray et al. have reviewed some of the factors which regulate the activity of adenine phosphoribosyltransferase in rodent liver [10]. In none of these previous studies could we find a suitable explanation for the initial-burst reaction. A procedure was developed, therefore, for the extensive purification of rat liver adenine phosphoribosyltransferase to apparent homogeneity. Indentity of the catalyst responsible for the initial-burst synthesis of AMP with the catalyst responsible for steady-state synthesis of AMP was thereby established.

Similar results have recently been found by Thomas et al. [5] with purified human adenine phosphoribosyltransferase. The present study was also undertaken to provide further information on the two phases of the reaction and to investigate the effect of sulfhydryl reagents on the enzymatic reaction.

Materials and Methods

Materials

[8-^{14}C]Adenine (51.5 Ci/mol) was obtained from Nuclear Chicago Corporation. *PP*-Ribose-*P* (Mg^{2+} salt) and all nucleotides were obtained from P-L Biochemicals. Sephadex G-100 and DEAE-Sephadex A-50 were purchased from Pharmacia. Horse heart cytochrome *c* was obtained from Pierce Chemical, and bovine albumin from Armour. Isoelectrofocusing ampholines, pH range 5—8, were obtained from LKB. Sucrose was purified by adding 30 mg of acid-washed Norite to 1 l of a 50% (w/v) solution and heating the mixture at 95°C for 30 min. After filtration through Whatman No. 40 filter paper, the solution was stored frozen at —20°C. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) and *p*-chloromercuribenzoate (PCMB) were obtained from Sigma Chemical Company. PCMB was purified by precipitation in acid as described by Boyer [11]. Stock solutions of PCMB were prepared by dissolving the solid after precipitation in 1.0 ml of 1.0 M NaOH, addition of 19.0 ml 0.05 M K_2HPO_4 (pH 7.5) and adjustment to pH 7.5 with 2.0 M HCl. The concentration of the solution was calculated from the absorbance at 232 nm using an extinction coefficient of $1.69 \cdot 10^4$ $cm^{-1} \cdot M^{-1}$. Acrylamide and methylenebisacrylamide were obtained from Eastman Organic Chemicals and recrystallized from chloroform and acetone, respectively, as described by Loening [12].

Enzyme assays

Two modifications of the assay method of Hori and Henderson [3] were utilized. In the first modification the reaction mixture contained in 0.1 ml of final volume: Tris · HCl buffer (50 mM) at pH 7.5, $MgCl_2$ (2 mM), *PP*-ribose-*P* (0.2 mM), [8-^{14}C]adenine (0.05 mM), bovine albumin (0.005 mg), and a preparation of adenine phosphoribosyltransferase containing 0—1.0 milliunits of activity. After incubation for 3 min at 37°C, the reaction was stopped by the addition with mixing of 2 ml of water-saturated 1-butanol. Water (0.4 ml) was added with mixing. After phase separation had occurred, the butanol was removed by aspiration. The butanol extraction was repeated five additional times. Residual butanol was removed by ether extraction. The conversion of butanol-extractable [8-^{14}C]adenine into [8-^{14}C]AMP was measured by liquid scintillation spectrometry. In the second modification the purified enzyme was added to an initial mixture consisting of Tris · HCl buffer at pH 7.5 (50 mM), bovine plasma albumin (0.3 μM), *PP*-ribose-*P* (0.125 mM), $MgCl_2$ (2.5 mM), plus chemical modifiers as specified in Results, in a total volume of 0.6—2.4 ml. Reaction was initiated, after preincubation in some cases as specified, by the addition of 0.2 ml of the above mixture to 50 μl of a solution containing 0.25 mM [^{14}C]adenine in 0.05 M Tris · HCl, pH 7.5. In different experiments as explained in Results, $MgCl_2$, *PP*-ribose-*P*, or both were excluded from the initial mixture described above. In these cases the omitted substance(s) was included in the 50-μl [^{14}C]adenine solution at the appropriate concentration to yield final concentrations identical to the above. At various times following reaction initiation, 100-μl aliquots were removed and rapidly pipetted into tubes at 0°C containing 2.0 ml cold water-saturated butanol plus 0.4 ml distilled water. The tubes were immediately mixed and extracted six times as above. Protein was measured by the procedure of Lowry et al. [13] after precipitation and washing with 10% trichloracetic acid. 1 activity unit is defined as the conversion of 1 μmol [8-^{14}C]adenine to AMP per min at 37°C during the steady-state phase (Fig. 5). Potassium fluoride (50 mM) was added when the crude extracts were employed. In these assays selected samples were cochromatographed with carrier [8-^{14}C]AMP to confirm that AMP was the major radioactive compound produced. Greater than 95% of the ^{14}C present in every water sample after butanol extraction was recoverable as [8-^{14}C]AMP.

Analytical disc gel electrophoresis

Preparations of adenine phosphoribosyltransferase were examined by vertical analytical disc gel electrophoresis. The Tris/glycine system (pH 8.3) described by Davis [15] was used with the 7% gels. Other analytical gels (3%) were prepared containing 1% ampholytes (pH 5—8) as described by Florini et al. [16]. The gels were made up in glass tubes (0.5 × 7.5 cm), and a current of 3 mA per gel was maintained for approx. 60 min for the 7.0% gels and an initial current of 10 mA per gel for 6—7 h for the 3% gels. Gels were immersed in coomassie blue for 60 min and after destaining were scanned in a Gilford linear transport. When molecular weight determinations of the adenine phosphoribosyltransferase were made on acrylamide gels containing sodium dodecylsulfate, gel buffer and acrylamide solutions were prepared according to Weber and Osborn [17]. The protein samples were treated as described by Dunker and Rueckert [18].

Zone sedimentation in sucrose density gradients

Linear 5—20% (w/v) sucrose gradients were made as described by Martin and Ames [19]. The 4.5-ml gradients contained Tris · HCl buffer (50 mM, pH 7.5) and $(NH_4)_2SO_4$ (150 mM). To the top of each gradient was added 50 μl of a solution containing 30—50 μg of adenine phosphoribosyltransferase (isoelectrofocused Fraction 4a) and 0.5 mg of cytochrome *c*. After centrifugation at 4°C for 23 h at 35 000 rev./min, the bottoms of the tubes were punctured, and four-drop fractions were collected and assayed for adenine phosphoribosyltransferase as described above and for cytochrome *c* by its absorbance at 410 nm. Apparent molecular weights for adenine phosphoribosyltransferase were calculated with reference to cytochrome *c* (M_W = 12 000) [20].

Sulfhydryl content

The sulfhydryl content of the purified enzyme was determined by titrating a sample of the enzyme with PCMB and plotting the increase in absorbance at 250 nm against the amount of PCMB added; or by the addition of excess PCMB, measurement of the change in absorbance at 250 nm, and calculation of the sulfhydryl content using the extinction coefficient of 7600 $cm^{-1} \cdot M^{-1}$ [11].

Sulfhydryl content was also measured by reaction with DTNB as described by Ellman [21] using an extinction coefficient of 13 600 $cm^{-1} \cdot M^{-1}$ at 412 nm.

Half-cystine content

Half-cystine content was determined by amino acid analysis following performic acid oxidation according to the method of Moore [22].

Animo-terminal analysis

0.2—0.4 mg of highly purified enzyme preparations were precipitated with 10% trichloroacetic acid and washed thoroughly with the same precipitant. The protein pellet was dried in vacuo over H_2SO_4. The Edman degradation was carried out essentially as described by Mills et al. [23]. The thiazolinone derivative was extracted with chlorobutane and rearranged to the phenylthiohydantoin by heating at 60°C with 0.2 M acetic acid for 10 min. The number of identifiable amino termini was evaluated by thin-layer chromatography [24]. Identification was confirmed by gas chromatography [25].

Results

Purification of rat liver adenine phosphoribosyltransferase

All steps in the purification were carried out at 2—4°C, and all solutions contained 5 mM β-mercaptoethanol unless stated otherwise.

Crude extract. Four to six female rats (150—250 g) were fasted overnight. The rats were decapitated. The livers were removed, cut into small pieces, and homogenized with a teflon homogenizer in 3 vol. of 30 mM Tris · HCl buffer, pH 7.5. The homogenate was centrifuged for 90 min at 30 000 rev./min in a No. 30 rotor in the Spinco Model L ultracentrifuge. The supernatant solution contained essentially all of the activity in the homogenate.

Ammonium sulfate fractionation. The supernatant fraction of the homogenate was adjusted to 45% saturation by the addition of saturated ammonium sulfate prepared in Tris · HCl buffer, 50 mM, pH 7.5. After several hours the precipitate was collected by centrifugation and discarded. The ammonium sulfate concentration was then raised to 75% of saturation, and the precipitate was collected by centrifugation and dissolved in a minimal amount (approx. 10 ml) of 1 mM phosphate buffer, pH 7.0. This solution was desalted by passage through a column (4 × 30 cm) of Sephadex G-25, which had been previously equilibrated with 1 mM phosphate buffer, pH 7.0. The specific activity of the enzyme was increased approx. 3-fold by the procedure.

DEAE-Sephadex A-50 Chromatography. The desalted protein solution above was percolated at a flow rate of 1—3 ml/min through a column of DEAE-Sephadex A-50 (4 × 5 cm) which had been equilibrated with 1 mM phosphate buffer, pH 7.0. The column was washed with 250 ml of 1 mM phosphate buffer, pH 7.0, containing 5 mM $(NH_4)_2SO_4$. The column was then eluted with 1 mM phosphate buffer, pH 7.0, containing 150 mM $(NH_4)_2SO_4$ at a flow rate of 1 ml/min. The first 50 ml of eluant contained little adenine phosphoribosyltransferase activity. Most of the activity was eluted from the column in ml 50—100 of eluant. After precipitation of the enzyme activity with $(NH_4)_2SO_4$ (75% saturation), the precipitated protein was dissolved in approx. 4 ml of 1 mM phosphate buffer, pH 7.0, containing 150 mM $(NH_4)_2SO_4$ (Fraction 2, Table I).

Sephadex G-100 chromatography. Fraction 2 was layered on the top of a Sephadex G-100 column (2 × 140 cm) equilibrated with 1 mM phosphate buffer, pH 7.0, containing 150 mM $(NH_4)_2SO_4$. The flow rate of the column was maintained at 10—15 ml/h (Fig. 1). The adenine phosphoribosyltransferase activity in ml 200—230 was concentrated by precipitation with $(NH_4)_2SO_4$ (75% of saturation). After dissolving the protein in 1—2 ml of 50 mM Tris · HCl buffer, pH 7.5, the solution was dialyzed for 2 h against 2 l of 20 mM Tris · HCl buffer, pH 7.5, containing 20 mM $(NH_4)_2SO_4$ (Fraction 3, Table I).

TABLE I

PURIFICATION OF RAT LIVER ADENINE PHOSPHORIBOSYLTRANSFERASE

Fraction	Volume (ml)	Protein (mg)	Specific activity* (units per mg protein)	Yield (%)	Purification (-fold)	Ratio *PP*-ribose-*P* burst: steady-state reaction**
1. Crude supernatant fraction	114	2600	0.001	100	1.0	0.31
2. DEAE-Sephadex A-50	30	248	0.01	68	10.0	0.28
3. Sephadex G-100	30	28	0.04	33	40.0	0.26
4. Isoelectrofocused	5.2	0.63	1.10	4	1100.0	0.39
a. Peak tube (No. 49)***	1.3	0.31	1.12	2		
b. Side tubes (Nos 48, 50, 51)	3.9	0.32	1.08	2		

* Assays carried out as described in the text using between 0.05—0.5 milliunit of enzyme per ml. Initial wet weight liver = 32.5 g.

** See text for explanation,

*** Tube numbers refer to fractions obtained from isoelectrofocusing experiments presented in Fig. 2.

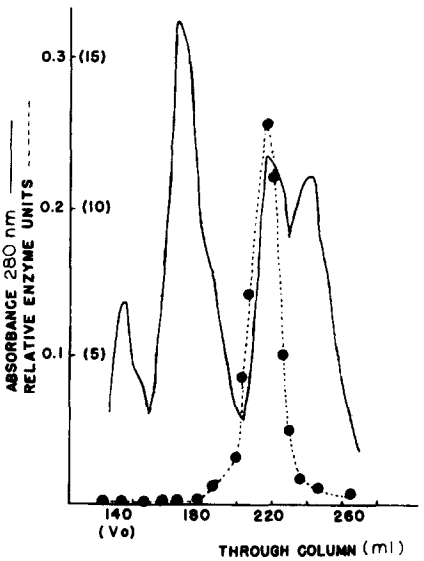

Fig. 1. Chromatography of rat liver adenine phosphoribosyltransferase on Sephadex G-100 (see text). The external volume (V_0) of the column was approx. 140 ml as predetermined with dextran blue. The effluent absorbance at 280 nm was measured in a Gilford 2000 modified spectrophotometer equipped with flow-through cuvettes (solid line). Assays for the enzyme activity (dotted line) which appeared in an effluent volume of 180—220 ml were carried out as described in Methods.

Isoelectrofocusing in an ampholyte pH gradient. An LKB electrofocusing column (110 ml capacity) was siliconized before use with Siliclad. The cathode was put on the bottom of the column, 1.5% ampholine solution (pH range 5—8) in a sucrose density gradient was used in the middle, and the anode was arranged at the top. β-Mercaptoethanol (5 mM) was included in the ampholine solution, otherwise the detailed directions in the instruction manual from LKB were followed in filling the column. Before adding Fraction 3 to the column, the ampholine gradient was prefocused for 12 h at 300 V and an additional 36 h at 500 V. A piece of 0.5 mm polyethylene tubing connected to a 5-ml syringe was then carefully inserted through the gradient to a predetermined point at which the pH of the solution was 5.6—5.7. 2 ml of the solution were drawn into the syringe. The polyethylene tubing was left inserted in the gradient, and Fraction 3 was combined with the ampholine solution in the syringe. Sufficient sucrose was added to Fraction 3 so that its density was equivalent to the ampholine solution. After adjusting the pH of the mixture to 5.6—5.7 with 0.1 M HCl, any precipitate which formed was removed by centrifugation. The mixture was then carefully reinserted into the ampholine gradient in the column. Electrofocusing of the column was then resumed for an additional 48 h at 500 V. The contents of the isoelectrofocusing column were drained at a flow rate of 1 ml/min using a Gilford Model 2000 spectrophotometer with flow-through cuvettes to monitor the regions of the gradient for protein (Fig. 2). Fractions of 1.3 ml were collected. Fractions containing adenine phosphoribosyltransferase activity were stored at 2°C. Several other methods of purification, including hydroxylapatite chromatography and $Ca_3(PO_4)_2$ gel absorption and elution of Fractions 2, 3 and 4 (Table I); preparative disc gel

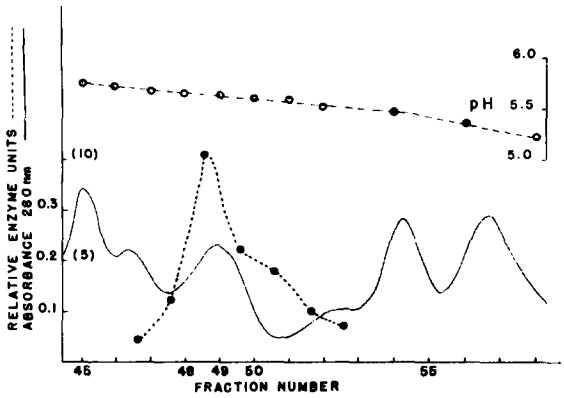

Fig. 2. Isoelectrofocusing of rat liver adenine phosphoribosyltransferase on a pH 5—8 sucrose gradient. The isoelectrofocusing was performed on an enzyme preparation after Sephadex G-100 chromatography. See the Results for a complete description of the procedure employed. After isoelectrofocusing the column contents were removed from the bottom and were monitored at 280 nm as in Fig. 1 (solid line). Only the pH region (5.25—5.75) of the gradient in close proximity to the enzyme activity (dotted line) is presented.

electrophoresis of Fraction 3 (as an alternative to isoelectrofocusing); and alumina Cγ absorption and elution of Fractions 2, 3 and 4, were ineffective as means of improving overall yield of enzyme activity or increased specific activity. The overall yield of enzyme activity can readily be increased to approx. 10% by taking wider cuts of the eluates from the Sephadex G-100 and isoelectrofocusing columns, however, preparations with lower specific activity and homogeneity result. Fig. 3 shows the protein pattern of Fractions 3, 4a and 4b after having been subjected to analytical disc gel electrophoresis at pH 8.3. Analytical 3% gels which were prepared containing ampholines in the pH range 5—8 yielded patterns for Fractions 4a and 4b similar to those of Fig. 3. Gels were scanned at 550 nm in a Gilford linear transport. Examination of the

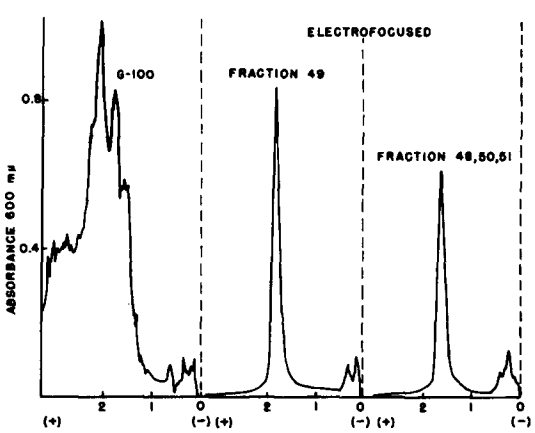

Fig. 3. Analytical polyacrylamide gel electrophoresis of rat liver adenine phosphoribosyltransferase at pH 8.3. Right: 40 μg of protein from the combined indicated side-tube fractions (48, 50, 51) from the isoelectrofocusing run presented in Fig. 2. Middle: 50 μg of protein from peak-tube fraction (49) (a single band was obtained at loads up to 200 mg of protein). Left: 300 μg of protein after Sephadex G-100 chromatography. (—): cathode; (+): anode.

protein pattern of Fractions 4a and 4b from these analytical gels indicates only one major band was present in the isoelectrofocused preparation. Duplicate unstained gels were divided into 2-mm sections with a Gilson Aliquogel fractionator; each section was collected in 0.5 ml of 50 mM Tris · HCl buffer, pH 7.5, and the sections were allowed to stand overnight at 2° C. Enzyme activity was recovered only in two sections possessing protein with the identical mobility of the band which appeared in the stained gel. The isoelectric point of the highly purified rat liver adenine phosphoribosyltransferase as determined in the isoelectrofocusing column is at pH 5.65 ± 0.03.

Enzyme stability. Purified adenine phosphoribosyltransferase as prepared in the isoelectrofocusing column had an average half-life of 2—4 weeks when stored in ice at 2—4° C. Both dialysis (against 20 mM Tris · HCl buffer, pH 7.5, containing 20 mM $(NH_4)_2SO_4$ plus 5 mM β-mercaptoethanol) and dilution accelerated inactivation of the enzyme.

Properties of the purified enzyme

Estimates of molecular weight. The isoelectrofocused adenine phosphoribosyltransferase was analyzed by zone sedimentation in a sucrose density gradient [19] using cytochrome *c* as the internal standard ($S_{20,w}$ = 1.9). The sedimentation coefficient of the adenine phosphoribosyltransferase was estimated to be 2.85 S. This sedimentation coefficient gave a calculated apparent molecular weight of 22 000.

The purified enzyme was also analyzed by disc gel electrophoresis in the presence of sodium dodecylsulfate. The single band of protein which appeared in these gels after staining had an apparent molecular weight of 17 500 when it was compared to internal standards of trypsin, avidin, and lysozyme [19].

Amino-terminal analysis. The highly purified enzyme preparations contained a single amino terminal which was identified as the phenylthiohydantoin (PTH) derivative of phenylalanine. Authentic phenylthiocarbamyl-phenylalanine (PTC-phenylalanine) migrated on thin-layer plates [24] and on the gas chromatograph [25] with an identical mobility to that prepared from adenine phosphoribosyltransferase. PTC-phenylalanine accounted for a minimum of 85% of the total PTH derivatives seen in both systems after a single round of the Edman degradation [23].

Apparent K_m values. Kinetic constants for adenine and *PP*-ribose-*P* were obtained for the liver adenine phosphoribosyltransferase after DEAE-Sephadex chromatography (Fraction 2) and after isoelectrofocusing (Fraction 4) [26]. K_m (*PP*-ribose-*P*) was 5 ± 2 μM and 5 ± 1 μM for Fractions 2 and 4, respectively. K_m (adenine) was 1 ± 0.3 μM and 0.8 ± 0.2 μM for Fractions 2 and 4, respectively. Three preparations of enzyme were used for these measurements. *PP*-ribose-*P* concentration was varied between 1 and 16 μM, and adenine was varied between 0.1 and 3 μM.

pH optima. The influence of pH upon the steady-state reaction between *PP*-ribose-*P* and adenine catalyzed by adenine phosphoribosyltransferase is presented in Fig. 4 (solid lines). The enzyme was active over a broad pH range increasing progressively in activity from pH 5.5 to 10. The *PP*-ribose-*P* burst reaction measured at 0° C, however, was relatively independent of pH under these conditions. Both Fractions 2 and 4 were evaluated and the same pH dependence was obtained with both fractions.

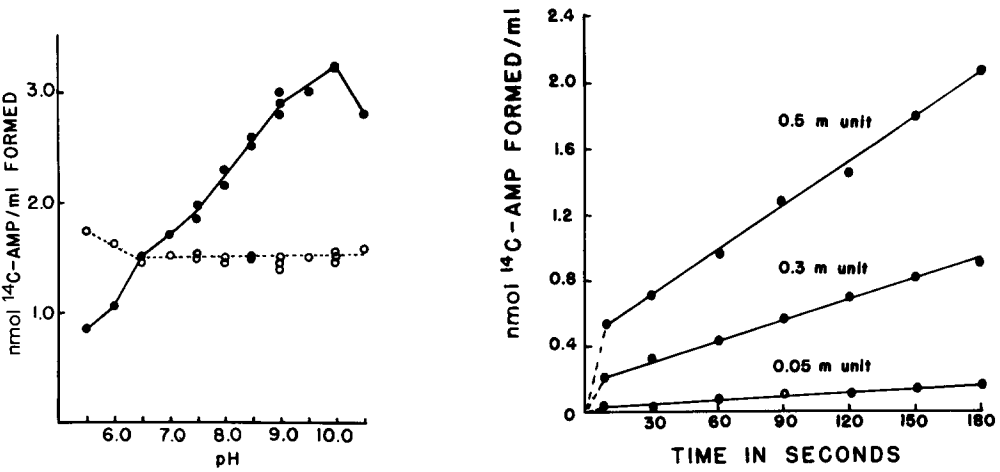

Fig. 4. Effect of pH upon adenine phosphoribosyltransferase activity (solid lines), and the *PP*-ribose-*P* initial burst (10 s at 0°C, dotted line). The *PP*-ribose-*P* initial-burst synthesis of [^{14}C] AMP was subtracted from the steady-state activity of each pH. Three overlapping buffers: Tris/maleate, pH 5.5—9.0; Tris/HCl, pH 7.5—9.5; and bicarbonate, pH 9.0—10.5; at a final concentration of 20 mM were used for the steady-state activity and the *PP*-ribose-*P* initial-burst activity. The enzyme was diluted with cold glass-distilled water immediately before use. The final concentration was 35 μg/ml of reaction mixture.

Fig. 5. Effect of enzyme concentration and time upon enzyme activity. Isoelectrofocused enzyme solution (0.25 mg/ml) was freshly diluted with 50 mM Tris · HCl (pH 7.5) containing 10 μg/ml of bovine albumin. The reaction was begun by the addition of appropriately diluted enzyme to reaction mixtures from which 100-μl aliquots were removed at the indicated times and assayed for [^{14}C] AMP as described in Methods. The concentration of enzyme per ml of reaction mixture is shown in milliunits.

Initial-burst kinetics observed under standard assay conditions. At all stages of purification of the rat liver enzyme, an initial burst of [8-^{14}C] AMP synthesis was obtained during the first 10 s of incubation. After the first 10 s, however, linear steady-state kinetics were observed for at least the next 180 s over a range of enzyme concentration between 0.05 and 0.50 milliunit of enzyme activity per ml (Fig. 5, Table I). The burst reaction was routinely measured at 0°C and is expressed as the nmol [8-^{14}C] AMP synthesized per milliunit of enzyme during the initial 10 s of incubation. "Zero" time controls (obtained by adding enzyme after first butanol addition) were routinely included and compared to "water blank" (no enzyme) controls. In every experiment no radioactive AMP was obtained in either type of control.

The identity of the initial-burst catalyst with adenine phosphoribosyltransferase (steady-state activity) was investigated by analysis of the two activities in individual fractions from purification runs in the isoelectrofocusing column (Fig. 6). The ratio of the initial-burst activity to the steady-state activity was relatively constant throughout the region in the gradient between ph 5.5 and 5.7.

In Table I the numerical value of the ratio of the initial-burst activity (10 s incubation at 0°C) to the steady-state activity (3 min incubation at 30°) are presented for all fractions obtained during enzyme purification. Similar fractional ratios were obtained with each enzyme fraction.

Effect of EDTA. Groth and Young [2] previously reported that although the steady-state synthesis of AMP from *PP*-ribose-*P* and adenine was Mg^{2+}

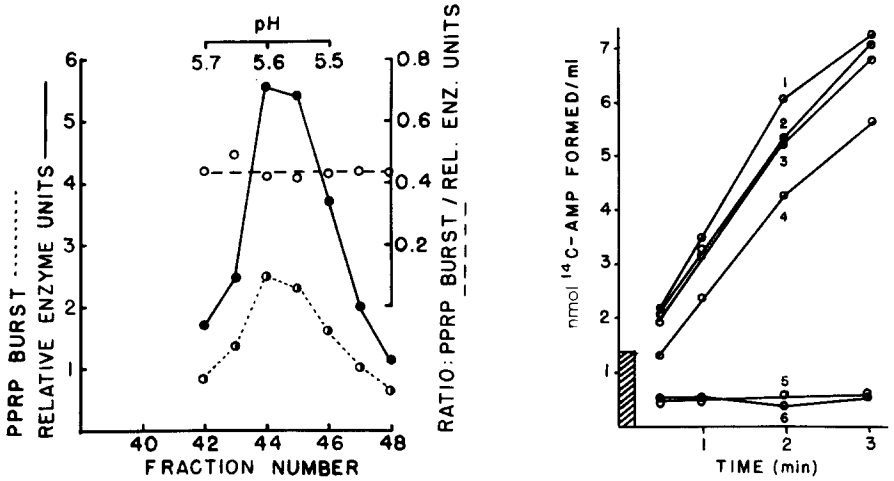

Fig. 6. Copurification by isoelectrofocusing of adenine phosphoribosyltransferase (steady-state) activity with the *PP*-ribose-*P* PPRP initial-burst activity. The isoelectrofocused fractions (42—48) containing the adenine phosphoribosyltransferase (solid line) were assayed individually for *PP*-ribose-*P* initial-burst activity (10 s at 0°C, dotted line) as described in the text. The ratio: *PP*-ribose-*P* initial burst per relative enzyme unit (dash-dotted line) has its ordinate numerals to the right of the figure. The pH region of the isoelectrofocused column is presented at the top.

Fig. 7. Titration with EDTA. Adenine phosphoribosyltransferase (1 μg) was preincubated for 1 min at 30°C in 450 μl of 50 mM Tris · HCl buffer, pH 7.5, containing the indicated concentrations of EDTA. AMP synthesis was initiated by the addition of 50 μl of a solution containing [8-^{14}C]adenine (0.25 mM), sodium *PP*-ribose-*P* (0.5 mM), and Tris · HCl, pH 7.5 (35 mM). At the indicated times of incubation at 30°C, 100 μl were removed and treated as described in Methods. 1, 0 mM EDTA; 2, 1 mM EDTA; 3, 2 mM EDTA; 4, 5 mM EDTA; 5, 8 mM EDTA; 6, 10 mM EDTA. The dashed bar is the AMP synthesized at 0°C (burst activity in the absence of EDTA).

dependent, the initial-burst synthesis of AMP showed no apparent Mg^{2+} requirement. To further investigate this difference an enzyme preparation was titrated with EDTA. Fig. 7 demonstrates that burst activity is still present at EDTA concentrations which completely eliminate steady-state activity. The burst activity (8—10 mm EDTA) was lower than the burst activity measured in the absence of EDTA. Addition of $MgCl_2$ to an enzyme pretreated with 10 mM EDTA resulted in essentially complete restoration of burst and steady-state activity to pretreatment levels. The Mg^{2+} concentration required to completely restore both activities was 10^{-2} M.

Effect of mercuric chloride. Mercuric chloride was used as a sulfhydryl modifier of the purified enzyme with respect to both the burst and steady-state phases of the reaction. The enzyme preparations used in all sulfhydryl-related studies were prepared as described above with the exception that β-mercaptoethanol was excluded from the dialysis solution in the step immediately preceding isoelectrofocusing and none was added to the isoelectrofocusing solutions. The activity and stability of these enzyme preparations was found not to differ significantly from those prepared in the presence of β-mercaptoethanol. The effect of $HgCl_2$ on the burst activity measured at 0°C is shown in Fig. 8 (top). The inhibitory effect of $HgCl_2$ was essentially complete in 15 s. The effect of identical concentrations of $HgCl_2$ on the steady-state phase of the reaction (Fig. 8, bottom) demonstrated that $HgCl_2$ addition decreased the

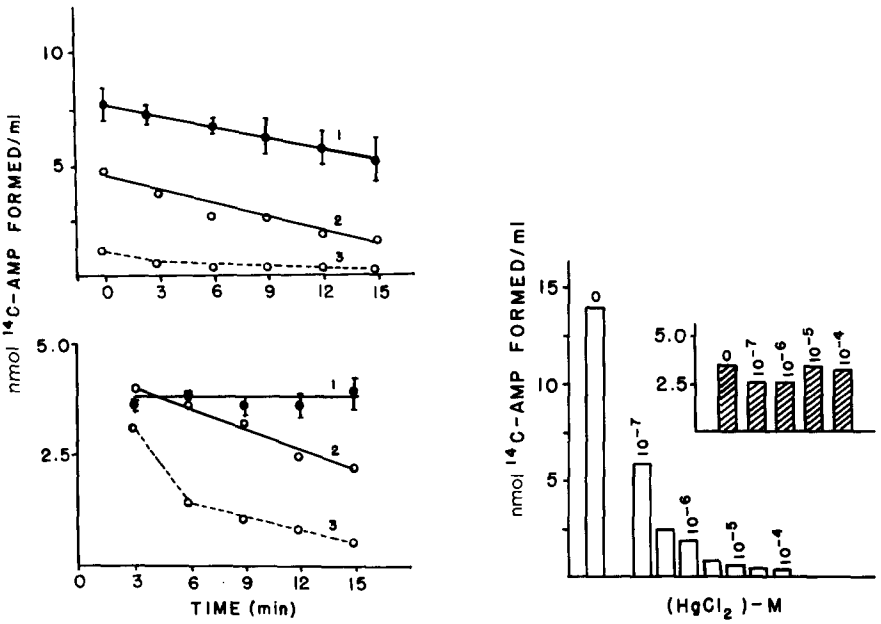

Fig. 8. Effect of mercuric chloride. Top: Inhibition of initial-burst activity. Adenine phosphoribosyltransferase (20 μg) was added to a preincubation mixture (final volume = 2.4 ml) containing 50 mM Tris · HCl buffer (pH 7.5), bovine plasma albumin (0.3 μm) and $HgCl_2$ at the indicated concentrations. At the preincubation time indicated as abscissas, 200-μl samples were removed and added to incubation tubes containing 50 μl of a mixture of 0.01 M $MgCl_2$, 0.5 mM *PP*-ribose-*P* and 0.3 mM [8-^{14}C]adenine. After 30 s, duplicate samples of 100 μl containing 0.67 μg (0.34 nmol enzyme per ml) were removed from incubation tubes and treated as in Methods. All operations were carried out at 0°C. 1, 0.0 M $HgCl_2$ (control) (●———●); 2, $1 \cdot 10^{-7}$ M $HgCl_2$ (○———○); 3, $1 \cdot 10^{-5}$ M $HgCl_2$ (○- - - - -○). Bottom: Inhibition of steadystate activity. As above except that 0.17 μg of enzyme was used per ml. Preincubation (abscissas) was performed at 0°C. Steady-state AMP synthesis was measured for 3 min at 30°C.

Fig. 9. Effect of mercuric chloride at different concentrations. Enzyme was preincubated with $HgCl_2$ for 3 min at 0°C. Initial-burst (open bars) and steady-state (dashed bars) AMP synthesis was measured as in the legend to Fig. 8. Enzyme concentrations are the same as in Fig. 8.

steady-state activity at a slower rate than that observed for the burst phase. Fig. 9 compares the activity of the burst and steady-state phases of the reaction in the presence of varying concentrations of $HgCl_2$. Addition of *PP*-ribose-*P* and/or $MgCl_2$ to the preincubation mixture at concentrations normally used in the assays had no effect on the observed rate of inactivation.

Effect of PCMB. PCMB was also used to study sulfhydryl related inactivation of adenine phosphoribosyltransferase. Rapid deactivation of the burst phase of the reaction was observed (Fig. 10, top). However, in the presence of *PP*-ribose-*P* and $MgCl_2$ at concentrations used in the regular assays some protection was obtained against PCMB inactivation. Similar protection was also observed with respect to the steady-state phase of the reaction (Fig. 10, bottom).

Reversal of mercurial inactivation by β-mercaptoethanol. Fig. 11 shows the effect of β-mercaptoethanol addition upon (the burst activity of) adenine phosphoribosyltransferase pretreated with $HgCl_2$ or PCMB. Burst activity was completely restored by β-mercaptoethanol with a PCMB-treated enzyme preparation and 80% of the original activity was recovered with a $HgCl_2$-treated enzyme preparation.

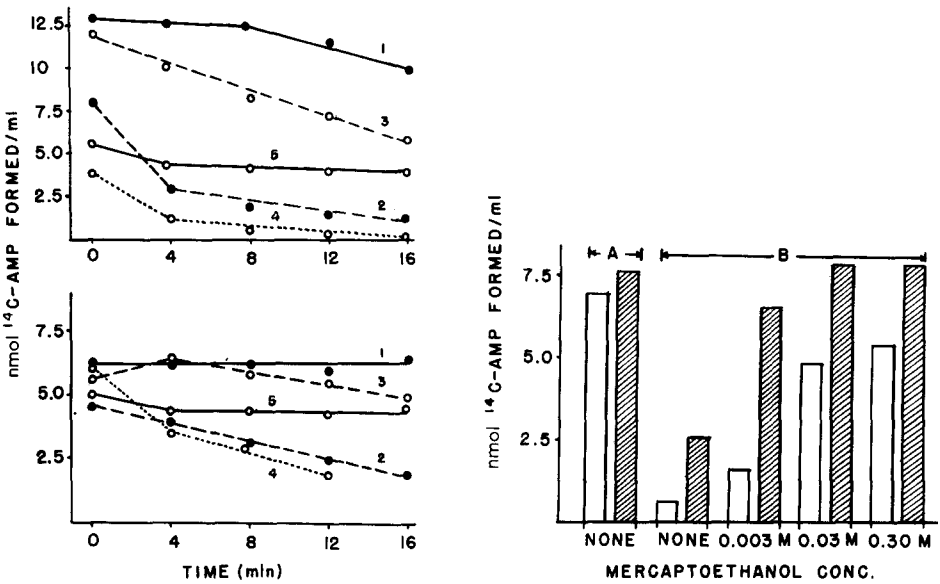

Fig. 10. Effect of PCMB. PCMB inhibition of initial-burst (top), and steady-state (bottom) AMP synthesis was measured as in Fig. 8. *PP*-ribose-*P* (0.125 mM) and $MgCl_2$ (2.5 mM) were included in the preincubation mixtures which were as follows: 1, 0.0 M PCMB (control) with or without *PP*-ribose*P* and $MgCl_2$ (●——●); 2, $0.8 \cdot 10^{-7}$ M PCMB (●- - - - -●); 3, $0.8 \cdot 10^{-7}$ M PCMB plus *PP*-ribose-*P* plus $MgCl_2$ (○- - - - -○); 4, $1 \cdot 10^{-6}$ M PCMB (○· · · · ·○); 5, $1 \cdot 10^{-6}$ M PCMB plus *PP*-ribose-*P* plus $MgCl_2$ (○——○).

Fig. 11. Reversal of $HgCl_2$ and PCMB inhibition of the burst reaction by β-mercaptoethanol. Adenine phosphoribosyltransferase was preincubated for 3 min at 0° C either with $1 \cdot 10^{-5}$ M $HgCl_2$ or $8.0 \cdot 10^{-4}$ M PCMB as in Figs 8 and 10. β-Mercaptoethanol at the indicated concentrations was added. After an additional 2 min 200-μl samples were added to 50 μl of a solution containing 0.01 M $MgCl_2$, 0.5 mM *PP*-ribose-*P*, and 0.25 mM [8-^{14}C]adenine. Initial-burst AMP synthesis was measured as before. ←A→ (NONE) control enzyme mixtures to which neither mercurial nor β-mercaptoethanol have been added. (Clear bar: control for $HgCl_2$; dashed bar: control for PCMB). ←B→ (NONE) β-mercaptoethanol has not been added but either $HgCl_2$ (clear bar) or PCMB (dashed bar) has been added. ←B→ 0.003 M, etc.: As above except that β-mercaptoethanol has been added.

Sulfhydryl content of purified enzyme. Sulfhydryl content as determined by the method of Boyer [11] is presented in Fig. 12. The enzyme preparation was dialyzed for 6 h against 0.02 M Tris · HCl containing 0.02 M $(NH_4)_2SO_4$, pH 7.5, It was then made 75% saturated with respect to ammonium sulfate and allowed to precipitate overnight. The precipitate was collected by centrifugation at 10 000 × *g* for 10 min and resuspended in 0.5 ml of 0.05 M K_2HPO_4, pH 7.5.

Fig. 12 shows the effect of enzyme titration with PCMB. A sharp break in the curve was observed at 3.39 mol added PCMB per mole of enzyme. When excess PCMB was added and the thiol content was calculated as described in Methods, an average value of 3.65 mol thiol per mol of enzyme was obtained.

Sulfhydryl content was also measured with DTNB. In initial studies the enzyme was dialyzed for 6 h against 1.0 l of 0.01 M K_2HPO_4, pH 7.0. Urea was added to certain samples to a final concentration of 4.9 M. Excess DTNB was added and the change in absorbance at 412 nm monitored in a Gilford Model 2000 recording spectrophotometer. Fig. 13 shows the results of such experiments. Complete reaction is shown to be significantly slower in the absence of

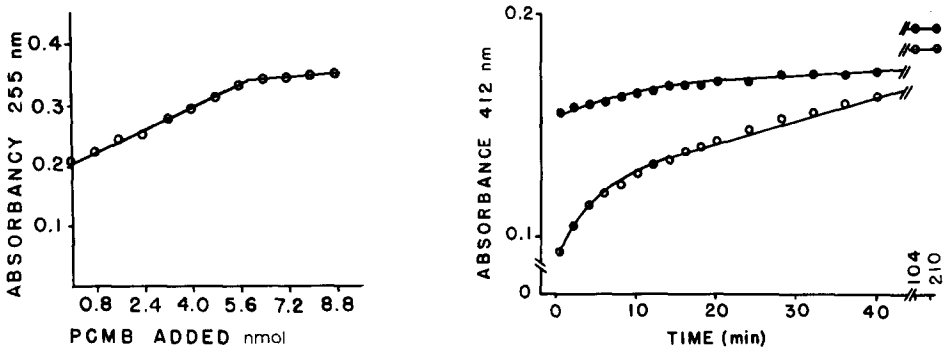

Fig. 12. Sulfhydryl titration with PCMB. The reaction mixture contained, in a final volume of 0.30 ml, 1.71 nmol enzyme protein and 15 μmol of potassium phosphate buffer, pH 7.5. PCMB was added in 0.8-nmol increments. The absorbance at 255 nm was measured 15 min after each addition. The plotted values are corrected for volume changes. The absence of contaminating low-molecular sulfhydryl compounds was demonstrated by the lack of increase in 255-nm absorbance when PCMB was added to a concentrated enzyme dialysate.

Fig. 13. Sulfhydryl determination with DTNB. Reaction mixtures contained initially 1.28 nmol enzyme protein in a volume of 0.3 ml. Solid urea (108 mg) was added to half of the reaction mixtures (final urea concn = 4.9 M). 10 μl of 0.01 M DTNB were added and the increase in absorbance at 412 nm was determined. Corrections were made for the absorbance contributed by the enzyme and DTNB solutions. ●———●, urea-treated enzymes; ○———○, non-urea-treated enzyme.

urea. Calculations utilizing the maximal values as shown in the figure indicate the presence of 3.6 mol sulfhydryl per mole of enzyme based on data from the urea-treated sample and a value of 3.11 mol sulfhydryl per mole of enzyme based on the non-urea-treated enzyme. Non-dialyzed enzyme was also subjected to this type of analysis and yielded essentially identical results.

The values obtained with both PCMB and DTNB compare favorably with a value of 3.3 cysteic acid residues obtained by amino acid analysis after performic acid oxidation. These results indicate the absence of any disulfide linkages in the molecule.

Discussion

Our procedure for the purification of rat liver adenine phosphoribosyltransferase resulted in a preparation which appeared homogeneous when analyzed by several technics. Previously published procedures for the purification of adenine phosphoribosyltransferase from Ehrlich ascites cells [3], from *Bacillus subtilis* [8], from *Escherichia coli* [9], and from the human red cell [4—6] all yielded preparations of reportedly high specific activity (0.4—14 units per mg protein). The overall yield of active enzyme was 4% with large losses of activity occurring in each step of the procedure. The unstable nature of adenine phosphoribosyltransferase during Sephadex chromatography has been noted earlier [3,4]. After isoelectrofocusing the rat liver enzyme has a half-life of 2 weeks at 4°C when stored in the electrofocusing solution. Dialysis, pH adjustment, or the addition of $MgCl_2$ and *PP*-ribose-*P* did not significantly increase the observed half-life. The isoelectrofocused enzyme slowly formed insoluble and inactive aggregates during storage. Since the ratio of the burst activity to

the steady-state activity (Table I), apparent K_m values, and pH optima (Fig. 4) remained relatively constant during purification, it appears that the isoelectrofocused fraction of the enzyme was representative of the total activity present in the whole liver. The isoelectrofocused rat liver adenine phosphoribosyltransferase has a specific activity of approx. 1.1 units per mg protein. The apparent homogeneity of our preparations, as evidenced by disc gel electrophoresis on buffered gels, gels containing ampholytes, and on gels containing sodium dodecylsulfate, has allowed us to coidentify the steady-state activity catalyzed by the phosphoribosyltransferase with the *PP*-ribose-*P* burst reaction. The determinations of apparent molecular weight as measured by sucrose density gradient sedimentation or by the sodium dodecylsulfate polyacrylamide gel electrophoresis technics, were 22 000 and 17 500, respectively. This observed molecular weight for the rat liver enzyme is considerably smaller than that reported by Berlin for the preparation from *B. subtilis* [8], that reported by Hochstadt-Ozer and Stadtman from *E. coli* [9] and that reported for the human red cell preparations [4—6]. The catalytic constant of adenine phosphoribosyltransferase at 37°C and pH 7.5 was calculated to be approx. 20 per min during the steady-state reaction. It is of interest to compare this small catalytic constant of the rat liver enzyme with the catalytic constant of other highly purified phosphoribosyltransferases, *N*-1-(5′-phosphoribosyl)-ATP synthetase and the adenine phosphoribosyltransferase from *E. coli.* The catalytic constant that can be calculated for the synthetase from the data of Voll et al. [27] is approx. 8 per min, and the turnover number for the *E. coli* adenine phosphoribosyltransferase was calculated to be 560 per min. The synthetase has been shown to function via an enzyme-bound phosphoribosyl group by Bell and Koshland [28,29].

The catalytic constant of the rat liver adenine phosphoribosyltransferase is undoubtedly much higher during the initial-burst reaction, however, accurate rate measurements for the initial-burst reaction have not yet been obtained in our laboratory.

Our earlier report [2] of two phases in the reaction sequence of AMP synthesis catalyzed by adenine phosphoribosyltransferase have been reinforced by the results reported here as well as by the recent report of Thomas et al. [5]. Studies using sulfhydryl ligands indicate that definite differences in effect upon burst-phase inactivation as compared with steady-state-phase inactivation are seen with both $HgCl_2$ and PCMB. Removal of endogenous $MgCl_2$ by EDTA titration also demonstrated a delineation between the burst and steady-state phases.

We previously reported [2] that our results were consistent with a mechanism involving a covalent enzyme-substrate intermediate. Attempts to demonstrate such an intermediate by use of ^{14}C-labeled *PP*-ribose-*P* or by the demonstration of a ^{32}P-labeled pyrophosphate exchange into *PP*-ribose-*P* in the absence of adenine and AMP were not successful. These experiments did, however, provide evidence that burst activity was not due to contamination with varying levels of pyrophosphatase activity. ^{32}P-labeled monophosphate did not accumulate as a product when ^{32}P-labeled pyrophosphate was incubated with the purified adenine phosphoribosyltransferase.

Both the rat liver and the human red cell adenine phosphoribosyltrans-

ferase preparations catalyze AMP synthesis in two distinct reaction phases: an initial-burst phase which is followed by a steady-state phase. The rat liver enzyme studied here, however, differs from the human enzyme studied by Thomas et al. [5]. The isoelectric points are 5.65 and 4.8 and the apparent molecular weights are 20 000 and 34 000, respectively. In addition sodium dodecylsulfate gel electrophoresis has indicated that the human enzyme may be a trimer consisting of subunits with a molecular weight of 11 000. We have not found any evidence for subunits in the rat liver enzyme.

Acknowledgments

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