Phosphoramidates. V. Probable Identity of Rat Liver Microsomal Glucose 6-Phosphatase, Phosphoramidase, and Phosphoramidate–Hexose Phosphotransferase*

R. Parvin and Roberts A. Smith

ABSTRACT: The microsomal fraction from rat liver and from kidney exhibited phosphoramidase and phosphoramidate–hexose phosphotransferase activities. A comparison of these two enzyme activities with that of glucose 6-phosphatase revealed that all three activities behaved alike under a variety of conditions. Evidence suggesting the involvement of a single enzyme catalyzing these different activities was also obtained by additive experiments and by kinetic data showing mutual competitive inhibitions.

Formation of phosphoryl–enzyme from ^{32}P-labeled glucose 6-phosphate supports the suggestion of a phosphoryl–enzyme intermediate in the over-all reaction mechanism.

It was reported previously that *Escherichia coli* extract had an enzyme that in addition to hydrolyzing phosphoramidate also catalyzed phosphotransferase reactions with relatively low concentrations of glucose as acceptor (Fujimoto and Smith, 1962). Subsequently Holzer *et al.* (1966) found that the microsomal fraction of liver and kidney also possessed similar enzymatic activities and suggested that these enzymatic activities were unrelated to the microsomal glucose 6-phosphatase activity which had been reported to catalyze pyrophosphatase and pyrophosphate glucose phosphotransferase reactions also (Stetten and Taft, 1964; Nordlie and Arion, 1964). Our recent work on the characterization of the microsomal phosphoramidase and phosphoramidate hexose phosphotransferase activities shows that both activities are catalyzed by one enzyme, which, contrary to the suggestion of Holzer *et al.* (1966), now appears identical with the microsomal glucose 6-phosphatase in its properties. The presumed common identity of these enzymatic activities is supported by several lines of evidence including kinetic parameters as well as similar responses to different treatments of the active preparation.

A reaction mechanism has been proposed for glucose 6-phosphatase (Segal, 1959; Hass and Byrne, 1960; Arion and Nordlie, 1964) involving formation of an enzyme–phosphoryl–substrate complex which dissociates leaving a phosphoryl–enzyme intermediate, the phosphoryl group of which is transferred either to water (hydrolysis) or to glucose or other acceptors (phosphotransferase). Evidence for the formation of the phosphoryl–enzyme from ^{32}P-labeled glucose 6-phosphate is described here.

Experimental Section

Materials. Phosphoramidate was prepared by the method of Stokes (1893). ^{32}P-Labeled phosphoramidate was prepared as described earlier (Holzer *et al.*, 1962). Glucose-6-^{32}P[1] was prepared from γ-labeled AT^{32}P by incubating with glucose, Mg^{2+} and purified hexokinase. Glucose-6-^{32}P was separated on a DEAE-cellulose column using a linear gradient from 0 to 0.3 M triethylammonium bicarbonate (pH 8.0). Labeled pyrophosphate was prepared by pyrolysis (Bergmann, 1962) and purified on a DEAE-cellulose column in the bicarbonate form with a linear gradient (0–0.35 M) of triethylamine bicarbonate (pH 7.5). Glucose 6-phosphate, yeast glucose 6-phosphate dehydrogenase, Nessler's reagent, and TPN^+ were Sigma Products.

Enzyme Preparation. Rat liver homogenates (10% w/v in 0.25 M sucrose containing 0.001 M EDTA) were prepared in a Potter-Elvehjem homogenizer. The microsomal fraction sedimenting between 12,500*g* (10 min) and 105,000*g* (1 hr) was suspended in 0.25 M sucrose, containing 0.001 M EDTA and 0.005 M mercaptoethanol. Part of the preparation not used for immediate work was divided in small batches and stored at −20° until further use. Gradual loss in enzyme activity was noticed on storing the microsomes at 4 or −20°. Repeated freezing and thawing affected the enzyme activities considerably.

Enzyme Assays. The glucose 6-phosphatase assay system in a final volume of 0.5 ml contained, 0.1 M Tris-maleate (pH 6.5), 20 mM glucose 6-phosphate, and microsomal protein. Reaction was stopped with 0.1 ml of 12% trichloroacetic acid. P_i was estimated either by the method of Marsh (1959) or by a modification of the Michaelsen method (Parvin and Smith, 1969).

The phosphoramidase assay system in a final volume of 0.5 ml contained, 0.1 M Tris-maleate (pH 6.5),

* From the Chemistry Department, Biochemistry Division, University of California at Los Angeles, Los Angeles, California 90024. *Received June 26, 1968.* This work was supported by a grant from the U. S. Public Health Service (GM 13407) and by the University of California Committee on Cancer Research.

[1] Glucose-6-^{32}P refers to ^{32}P-labeled glucose 6-phosphate.

20 mM phosphoramidate, and microsomal protein. At the end of the incubation period the reaction was terminated with an equal volume of ethanol. After mixing, aliquots were made alkaline and liberated ammonia was trapped in 0.5 ml of saturated boric acid. Either a Conway microdiffusion dish or test tube with a self-sealing serum stopper with an attachment to hold a boric acid trap was used. Ammonia trapped in boric acid was estimated using Nessler's reagent containing KCN according to Minari and Zilversmith (1963) except that the volume of Nessler's reagent was reduced to one-fifth. Although the 20 mM phosphoramidate concentration employed in some experiments was below the saturating level, the initial reaction rate was linear for up to 20-min incubation. At still greater phosphoramidate levels, the blank value of ammonia obtained in controls became undesirably high.

For the phosphoramidate–hexose phosphotransferase assay, glucose was used as acceptor. The reaction was followed spectrophotometrically at 340 mμ by coupling with glucose 6-phosphate dehydrogenase. Cuvets with a 1-cm light path contained, in 1.0 ml, 0.1 M acetate or Tris-maleate (pH 5.5 or 6.5), 0.2–0.4 M glucose, 20 mM phosphoramidate, 0.5 mM TPN^+, 1 unit of glucose 6-phosphate dehydrogenase, and microsomal protein. In some experiments reaction was first stopped with trichloroacetic acid, protein was centrifuged, and the pH of the supernatant fluid was brought to 7.5 by adding a predetermined amount of alkali. Glucose 6-phosphate was measured by the formation of reduced TPNH after incubating with glucose 6-phosphate dehydrogenase and TPN^+ for 30 min at 30°. Pyrophosphate–hexose phosphotransferase assay was similar to phosphoramidate–hexose phosphotransferase assay except that pyrophosphate was substituted for phosphoramidate.

The buffer used was adequate to maintain the pH and there was no change in pH at the end of incubation period. All enzyme incubations were for 10 min at 30° unless otherwise mentioned.

Protein was estimated by the differential absorbancy Biuret method (Parvin *et al.*, 1965).

Results

Activities in Liver and Kidney Microsomes. The data in Table I show the specific activities of phosphoramidase, phosphoramidate–hexose phosphotransferase, and glucose 6-phosphatase. As may be seen for both the liver and kidney microsomal preparations, the relative ratios of the three enzymatic activities were nearly the same. As also seen from Table I, the kidney microsomal preparations were nearly as active as liver microsomes in exhibiting glucose 6-phosphatase activity. This observation is in agreement with the work of Nordlie and Arion (1965) and Freedland (1962).

Acid Inactivation. Glucose 6-phosphatase is known to exhibit considerable activity even at 0° (Stetten and Taft, 1964), and this was found to be true for phosphoramidase and phosphoramidate–hexose phosphotransferase activities as well. Here again, the relative ratios of the three enzyme activities remained constant up to 5-hr incubation. Microsomal glucose 6-phosphatase is a very labile enzyme; complete loss of catalytic activity results by warming microsomal preparations for 10 min at 30° at pH 5 in the absence of glucose 6-phosphate. Since acid phosphatase remains stable under these conditions (De Duve and Heaufays, 1951), this method has been used for determining the extent of glucose 6-phosphate hydrolysis due to glucose 6-phosphatase and to acid phosphatase in different preparations (Freedland, 1962). The effect of such acid pH treatment on phosphoramidase, on phosphoramidate–hexose phosphotransferase, and on glucose 6-phosphatase was studied and the results in Table II show that like glucose 6-phosphatase both phosphoramidase and phosphoramidate–hexose phosphotransferase activities were also very labile to low pH. All three activities were reduced by this acid treatment to about the same relative extent.

TABLE I: Specific Activities of Phosphoramidate–Hexose Phosphotransferase, Phosphoramidase, and Glucose 6-Phosphatase.[a]

Enzymic Activity	Liver Microsomes	Kidney Microsomes
Phosphoramidate–hexose phosphotransferase	0.079	0.074
Phosphoramidase	0.181	0.160
Glucose 6-phosphatase	0.131	0.126

[a] All assays were carried out at pH 6.5 as described in Methods; 0.4 M glucose was used in phosphoramidate–hexose phosphotransferase assay. Enzyme activities are expressed as micromoles of product formed per minute per milligram of protein.

Effect of Prior Alkaline Treatment on Enzyme Stability and Affinity of Enzyme. Recently Stetten and Burnett (1966) reported that prior exposure of microsomes to pH 9.5–9.8 led to increased glucose 6-phosphatase, pyrophosphatase, and pyrophosphate–glucose phosphotransferase activities. This effect of alkaline treatment on glucose 6-phosphatase was confirmed and found to apply for phosphoramidase and phosphoramidate–hexose phosphotransferase activities as well (Table III). It was also reported that prior alkaline treatment had a stabilizing effect on glucose 6-phosphatase activity, as the susceptibility of this enzyme to inactivation by varying pH treatment was reduced if the microsomes were first exposed to an alkaline pH of 9.5–9.8. The data shown in Figure 1a–c reveal that such prior alkaline treatment of the microsomes stabilized phosphoramidase, phosphoramidate–hexose phosphotransferase, and glucose 6-phosphatase activities in a similar way.

Stetten and Burnett (1966) further noted that alkaline treatment considerably reduced the Michaelis constant of glucose 6-phosphatase for glucose 6-phosphate. As seen from Figure 2, the affinity of microsomal phosphoramidase for phosphoramidate was also

TABLE II: Inactivation of Glucose 6-Phosphatase, Phosphoramidase, and Phosphoramidate–Hexose Phosphotransferase by pH 5.0 Treatment.[a]

Activity	μmoles/mg of Protein		
	Untreated	pH 5.0 Pretreated	Untreated/ pH 5.0 Pretreated
Glucose 6-phosphatase	0.625	0.025	25.0
phosphoramidase	0.94	0.037	25.5
Phosphoramidate–hexose phosphotransferase	0.48	0.020	24.0

[a] Where pretreated, microsomes were exposed to pH 5.0 for 10 min at 30° and then assayed. All assays were performed at pH 6.5 under standard assay conditions as given under enzyme assay. Glucose (0.2 M) was used in Phosphoramidate hexose phosphotransferase assay.

TABLE III: Enhancement of Catalytic Activities as a Result of Alkaline Treatment.[a]

	Before pH 9.7 Exposure	After pH 9.7 Pretreatment	Untreated/ Alkaline Treated
Glucose 6-phosphatase	0.46[b]	0.63	1.37
Phosphoramidase	0.62	0.88	1.40
Phosphoramidate–hexose phosphotransferase	0.39	0.51	1.30

[a] For alkaline treatment microsomes were brought to pH 9.7 by adding glycine–KOH buffer and maintained for 15 min at 0°. All assays were then carried out at pH 6.5 as outlined in the text. Glucose (0.2 M) was used in phosphoramidate hexose phosphotransferase assay. [b] μmoles/mg of protein.

affected by prior alkaline treatment in an analogous manner; the K_m value for phosphoramidate decreased from an initial 13–2.8 mM after prior alkaline (pH 9.7) exposure of the preparation.

Molybdate Inhibition. Since molybdate ions at low concentration inhibit glucose 6-phosphatase (Nordlie and Arion, 1964), it was of interest to compare the effects of varying molybdate concentration on the inhibition of glucose 6-phosphatase and pyrophosphate–hexose phosphototransferase activities with that of phosphoramidase and phosphoramidate phosphohexose transferase activities. As is evident from the results obtained (Figure 3), all four enzymatic activities were inhibited in a parallel manner at varying molybdate levels.

Kinetic Studies. The foregoing results show that rat liver microsomal phosphoramidase, phosphoramidate–hexose phosphotransferase, and glucose 6-phosphatase activities all behaved alike under different experimental conditions, suggesting that the same or very similar enzymes are responsible for these activities. If the enzyme catalyzing these reactions is identical, then both substrates, that is, phosphoramidate and glucose 6-phosphate, may be expected to combine with the same site on the enzyme. Thus, if the hydrolysis of phosphoramidate were being followed by the measurement of liberated ammonia, then the presence of glucose 6-phosphate should competitively inhibit the phosphoramidase reaction, since hydrolysis of both substrates would be expected to involve the same site on the enzyme. Results of such an experiment are presented in Figure 4. As seen from the Dixon (1953) plot (Figure 4) glucose 6-phosphate competitively inhibited the phosphoramidase activity.

As mentioned earlier it is now known from the work of Nordlie and Arion (1964) and of Stetten and Taft (1964) that microsomal glucose 6-phosphatase acts also as a pyrophosphatase and as a pyrophosphate phosphotransferase. Therefore, the presence of pyrophosphate also may be expected to competitively inhibit the phosphoramidase reaction, and the results obtained (Figure 5) are fully in accord with this view, as the pyrophosphate inhibition of phosphoramidase was similar to the inhibition by glucose 6-phosphate described above. Similarly if phosphoramidase and the phosphoramidate–hexose phosphotransferase activities are also associated with the same enzyme, then pyrophosphate should inhibit the phosphoramidate–hexose phosphotransferase activity as well. For these experiments, the formation of ^{32}P-labeled glucose 6-phosphate from ^{32}P-labeled phosphoramidate was determined in the presence and absence of pyrophosphate. The double-reciprocal plots of the results obtained (Figure 6) show a competitive inhibition of phosphoramidate–hexose phosphotransferase activity by pyrophosphate.

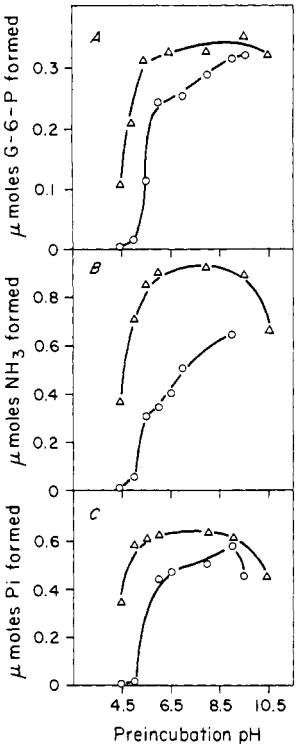


FIGURE 1: Effect of alkaline exposure of microsomes on the stability of phosphoramidate-hexose phosphotransferase (A), phosphoramidase (B), and glucose 6-phosphatase (C). Microsomal preparation (2 ml) was brought to pH 9.7 by the addition of 0.1 ml of 0.3 M glycine-KOH buffer. In the control water was added in a place of glycine-KOH buffer. After incubating for 10 min at 0°, 25 μl of enzyme aliquots was added to tubes containing 8 μmoles of desired buffer (from pH 4.5 to 10.5) and H_2O to give 0.2 ml of final volume. In a duplicate set, the final pH in each tube was checked at each stage. Following 30-min incubation at 30° reaction was started by the addition of a preincubated assay system mixture, containing 0.2 M Tris-maleate (pH 6.5), 20 mM of either glucose 6-phosphate (glucose 6-phosphate assay) or phosphoramidate (phosphoramidase and phosphoramidate-hexose phosphotransferase assay), and 0.2 M glucose (phosphoramidate-hexose phosphotransferase assay). Reaction was terminated after 10 min and glucose 6-phosphate, P_i, or NH_3 was estimated as described in the text. (○—○—○) With microsomes not exposed to pH 9.7 and (△—△—△) using microsomes previously exposed to pH 9.7.

Since prior exposure of microsomes to alkaline pH stabilizes the glucose 6-phosphatase and gives consistently reproducible kinetic constants, such preparations were used to obtain this data. It may be pointed out that the K_m values obtained by using these alkaline pH-treated microsomes were lower (Figure 2) than those obtained using fresh microsomes as also noted by Stetten and Burnett (1966, 1967). Further the K_m values thus obtained were comparable with those reported by previous workers using either detergent-treated microsomes (Nordlie and Arion, 1964) or by assaying in the presence of detergent (Stetten and Taft, 1964). The Michaelis constant for the hydrolase activity for phosphoramidate and for glucose 6-phosphate and pyrophosphate was determined from Lineweaver and

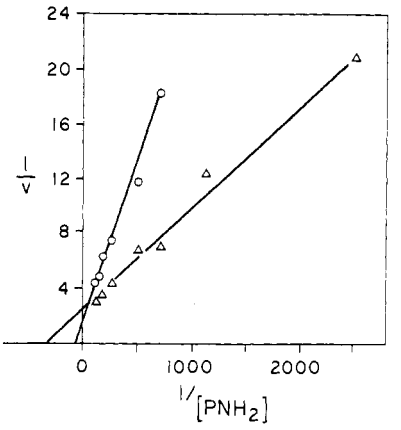


FIGURE 2: Effect of alkaline treatment on Michaelis constant for phosphoramidate. The assay was carried out as described in methods by determining liberated ammonia. Phosphoramidate concentration was varied as indicated. The microsomal preparation (0.6 mg of protein) was used either untreated (○—○—○) or after 30-min prior exposure to pH 9.7 in 50 mM glycine-NaOH buffer at 0° (△—△—△). Velocity is expressed as micromoles of ammonia liberated.

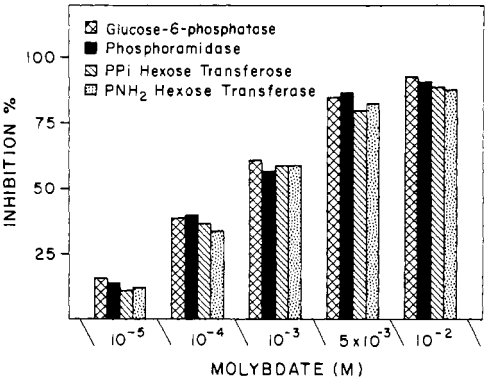


FIGURE 3: Percentage inhibition of phosphohydrolase and phosphotransferase activities at different concentrations of sodium molybdate. All assays were carried out at pH 5.3.

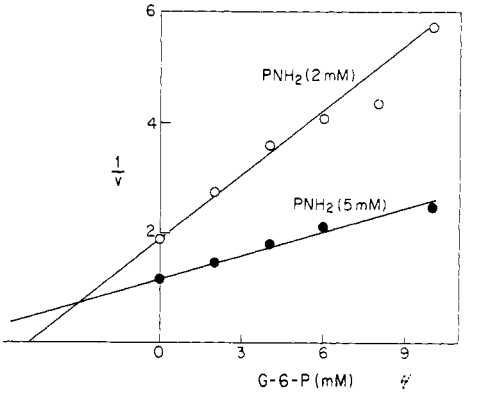


FIGURE 4: Competitive inhibition of phosphoramidate hydrolysis by glucose 6-phosphate. Dixon plot (Dixon, 1953). Activity was followed by measurement of liberated ammonia. Standard assay conditions given in the text were employed with 0.68 mg of microsomal protein (preincubated at pH 9.7 for 1 hr). Assay was performed at pH 6.5.

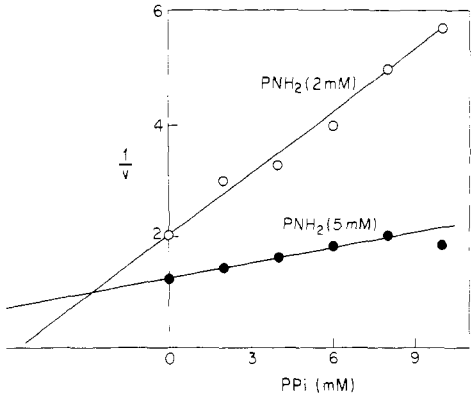


FIGURE 5: Competitive inhibition of phosphoramidate hydrolysis by pyrophosphate. Dixon plot (Dixon, 1953). Assay was carried out as in Figure 4.

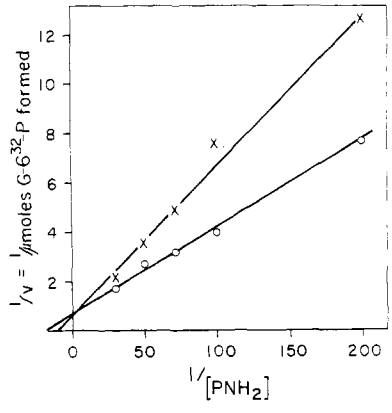


FIGURE 6: Inhibition of phosphoramidate–hexose phosphotransferase activity by pyrophosphate. The assay system in 0.5 ml contained, 0.1 M acetate (pH 5.5), 0.2 M glucose, 2.3 mg of microsomal protein, and ^{32}P-labeled phosphoramidate as indicated, without (○—○—○) or with 40 mM PP_i (x—x—x); all reactants except microsomes were preincubated for 5 min at 37°. The reaction was started by the addition of microsomal protein. After 10 min, the reaction was terminated with 0.1 ml of 25% trichloroacetic acid, protein was centrifuged, and aliquots were used for separating organic phosphate formed by the method of Neilsen and Lehninger (1955). The nonextractable organic phosphate was plated and radioactivity was detected with an end-window counter. Velocity is given as micromoles of glucose-6-P formed per minute.

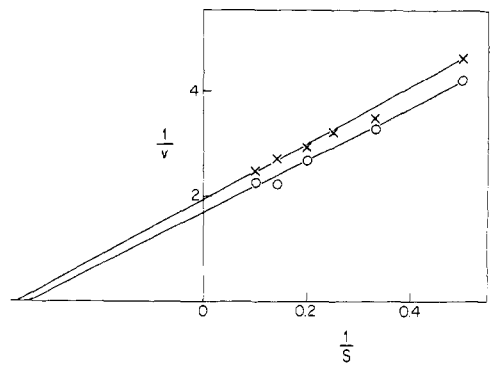


FIGURE 7: Reciprocal plots of rate of pyrophosphate and glucose 6-phosphate hydrolysis against substrate concentration. The assay system in 0.5 ml contained, 0.2 M Tris-maleate (pH 6.5), 0.68 mg of microsomal protein (preincubated at pH 9.7 for 1 hr), and pyrophosphate (○—○) or glucose 6-phosphate (x—x) as indicated. *S* is expressed as millimolar and velocity is given as micromoles of P_i liberated under assay condition.

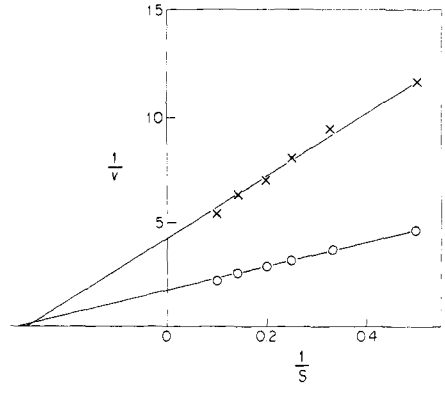


FIGURE 8: Reciprocal plots of rate pf pyrophosphate and phosphoramidate–hexose phosphotransferase against substrate concentration. The assay system in 0.5 ml contained 0.2 M Tris-maleate (pH 6.5), 0.2 M glucose, 0.65 mg of alkali-treated microcomal protein (preincubated at pH 9.7 for 1 hr), and pyrophosphate (x—x) or phosphoramidate (○—○) varied from 2 to 10 mM as indicated. Velocity is given as micromoles of glucose-6-P formed.

Burk (1934) plots given in Figure 2 and 7, respectively. Michaelis constants for phosphotransferase activity using phosphoramidate and pyrophosphate as phosphoryl donors were determined from the reciprocal plots given in Figure 8. All this data is compiled in Table IV. The K_m value for phosphoramidate in the hydrolase reaction was similar to the K_m for phosphoramidate in the phosphotransferase reaction as expected if the same enzyme is involved for these two activities. K_i for pyrophosphate (Figure 5) determined by the method of Dixon (1953) was identical with the K_m for pyrophosphate in the hydrolase and phosphotransferase reactions, while K_i for glucose 6-phosphate (Figure 4) determined from the Dixon (1953) plot of the inhibition of phosphoramidate hydrolysis by glucose 6-phosphate and K_m for glucose 6-phosphate hydrolysis also agreed as predicted for the same enzyme being involved in these reactions.

The results of an experiment in which glucose 6-phosphate formation was measured using pyrophosphate and phosphoramidate as phosphoryl donors separately and together are given in Table V. If these reactions were catalyzed by more than one enzyme, then in the presence of both phosphoryl donors together the rate of glucose 6-phosphate formation should have been greater than that observed with either phosphoryl donor alone. But the data of Table V show that the results obtained were close to the expected theoretical value. These kinetic data thus support the view that it is the same enzyme protein that catalyzes the phosphoramidase, phosphoramidate–

TABLE IV: Comparison of Kinetic Constants for the Reactions Catalyzed by Glucose 6-Phosphatase.[a]

Reaction Studied	Product Measured	Data Given in Figure	Michaelis Constant
Phosphoramidate hydrolysis	NH_3	2	K_m (phosphoramidate) = 2.80×10^{-3}
Glucose 6-phosphate hydrolysis	P_i	7	K_m (glucose 6-phosphate) = 3.05×10^{-3}
PP_i hydrolysis	$P_i/2$	7	K_m (PP_i) = 2.78×10^{-3}
Inhibition of phosphoramidate hydrolysis by PP_i	NH_3	5	K_i (PP_i) = 2.80×10^{-3}
Inhibition of phosphoramidate hydrolysis by glucose 6-phosphate	NH_3	4	K_i (glucose 6-phosphate) = 2.90×10^{-3}
PP_i–hexose phosphotransferase	Glucose 6-phosphate	8	K_m (PP_i) = 3.3×10^{-3}
Phosphoramidate–hexose phosphotransferase	Glucose 6-phosphate	8	K_m (phosphoramidate) = 3.1×10^{-3}

[a] Assays were carried out at pH 6.5 using microsomes preincubated at pH 9.7 for 1 hr.

TABLE V: Phosphorylation of Glucose by Rat Liver Microsomal Phospho–Hexose Transferase Using Phosphoramidate and Pyrophosphate as Phosphoryl Donors, Separately and Together.[a]

Substrate (mM)	μmoles of Glucose 6-Phosphate Formed: Found	Expected
Phosphoramidate (10)	0.488	
Pyrophosphate (10)	0.199	
Phosphoramidate + pyrophosphate (10) each	0.326	0.3[b]

[a] Assay system contained 0.2 M Tris-maleate (pH 6.5), 0.2 M glucose, 0.67 mg of microsomal protein (preincubated at pH 9.7 for 1 hr), and phosphoryl donors as given in the table. Final volume 0.5 ml, incubation period 10. Reaction was terminated by placing the tube in boiling water bath for 3 min. Glucose 6-phosphate formed was determined by coupling to glucose 6-phosphate. [b] This theoretical value was obtained by using the equation described in Dixon and Webb (1958, p 92) assuming one enzyme involvement.

hexose phosphotransferase, and glucose 6-phosphatase reactions.

Possible Mechanism of Phosphohydrolase and Phosphotransferase Activities. To explain the glucose 6-phosphatase, pyrophosphatase, and pyrophosphate–hexose phosphotransferase activities of microsomal glucose 6-phosphatase, a reaction mechanism which involves the formation of a phosphoryl–enzyme intermediate has been proposed (Segal, 1959; Hass and Byrne, 1960; Arion and Nordlie, 1964). The possibility of the formation of phosphohistidine in the glucose 6-phosphatase reaction has also been suggested by Nordlie and Lygre (1966). To accommodate the finding that glucose 6-phosphatase also catalyzes phosphoramidase and phosphoramidate–hexose phosphotransferase reactions, one must assume that phosphoramidate also leads to the formation of the same phosphoryl–enzyme intermediate as that formed by the interaction of pyrophosphate or glucose 6-phosphate and enzyme.

If a phosphoryl–enzyme is involved as an intermediate in the reaction mechanism, as proposed by other investigators, incorporation of radioactivity from ^{32}P-labeled glucose 6-phosphate, pyrophosphate, or phosphoramidate into the enzyme protein following incubations with any of these labeled substrates, may be expected. Results of one such experiment are presented in Table VI. The microsomal protein obtained by the phenol-extraction procedure of Beiber and Boyer (1966) contained considerable radioactivity following preincubation with ^{32}P-labeled glucose 6-phosphate. Since most of this incorporated radioactivity was lost on subjecting the labeled microsomal protein to mild acid hydrolysis, and only a smaller fraction (15–18%) of the total radioactivity incorporated into microsomal protein, remained after mild acid hydrolysis, it is possible that ^{32}P was incorporated in some group such as phosphohistidine which is known to be markedly acid labile (Beiber and Boyer, 1966). Although these incorporation experiments were performed with microsomal proteins, rather than a pure enzyme preparation, the finding that incorporation of ^{32}P from glucose 6-phosphate into an acid-labile group of microsomal protein was markedly lowered by the presence of either unlabeled phosphoramidate or pyrophosphate (Table VI) suggests the possibility that the labeling of the microsomal protein resulted from phosphorylation in the active site of the enzyme.

Discussion

As is evident from the data obtained in this study the microsomal phosphoramidase as well as phosphor-

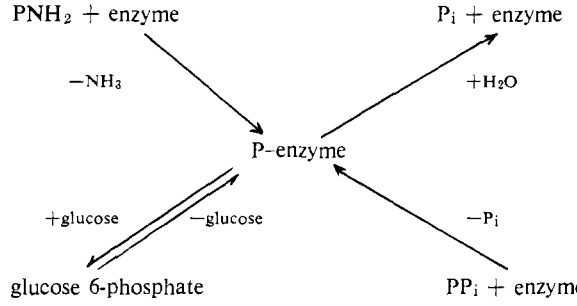
TABLE VI: Labeling of Microsomal Protein by Glucose-6-^{32}P and Its Inhibition by Pyrophosphate and Phosphoramidate.[a]

Additions to Complete System (mM)	Protein-Bound ^{32}P Released by Acid (mμmoles)	Inhibn (%)
None	4.15	
None	3.73	
Phosphoramidate (40)	1.06	72
Phosphoramidate (20)	1.2	69
PP_i (30)	0.61	84
PP_i (20)	0.88	77
Heated control[b]	0.48	

[a] To 5 mg of microsomal protein buffered to pH 6.5 with 50 μmoles of Tris-maleate, 10 μmoles of glucose-6-^{32}P (55,650 cpm/μmole) was added. Final volume 0.5 ml. The components were mixed thoroughly at 4° and after 30 sec the reaction was stopped by adding 1.0 ml of phenol (containing 10 mM EDTA) buffered to pH 8.3 with ammonia. The phenol layer containing labeled protein was washed five times by mixing with aqueous phenol-saturated solution of ammonium acetate containing 0.01 M EDTA (pH 8.3). ^{32}P-containing lipids were removed by precipitating proteins and phospholipids with seven volumes of acetone and extracting the acetone-precipitated residue. Protein-bound acid-hydrolyzable phosphate which has been described as phosphohistidine by Beiber and Boyer (1966) determined on aliquots, after boiling the lipid-free protein for 5 min with 0.3 M trichloroacetic acid containing 1 mM P_i. [b] Complete system except that microsomes were heated (3 min at 100°) before addition.

amidate–hexose phosphotransferase activities behaved exactly alike under diverse experimental conditions and these behaviors corresponded closely to that of glucose 6-phosphatase and its associated pyrophosphatase and pyrophosphate–hexose phosphotransferase reactions. In addition to these striking similarities, the lack of additive effects on either hydrolytic or on phosphotransferase activities by inclusion of pyrophosphate and phosphoramidate together (Tables IV and V) also suggests involvement of a single enzyme for the several reactions discussed here. The kinetic data obtained showing mutual competitive inhibitions on both phosphohydrolase activities as well as phosphotransferase activities suggest that both phosphoramidase and phosphoramidate–hexose phosphotransferase activities are indeed possessed by the enzyme that also catalyses glucose 6-phosphate and pyrophosphate hydrolysis as well as the pyrophosphate–hexose phosphotransferase reaction.

The different activities of glucose 6-phosphatase are presented in the following scheme. In the presence of glucose and a suitable phosphoryl donor this enzyme acts as a phosphotransferase. Otherwise in the absence of glucose water serves as phosphoryl acceptor and enzyme catalyzes phosphohydrolase reaction.



The conclusion of Holzer *et al.* (1966) that phosphoramidase and phosphoramidate–hexose phosphotransferase activities are distinct from that of glucose 6-phosphatase appears to have resulted on meager and incomplete data and is untenable in view of the results described in detail in this paper. While it is certain that at least the glucose 6-phosphate hydrolyzing activity of this enzyme plays an essential part in the process of gluconeogenesis (Krebs, 1963; Weber *et al.*, 1964), it is by no means established if other hydrolytic or phosphohexose transferase (Nordlie and Arion, 1965) activities are metabolically relevant. Assigning any possible metabolic significance to the observed phosphoramidase and phosphoramidate–hexose phosphotransferase reactions would be, at present, a matter of speculation alone since a clear-cut demonstration of the occurrence of phosphoramidate in nature has not been successful to date. However, Correll (1966) does present evidence for the occurrence of imidodiphosphate bonding in polyphosphate fractions of *Chlorella*; therefore, the possibility of the formation of simpler molecules with a P–N bond of the type present in phosphoramidate is not entirely ruled out. In this connection we have observed that rat liver microsomal preparation effectively hydrolyzes diimidotriphosphate as judged by the liberation of P_i. Recently Dowler and Nakada (1968) have described an enzyme from yeast which utilizes phosphoramidate for the phosphorylation of ADP and consider the possibility that this may be analogous to the formation of ATP during oxidative phosphorylation. It may be pointed out in this connection that the presently studied microsomal enzyme did not utilize ADP as a phosphoryl acceptor.

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Different Allosteric Properties of Nucleoside Diphosphatase Isoenzymes from Rat Liver*

R. Parvin and Roberts A. Smith

ABSTRACT: The nucleoside diphosphatase activity of the rat liver microsomal fraction behaved differently from the nucleoside diphosphatase activity of the rat liver supernatant fraction. The microsomal enzyme was effectively inhibited by adenosine triphosphate; several other nucleoside phosphates were much less inhibitory. This adenosine triphosphate inhibition, was found to be competitive with respect to substrate inosine diphosphate, as it decreased the affinity of the enzyme for substrate without affecting the maximum velocity. The plot of percentage inhibition against adenosine triphosphate concentration was sigmoidal showing cooperative effects in the inhibition. The nucleoside diphosphatase activity of the supernatant fraction, on the other hand, was markedly stimulated by adenosine triphosphate. In this case adenosine triphosphate increased both the maximum velocity as well as the affinity of the enzyme for its substrate, inosine diphosphate. From these and other differences noted it is concluded that the microsomal and supernatant nucleoside diphosphatase activities are due to two different enzymes. The possible metabolic significance of opposite modulatory effects of adenosine triphosphate on these rat liver isoenzymes is discussed.

During recent studies with rat liver microsomal glucose 6-phosphatase and other activities associated with it, a very active nucleoside diphosphatase was observed. Later studies showed that this nucleoside diphosphatase was different from the one reported by Nordlie and Arion (1965) as it could be separated from glucose 6-phosphatase activity by deoxycholate treatment and ammonium sulfate fractionation. Furthermore this preparation (after glucose 6-phosphatase removal) was not able to catalyze phosphoryl transfers from nucleoside di- or triphosphates to glucose.

Nucleoside diphosphatase which catalyzes the hydrolysis of IDP, GDP, and UDP, but not of ADP or CDP, to the corresponding nucleoside monophosphate and P_i has been studied earlier by Plaut (1955), by Gibson *et al.* (1955), by Novikoff and Heus (1963), and by Ernster and Jones (1962). An allosteric activation by ATP of a nucleoside diphosphatase preparation

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