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# PROTEIN-BOUND PHOSPHORYL HISTIDINE: A PROBABLE INTERMEDIATE IN THE MICROSOMAL GLUCOSE-6-PHOSPHATASE/INORGANIC PYROPHOSPHATASE REACTION

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SUMMARY

1. Several types of evidence have suggested the occurrence of a common phosphoryl enzyme intermediate in the reactions catalyzed by rat liver microsomal glucose-6-phosphatase (D-glucose 6-phosphate phosphohydrolase, EC 3.1.3.9). We have identified *N*-3-phosphoryl histidine in an alkaline digest of rat liver microsomes denatured by phenol extraction after brief exposure to glucose 6-[$^{32}$P]phosphate or $^{33}PP_i$, an alternative substrate for microsomal glucose-6-phosphatase.

2. This phosphorylated histidine derivative appears to meet criteria for an obligatory intermediate in the phosphohydrolase and phosphotransferase reactions catalyzed by glucose-6-phosphatase.

3. Comparison of isotope exchange reactions and labeling patterns of this enzyme with those of other enzymes in which phosphoryl histidine has been detected suggest that the free energy of hydrolysis of the phosphoryl enzyme in this reaction is relatively low.

4. Denatured microsomal protein binds $^{32}PP_i$ (but not $^{32}P_i$ or glucose 6-[$^{32}$P]-phosphate) very strongly, but non-covalently.

## INTRODUCTION

The classical microsomal enzyme, glucose-6-phosphatase (D-glucose 6-phosphate phosphohydrolase, EC 3.1.3.9), catalyzes a variety of phosphohydrolase and phosphotransferase activities[1–5], all of which can be accommodated by a mechanism involving a common phosphoryl enzyme intermediate from which the phosphoryl group may be transferred to water or other acceptors[1]. The probable occurrence of such an intermediate was indicated by the observation of [$^{14}$C]glucose exchange into glucose 6-phosphate[6], and the lack of exchange of $^{18}$O from water into the phosphoryl group of glucose 6-phosphate[7]. Independent kinetic studies demonstrated the importance in the catalytic reaction of a protein ionizable group with a p$K$ between 6

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and 7, and led Nordlie and Lygre[8] to suggest that a histidine residue may be involved, possibly as phosphoryl histidine. In the accompanying paper we have presented photoinactivation studies which are consistent with a crucial role for histidine[9]. Parvin and Smith[3] have briefly reported apparent incorporation of $^{32}P$ from glucose 6-[$^{32}P$]phosphate into microsomal protein in an acid-labile form, and suggested that the chemical form of the material might be phosphoryl histidine.

We have, as briefly reported earlier[10], demonstrated that protein-bound *N*-3-phosphoryl histidine is formed when the microsomal enzyme is incubated with glucose 6-[$^{32}P$]phosphate, a "low-energy" substrate. In this paper we document the apparent turnover rates of this material, and the formation of the corresponding protein-bound phosphoryl histidine from an alternative "high-energy" substrate, inorganic pyrophosphate[1,2]. We also demonstrate the lack of exchange of $^{32}P$ from $^{32}P_i$ with either substrate. The $^{32}P$-labeled protein formed from glucose 6-[$^{32}P$]phosphate and $^{32}PP_i$ appears to be chemically and mechanistically equivalent, and has the properties expected of a low-energy intermediate in the enzyme reaction.

## METHODS

### *Isotope and other materials*

$^{32}P_i$ (carrier-free) was obtained from ICN Corporation and used without further purification. $^{32}PP_i$ (spec. act. $>$ 2000 mCi/mmole) was obtained from New England Nuclear Corporation. Glucose 6-[$^{32}P$]phosphate was prepared from $^{32}P_i$ via [$^{32}P$]ATP by modification of the method of Glynn and Chappell[11]. Modifications included addition to the incubation mix of $1 \cdot 10^{-5}$ M NAD (ref. 12), 0.1 M glucose, and 25 units of yeast hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1). After 60 min at room temperature with constant stirring, the mixture (10 ml) was applied to a 5 cm $\times$ 1 cm column of Dowex AG 1-X8 (formate) which had been thoroughly washed with water. Elution was with a linear gradient of formic acid, 0.3 M to 1.0 M. Under these conditions glucose 6-[$^{32}P$]phosphate is eluted first, followed by $^{32}P_i$; [$^{32}P$]ATP remains on the column but can be eluted with 2.0 M formic acid. Peak fractions containing glucose 6-[$^{32}P$]phosphate were lyophilized and stored at $-20$ °C. Authentic *N*-3-phosphoryl histidine was synthesized from phosphoramidate[13] and purified as described by Hultquist *et al.*[14].

Biogel P-2 and Dowex AG 1-X8 were obtained from Bio-Rad Laboratories, and prepared by thorough recycling and washing[15]. All other materials were analytical grade reagents or the highest grade available, and were used without further purification.

### *Enzymes*

The preparation, activation, and assay of microsomal glucose-6-phosphatase have been described in the accompanying paper[9]. Crystalline yeast inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) (spec. act. 650 $\mu$moles $PP_i$ cleaved per min per mg protein) was prepared in this laboratory by J. Ridlington. Pronase, (B grade), trypsin (EC 3.4.4.4) and yeast phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) were from Calbiochem. Muscle glyceraldehyde 3-phosphate dehydrogenase and yeast hexokinase were from Sigma Chemical Company. Ribonuclease (ribonucleate pyrimidine nucleotide-2'-transferase

(cyclizing), EC 2.7.7.16) was from Worthington; lyophilized venom of *Trimerasaurus flavoviridis* was from Miami Serpentarium Laboratories.

*$^{32}P$-Analyses*

Analysis of $^{32}P_i$ produced from glucose 6-[$^{32}$P]phosphate, $^{32}PP_i$, or phosphorylated peptides was by a modification of the Martin and Doty[16] isobutanol–benzene extraction procedure. In our analyses, 1.0 ml sample (containing 0.1–2.0 μmoles carrier phosphate) was added to 3 ml isobutanol–benzene (1:1, v/v) and 3 ml acid molybdate (0.012 M $(NH_4)_6Mo_7O_{24}\cdot 4\ H_2O$ in 0.25 M $H_2SO_4$), mixed vigorously for 30 s and the two layers permitted to separate in the cold. After removal of an aliquot of the upper layer for counting, the remaining upper layer was discarded and the aqueous lower layer was reextracted with an additional 3 ml of isobutanol–benzene. An aliquot of the lower layer was then removed for counting. The organic upper layer contains exclusively $^{32}P_i$, with all other $^{32}$P compounds present in the lower layer. $^{32}$P was measured in a Nuclear-Chicago gas flow planchet counter.

*Isotope labeling of protein*

Incubation of microsomes (25–35 mg protein/ml) with glucose 6-[$^{32}$P]phosphate or $^{32}PP_i$ was done at 2–3 °C in 0.5 M sodium acetate, pH 6.0. Incubation was terminated by addition of 2–4 vol. of 88% aqueous phenol into which the labeled protein is quantitatively and irreversibly extracted[17]. $^{32}$P not associated with protein was removed by repeated (6 ×) extraction of the phenol layer with aqueous buffer containing 10 mM EDTA, 1 mM glucose 6-phosphate, 10 mM $P_i$, and 16% phenol (pH 9). For determination of the level of isotope incorporated into protein, the phenol extracts were quantitatively transferred to planchets, dried and counted on an automatic Nuclear-Chicago gas-flow counter. For characterization of labeled protein, phenol was omitted from the last three extractions so that the protein precipitated due to removal of phenol.

*Column chromatography*

Column effluents were continuously monitored for both radioactivity and 280 nm absorbing materials by pumping through a Teflon flow cell of the ISCO optical unit attached to ISCO Ultraviolet Analyzer Model UA 2 (instrumentation Specialties Co., Inc.) to record the absorbance, then through a flow-through counter cell (Model 8-711, Baird Atomic) electronically connected a count-rate meter (Nuclear Chicago Model 1620 B) which detected the $^{32}$P and recorded the count rate on a Sargent linear recorder (SRLG). The effluent was then deposited in tubes in a fraction collector.

## RESULTS

*Rapid turnover of the protein-bound phosphoryl histidine*

A time course of incorporation of $^{32}$P from glucose 6-[$^{32}$P]phosphate into microsomal protein at 2 °C is shown in Fig. 1. It has previously been demonstrated that the $^{32}$P incorporated under these conditions is covalently bound to protein as *N*-3-phosphoryl histidine[10]. Near-maximal labeling is observed within 15 s at this low temperature, and subsequent addition of excess non-radioactive glucose 6-phosphate or $PP_i$, an alternative substrate, similarly results in rapid loss of the $^{32}$P due to the greatly

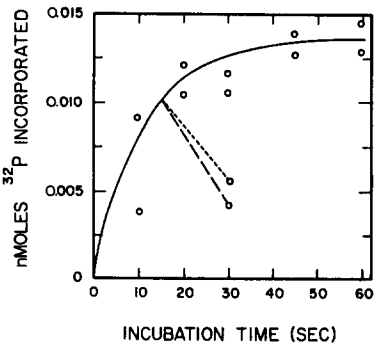


Fig. 1. Time course of $^{32}P$-incorporation into microsomal protein. Each sample (0.5 ml) contained 6 mg microsomal protein, 0.5 M acetate buffer, pH 6.0; and $2.5 \cdot 10^{-4}$ M glucose 6-[$^{32}P$]phosphate ($3.6 \cdot 10^{7}$ cpm/$\mu$mole). At 15 s incubation time certain samples were made a $2.5 \cdot 10^{-3}$ M in non-radioactive glucose 6-phosphate (------) or $PP_i$ (— — —). Incubation was at 1–3 °C; assays for $^{32}P$ incorporation into protein are described in Methods. Incorporation is reported per experimental mixture. The results reported here have been corrected for non-enzymatic binding of $^{32}P$ to denatured microsomal protein by subtraction of the value observed when the order of addition of glucose 6-[$^{32}P$]phosphate and the phenol stop was reversed so that the enzyme was denatured before exposure to $^{32}P$.

diminished specific activity of the pool of phosphoryl groups of substrates, although the total amount of phosphorylated protein may be increased. The data demonstrate that the protein-bound phosphoryl group is in rapid equilibrium with phosphoryl groups of both substrates. In experiments reported earlier it was shown that orthophosphate and $\beta$-glycerol phosphate, neither of which are substrates, are not in equilibrium with the phosphoryl group on the enzyme[10].

*Labeling of the enzyme using $^{32}PP_i$*

If the reaction mechanism of this enzyme includes a covalent phosphoryl enzyme as an obligatory intermediate, all substrates should be effective in phosphorylation of the microsomal protein. In particular, since the enzyme utilizes both high-energy and low-energy substrates, a comparison of the nature and extent of phosphorylation of the enzyme by representatives of the two types of substrates was of interest. Microsomes incubated with $^{32}PP_i$ under conditions similar to those used to measure $^{32}P$ incorporation from glucose 6-[$^{32}P$]phosphate yielded much larger amount of $^{32}P$ which extracted with the protein into phenol than was observed with glucose 6-[$^{32}P$]phosphate[10]. Thus, the extent of labeling was apparently increased by using a high energy substrate. Control experiments indicated, however, that this high level of apparent incorporation is an artifact of the assay, for similarly large amounts of $^{32}P$ from $^{32}PP_i$ but not glucose 6-[$^{32}P$]phosphate became phenol extractable using microsomal preparations which had been previously denatured by extraction into phenol before addition of $^{32}P$-substrates. Thus, even phenol-denatured microsomal preparations bind considerable $^{32}P$ from $^{32}PP_i$ substrates. Therefore further characterization was necessary to determine the extent, as well as the nature, of any covalent $^{32}P$ incorporation from $^{33}PP_i$ which is specifically due to enzymatic activity.

*Isolation and characterization of $^{32}P$-peptides from $^{32}PP_i$*

Digestion with pronase of the insoluble $^{32}P$-protein which had been labeled from

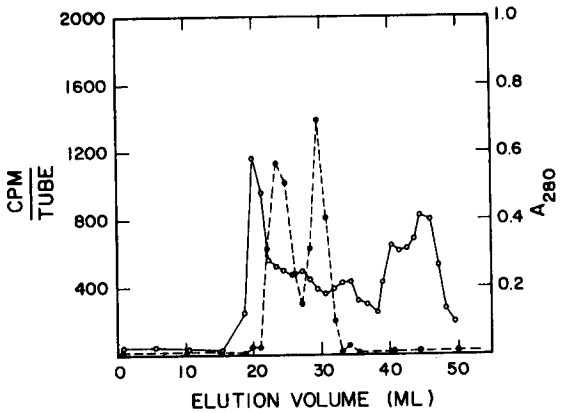


Fig. 2. Gel filtration of pronase digest of $^{32}$P-labeled protein. Insoluble $^{32}$P-labeled protein (36 mg containing 2 $\mu$Ci $^{32}$P, 1.4 nmoles P), prepared from $^{32}PP_i$ (1.4 $\mu$moles, 1420 mCi/mmole) by phenol extraction as described in Methods, was digested with 2 mg pronase in 1 ml 5 mM phosphate buffer, pH 8.5, for 18 h at room temperature. The remaining insoluble pellet, containing approx. 80% of the original $^{32}$P, was removed by centrifugation and the entire solubilized extract was chromatographed on a 0.8 cm × 100 cm column of Biogel P2 which had been equilibrated with 0.1 M ammonium formate, 1 mM $P_i$, 1 mM $PP_i$, pH 9. The column was eluted with the same buffer at a flow rate of 11.5 ml/h; fraction size was 0.96 ml. cpm (●---●); $A_{280\ nm}$ (○—○).

$^{32}PP_i$ and obtained by phenol extraction released, in several experiments, approx. 20% of the $^{32}$P in a soluble form. In contrast, 90% of the $^{32}$P was solubilized under similar conditions when labeled from glucose 6-[$^{32}$P]phosphate[10]. Gel filtration of the solubilized material on Biogel P-2 gave the results shown in Fig. 2. Considerable non-radioactive material was eluted in the void volume of the column followed by two radioactive fractions. A similar pattern was found previously with labeling from glucose 6-[$^{32}$P]phosphate (compare with Fig. 2 in ref. 10), As seen in Table I, the second peak of radioactivity consisted almost exclusively of $^{32}P_i$. The first radioactive peak contained no $^{32}P_i$ but could be readily and quantitatively converted to $^{32}P_i$ by a mild acid hydrolysis, as would be expected for either $^{32}PP_i$ or a [$^{32}$P]phosphoryl histidine derivative[14], but not for phosphoryl serine[18]. In order to distinguish between these possibilities an enzymatic test utilizing the high substrate specificity of yeast

TABLE I

ANALYSIS OF $^{32}$P PEAKS FROM BIOGEL P2

Samples (50 $\mu$l) from the peak tubes in Fig. 2 were analyzed by the isobutanol–benzene distribution assay as described in Methods. Acid hydrolysis was 0.5 M $H_2SO_4$, 100 °C, 10 min. Pyrophosphatase treatment was with the crystalline yeast enzyme, 50 $\mu$l of $3.6 \cdot 10^{-6}$ M, in 2 mM $MgCl_2$, 0.1 M $NH_4HCO_3$, pH 8.5, incubated with 50 $\mu$moles of $^{32}$P-labeled sample for 20 min at 3 °C.

| *Peak* | *% of total $^{32}$P as $^{32}P_i$* |
|---|---|
| I | 0 |
| II | 96.7 |
| I (acid hydrolyzed) | 100 |
| I (treated with pyrophosphatase) | 0 |

inorganic pyrophosphatase[19] was developed. Under the conditions employed synthetic *N*-3-phosphoryl histidine was not hydrolyzed by the yeast enzyme, which was highly active toward $PP_i$. All material to be tested for the presence of $^{32}PP_i$ was treated with the yeast enzyme until no further $^{32}P_i$ was produced. As seen in Table I, the first peak of radioactivity from the Biogel P-2 column was not susceptible to cleavage by inorganic pyrophosphatase, suggesting it contained the expected enzyme intermediate, phosphoryl histidine.

*Identification of N-3-phosphoryl histidine*

Ion exchange chromatography on Dowex AG 1-X8 ($OH^-$) indicated the $^{32}P$-labeled material of the first radioactive peak (Fig. 2) was heterogeneous; it was therefore subjected to alkaline hydrolysis to convert any peptides to free amino acids[20]. After hydrolysis in the presence of non-radioactive carrier $PP_i$ and synthetic *N*-3-phosphoryl histidine, the alkaline hydrolysate was chromatographed on Dowex AG 1-X8 ($OH^-$)[21] with the results seen in Fig. 3. The first radioactive peak consisted of at least 94% $^{32}P_i$, and coincided with a small $P_i$ peak which presumably arose from hydrolysis of the non-radioactive carriers. In control experiments it was found that

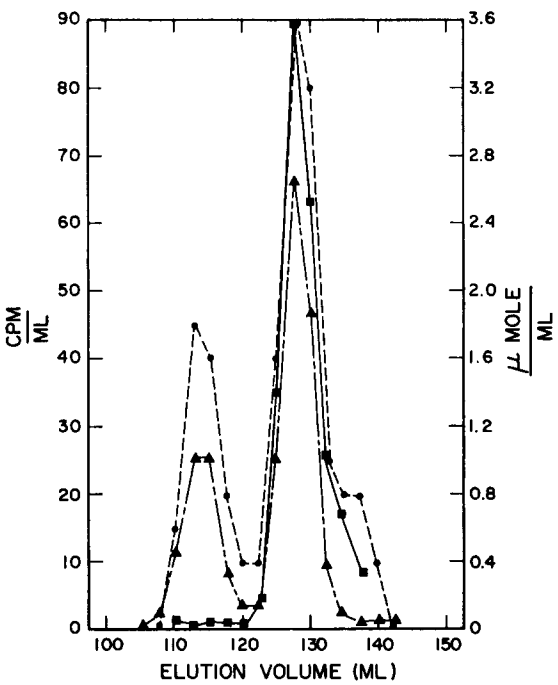


Fig. 3. Apparent chromatographic identity of alkali-digested $^{32}P$-labeled component with synthetic *N*-3-phosphoryl histidine. The fractions of the first radioactive peak of Fig. 2 were combined, 20 μmoles of synthetic *N*-3-phosphoryl histidine was added, and the solution was made 3 M in KOH and heated for 3 h at 100 °C in a sealed tube[20]. After neutralization to pH 9 with $HClO_4$ and removal of the precipitated $KClO_4$ by centrifugation an additional 10 μmoles of synthetic *N*-3-phosphoryl histidine and 20 μmoles of non-radioactive $PP_i$ were added to provide internal standards. The hydrolysate was chromatographed at room temperature on a Dowex AG 1-X8 ($OH^-$) column (2.3 cm × 10 cm) with a linear gradient of 0.2 M–1.5 M $NH_4HCO_3$, pH 8.5 (ref. 21). cpm/ml (●---●); $P_i$ (▲---▲)[22]; histidine (■—■)[23].

$PP_i$ is eluted between the first and second radioactive peaks. The migration of the second radioactive peak was coincident with that of the authentic *N*-3-phosphoryl histidine, as previously described for the material labeled from glucose 6-[$^{32}$P]phosphate[10]. Under these conditions *N*-3-phosphoryl histidine is resolved from the L-isomer[21]. Because of the close correspondence of this result with the previous identification of *N*-3-phosphoryl histidine, and because of the relatively low amount of radioactivity in the preparation, additional analyses to verify the identification were not carried out.

*Identification of the $^{32}$P not solubilized by pronase digestion*

The fraction of radioactivity solubilized by pronase digestion, from which phosphoryl histidine was identified, amounted to onyl 20% of the total $^{32}$P in the insoluble pellet obtained from the phenol extract, necessitating rigorous identification of the remaining material to ensure detection of all possible intermediates. Repeated treatment with pronase (4 mg/ml, 15 h) released less than 2% of the $^{32}$P remaining in the pellet. Further treatment for several hours with 4 mg/ml of either trypsin,

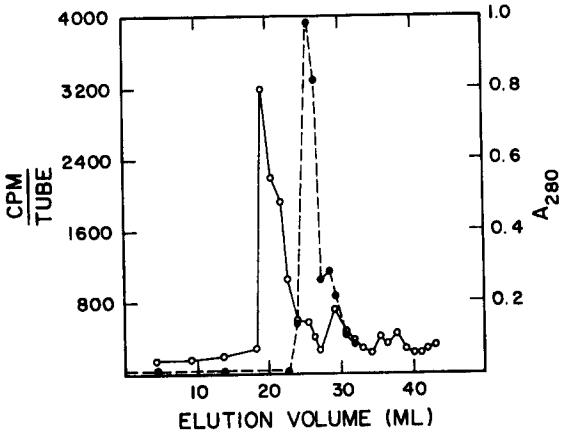


Fig. 4. Gel filtration of the alkaline extract of the $^{32}$P-labeled protein not solubilized by pronase digestion. The insoluble pellet remaining after pronase digestion of $^{32}$P-labeled protein (see legend for Fig. 2) was extracted with 0.4 ml 0.1 M KOH and 0.1 ml 0.1 M $PP_i$. After centrifugation, the extract (containing 25% of the $^{32}$P initially present in the protein) was subjected to gel filtration on Biogel P2 as described for Fig. 2. cpm (●---●); $A_{280\ nm}$ (○—○).

phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4) in lyophilized snake venom (*Trimerasaurus flavoviridis*) or ribonuclease was equally ineffective in solubilizing additional $^{32}$P.

Extraction with 0.4 ml 0.1 M KOH, 0.1 M $Na_4PP_i$ released 25% of the total initial radioactivity in a form which would not sediment when centrifuged at 5000 × *g* for 10 min. Gel filtration on Biogel P2, shown in Fig. 4, gave two incompletely resolved radioactivity peaks well separated from the bulk of the protein. Only the second peak contained $^{32}P_i$; the first peak consisted of an acid labile material which was completely converted to $^{32}P_i$ by digestion with yeast inorganic pyrophosphatase. Thus, the unknown material could be either $^{32}PP_i$ or a [$^{32}$P]pyrophosphoryl amino acid derivative, at least some of which are substrates for this yeast enzyme[24].

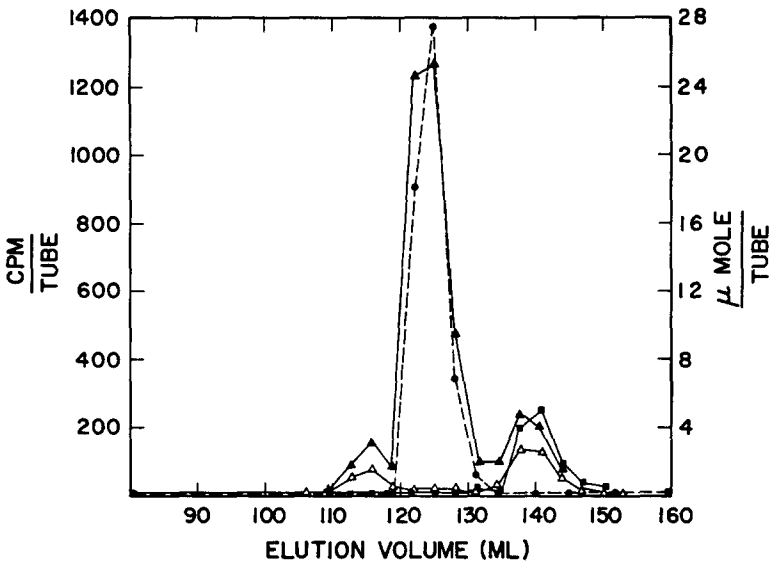


Fig. 5. Apparent chromatographic identity of alkali-released $^{32}$P-labeled material with authentic pyrophosphate. Non-radioactive carrier $PP_i$ (20 $\mu$moles) and synthetic *N*-3-phosphoryl histidine (10 $\mu$moles) were added to the combined fractions containing the major radioactive peak of Fig. 4, and the solution was diluted five-fold with 0.01 M potassium bicarbonate buffer, pH 8.5, to lower the ionic strength. Chromatography was on Dowex AG 1-X8($OH^-$); elution was with a linear gradient of 0.2 M–1.5 M $NH_4HCO_3$, pH 8.5. Radioactivity (●---●); total histidine (■—■); and $P_i$ before (△—△) and after (▲—▲) acid hydrolysis (0.9 M $H_2SO_4$, 100 °C 30 min).

The identification of the material in the first peak as $^{32}PP_i$ was confirmed by ion exchange chromatography on Dowex AG 1-X8 ($OH^-$) in the presence of non-radioactive carrier $PP_i$ and phosphoryl histidine, as shown in Fig. 5. The single radioactive peak was coincident with $PP_i$, and 15 ml ahead of phosphoryl histidine. These data argue against the occurrence of a pyrophosphoryl enzyme, as postulated in other systems[25–27].

The remainder of the $^{32}$P in the pellet which was not extracted by the above treatments was solubilized using 10% sodium dodecyl sulfate, pH 8, and subjected to gel filtration as above. Like the previous fraction, all the remaining radioactivity was demonstrated to be present in the form of $^{32}PP_i$. Thus all of the protein-bound $^{32}$P from $^{32}PP_i$, as detected by the phenol extraction assay, has been accounted for; over 80% is present as non-covalently bound $^{32}PP_i$.

*Association of $^{32}PP_i$ with inactive microsomes*

In order to determine the significance of the apparent incorporation into microsomal protein of relatively large amounts of $^{32}$P as $^{32}PP_i$, parallel controls were run in which microsomes were exposed to $^{32}PP_i$ only after the addition of phenol to denature and inactivate the enzyme. The protein was isolated by phenol extraction and digested with pronase as previously described. In contrast to the preceding experiments only 6% of the $^{32}$P was released in soluble form by the pronase, and this $^{32}$P was entirely in the form of $^{32}PP_i$ which was rapidly converted to $^{32}P_i$ by pyrophosphatase activity in the pronase preparation. The $^{32}$P remaining in the pellet was

dissolved in 10% sodium dodecyl sulfate, subjected to gel filtration, and identified as $^{32}PP_i$. As expected, therefore, this apparent incorporation is not due to an enzymatic reaction.

Several other conditions for stopping the reaction were investigated in an attempt to circumvent this $^{32}PP_i$ binding which interferes with the phenol assay. Reaction was stopped by addition of cold trichloroacetic acid, alkali, sodium dodecyl sulfate at pH 10, or a urea–Triton–EDTA mix at neutral pH[28]. Instead of the desired diminution of non-covalent $^{32}PP_i$ binding it was found that approximately equal amounts of $^{32}PP_i$ were bound by denatured protein in all conditions, indicating that this strong binding of $^{32}PP_i$ is a general property of denatured microsomal protein. Moreover, phosphoryl histidine could be detected only when the reaction was stopped by addition of phenol. Apparently the effective removal of water by dilution into phenol is necessary to prevent hydrolysis of the phosphoryl enzyme before denaturation is complete. This is not unexpected, from the hydrolytic nature of the reaction.

*Characterization of $^{32}P$-labeled protein*

Because glucose-6-phosphatase has never been satisfactorily separated from the bulk of microsomal protein[5] we sought to do this by utilizing the $^{32}P$ label covalently incorporated into this specific protein, recognizing that because the protein was denatured, only limited characterization might be possible. Protein labeled from glucose 6-[$^{32}P$]phosphate, rather than $^{32}PP_i$, was utilized because of less extensive non-covalent binding of the former to denatured protein. The $^{32}P$-labeled protein pellet, obtained by removal of phenol from the phenol extract, was dissolved in 1 ml 10% sodium dodecyl sulfate titrated to pH 8.6 for molecular weight analysis by sodium dodecyl sulfate gel electrophoresis[29]. Examination of the distribution of radioactivity and protein on duplicate series of gels revealed the presence of at least 10 different protein bands, none of which contained radioactivity. A tiny fraction of radioactivity, presumably $^{32}P_i$ or glucose 6-[$^{32}P$]phosphate, moved with the tracking dye, but the bulk of the $^{32}P$ was found at the top of the gel, along with considerable protein which did not penetrate the gel. Electrophoresis in more porous gels, 4% acrylamide instead of 10%, produced a different pattern of protein bands but again the $^{32}P$-labeled protein did not penetrate the gel. Gel electrophoresis in the presence of 8 M urea produced the same result. Thus some fractionation of labeled protein from other proteins was achieved, but the form of the $^{32}P$-labeled denatured enzyme was not amenable to characterization.

*$^{32}P_i$–Substrate exchange study*

The possible exchange of $^{32}P_i$ with $PP_i$ and with glucose 6-phosphate was examined from pH 3 to 9, with substrate concentrations from 0.1 mM to 1 mM. These experiments were designed to maximize the opportunity for detecting isotope exchange by utilizing very high specific radioactivity $^{32}P_i$ (up to 130 Ci/mmole), and by taking samples at very short reaction time (less than 10% reaction) to minimize dilution of the specific radioactivity of $^{32}P_i$ by product $P_i$. No exchange was observed under any conditions. These experiments indicate that phosphoryl enzyme does not phosphorylate $P_i$ to form $PP_i$ to any significant extent (Reaction 2 in Fig. 6), and provide further evidence that, as shown previously by the lack of incorporation of

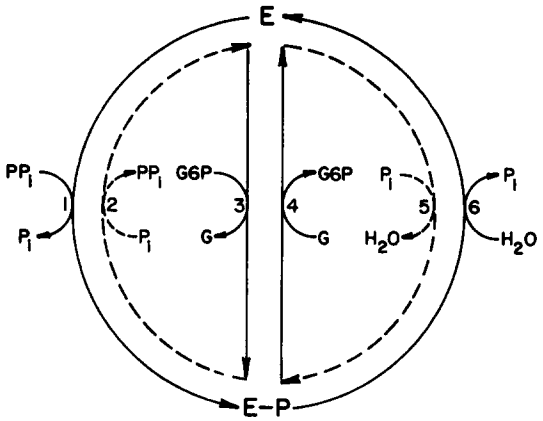


Fig. 6. Reactions catalyzed by microsomal glucose-6-phosphatase. Abbreviations: E, non-phosphorylated enzyme; E–P, enzyme containing phosphoryl histidine; G, glucose; and G6P, glucose 6-phosphate. (Adapted from Arion and Nordlie[39].)

$^{32}P_i$ into the enzyme[10], $^{32}P_i$ does not phosphorylate the active site (Reaction 5 in Fig. 6). However, $P_i$ does bind without phosphorylating the site, for it is a competitive inhibitor[6,9] and provides protection against photoinactivation[9].

## DISCUSSION

### *Evidence for role as intermediate*

If the incorporation of $^{32}P$ from substrate into protein represents formation of an obligatory intermediate in the enzymatic reaction, then incorporation should be observed from all substrates but not from inhibitors. Although not all substrates for this versatile enzyme have been tested for capacity to label the active site, the previous and present observations are in accord with the suggested role of the same phosphoryl enzyme as an obligatory intermediate in its several catalytic activities.

Moreover, an obligatory intermediate should incorporate and discharge $^{32}P$ at a rate which is at least as rapid as the overall rate of the reaction. Comparison of the overall rate of $^{32}P$ incorporation shown in Fig. 1 is complicated by several factors such as the possibility of a rate-limiting accessibility of substrate to the active site of the membraneous enzyme[30,31] and by the possible presence near the active site of endogenous substrates which would dilute the specific activity of the added $^{32}P$-labeled substrate, as well as by the limitations of the assay. It is not known, for example, what proportion of the phosphoryl enzyme present is actually trapped by the phenol stop technique. Within these limitations the labeling data as well as the photooxidation studies[9] do appear to be consistent with the proposed role as obligatory intermediate. Our data further substantiate the presence of both glucose 6-phosphatase and inorganic pyrophosphatase activity in the same protein, as illustrated in Fig. 6. It is conceivable but unlikely that the protein which becomes labeled is a non-specific microsomal phosphatase, rather than glucose-6-phosphatase.

In contrast with the rate of protein labeling, the steady-state level of $^{32}P$ incorporation is less ambiguous; only the last of the complications mentioned above

is likely to have a significant effect. Comparison of the apparent steady-state level of $^{32}P$-labeled protein observed in Fig. 1, 2 pmoles/mg protein, with the observed overall rate of the reaction as determined in the same experiment, $5.6 \cdot 10^3$ pmoles per min per mg protein, yields an apparent turnover number of $2.8 \cdot 10^3$ per min per phosphorylated site detected under these conditions. This is a maximum value; the true turnover number will be lower by a factor equal to the proportion of active sites not trapped in the phosphorylated form in the phenol assay. It is nevertheless clear that the turnover is far too rapid to accurately measure the incorporation rate with the present techniques.

Assuming that 10% of the active sites are trapped in the phosphorylated form (the concentration of glucose 6-[$^{32}P$]phosphate utilized in the experiment described in Fig. 1 is approx. 10% of the $K_m$ for glucose 6-phosphate[5]) the steady-state level of $^{32}P$ incorporation indicates an apparent "molecular weight" of $5 \cdot 10^7$ g microsomal protein per active site. This too is an upper limit, but it suggests that glucose 6-phosphatase is only a minor component of microsomal membranes.

*Free energy of hydrolysis of phosphoryl enzyme*

By analogy with other systems in which it readily phosphorylates ADP to ATP (ref. 32,33), protein bound *N*-3-phosphoryl histidine might be expected to have a high free energy of hydrolysis. Several considerations suggest that, in contrast, in this system as well as a few others[34,35] it has a relatively low free energy of hydrolysis.

The extent of incorporation of $^{32}P$ into a "high energy" intermediate from glucose 6-[$^{32}P$]phosphate should be far lower than that observed from $^{32}PP_i$, which has a relatively high free energy of hydrolysis[36], yet we have repeatedly observed similar degrees of labeling by the two substrates, suggesting that each is energetically capable of essentially saturating the site.

Moreover, the observed isotope exchange patterns are consistent with a "low energy" but not "high energy" intermediate. The various activities of this enzyme are diagrammed in Fig. 6, which shows the interconversion of the non-phosphorylated form of the enzyme, *E*, and the form which contains *N*-3-phosphoryl histidine, *E*-P, by several alternative pathways. Glucose 6-phosphatase activity (Fig. 6, Reactions 3 and 6), inorganic pyrophosphatase (Fig. 6, Reactions 1 and 6), $PP_i$–glucose phosphotransferase (Fig. 6, Reactions 1 and 4), and the exchange of [$^{14}C$]glucose into glucose 6-phosphate (Fig. 6, Reactions 3 and 4) are accounted for by solid lines; the dotted lines (Fig. 6, Reactions 2 and 5) represent other possible reactions which we have demonstrated not to occur at a significant rate.

The lack of phosphorylation of the enzyme by $^{32}P_i$ (ref. 10) and the lack of $^{32}P$ exchange from $^{32}P_i$ into glucose 6-phosphate show the absence of Reaction 5. Thus, the free energy of *E*-P (*N*-3-phosphoryl histidine) is higher than the free energy of non-phosphorylated enzyme *plus* $P_i$. This result, although expected, is not universally observed; under conditions similar to those used here *E. coli* alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) is phosphorylated by $^{32}P_i$ (ref. 36), and formation of protein-bound phosphoryl histidine from $^{32}P_i$ has been reported for a rat liver acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2)[35]. We have looked for exchange of $^{32}P$ from $^{32}P_i$ in the presence of either substrate into $PP_i$ (Fig. 6, Reaction 2) as expected if the phosphoryl enzyme intermediate has a free energy of hydrolysis similar to that of $PP_i$. No evidence of

such exchange was found (exchange rate at least 4 orders of magnitude slower than overall reaction rate), utilizing a variety of conditions. We conclude that Reaction 2 does not occur, presumably because the free energy of hydrolysis of the phosphoryl enzyme intermediate is significantly lower than that of $PP_i$ although mechanistic as well as thermodynamic considerations may be involved. It is possible that lack of this exchange is due to the absence of a binding site for $P_i$ on the phosphorylated enzyme, although this appears unlikely due to the principle of microscopic reversibility. The intermediate appears to be energetically lower than in those enzymes that transfer the phosphoryl group to ADP, but higher than those which can be labeled by $^{32}P_i$ alone.

We may speculate that differences in environment surrounding the phosphorylated histidine are responsible for the apparent differences in the free energy of hydrolysis of this material in various enzymes. Comparison of amino acid sequences in phosphorylated peptides from the appropriate enzymes might be helpful in understanding these differences, but because of the problems inherent in working with substrate levels of a particulate, unpurified enzyme[37], no sequence analyses are contemplated in our laboratory. Interestingly, since both free *N*-3-phosphoryl histidine and peptides and denatured protein containing this material all seem to have similar hydrolytic properties which are characteristic of high energy phosphates[38,10], the free energy of hydrolysis of the phosphorylated form of this enzyme in its native state appears to increase on denaturation.

*Relationship to microsomal membrane*

The present experiments reveal interesting relationships, of as yet unknown significance, between the glucose-6-phosphatase enzyme and the microsomal membrane in which it occurs. The denatured protein bearing the phosphorylated active site is completely available for protease digestion in the insoluble pellet after removal of phenol, but nonhydrolytic "solubilization" by alkali or detergent does not permit the $^{32}P$-labeled protein to enter a polyacrylamide gel, although several other of the denatured microsomal proteins do enter the gel after this treatment. Alternative methods of purification were no more successful; for instance the material invariably precipitated at its isoelectric point in isoelectric focussing experiments. Glucose 6-phosphatase appears to be an integral part of the "basement membrane" of the microsome, accounting for the lack of ability to separate the enzyme activity from the bulk of microsomal protein[9].

In addition to covalently bound phosphoryl group at the glucose-6-phosphatase active site, denatured microsomes contain a class of sites which bind $^{32}PP_i$, but not $^{32}P_i$ or glucose 6-[$^{32}$P]phosphate, very tightly but non-covalently. In the phenol-denatured protein these sites are not destroyed by pronase digestion, and the bound $^{32}PP_i$ is not available for enzymatic hydrolysis, but is readily released by alkali or detergent "solubilization". The occurrence of such sites necessitates careful identification of any apparent covalent incorporation of $^{32}P$ in order to substantiate formation of a phosphoryl enzyme intermediate in this system.

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