ABSTRACT

PURIFICATION AND ROLE OF THE INDUCIBLE SOLUBLE PROTEIN COMPONENT OF THE PHOSPHOENOLPYRUVATE:D-FRUCTOSE 1-PHOSPHOTRANSFERASE SYSTEM OF AEROBACTER AEROGENES

By

Richard Webb Walter, Jr.

An inducible soluble protein, involved in phosphoenolpyruvate (PEP)-dependent phosphorylation of D-fructose, was purified to homogeneity by 100,000 x g centrifugation, differential ammonium sulfate precipitation, chromatography by DEAE cellulose, Sephadex G100, hydroxylapatite columns, and polyacrylamide disc gel electrophoresis. This protein was termed a D-fructose phosphoryl transfer protein (PTP_{fru}). The molecular weight of PTP_{fru} was determined to be 52,000 by Sephadex G100 chromatography and the protein contains two 26,000 molecular weight subunits as determined by SDS polyacrylamide disc gel electrophoresis. PTP_{fru} is required for growth of Aerobacter aerogenes PRL-R3 on low concentrations of D-fructose.

PTP_{fru} significantly increased only the activity of the enzymes II (membrane-bound component of the PEP: D-fructose 1-phosphotransferase system) obtained

from D-fructose-grown cells when it was added to various enzymes II obtained from cells grown on a variety of substrates. Only 100,000 x g supernatants obtained from D-fructose-grown cells activated enzyme II_{fru}. Thus, D-fructose specifically induces both an enzyme II and a soluble component, PTP_{fru}, which function in a PEP:D-fructose 1-phosphotransferase system. This system has a low K_m (1.6×10^{-5} M) for D-fructose and does not require HPr for activity. A second system that does require HPr for activity is constitutive and has a high K_m (7.1×10^{-3} M) for D-fructose.

Assays were developed to determine the individual enzyme II activities in the presence of each other. The amount of the inducible enzyme II is from 5 to 20 percent of the total activity in enzyme II preparations obtained from cells grown on substrates other than D-fructose, whereas it comprises from 50 to 90 percent of the total activity in enzyme II preparations obtained from cells grown on D-fructose. The inducible enzyme II_{fru} is activated by 2-mercaptoethanol.

[^{32}P] PEP was formed enzymatically from $^{32}P_i$ and L-malate and was identified by paper chromatography in two solvent systems and by enzymatic reactions involving the formation of [^{32}P] ATP and D-fructose 1-[^{32}P] phosphate.

A phosphoryl transfer from [^{32}P] PEP to PTP_{fru} was catalyzed by enzyme I and did not require HPr. The

[^{32}P] phospho-PTP_{fru} formed had two moles ^{32}P bound per mole of 52,000 molecular weight protein, or one mole bound per monomer of 26,000 molecular weight. Enzyme II_{fru} catalyzed the phosphoryl transfer from [^{32}P] phospho-PTP_{fru} to D-fructose, forming D-fructose 1-[^{32}P] phosphate.

Although HPr is not required either for D-fructose phosphorylation by the inducible system or for phosphorylation of PTP_{fru} by enzyme I, it does affect the activity of the inducible system. When PTP_{fru} is limiting, HPr increases the velocity of this system; however, at saturating levels of PTP_{fru} the apparent V_{max} is decreased by HPr. The K_A for HPr of the inducible system is 1/50 the K_m of the constitutive system for HPr. Increasing the concentration of PTP_{fru} does not affect the apparent K_A, whereas increasing HPr decreases the apparent K_m for PTP_{fru}.

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By

Richard Webb Walter, Jr.

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ABBREVIATIONS

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
Bicine	N,N-bis(2-hydroxyethyl)glycine
BSA	bovine serum albumin
DEAE	diethylaminoethyl-
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
E_I	enzyme I of PEP-dependent phosphotransferase system
E_{II}	enzyme II of PEP-dependent phosphotransferase system
enzyme II_{fru}	enzyme II obtained from cells induced on D-fructose
enzyme II_{glu}	enzyme II obtained from cells induced on D-glucose
enzyme II_{gly}	enzyme II obtained from cells induced on glycerol
enzyme II_{mtl}	enzyme II obtained from cells induced on D-mannitol
enzyme II_{NB}	enzyme II obtained from cells induced on nutrient broth
F-1-P	D-fructose 1-phosphate
GDP	guanosine 5'-diphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPr	histidine-containing protein
HPr_{fru}	HPr obtained from cells induced on D-fructose

HPr_{mtl}	HPr obtained from cells induced on D-mannitol
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
^{32}P	labeled phosphoryl group
P_i	orthophosphate
PEP	phosphoenolpyruvate
PGA	phosphoglyceric acid
PIPES	piperazine-N,N'-bis(2-ethane-sulfonic acid)
PTP_{fru}	D-fructose phosphoryl transfer protein
SDS	sodium dodecyl sulfate
TEMED	N,N,N'N'-tetramethylenediamine
TES	N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid
Tricine	N-tris(hydroxymethyl)methyl glycine
Tris	tris(hydroxymethyl)aminomethane

INTRODUCTION

The first step in D-fructose metabolism in Aerobacter aerogenes is phosphorylation with phosphoenolpyruvate to yield D-fructose 1-phosphate (35, 100). The enzyme system that catalyzes this reaction has been resolved by Hanson and Anderson (35) into four protein components. Three of the components, called enzyme I, enzyme II, and HPr according to the terminology of Kundig, Ghosh, and Roseman (61), were believed to be constitutive and to participate in the following reactions:

$$\text{PEP+HPr} \xrightarrow{E_I} \text{pyruvate+phospho-HPr}$$

$$\text{Phospho-HPr+D-fructose} \xrightarrow{E_{II}} \text{HPr+D-fructose 1-phosphate}$$

The fourth component, which was specifically induced by D-fructose, was termed a "D-fructose specifier protein" or a "K_m factor" because it increased the maximal velocity and the affinity of the system for D-fructose. A mutant (CQ17) lacking this "specifier protein" had impaired growth on D-fructose but not on other sugars (33, 35).

The purpose of this thesis investigation was to further elucidate the roles of the four components

involved in this PEP-dependent phosphotransferase reaction, particularly with respect to the "specifier protein". The results indicate that there are actually two separate systems that utilize phosphoenolpyruvate to phosphorylate D-fructose at C-1. One system has a low affinity for D-fructose; it involves enzyme I, HPr, and a constitutive enzyme II. The other system has a high affinity for D-fructose; it involves enzyme I, the inducible "specifier protein", and an enzyme II which is also induced by growth on D-fructose. In this thesis, the "specifier protein" has been termed a "phosphoryl transfer protein" (PTP_{fru}) because, as the data will show, this more accurately describes its function. Enzyme I catalyzes a phosphoryl transfer from PEP to PTP_{fru}; however, HPr is not required for this phosphorylation and, further, seems not to be required for the enzyme II_{fru}-catalyzed phosphorylation of D-fructose in the presence of PEP, enzyme I, and PTP_{fru}.

LITERATURE REVIEW

The phosphoenolpyruvate:sugar phosphotransferase system was first discovered in 1964 by Kundig, Ghosh, and Roseman (61). Since then there has been an extensive amount of research done in this field, attempting to further explain this complex system which is involved in both the phosphorylation of sugars and their transport into the cell. Much of this work has recently been reviewed (3, 47-49, 83, 93). Consequently, this literature review will only attempt to summarize the major findings, cite critical similarities and differences, and update the previous reviews.

As first described, the system in Escherichia coli was comprised of three protein components: (i) Enzyme I, a cytoplasmic protein that catalyzes a phosphoryl transfer from PEP to a histidine residue of HPr, (ii) HPr, a heat stable, low molecular weight (9,500 daltons) (2) protein that is found in the periplasmic space (62), and (iii) enzyme II, a membrane-bound protein that catalyzes the phosphoryl transfer from phospho-HPr to the sugar (61, 62). In most cases the products formed are 6-phosphate esters (62); however, both D-fructose

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(25, 35) and L-sorbose (54) are converted to 1-phosphate esters.

Mutants missing either enzyme I or HPr are pleiotropic in that they do not grow on a wide variety of sugars. Pleiotropic mutants isolated from Aerobacter aerogenes and Escherichia coli by Tanaka and co-workers (107, 109) lack the ability to utilize D-glucose, D-fructose, D-mannose, D-glucitol, and D-mannitol; however they do grow on D-galactose. Fox and Wilson (24) reported that an Escherichia coli enzyme I negative mutant lacks the ability to utilize twelve sugars and related compounds, including lactose, succinate, and D-galactose. Pleiotropic mutants of Staphylococcus aureus isolated by Egan and Morse (19) do not grow on eight different sugars including D-galactose and lactose, whereas Salmonella typhimurium mutants isolated by Simoni and co-workers (104) have been shown to lack the ability to grow on nine different sugars.

Some pleiotropic mutants do not grow on glycerol or succinate even though these compounds are not phosphorylated by the PEP-dependent phosphotransferase system. This could implicate a control mechanism involving the phosphotransferase system (9). Other studies have linked enzyme I with both transient repression (116) and inducer exclusion (94), and enzyme II with catabolite repression (17, 84). Addition of cyclic AMP relieves both types of repression. This

permits enzyme I negative Escherichia coli mutants to grow on lactose (84) and on glycerol (10).

Whereas enzyme I and HPr are constitutive and are utilized in the phosphorylation of many sugars, there is a family of enzymes II, some of which are constitutive (62), while a majority are inducible and sugar-specific (41, 62, 105).

Mutants lacking functional enzymes II only lose the ability to grow on a single sugar or closely related sugars, such as lactose (41), D-fructose (21), β-glucosides (24), or D-mannitol (8, 108), and therefore are missing components of sugar-specific enzymes II, rather than constitutive enzymes II.

In 1968 Hanson and Anderson (35) described an inducible fourth component ("K_m factor", "D-fructose specifier protein") which increased the V_{max} and decreased the K_m of a D-fructose phosphotransferase system in Aerobacter aerogenes. A mutant, QQ17, missing this component exhibited defective growth on D-fructose but grew normally on other substrates. Crude extracts of this mutant induced on D-fructose retained their high K_m constitutive-type enzyme II activity for D-fructose. This activity was converted to a normal wild-type activity with low K_m for D-fructose by addition of partially purified "K_m factor". Enzyme II isolated from D-mannitol-grown cells contained a constitutive high K_m activity for D-fructose phosphorylation which was

inhibited by addition of D-mannitol; however, addition of "K_m factor" to this enzyme II had no effect on the K_m or V_{max}. Further, D-mannitol did not inhibit D-fructose phosphorylation by enzyme II which had been isolated from D-fructose-grown cells. Thus, enzymes II which were isolated from D-mannitol- and D-fructose-grown cells had different properties. It was proposed that the enzyme II "heavy protein" was constitutive and that various "factors" induced on different substrates ascribed sugar-specificity to the system. If the D-mannitol-induced factor was tightly bound to the heavy protein, it would not easily be displaced by the D-fructose specifier protein and thus no change in K_m would be seen (33, 35).

Two four-component systems have been isolated from Staphylococcus aureus. They involve specifically induced enzymes II for lactose and D-mannitol as well as specifically induced fourth components (Factors III) for these two sugars (40, 41, 105). There is a nearly absolute requirement for both components for phosphorylation of the sugar for which they are specific. Further investigations of the lactose-specific Factor III have shown that it is a phospho-protein (78) with a molecular weight of 36,000 daltons with three or four subunits of 9,000 to 12,000 daltons (101). It contains two phosphates per protein molecule and functions as an intermediate between phospho-HPr and enzyme II^{lac} (78).

Hengstenberg (37) has solubilized the lactose-specific enzyme II and found that it retains activity after most of the lipid appears to be removed.

In investigations of a constitutive phosphotransferase system in Escherichia coli, Kundig and Roseman (64-66, 93) have fractionated a solubilized enzyme II isolated from D-glucose-grown cells into two protein components (II-A and II-B) and phosphatidylglycerol. II-A has been further separated into three proteins which specify phosphorylation of D-glucose, D-fructose, and D-mannose, respectively. II-B is a protein with a molecular weight of 36,000 daltons and forms an active complex with any of the II-A proteins in the presence of phosphatidylglycerol. This is another four-component system that has sugar-specificity, although the individual II-A proteins are not specifically induced. Also, both protein components are originally membrane-bound, whereas Factors III for lactose and D-mannitol (105) and the "K_m factor" (35) for D-fructose are soluble components.

Rose and Fox (24, 91, 92) have solubilized a β-glucoside enzyme II from Escherichia coli; however, they have not detected any corresponding fourth components. This solubilized enzyme II apparently does not require lipid for activity (91), whereas phosphatidylglycerol is required for formation of an active enzyme II complex in Roseman's studies (64-66). Palmer

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(81) has shown that Aerobacter aerogenes does not contain a PEP-dependent phosphorylation system for the β-glucoside cellobiose; however, it does have an ATP-dependent β-glucoside kinase (81, 82).

Aside from phosphorylation as the initial step in the metabolism of sugars that utilize this system, the phosphotransferase system also functions in the unidirectional transport of the sugars it phosphorylates across the bacterial membrane. This dual function has been termed both group translocation (93) and vectorial phosphorylation (46, 48). Many studies done with whole cells have related uptake of substrates or accumulation of phosphate esters to the phosphotransferase system (19, 28, 29, 31, 38-41, 53, 55, 102, 107, 111, 120, 129). Kaback (45-47) has shown PEP-dependence of uptake of sugars as their phosphorylated derivatives into membrane vesicles. Treatment of vesicles with phospholipase D, which specifically hydrolyzes phosphatidylglycerol, inhibits vectorial phosphorylation of α-methylglucoside. Membrane vesicles isolated from mutants missing enzyme I or HPr were also shown to lack the ability to transport sugars. Osmotically shocked (42, 79, 80) cells have decreased levels of both uptake and phosphorylation activities (43). Addition of HPr to these shocked cells restores both of these activities (62).

This phosphotransferase system that functions both in the phosphorylation and translocation of sugars has

mainly been studied in Aerobacter aerogenes (33, 35, 52, 53, 109), Escherichia coli (1, 2, 20-22, 24-26, 28, 29, 45-48, 60-66, 91, 92, 102, 108, 119-122, 129), Salmonella typhimurium (8, 70, 95, 96, 104, 111), and Staphylococcus aureus (19, 37-41, 76-78, 101, 105). It has also been detected in many other anaerobes or facultative anaerobes including Achromobacter parvulus (90), Bacillus cereus (90), Bacillus megaterium (90), Bacillus subtilis (27, 45, 62, 64, 90), Clostridium perfringens (31), Clostridium thermocellum (85, 86), Corynebacterium ulcerans (90), Lactobacillus arabinosus (61), and Streptococcus lactis (73). The strictly aerobic bacteria investigated by Romano and co-workers (90) showed either very low or no PEP-dependent phosphorylation of 2-deoxyglucose. Pseudomonas aeruginosa (89) and the fungus Aspergillus nidulans (12) have also been shown to lack the phosphotransferase system. Leighton (68) has presented some qualitative data which imply that Microsporum gypseum contains a PEP-dependent inducible system that phosphorylates D-fructose and D-mannitol; however, the method by which these data were acquired, and thus their validity, are not clear.

Van Steveninck (117) has shown evidence for a maltose-induced transport-associated phosphorylation of α-methylglucoside in Saccharomyces cerevisiae, but the phosphoryl donor has not been characterized. Recently, Saier and co-workers (96) reported on a PEP-dependent

phosphorylation of D-fructose in photosynthetic bacteria (Rhodospirillum rubrum and Rhodopseudomonas spheroides) which utilized two membrane-bound components, one of which is solubilized by low ionic strength buffer. Neither of these proteins complemented Salmonella typhimurium enzyme I or HPr mutants for α-glucoside phosphorylation. Also, enzyme I and HPr isolated from Salmonella did not substitute for either of the Rhodospirillum rubrum components in D-fructose phosphorylation. It has not been demonstrated that this system functions in vectorial phosphorylation.

Weiser (125, 126) has described a PEP-dependent phosphorylation of D-fructose to D-fructose 1-phosphate in rat intestine. No evidence has been obtained to indicate that this PEP-activated system is involved in intestinal sugar transport.

Ferenci and Kornberg (21, 22) have reported that extracts of Escherichia coli grown on D-fructose contain a PEP-dependent D-fructose phosphotransferase system that forms D-fructose 1-phosphate at a low concentration of D-fructose (0.04 mM); however at a high concentration of D-fructose (50 mM), D-fructose 6-phosphate is also formed. Neither D-fructose 1-phosphate nor D-fructose 6-phosphate are formed by a mutant apparently missing an enzyme II specific for D-fructose.

Another system that takes up phosphorylated hexoses has been studied by Winkler (130, 121) and Ferenci (23).

This hexose 6-phosphate transport system is induced by growth on D-glucose 6-phosphate, D-mannose 6-phosphate, or D-fructose 6-phosphate, but not by D-fructose 1-phosphate. Induced cells, however, were found to transport D-fructose 1-phosphate for one to one-and-one-half generations. Mutants lacking phosphoglucose isomerase are not induced by D-fructose 6-phosphate (130) and double mutants lacking phosphoglucose isomerase and D-glucose 6-phosphate dehydrogenase are not induced by growth on D-glucose (130). Thus, the true inducer is exogenous D-glucose 6-phosphate (18). There has been no evidence presented to relate this hexose phosphate transport system to the PEP-dependent phosphotransferase system.

Other transport systems have been elucidated and are discussed in the review articles cited (3, 47-49, 83, 93). The most pertinent of these transport systems is the "D-galactose permease" system originally described by Horecker (44). It has more recently been shown to be an active transport system coupled primarily to a membrane-bound D-lactate dehydrogenase (6, 7, 50, 56, 127, 128). Such coupled transport systems have been characterized in vesicles isolated from a wide variety of aerobic and facultatively anaerobic bacteria (59). This system could be the initial step in the metabolism of sugars in aerobic bacteria replacing the PEP-dependent phosphotransferase system which serves this function

in anaerobic and facultative anaerobic bacteria. Von Meyenburg (118) has isolated a mutant that has transport-limited growth rates for sugars, amino acids, and the anions PO_4^{3-} and SO_4^{2-}. The defect in this mutant has not been characterized; however, it could be related to the D-lactate dehydrogenase-coupled system.

As indicated by this literature review, the PEP-dependent phosphotransferase system is a complex system which varies widely in its specificity in different species. The research reported in this thesis further elucidates the roles of the four components involved in the PEP:D-fructose 1-phosphotransferase system in Aerobacter aerogenes.

EXPERIMENTAL METHODS

Bacterial Strains

Aerobacter aerogenes PRL-R3 (wild type) and a uracil auxotroph, PRL-R3(U^-), derived from it were used as the parental organisms. A mutant (strain DD31) missing D-fructose 1-phosphate kinase (53) and a mutant (strain QQ17) missing the D-fructose "specifier protein" (35), more correctly termed D-fructose "phosphoryl transfer protein" (PTP_{fru}), were both isolated by mutagenesis of PRL-R3 with ethyl methanesulfonate, treatment with penicillin D in D-fructose mineral media, and selection of small colonies on mineral-agar plates containing 0.5 percent D-fructose and 0.005 percent D-glucose. A mutant lacking D-fructose 6-phosphate kinase (strain A9-1) (100) was isolated from PRL-R3(U^-) by mutagenesis with ethyl methanesulfonateate and selection for positive growth on D-fructose and slow growth on D-glucose and D-mannose by replica plating techniques.

Pleiotropic mutants were isolated from PRL-R3(U^-). Cells from an overnight 7.0 ml nutrient broth culture were harvested by centrifugation, washed twice with 14 ml of mineral medium, and resuspended in 7.0 ml of mineral medium. Ethyl methanesulfonate (0.03 ml) was

added to 2.0 ml of the resuspended cells which were then incubated at 32°C on a reciprocal shaker. After 2 hours the cells were harvested, washed three times with 7.0-ml volumes of mineral medium, resuspended in 7.0 ml of mineral medium containing 0.5 percent D-galactose, and grown for 10 generations. The expressed cells were diluted serially to 500 cells per 0.1 ml, assuming 10^9 cells per ml in a maximally grown culture, and 0.1-ml amounts were plated on MacConkey agar containing 0.5 percent D-mannitol. Nonfermenting pale colonies were then streaked on five individual MacConkey agar plates containing 0.5 percent D-fructose, D-galactose, D-mannose, D-mannitol, and D-glucitol, respectively. Several strains failed to ferment all the tested sugars except D-galactose and were considered pleiotropic mutants. Crude extracts of each strain, induced on D-fructose mineral medium for 4 hours after growth on nutrient broth overnight, were assayed for their various phosphotransferase components by assaying for PEP-dependent D-fructose 1-phosphate formation in the presence of added enzyme I, enzyme II, HPr, PTP_{fru}, or no addition.

Only the addition of enzyme I stimulated D-fructose phosphorylation, and thus it was concluded that all the pleiotropic strains were missing enzyme I. Strain PL-122 (52) had the highest level of activation and was the strain utilized in this study.

Spontaneous revertants were obtained by plating an overnight broth culture of PL-122 on D-mannitol MacConkey agar and selecting red colonies that developed. These revertants were then tested for their uracil requirement for growth to eliminate the possibility of contamination. The revertant (PL-122R) selected for further study phosphorylated D-fructose at wild-type rates without added enzyme I and required uracil for growth.

Media

For growth of uracil auxotrophs, 0.005 percent uracil (Sigma) was added to all media described. The pH of all media was adjusted to 7.0 before autoclaving.

Mineral Medium

This medium consisted of the following components: 0.71 percent of Na_2HPO_4, 0.15 percent of KH_2PO_4, 0.3 percent of $(NH_4)_2SO_4$, 0.01 percent of $MgSO_4$, 0.0005 percent of $FeSO_4 \cdot 7\ H_2O$, and 0.5 percent of a specified sugar (autoclaved separately).

Nutrient Broth Medium

This medium consisted of 5.0 g of Bactopeptone (Difco) and 3.0 g of Bacto beef extract (Difco) or 8.0 g of Bacto nutrient broth (Difco) in 1.0 l of water.

Nutrient Agar Medium

This medium consisted of 23 g of Bacto nutrient agar (Difco) in 1.0 l of water.

MacConkey Agar Medium

This medium consisted of 40 g of MacConkey agar base (Difco) in 1.0 l of water mixed with an equal volume of 1 percent of a specified sugar after being autoclaved separately.

Growth of Cultures

Growth curves were done in 18 x 150-mm culture tubes containing 7.0 ml of mineral medium which contained 0.3 percent sugar except where specified otherwise. Inocula were 0.1-ml amounts of overnight nutrient broth cultures. The tubes were incubated at 32°C on a reciprocal shaker and optical density readings were made at 520 nm with a Coleman Jr. spectrophotometer. Growth of cells for enzyme purification was done either in 1.0 l of mineral medium in Fernbach flasks on a rotatory shaker at 32°C, in 40 l of mineral medium in carboys at 32°C with cotton-filtered air bubbling through at 8 lb pressure, or in 80 l of mineral medium in a New Brunswick Model 130 Fermacell fermenter.

For induction studies, cells from overnight nutrient broth cultures were collected by centrifugation and resuspended in mineral medium plus 0.5 percent inducer.

These suspensions were then incubated with agitation at 32°C for 4 hours.

Preparation of Cell Extracts

Cells were harvested by centrifugation at 16,000 x g in a Sorvall refrigerated centrifuge when grown in tubes or Fernbach flasks. Larger quantities of cells were harvested in a Sharples centrifuge. The cells were washed twice with 0.85 percent NaCl and resuspended in 0.02 M Tris-HCl buffer (pH 7.5) containing 0.028 M 2-mercaptoethanol. For purification of enzymes other than those involved in the phosphotransferase system the cells were resuspended in 0.02 M potassium phosphate buffer (pH 7.5). Small volumes of cells were broken by ultrasonic vibration for 10 minutes in a Raytheon 250-watt, 10-kHz sonic oscillator equipped with an ice-water cooling jacket. Larger cell preparations were broken in a Manton-Gaulin Laboratory homogenizer (Model 15M-8TA) at 6,000 lb pressure. The broken cell suspensions were centrifuged at 45,000 x g for 15 minutes and the resulting supernatant was the crude extract. All purifications were carried out at 0 to 4°C.

Preparation of Enzymes

Preparation of Enzyme II

The crude extract was centrifuged in a Spinco L2 ultracentrifuge either at 38,000 rpm (100,000 x g) for 2 hours in a Beckman 40 rotor, or at 28,000 rpm (68,000 x g) for 3 hours in a Beckman 30 rotor. The resulting pellet was resuspended in 0.02 M Tris-HCl (pH 7.5) containing 0.028 M 2-mercaptoethanol and recentrifuged at 100,000 x g for 2 hours. The resulting pellet was resuspended in the same buffer and was the enzyme II preparation used in this study. Where noted, this preparation was resonicated for 10 minutes and chromatographed on Sephadex G200. The enzyme II activity was eluted in the void volume with 0.02 M Tris-HCl (pH 7.5) containing 0.028 M 2-mercaptoethanol and recentrifuged at 100,000 x g for 90 minutes. The resulting pellet was resuspended in the same buffer and used as purified enzyme II. These preparations were stored at $0^{o}C$ and used for a period of 1 month.

Preparation of Enzyme I

Enzyme I was prepared from both D-fructose- and D-mannitol-grown cells. The first 100,000 x g supernatant from the above enzyme II preparation was recentrifuged at 100,000 x g. The resulting supernatant was fractionated by ammonium sulfate precipitation. The 40 to 70 percent saturation precipitate was dissolved

in 0.02 M potassium phosphate buffer (pH 8.0) containing 0.001 M DTT and 10 percent glycerol, dialyzed overnight against the same buffer, and layered on a 4.8 x 12.5-cm DEAE cellulose column. The column was eluted with a 0 to 0.4 M KCl linear gradient in a 0.02 M potassium phosphate buffer (pH 8.0) containing 0.001 M DTT and 10 percent glycerol. The active fractions (0.3 M KCl) were concentrated by ultrafiltration, layered on a 4.8 x 35-cm Sephadex G200 column, and eluted with the same buffer. The pooled activity was concentrated by ultrafiltration, dialyzed against 0.02 M potassium phosphate buffer (pH 7.5) containing 0.001 M DTT and 0.001 M EDTA, and stored at -10°C. This 60-fold purified enzyme I preparation was devoid of enzyme II, HPr, PTP_{fru}, D-fructose 1-phosphate kinase, and D-fructose 1-phosphate phosphatase. Enzyme I was stored at -10°C and was used for periods of up to 6 months with a loss in activity of 50 percent.

Preparation of HPr

The 40 to 70 percent saturation supernatant from the enzyme I preparation was saturated with ammonium sulfate (30) and after equilibrating for 1 hour was centrifuged at 40,000 x g. The resulting precipitate was dissolved in 0.02 M potassium phosphate buffer (pH 7.5) and layered on a 4.8 x 35-cm Sephadex G75 column. The activity was eluted with the same buffer

and layered directly on a 4.8 x 12.5-cm DEAE cellulose column. The activity eluted at 0.1 M KCl with a 0 to 0.2 M KCl linear gradient in a 0.02 M potassium phosphate buffer (pH 7.5) and was concentrated by lyophilization. The dried protein was dissolved in distilled water and dialyzed against 0.02 M potassium phosphate buffer (pH 7.5). This 130-fold purified HPr was devoid of enzyme I, enzyme II, PTP_{fru}, D-fructose 1-phosphate kinase, and D-fructose 1-phosphate phosphatase. HPr was stored at -10°C and used over a period of 2 months.

Preparation of D-Fructose 1-Phosphate Kinase

D-fructose 1-phosphate kinase was purified by the method of Sapico (97, 98) through the calcium phosphate step from crude extracts of strain A9-1 grown on D-fructose. Some D-fructose 1-phosphate kinase preparations were obtained as by-products of the PTP_{fru} preparation.

D-fructose 1-phosphate kinase nearly cochromatographs with PTP_{fru} on both DEAE cellulose and Sephadex G200 columns as well as precipitating in the same ammonium sulfate fractions. However, D-fructose 1-phsophate kinase separates from PTP_{fru} on the hydroxylapatite column. D-fructose 1-phosphate kinase activity was monitored during the purification of PTP_{fru} both as a marker for the location of PTP_{fru} and as a measure of its contamination in the PTP_{fru} pools. If any ATP

was present in the assay for PTP_{fru}, the presence of D-fructose 1-phosphate kinase could convert some of the D-fructose 1-phosphate formed to D-fructose 1,6-diphosphate, and thus affect the measured PTP_{fru}-dependent activity.

General Assay Procedures

All enzyme and end-point assays involved the use of a Gilford Model 2400 absorbance recording spectrophotometer thermostatically regulated at 30°C. Microcuvettes with a 1-cm light path were used. The oxidation and reduction of pyridine nucleotide was measured by monitoring the optical density changes at 340 nm. In all cases the enzymes were assayed at levels that gave rates proportional to the enzyme concentration.

Substrate Assays

Assay for D-Fructose 1-Phosphate

A quantitative end-point assay for D-fructose 1-phosphate consisted of the following components in a total volume of 0.15 ml: 0.05 μmole of NADH, 10 μmoles of glycylglycine buffer (pH 7.5), 1.0 μmole of $MgCl_2$, 0.5 μmole of ATP, 5.0 μmoles of KCl, and excesses of D-fructose 1,6-diphosphate aldolase, triose phosphate isomerase, α-glycerophosphate dehydrogenase, and D-fructose 1-phosphate kinase purified through the calcium phosphate gel step, or an hydroxylapatite column.

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An aliquot of a solution containing D-fructose 1-phosphate was added and the reaction started by the addition of D-fructose 1-phosphate kinase or ATP. An optical density change of 0.82 is equivalent to 0.01 μmole of D-fructose 1-phosphate (35).

Assay for D-Fructose

This end-point assay contained the following components in a total volume of 0.15 ml: 10 μmoles of glycylglycine buffer (pH 7.5), 1.0 μmole of $MgCl_2$, 0.5 μmole of ATP, 0.2 μmole of $NADP^+$, and excesses of hexokinase, phosphoglucose isomerase, and D-glucose 6-phosphate dehydrogenase. The reaction was started by the addition of ATP. An optical density change of 0.41 is equivalent to 0.01 μmole of D-fructose.

Assay for D-Fructose 6-Phosphate

This end-point assay was similar to the D-fructose end-point assay except that hexokinase and ATP were omitted. The reaction was started by the addition of phosphoglucose isomerase.

Assay for D-Glucose 6-Phosphate

This end-point assay was similar to the D-fructose assay with ATP, hexokinase, and phosphoglucose isomerase being omitted. The reaction was started by the addition of D-glucose 6-phosphate dehydrogenase.

Assay for D-Fructose 1,6-Diphosphate

This end-point assay was similar to the D-fructose 1-phosphate assay except that D-fructose 1-phosphate kinase, ATP, and $MgCl_2$ were omitted. The reaction was started by the addition of aldolase.

Assay for Phosphoenolpyruvate

This end-point assay contained the following components in a total volume of 0.15 ml: 10 μmoles of glycylglycine buffer (pH 7.5), 0.05 μmole of NADH, 1.0 μmole of $MgCl_2$, 0.5 μmole of ADP, and excesses of pyruvate kinase and D-lactate dehydrogenase. The reaction was started by the addition of ADP. An optical density change of 0.41 is equivalent to 0.01 μmole of phosphoenolpyruvate.

Enzyme Assays

Assay for Aldolase

This enzyme assay contained the same components as the D-fructose 1,6-diphosphate assay with the exception of aldolase which was limiting. The reaction was started by the addition of 1.0 μmole of D-fructose 1,6-diphosphate.

Assay for D-Fructose 1-Phosphate Kinase

This assay contained the same components as the D-fructose 1-phosphate end-point assay except that 1.0 μmole of D-fructose 1-phosphate was added and the rate

of NADH oxidation was proportional to the amount of D-fructose 1-phosphate kinase added (34, 99). Activity was defined as the μmoles of D-fructose 1,6-diphosphate formed per minute per ml.

Assay for Malate Dehydrogenase

This assay contained the following components in a total volume of 0.15 ml: 2.5 μmoles of potassium phosphate buffer (pH 7.4), 0.05 μmole of NADH, and 0.15 μmole of oxaloacetic acid which was used to start the reaction. An optical density change of 0.41 is equivalent to reduction of 0.01 μmole of oxaloacetic acid.

Assay for D-Glucose 6-Phosphate Dehydrogenase

This assay was similar to the end-point assay for D-glucose 6-phosphate except that 0.5 μmole of D-glucose 6-phosphate was added to start the reaction and the rate of reduction of $NADP^+$ was proportional to the amount of D-glucose 6-phosphate dehydrogenase added.

Assay for Hemoglobin

The elution of hemoglobin from a Sephadex G100 column, used for molecular weight determination, was monitored by measuring the absorbance at 415 nm (4).

Assay for Enzyme I (D-mannitol continuous)

This assay consisted of the following components in a total volume of 0.15 ml: 8.0 μmoles of Tris-HCl buffer (pH 9.0), 0.2 μmole of $NADP^+$, 0.2 μmole of NAD^+,

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1.0 μmole of $MgCl_2$, 1.0 μmole of PEP, 0.5 μmole of D-mannitol, 0.57 μmole of 2-mercaptoethanol, 5.0 μl of enzyme II_{mtl} and excesses of HPr, D-mannitol 1-phosphate dehydrogenase (either purified by the method of Liss (71) or 1.0 μl of a 100,000 x g supernatant from crude extracts induced on D-mannitol), phosphoglucose isomerase, and D-glucose 6-phosphate dehydrogenase. The reaction was routinely started by the addition of PEP and after a lag of from 1 to 10 minutes (depending on the enzyme II preparation) the observed rate of increase in absorbance at 340 nm was dependent on enzyme I concentration. An optical density change of 0.41 was equivalent to 0.01 μmole of D-mannitol 1-phosphate formed since the enzyme II preparation contained NADH oxidase. For a more accurate measure of enzyme I activity, sealed cuvettes were evacuated and oxygen present in the reaction mixture was displaced with nitrogen. This process was repeated three times, the reaction was equilibrated at 30°C in a thermostated cuvette positioner, and PEP was added to start the reaction. With this anaerobic system there was less of a lag period and 0.01 μmole of D-mannitol 1-phosphate gave a change in optical density of 0.82. A unit of activity is defined as μmoles D-mannitol 1-phosphate formed per minute per ml.

Assay for HPr (D-mannitol continuous)

This assay was the same as the enzyme I (D-mannitol continuous) assay except that saturating levels of enzyme I and limiting levels of HPr were used.

General Assay for PEP-Dependent D-Fructose 1-Phosphate Formation

This assay consisted of the following components in a total volume of 0.2 ml in a 6 x 50-mm test tube: 8.0 μmoles of Tris-HCl (pH 7.5), 0.57 μmole of 2-mercaptoethanol, 1.0 μmole of PEP, and amounts of $MgCl_2$, D-fructose, enzyme I, HPr, enzyme II, and PTP_{fru} as noted in the individual assays for the separate components. The assay was started by the addition of PEP or crude extract (when included in the assay), incubated at 30°C for 10 minutes, and stopped by placing the tubes in a 95°C bath for 7 minutes. The denatured protein was removed by centrifugation at 6,000 x g for 10 minutes and a 50 μl aliquot was assayed for D-fructose 1-phosphate.

The amount of D-fructose 1-phosphate formed in the total reaction was calculated and related to the component being assayed in the form of nmoles of D-fructose 1-phosphate formed per minute per ml or mg of that component.

When crude extracts were assayed, low levels of $MgCl_2$ (0.01 μmole per 0.2 ml assay) were added to decrease the activity of D-fructose 1-phosphate kinase, and NaF

(3.0 μmoles per 0.2 ml assay) was added to inhibit the activities of enolase and D-fructose 1-phosphatase. Endogenous contamination by these enzymes would decrease the measured rate of D-fructose 1-phosphate formation dependent on PEP.

In reconstituted assays with purified components, which were devoid of D-fructose 1-phosphatase, ATP, and 2-phosphoglycerate, 1.0 μmole of $MgCl_2$ was added and NaF was omitted, because low concentrations of $MgCl_2$ and the presence of NaF in these reconstituted assays were found to inhibit D-fructose 1-phosphate formation.

Various D-fructose concentrations were used throughout this thesis research and are noted with each individual experiment. Initially, assays for PTP_{fru} were run at 2.0, 20, and 200 mM D-fructose to obtain a relative measure of its effect on the K_m of the system, and assays for enzyme I, HPr, and enzyme II were run at saturating (200 mM) D-fructose concentrations. Later in the study, when it became apparent that there were two enzyme II activities with high (7 mM) and low (0.02 mM) K_m's for D-fructose, 100 mM D-fructose was used in assaying for levels of enzyme I, HPr, and the constitutive high K_m enzyme II, whereas 1.0 mM and 0.5 mM D-fructose were used in assaying for the inducible low K_m enzyme II_{fru} and PTP_{fru} in the presence of enzyme II_{fru}.

The inducible system is nearly saturated at 0.5

mM D-fructose and so, by altering the concentration from 2.0 to 1.0 to 0.5 mM would not affect the actual activity of the inducible system; however, the apparent inducible activity at 2.0 mM would increase because of the presence of the constitutive system whose activity would necessarily increase.

A problem inherent in assaying for PTP_{fru} and in determining the K_m of the inducible system for D-fructose was the low level of substrate. In the normal D-fructose 1-phosphate end-point assay utilizing a 50 µl aliquot of the D-fructose 1-phosphate-forming assay solution, utilization of 25 percent of 0.5 mM D-fructose would give an optical density change of 0.51. Thus, all assays had to be performed at low levels of enzyme II activity to prevent depletion of D-fructose and consequent nonlinear formation of D-fructose 1-phosphate. In the experiment described by Figure 17, where the K_m for D-fructose of the inducible system was determined, the effect of depletion of D-fructose on the rate of the reaction was corrected for by measuring the initial and final D-fructose concentrations of each assay, taking their averages, and using these average concentrations in plotting the rates of D-fructose 1-phosphate formation (67).

For more accurate determination of D-fructose 1-phosphate formed at low D-fructose concentrations, a radioactive assay is being developed in this laboratory.

Employing high specific activity-D-[U-^{14}C] fructose as the substrate, low levels of enzyme II can be used to measure accurate rates of D-fructose 1-phosphate formation without utilizing a large percentage of the available substrate.

a. Assay for Enzyme I

When purified components were not available, a specific assay for enzyme I included the components of the crude extract assay outlined in the general assay above, 50 µl of a fresh crude extract of PL-122 induced on D-fructose, and the sample containing enzyme I.

When purified components were available, saturating amounts of HPr and enzyme II_{fru} were included with the components of the reconstituted assay described above, and D-fructose 1-phosphate formation was measured at 100 mM D-fructose. A unit of activity is defined as the amount of enzyme I that catalyzes the formation of 1.0 nmole D-fructose 1-phosphate per minute.

b. Assay for HPr

This assay was the same as the reconstituted enzyme I assay; however, enzyme I was saturating, and limiting levels of HPr were used. A unit of activity is defined as the amount of HPr that catalyzes the formation of 1.0 nmole D-fructose 1-phosphate per minute. A more accurate assay for HPr would be similar to the "half-maximal saturation" assay developed for PTP_{fru},

since both HPr and PTP_{fru} function as substrates for their respective enzymes II (inducible and constitutive).

c. Assays for Enzymes II

There are two enzyme II activities (which can be assayed independently) in extracts of D-fructose-grown cells. The constitutive activity was found in extracts of cells grown on a wide variety of substrates and was assayed at 100 mM D-fructose (20 μmoles in a 0.2 ml assay) using the components of the general reconstituted assay described above, saturating enzyme I and HPr, and no added PTP_{fru}. The controls for this activity were assays minus PEP at 100 mM D-fructose and minus HPr at 0.5 mM D-fructose (which would correct for activity of the inducible system).

The enzyme II_{fru} (inducible) activity was assayed at 0.5 mM D-fructose (0.1 μmole in a 0.2 ml assay) in the presence of saturating PTP_{fru} and enzyme I. The activity in the absence of PTP_{fru} at 0.5 mM D-fructose was used as the control and was equivalent to the activity in the absence of PEP. A unit of activity is defined as the amount of enzyme II_{fru} that catalyzes the formation of 1.0 nmole D-fructose 1-phosphate per minute.

d. Assays for D-Fructose Phosphoryl Transfer Protein (PTP_{fru})

In the course of this thesis investigation, three methods of quantifying the amount of PTP_{fru} were

developed. The presence of PTP_{fru} in fractions from columns was measured by the increase in D-fructose 1-phosphate formed per minute per ml in an assay that contained the general components of the reconstituted assay, saturating levels of enzyme I and HPr, and limiting levels of PTP_{fru} and PRL-R3 enzyme II_{fru}. These assays were initially run at 2.0 mM D-fructose; however, in later experiments, 1.0 mM D-fructose was used. By lowering the D-fructose concentration, the activity in the absence of PTP_{fru} was decreased; however, the D-fructose 1-phosphate formed that was dependent on PTP_{fru} remained the same, since the K_m of the inducible enzyme II_{fru} which requires PTP_{fru} for activity is 0.02 mM D-fructose.

The background activity of the PRL-R3 enzyme II_{fru} is a result of both the constitutive activity (which has a K_m of 7 mM) and residual levels of PTP_{fru} in the enzyme II_{fru} 100,000 x g precipitate preparation which allows the enzyme II_{fru} (inducible) to function at a low level. Changing the amount of inducible enzyme II alters the amount of D-fructose 1-phosphate formed when a given amount of PTP_{fru} is added. Thus, this assay is only relatively quantitative and units cannot be correlated from one enzyme II_{fru} to another; however, it is a quick and simple method for detecting the presence of PTP_{fru} in various fractions from columns or other steps of purification.

To correct for the variability in the above assay, activity was calculated as the "fold increase" over the background enzyme II_{fru}-dependent D-fructose phosphorylation. The net increase in units divided by the background units represents the "fold increase" in activity. The amount of PTP_{fru} that increases a background of 0.5 units of enzyme II_{fru} activity per reaction to 3.0 units, yields a net increase of 2.5 units. This is a 5-fold increase and thus, the amount of PTP_{fru} that effects this net increase contains 5 "fold increase" units. These "fold increase" units were utilized throughout a majority of this thesis study; however, when it became apparent that there were two separate systems that utilize PEP to phosphorylate D-fructose in Aerobacter aerogenes, a new method of calculating units of PTP_{fru} was devised.

The inducible enzyme II_{fru} has an absolute requirement for PTP_{fru} which acts as a substrate for enzyme II_{fru} and displays saturation-type kinetics. QQ17 enzyme II_{fru} is used in assays at 0.5 mM D-fructose and the amount of PTP_{fru} that gives one-half the maximal activity of a single concentration of enzyme II_{fru} is defined as a "half-maximal saturation" unit. This value was determined by assaying four or five different concentrations of PTP_{fru}, plotting the reciprocals of the velocities obtained versus the reciprocals of PTP_{fru}

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concentrations, and extrapolating the curves to obtain the apparent K_m. This derived amount of PTP_{fru} is defined as one unit of PTP_{fru}. To obtain units per ml, this amount (in µl) is divided into one ml. Doubling the amount of enzyme II doubles the V_{max} of the system; however, the apparent K_m for PTP_{fru} remains the same. This assay is only valid when QQ17 enzyme II_{fru} is used, since other enzymes II prepared as 100,000 x g precipitates contain residual levels of PTP_{fru} that cause an endogenous background activity.

Using QQ17 enzyme II_{fru} the D-fructose 1-phosphate formed at 0.5 mM D-fructose is entirely dependent on the PTP_{fru} added and thus, the "half-maximal saturation" units are independent of the individual QQ17 enzyme II_{fru} preparation and are more accurate than the "fold increase" units which are affected by the level of background activity. If a certain PRL-R3 enzyme II preparation had more residual PTP_{fru} bound than another, it would have a higher background activity, the inducible enzyme II_{fru} would be more saturated, and thus, the amount of PTP_{fru} that gave a 5-fold increase in activity with one enzyme II might only give a 3- or 4-fold increase in activity with another enzyme II_{fru}. Although the "half-maximal saturation" unit is more accurate, its determination is more burdensome since a Lineweaver-Burk plot must be obtained for each sample of PTP_{fru}, whereas with the "fold increase" units only two levels of PTP_{fru}

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in the linear portion of the saturation curve must be assayed.

Thus, in assaying for PTP_{fru} a general assay was utilized for detecting PTP_{fru} in column fractions. The units of this assay were a measure of the increase in nmoles D-fructose 1-phosphate formed per minute per ml of PTP_{fru} in the reconstituted 0.2 ml reaction. A more specific value, the "fold increase" in activity over the background activity, was calculated and used as a measure of units of activity per mg of PTP_{fru} in the pools of activity when PRL-R3 enzyme II_{fru} was used. A third, more accurate and reproducible, method can be used to calculate the PTP_{fru} activity when QQ17 enzyme II_{fru} is used in a purified assay system. In this case, the amount of PTP_{fru} that gives one-half the maximal enzyme II_{fru} activity is defined as a "half-maximal saturation" unit of activity.

Preparation of [^{32}P] Phosphoenolpyruvate

The method for the preparation of [^{32}P] PEP was a modification of the procedure described by Mendicino and Utter (74).

Isolation of Chicken Liver Mitochondria

One and one-half week-old Leghorn Cockerels, donated by Dr. W. W. Wells, were starved for 15 hours. Their livers were excized, minced, and homogenized with a Potter-Elvehjem homogenizer in four volumes of a cold

solution (pH 7.5) consisting of 0.25 M sucrose and 2×10^{-4} M EDTA. The suspension was centrifuged at 755 x g for 20 minutes. The resulting supernatant was centrifuged at 10,800 x g for 15 minutes. The pellet was resuspended in the above solution, centrifuged at 755 x g for 15 minutes and the supernatant was recentrifuged at 10,800 x g for 15 minutes. The resulting pellet was resuspended in two volumes of 0.002 M HEPES buffer (pH 7.5) containing 0.07 M sucrose, 0.001 M EDTA, and 0.22 M D-mannitol. This suspension was centrifuged at 6,780 x g for 15 minutes and the pellet was resuspended in 2.0 ml of the same buffer. This suspension of mitochondria was estimated to contain 75 mg protein per ml.

Synthesis of [^{32}P] Phosphoenolpyruvate

Each of two [^{32}P] PEP-forming reactions included the following components in a total volume of 1.51 ml: 100 μmoles of Tris-HCl (pH 7.5), 100 μmoles of sucrose, 8.0 μmoles of $MgCl_2$, approximately 0.5 mCi of $^{32}P_i$ (carrier free), and 30 mg of coupled mitochondria in the main section of a Warburg flask; 0.1 ml of 10 percent KOH plus pleated filter paper were in the center well to absorb CO_2. The side arm contained 10 μmoles of L-malate, 0.1 μmole of ADP, and 0.1 μmole of GDP. After equilibration at 30°C the reaction was initiated by adding the contents of the side arm. The reaction

was terminated after 45 minutes by adding 3.0 ml of 0.6 M trichloroacetic acid. The reaction flasks were washed twice with buffer and twice with trichloroacetic acid to get total transfer of the radioactive compounds. This reaction could be run directly in a centrifuge tube, thus avoiding transfer of the product, since without added carrier P_i the amount of O_2 uptake was found to be too low to be measured by the Warburg apparatus. The protein was removed by centrifugation in an International desk top clinical centrifuge and trichloroacetic acid was removed from the supernatant by four extractions with anhydrous ether.

The ether was removed by bubbling nitrogen through the solution which was then titrated to neutrality with dilute ammonium hydroxide. The solution was applied to a 1.6 x 12-cm column of Dowex 1-X10 (200-400 mesh) in the bicarbonate form. The column was washed with 50 ml of water prior to eluting with a step-wise gradient consisting of 300 ml of 0.15, 0.30, 0.40, and 0.45 M (65) triethylammonium bicarbonate buffers (pH 7.5) which were prepared using the procedure of Smith and Khorana (106). The radioactivity in the fractions was measured by using a Geiger-Muller tube and three major peaks were detected as shown in Figure 24. In future preparations of [^{32}P] PEP it would be best to monitor the eluted radioactivity and ensure that the 0.40 M peak is completely eluted from the column prior to

adding the 0.45 M buffer.

The third major peak that was eluted with 0.45 M triethylammonium bicarbonate was identified as [^{32}P] PEP by its ability to make [^{32}P] ATP (Table VIII), by paper chromatography (Figure 25), and ability to make D-fructose 1-[^{32}P] phosphate using the constitutive enzyme II assay (Figure 26). Fractions 200 through 221 were pooled, concentrated at 30°C in a Büchi rotary evaporator, and excess triethylammonium bicarbonate was removed by repeated addition and removal of water. This labeled product, carrier free [^{32}P] PEP, was dissolved in 1.0 ml of water and stored at -10°C; it was diluted with unlabeled PEP immediately prior to the individual experiments in which it was utilized and the cpm's per nmole were measured using Čerenkov radiation in a liquid scintillation counter (36). These experiments extended over a 6-week period, thus only 12 percent of the original [^{32}P] PEP formed was present when the last experiment was performed.

Characterization of [^{32}P] Phosphoenolpyruvate

Assay for Formation of [^{32}P] ATP from [^{32}P] Phosphoenolpyruvate

Aliquots of fractions from the Dowex column of products from [^{32}P] PEP-forming reactions were assayed for their ability to form [^{32}P] ATP using pyruvate kinase. The complete assay contained 10 μmoles of

Tris-HCl (pH 7.5), 0.1 μmole of PEP, 4.0 μmoles of $MgCl_2$, 2.0 μmoles of ADP, 40 μmoles of KCl, 10 μl of a 1/10 dilution of pyruvate kinase, and a 50 μl aliquot of the fraction being assayed in a total volume of 0.5 ml. After 20 minutes at 30°C the reaction was stopped by adding 0.5 ml of 10 percent trichloroacetic acid. One-half of the resulting solution was mixed with 30 mg of charcoal and filtered through millipore filters. The filters were washed with 90 percent ethanol, dried, and radioactive [^{32}P] ATP bound to the charcoal (15) was measured in a liquid scintillation counter (Table VIII).

Other Analytical Procedures

Sephadex gels were prepared by the method recommended by Pharmacia (88). DEAE cellulose was fined and washed successively with 1 N NaOH, 1 N HCl, 1 N NaOH and water to neutrality (87). Dowex 1-X10 and Dowex 50W-X8 were fined and washed with 1 N HCl to convert them to the Cl^- and H^+ forms, respectively. They were then washed with water to neutrality.

D-fructose was recrystallized from a warm, saturated 95 percent ethanol solution by cooling on ice. Crystals were washed with cold 100 percent ethanol.

Ascending paper chromatography of phosphorylated derivatives was performed at 4°C on oxalic acid-washed Whatman No. 1 paper utilizing two solvent systems. The acid solvent was methanol-88 percent formic acid-water

(80:15:5, v/v) and the basic solvent was methanol-ammonium hydroxide-water (60:10:30, v/v) (6). Phosphates were detected with Hanes-Isherwood reagent (32) which contained 60 percent (w/w) perchloric acid-4 percent (w/v) ammonium molybdate-1 N hydrochloric acid-water (5:25:10:60, v/v). Sugars and sugar phosphates were also located by ammoniacal silver nitrate (115). Chromatograms were air dried, cut into strips, and scanned for radioactivity with a Nuclear-Chicago Model 1036 4-Pi Actigraph II scanner, Nuclear-Chicago Model 1620 CS analytical count rate meter connected to a synchronized Sargent Model SRL recorder. For an accurate measurement of total counts per minute, the radioactive spots were cut out, inserted in glass scintillation vials, and counted in a refrigerated Packard Tri-carb Model 3310 liquid scintillation spectrometer utilizing Čerenkov radiation without added water or scintillation fluid (36).

Polyacrylamide disc gel electrophoresis was run in Tris-glycine buffer (pH 9.0) with constant current of 1.5 ma per tube according to the method of Davis (16). Gels to be sliced and assayed for active PTP_{fru} were run at $4^{o}C$, while other gels were run at $25^{o}C$.

Gels were scanned at 280 nm with a Gilford Model 2410 linear transport gel scanner. The peaks were sliced and activity was eluted with 0.02 M potassium phosphate buffer (pH 8.0) containing 0.001 M DTT and 10 percent glycerol. Gels were also sliced with a

multiple razor slicer after freezing. For sensitive protein detection, gels were fixed with 12 percent trichloroacetic acid, stained with 12 percent trichloroacetic acid containing 0.05 percent Coomassie blue, and destained with 10 percent trichloroacetic acid (13). They were then scanned at 550 nm.

SDS polyacrylamide disc gels run by J. Markwell were polymerized in 0.01 M sodium phosphate buffer (pH 7.1) containing 0.1 percent SDS, 0.03 percent TEMED, 7.5 percent acrylamide, 0.2 percent methylenebisacrylamide, and 0.07 percent of ammonium persulfate. The protein samples were prepared by heating at 100°C for 10 minutes in a solution of 1 percent SDS, 1 percent 2-mercaptoethanol, 0.1 M sodium phosphate buffer (pH 7.1), and 10 percent glycerol. Aliquots containing 3.0 to 6.0 μg of each standard protein were layered on 8-cm gels. The electrophoresis buffer containing 0.01 M sodium phosphate (pH 7.1) and 0.1 percent SDS was added and the gels were electrophoresed at 8 ma per tube for 3.5 hours. Gels were removed, fixed with 10 percent trichloroacetic acid for 30 minutes, and stained overnight in 10 percent trichloroacetic acid, 33 percent methanol, and 0.4 percent Coomassie blue. After destaining in 10 percent trichloroacetic acid and 33 percent methanol, the gels were scanned at 550 nm and R_f values were calculated for each protein band with respect to the migration of a methylene blue dye front.

Protein concentrations were estimated spectrophotometrically with the aid of a nomograph (courtesy of Calbiochem) based on the data of Warburg and Christian (123) and by the absorption at 210 nm (114) using bovine serum albumin as the standard. The method of Lowry (72) was also used to check the protein concentration in crude extracts.

Proteins were concentrated by ultrafiltration through a UM-10 membrane in an Amicon Model 50 ultrafiltration cell.

Reagents

PEP, D-glucose 6-phosphate dehydrogenase, pyruvate kinase, lactic dehydrogenase, HEPES, aldolase, β-lactoglobulin, and DTT were purchased from Calbiochem, Los Angeles, California; phosphoglucose isomerase, L-malate, uracil, D-fructose, protamine sulfate, yeast hexokinase, 2-phosphoglyceric acid, 3-phosphoglyceric acid, D-fructose 1-phosphate, D-glucose 6-phosphate, GDP, D-fructose 1,6-diphosphate, ATP, D-galactose, D-glucitol (sorbitol), D-mannitol, L-sorbose, α-glycerophosphate, Trizma-Base, α-glycerophosphate dehydrogenase-triose-phosphate isomerase, lysozyme, trypsin, carbonic anhydrase, and 2-mercaptoethanol from Sigma Chemical Company, St. Louis, Missouri; ethyl methanesulfonate from Eastman Organic Chemicals, Rochester, New York; enzyme-grade ammonium sulfate from Schwarz/Mann, Orangeburg,

New York; NAD^+, NADH, $NADP^+$, NADPH, and ADP from P-L Biochemicals, Milwaukee, Wisconsin; Dowex 1-X10 (Cl^-) and Dowex 50W-X8 from J. T. Baker Chemical Company, Phillipsburg, New Jersey; D-mannose and D-ribose from General Biochemicals, Chagrin Falls, Ohio; L-arabinose from Pfanstiehl Laboratories, Waukegan, Illinois; hydroxylapatite was prepared by R. L. Anderson by the method of Levin (69, 113); calcium phosphate gel was prepared by the method of Hartree (14); carrier free $^{32}P_i$ (9.1 MCi per mole) in the form of $H_3{}^{32}PO_4$ in 0.02 N HCl from New England Nuclear Corporation, Boston, Massachusetts; malate dehydrogenase and lactate dehydrogenase from Worthington Biochemical Corporation, Freehold, New Jersey; acrylamide, N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylethylenediamine from Conalco, Bethesda, Maryland; D-fructose 6-phosphate from Boehringer Mannheim Corporation, New York, New York; D-mannitol 6-phosphate was prepared by D. Allison (51); SDS from Mallinckrodt Chemical Works, St. Louis, Missouri; triply distilled water was used for all buffers and other solutions.

RESULTS

Purification of D-Fructose Phosphoryl Transfer Protein

Throughout this study many different methods of purification were investigated and utilized in various sequences. The method reported here was used several times yielding equivalent results. Table I summarizes a typical purification. The assays in this purification scheme utilized PRL-R3 enzyme II, and thus PTP_{fru} activities are in terms of "fold increase" units as described in Methods. Figure 1 shows a validation of these units using different levels of enzyme II_{fru} and PTP_{fru}. Figure 1A shows the actual data with PTP_{fru} causing increases in the level of D-fructose 1-phosphate formed per assay. Figure 1B shows this data plotted as "fold increase" units versus the PTP_{fru} concentration. In the linear portion of the saturation curve, these "fold increase" units are proportional to the amount of PTP_{fru}. However, as saturation of the enzyme II_{fru} is reached, they no longer are proportional. In all cases where these units were used, the activity was measured in the linear portion of the saturation of the individual enzyme II_{fru} being used.

Table I. Purification of D-fructose phosphoryl transfer protein (PTP_{fru}).

Fraction	Total Protein (mg)[a]	Volume (ml)	Total Activity (units)[b]	Recovery %	Specific Activity[c]	Fold Purified
Diluted 100,000 x g supernatant	12,800	1,200	114,000	100	8.91	1.0
35-70% ammonium sulfate	6,550	172	108,000	94.7	16.5	1.85
DEAE cellulose (I)	3,740	380	92,300	81.0	24.7	2.77
45-65% ammonium sulfate	740	15	83,600	73.3	113	12.7
Sephadex G100 (I)	401	95	32,700	28.7	81.5	9.15
Hydroxylapatite pool	23.5	50	15,300	13.4	651	73.1
DEAE cellulose (II)	9.8	3.5	13,600	11.9	1,390	156
Sephadex G100 (II)	4.8	28	13,200	11.6	2,750	309

[a]Estimated by Lowry method.

[b]"Fold increase" unit defined as the amount of PTP_{fru} that gives a 1-fold increase in D-fructose 1-phosphate formed over the endogenous background activity in the standard 10 minute assay.

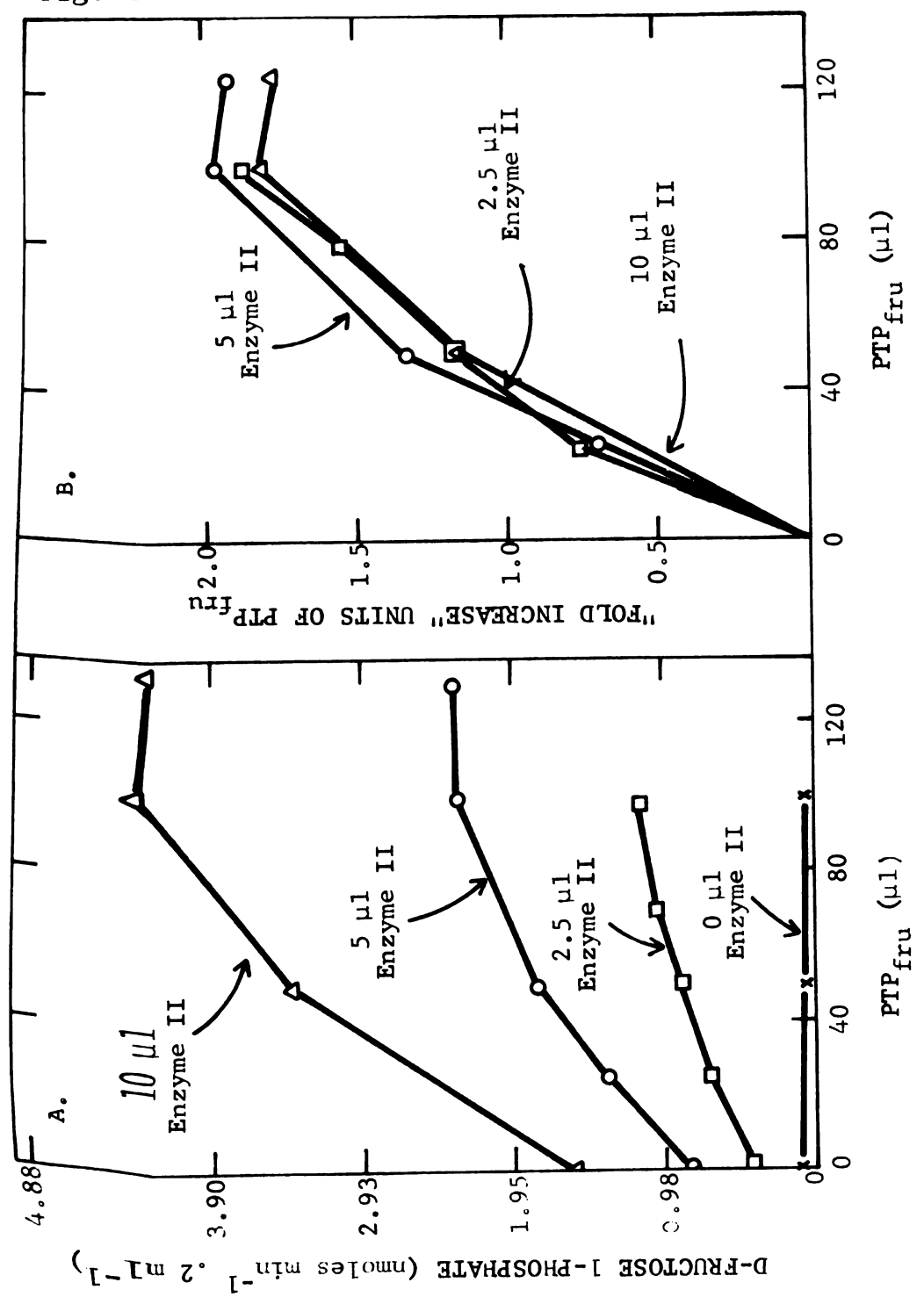
[c]Units/mg protein.

Figure 1. Effect of PTP_{fru} on PRL-R3 enzyme II_{fru}.

A. Saturation of various concentrations of PRL-R3 enzyme II_{fru} by PTP_{fru}.
B. Linearity of calculated "fold increase" units of PTP_{fru}.

Assays contained components of the reconstituted assay (general assay for PEP-dependent D-fructose 1-phosphate formation) described in Methods, including 0.4 μmoles of D-fructose, 13 ng of HPr, 33 ng of enzyme I, and the noted amounts of 33.8 mg/ml PRL-R3 enzyme II_{fru} and 2.69 mg/ml PTP_{fru} (from a Sephadex G200 column).

Figure 1


A.
B.
10 µl Enzyme II
5 µl Enzyme II
2.5 µl Enzyme II
0 µl Enzyme II
D-FRUCTOSE 1-PHOSPHATE (nmoles min^{-1} .2 ml^{-1})
"FOLD INCREASE" UNITS OF PTP_{fru}
PTP_{fru} (µl)
4.88
3.90
2.93
1.95
0.98
2.0
1.5
1.0
0.5
0
40
80
120

100,000 x g Centrifugation

Crude extracts of PRL-R3 cells grown on D-fructose were centrifuged at 38,000 rpm (100,000 x g) in a Beckman 40 rotor in a Spinco L2 ultracentrifuge for 2 hours. The resulting supernatant was recentrifuged for 2 hours and this second 100,000 x g supernatant was diluted with 0.02 M Tris-HCl buffer (pH 7.5) containing 0.028 M 2-mercaptoethanol to a protein concentration of 10 mg per ml.

This solution, termed the diluted 100,000 x g supernatant, was generally devoid of enzyme II. In some cases, where very heavy crude extracts were prepared in order to have a smaller volume, all of the enzyme II was not removed by this ultracentrifugation. The presence of this contaminating enzyme II, which makes the PTP_{fru} saturation curves nonlinear, was corrected for in calculating the "fold increase" units of PTP_{fru}.

35 to 70 Percent Ammonium Sulfate Fractionation

The ammonium sulfate concentration was brought to 35 percent saturation (30) by adding 18.8 g of powdered ammonium sulfate for each 100 ml of diluted supernatant over a period of 30 minutes. After stirring for another hour, the solution was centrifuged at 16,000 x g for 20 minutes. The resulting precipitate was discarded and the supernatant was brought to 70 percent saturation by slowly adding 23.7 g of ammonium sulfate per 100 ml of

original solution. The solution was equilibrated by stirring for 1 hour prior to centrifugation at 16,000 x g for 20 minutes. The resulting precipitate was dissolved in 0.02 M potassium phosphate buffer (8.0) containing 0.001 M DTT and 10 percent glycerol and was dialyzed overnight against this same buffer to remove ammonium sulfate which inhibits both the phosphotransferase activity and the binding of PTP_{fru} to the DEAE cellulose column.

The PTP_{fru} in this solution, termed the 35 to 70 percent ammonium sulfate fraction, was 1.85-fold purified over the 100,000 x g supernatant. This fraction contained enzyme I and D-fructose 1-phosphate kinase; however, a majority of contaminating HPr (which precipitates out mainly in the 70 to 100 percent ammonium sulfate fraction) was removed. Furthermore, any enzyme II which was not removed by ultracentrifugation was removed in the 0 to 35 percent ammonium sulfate fraction.

DEAE Cellulose Chromatography (I)

The 35 to 70 percent ammonium sulfate fraction was layered onto a 4.8 x 12.5-cm DEAE cellulose column which was then washed with 0.02 M potassium phosphate buffer (pH 8.0) containing 0.001 M DTT and 10 percent glycerol before starting a 2.0 l linear gradient of 0 to 0.4 M potassium chloride in the same buffer. Fractions of 16.5 ml were collected and PTP_{fru} was eluted under a broad

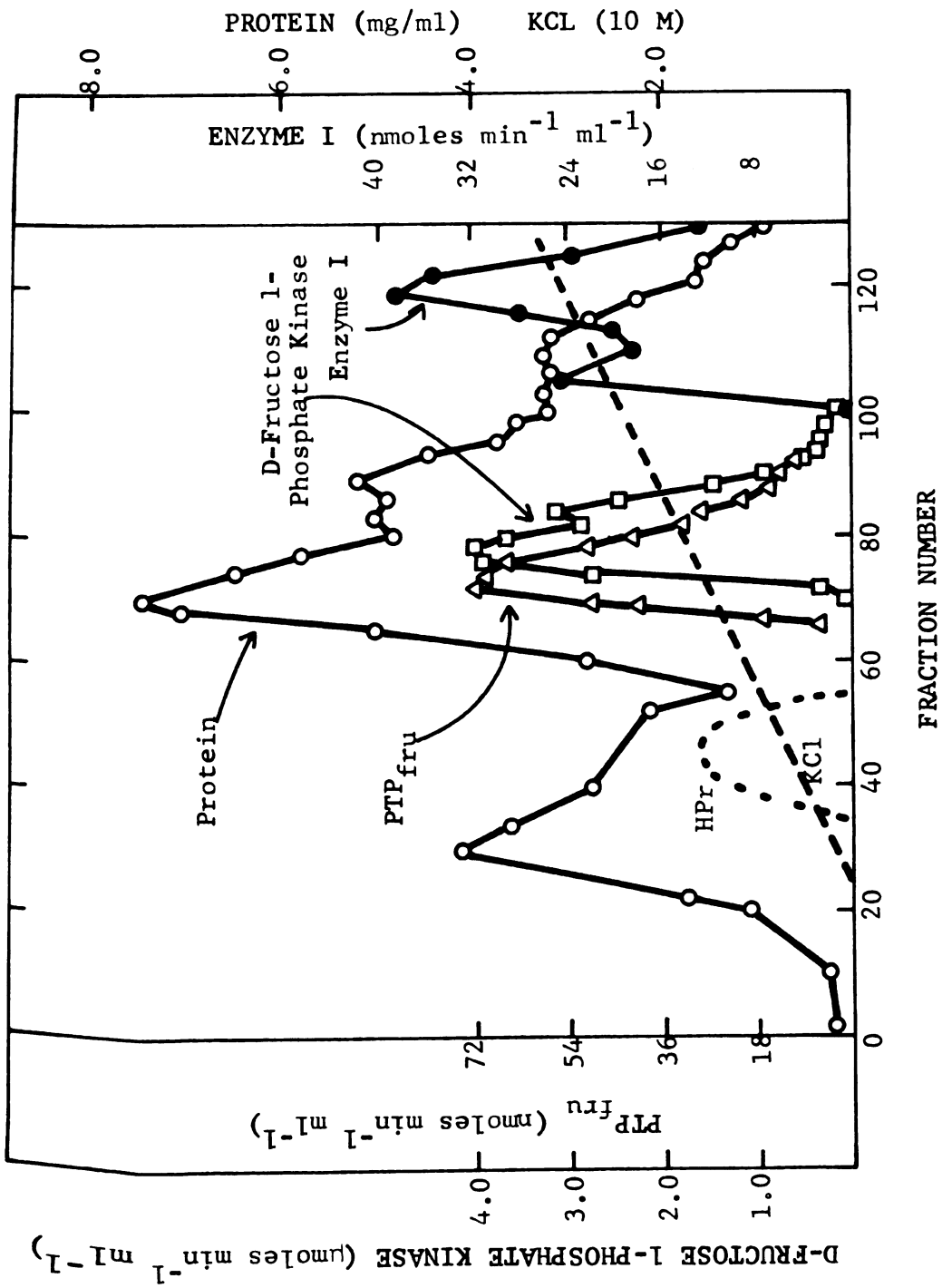
protein peak at 0.16 M potassium chloride, thus yielding very little purification. However, it was separated from contaminating HPr and enzyme I which elute at 0.1 M and 0.3 M potassium chloride, respectively. D-Fructose 1-phosphatase activity is also removed by this step; however, D-fructose 1-phosphate kinase is still a contaminant. Fractions 67 through 89 were pooled (Figure 2).

45 to 65 Percent Ammonium Sulfate Fractionation

A second ammonium sulfate step was performed at this point not only to purify the PTP_{fru}, but also to concentrate it prior to Sephadex G100 chromatography. To each 100 ml of pooled activity, 21.9 g of ammonium sulfate were slowly added to attain 40 percent saturation. After 1 hour equilibration, the resulting precipitate was removed by centrifugation at 48,200 x g for 10 minutes. The resulting supernatant was brought to 65 percent saturation by slowly adding 16.8 g of ammonium sulfate and equilibrated for 1 hour. The solution was centrifuged at 48,200 x g for 10 minutes and the resulting precipitate was dissolved in 0.01 M potassium phosphate buffer (pH 8.0) containing 0.001 M DTT and 10 percent glycerol. A sample of this fraction was dialyzed overnight against this same buffer and assayed for PTP_{fru} activity. This step yielded a four-fold purification of PTP_{fru} with very little loss in activity.

Figure 2. DEAE cellulose (I) chromatography of 35 to 70 percent ammonium sulfate fraction. Activities are in terms of μmoles or nmoles of product formed per minute per ml fraction added in the assay for the activity noted. The dotted line (·····) designates where HPr would elute if present.

Figure 2


PROTEIN (mg/ml)
KCL (10 M)
8.0
6.0
4.0
2.0
ENZYME I (nmoles min^{-1} ml^{-1})
40
32
24
16
8
D-Fructose 1-Phosphate Kinase Enzyme I
Protein
PTP_{fru}
HPr
KCl
120
100
80
60
40
20
0
FRACTION NUMBER
72
54
36
18
PTP_{fru} (nmoles min^{-1} ml^{-1})
4.0
3.0
2.0
1.0
D-FRUCTOSE 1-PHOSPHATE KINASE (μmoles min^{-1} ml^{-1})

Sephadex G100 Chromatography (I)

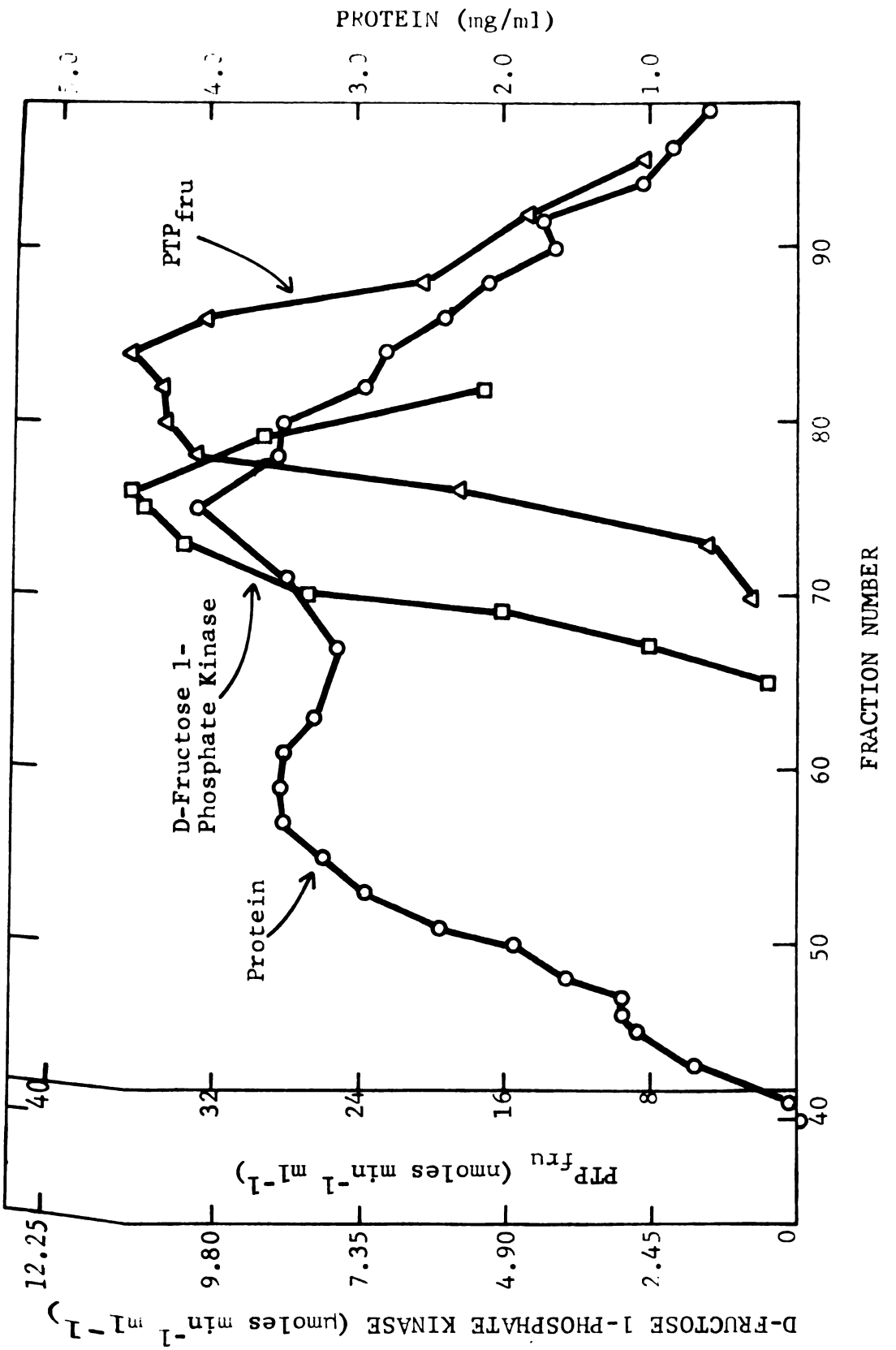
The 45 to 65 percent ammonium sulfate fraction was layered onto a 4.8 x 35-cm Sephadex G100 column, chromatographed with 0.01 M potassium phosphate buffer (pH 8.0) containing 0.001 M DTT and 10 percent glycerol, and 5.0-ml fractions were collected. This lower concentration of potassium phosphate was used so that the eluted Sephadex G100 pool of activity would have a lower phosphate concentration. This procedure would allow a shorter dialysis time for diluting the phosphate concentration to below 0.005 M so that the PTP_{fru} could bind to hydroxylapatite. However, at this lower concentration there was a great loss in PTP_{fru} activity resulting in a net decrease in the specific activity of this Sephadex G100 pool (fractions 77 through 95, Figure 3). Other purifications utilizing a Sephadex G200 column at 0.02 M potassium phosphate concentration had little loss in total units with a greater degree of purification.

Hydroxylapatite Chromatography

The Sephadex G100 (I) pool was concentrated and dialyzed against 0.002 M potassium phosphate buffer (pH 8.0) containing 0.001 M DTT and 10 percent glycerol using an Amicon UM-10 ultrafilter. The resulting protein solution was applied to a 2.5 x 9.5-cm hydroxylapatite column and 5.0-ml fractions were eluted with a 400 ml

Figure 3. Sephadex G100 (I) chromatography of DEAE cellulose (I) pool. Activities are in terms of μmoles or nmoles of product formed per minute per ml fraction added in the assay for the activity noted.

Figure 3


PROTEIN (mg/ml)
4.0
3.0
2.0
1.0
PTP$_{fru}$
D-Fructose 1-
Phosphate Kinase
Protein
D-FRUCTOSE 1-PHOSPHATE KINASE (μmoles min^{-1} ml^{-1})
PTP$_{fru}$ (nmoles min^{-1} ml^{-1})
12.25
9.80
7.35
4.90
2.45
0
40
32
24
16
8
0
10
40
50
60
70
80
90
FRACTION NUMBER

linear gradient of 0.005 to 0.07 M potassium phosphate buffer (pH 8.0) containing 0.001 M DTT and 10 percent glycerol. A second 400 ml linear gradient of 0.07 to 0.2 M potassium phosphate buffer (pH 8.0) containing 0.001 M DTT and 10 percent glycerol was added to elute the D-fructose 1-phosphate kinase.

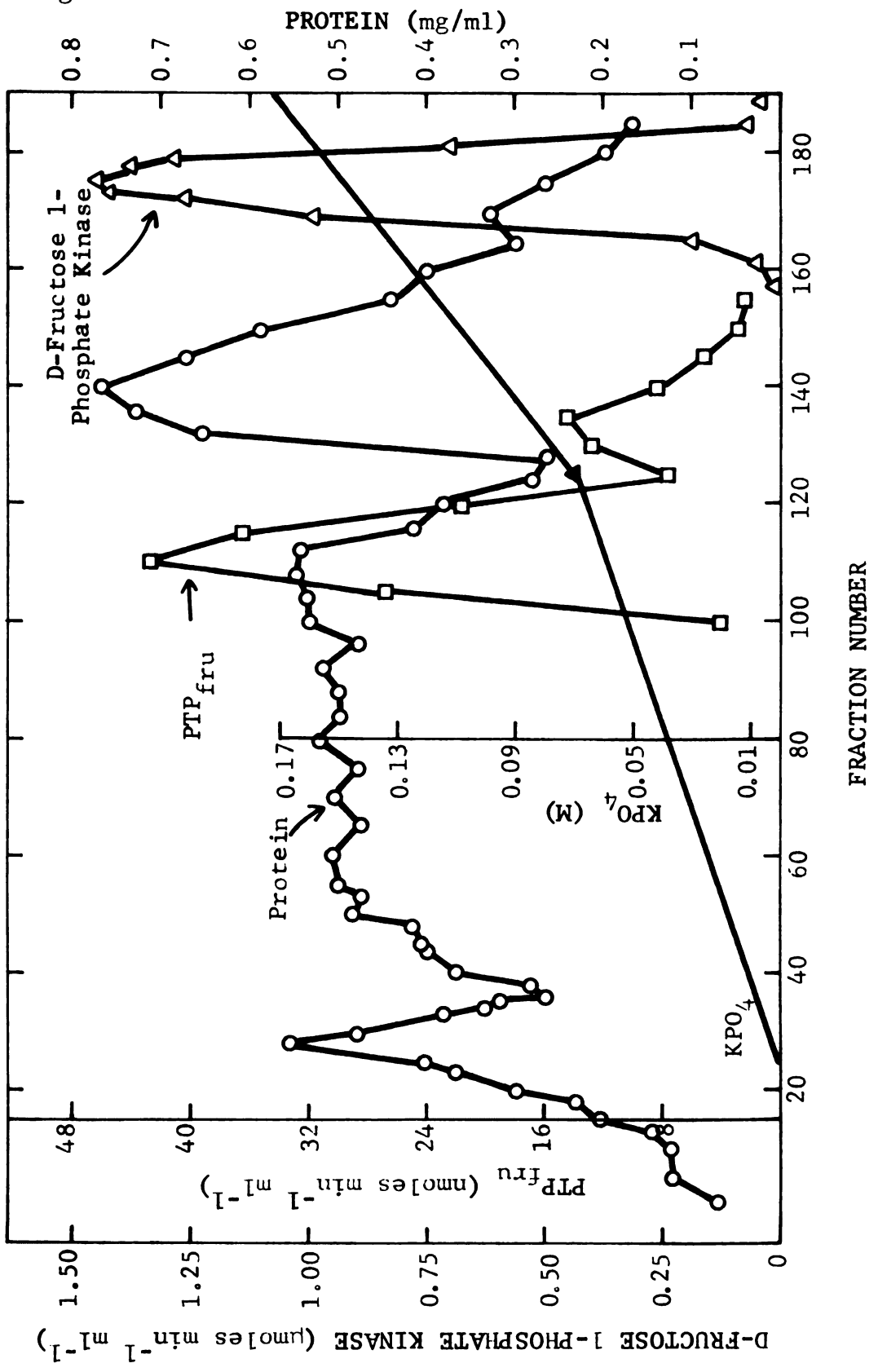
The pool of the peak fractions (105 through 125, Figure 4) contained only 50 percent of the total units applied to the column; however, an eight-fold purification of PTP_{fru} was attained. This column removed the contaminating D-fructose 1-phosphate kinase which elutes with 0.15 M potassium phosphate. This same column was used several times yielding from 2- to 10-fold purifications with varying levels of recovery. The cause of this variability was not determined. In general, the use of this column gave the best purification results of any method attempted. In one case, in which PTP_{fru} washed through the column without adsorbing it was subjected to the next two purification steps [DEAE cellulose (II) and Sephadex G100 (II)] columns and then rechromatographed on the hydroxylapatite column. This time the PTP_{fru} did bind; however, no further purification was achieved.

DEAE Cellulose Chromatography (II)

The hydroxylapatite pool preparation reported in Table I was layered on a 1 x 10-cm DEAE cellulose column and chromatographed with a 400 ml linear gradient of 0 to

Figure 4. Hydroxylapatite chromatography of Sephadex G100 (I) pool. Activities are in terms of μmoles or nmoles of product formed per minute per ml fraction added in the assay for the activity noted.

Figure 4


PROTEIN (mg/ml)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
D-Fructose 1-
Phosphate Kinase
PTP$_{fru}$
Protein
0.17
0.13
0.09
0.05
0.01
KPO$_4$ (M)
KPO$_4$
PTP$_{fru}$ (nmoles min^{-1} ml^{-1})
48
40
32
24
16
8
1.50
1.25
1.00
0.75
0.50
0.25
0
D-FRUCTOSE 1-PHOSPHATE KINASE (μmoles min^{-1} ml^{-1})
20
40
60
80
100
120
140
160
180
FRACTION NUMBER

0.3 M potassium chloride in a 0.02 M potassium phosphate buffer (pH 8.0) containing 0.001 M DTT and 10 percent glycerol. Five-ml fractions were collected and the peak fractions (46 through 54, Figure 5) were pooled and concentrated to 3.5 ml by ultrafiltration. Very little PTP_{fru} activity was lost and a greater than 2-fold purification was attained.

Sephadex G100 Chromatography (II)

The concentrated protein solution was then carefully layered on a 2.5 x 42-cm Sephadex G100 column and subsequently eluted with 0.02 M potassium phosphate buffer (pH 8.0) containing 0.001 M DTT and 10 percent glycerol, 3.2-ml fractions were collected, and fractions 30 through 38 were pooled (Figure 6). This step also resulted in very little loss in PTP_{fru} activity with another two-fold purification, yielding a total purification of 309-fold with 11.6 percent recovery of total PTP_{fru} activity. The actual yield and purification may have been greater if 0.02 M potassium phosphate buffer had been used for the first Sephadex G100 step.

Polyacrylamide Disc Gel Electrophoresis

Polyacrylamide disc gel electrophoresis at pH 9.0 with 7.5 percent acrylamide gels was performed on samples of PTP_{fru} from the final steps of a purification in which the second DEAE cellulose column and second Sephadex G100 column were run prior to a second hydroxylapatite

Figure 5. DEAE cellulose (II) chromatography of hydroxylapatite pool. PTP_{fru} activity is in terms of nmoles D-fructose 1-phosphate formed per minute per ml fraction added.

Figure 5

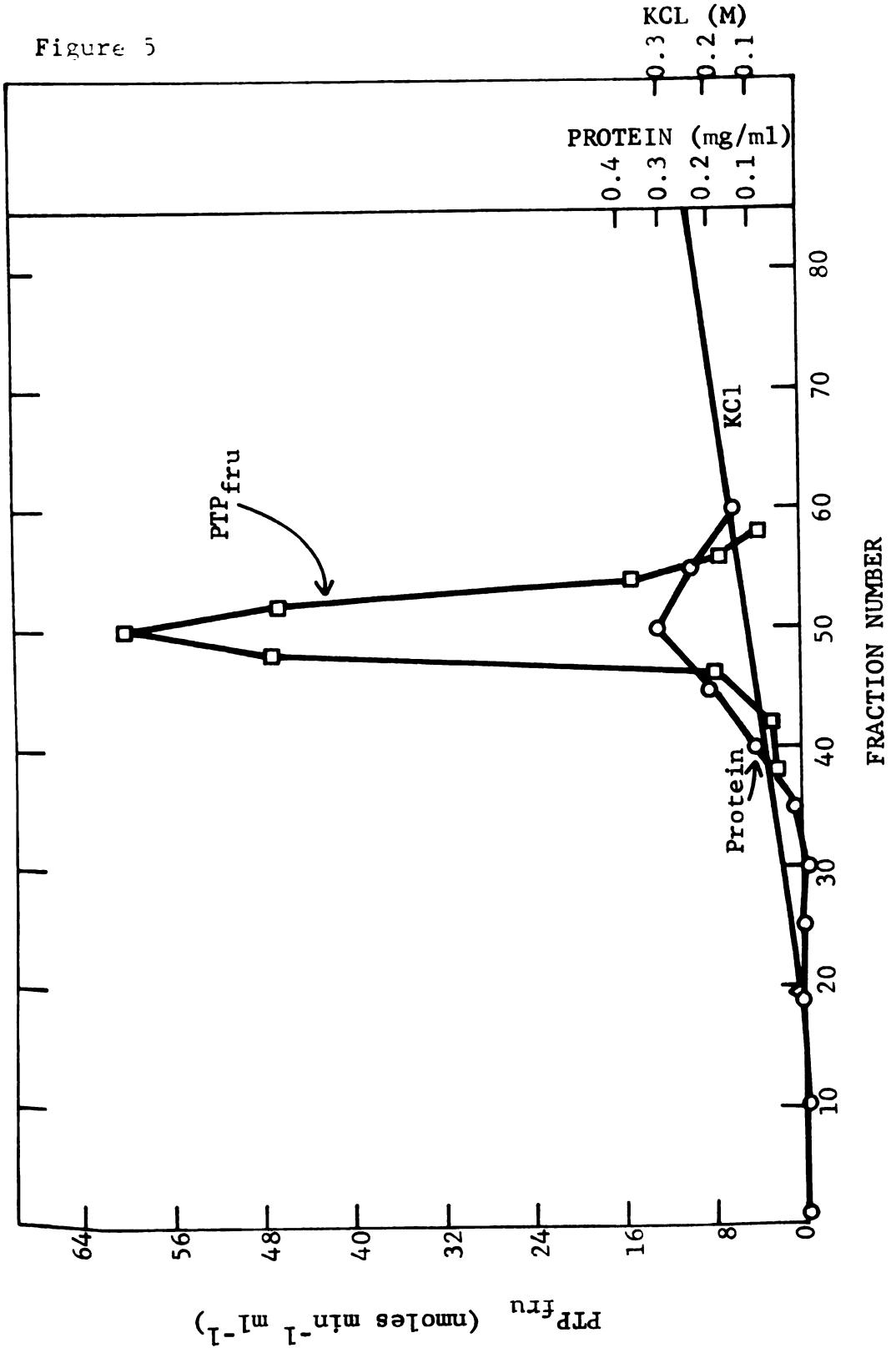
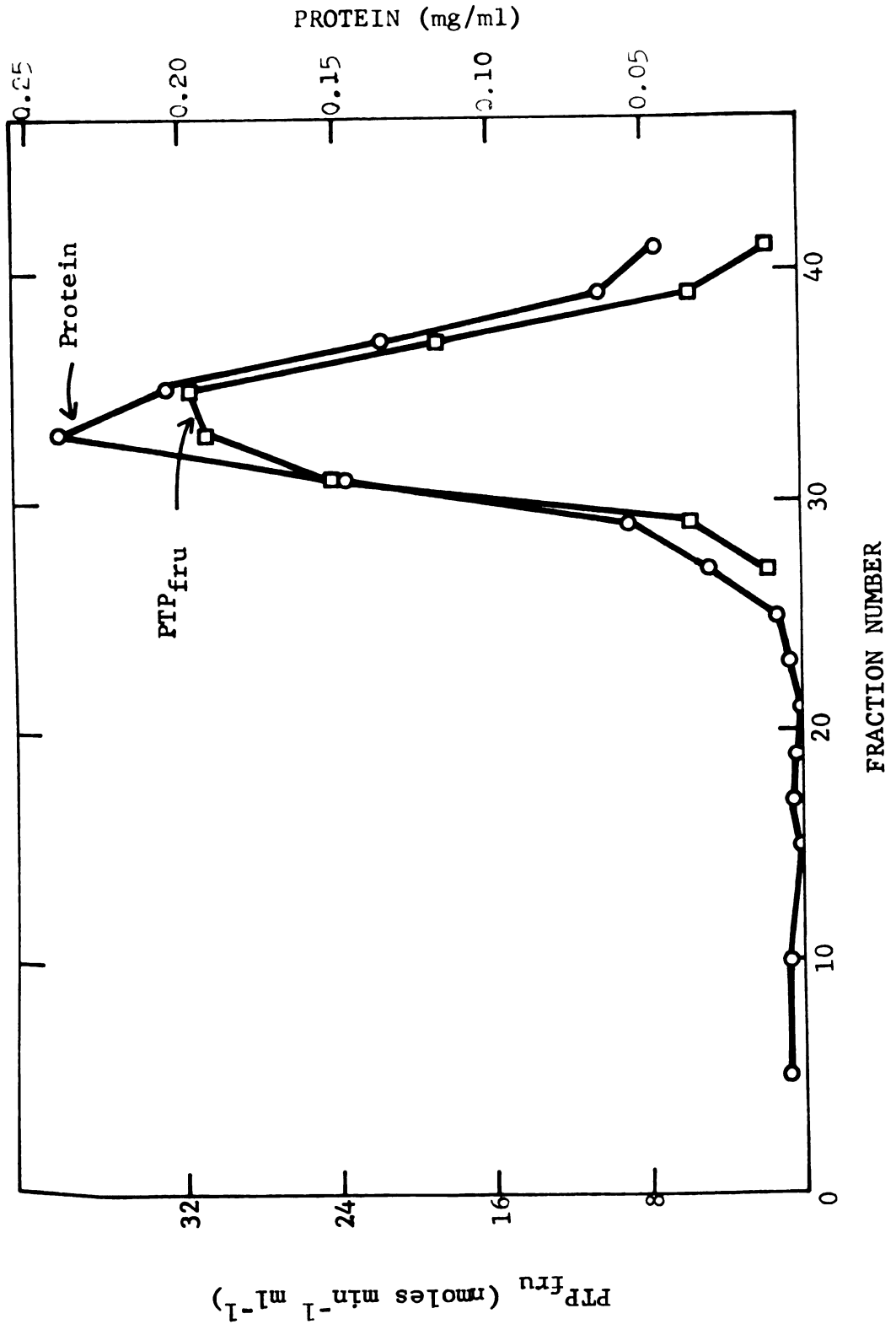

KCL (M)
0.3
0.2
0.1
PROTEIN (mg/ml)
0.4
0.3
0.2
0.1
KCl
Protein
PTP fru
FRACTION NUMBER
10
20
30
40
50
60
70
80
0
8
16
24
32
40
48
56
64
PTP fru (nmoles min-1 ml-1)

Figure 6. Sephadex G100 (II) chromatography of DEAE cellulose (II) pool. PTP_{fru} activity is in terms of nmoles D-fructose 1-phosphate formed per minute per ml fraction added.

Figure 6


PROTEIN (mg/ml)
0.25
0.20
0.15
0.10
0.05
Protein
PTP_{fru}
32
24
16
8
0
40
30
20
10
FRACTION NUMBER
PTP_{fru} (nmoles min^{-1} ml^{-1})

column (see hydroxylapatite chromatography step).

The gel shown in Figure 7A contained 70 μg of protein from a DEAE cellulose (II) pool, and the gel in Figure 7B contained 18 μg of protein from the hydroxylapatite pool and was similar to a gel of the Sephadex G100 pool (not shown). Another gel was overloaded with 200 μg of protein from the hydroxylapatite pool, sliced, and protein was eluted with 0.02 M potassium phosphate buffer (pH 8.0) containing 0.001 M DTT and 10 percent glycerol. The eluted fractions were assayed for PTP_{fru} activity and a sample (containing approximately 25 μg) of the active fraction was concentrated by lyophilization and run on a second gel. The specific activity and recovery of this homogeneous PTP_{fru} (Figure 7C) was not calculated due to the low concentrations of protein.

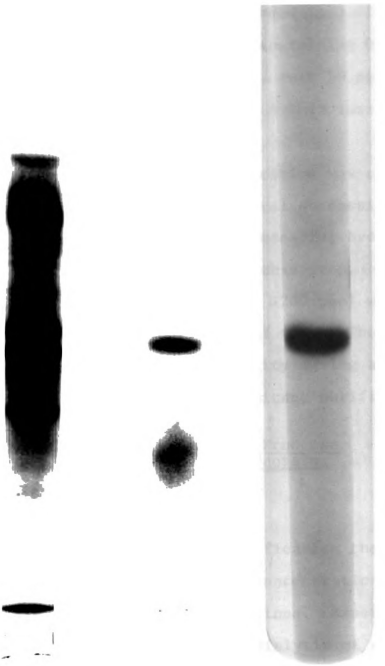
Other Attempted Purification Procedures

Other purification steps which were investigated included carboxymethyl cellulose and phospho-cellulose columns. A Sephadex G200 pool of PTP_{fru} was adjusted to pH 6.5 and applied to both types of columns. In both cases all of the protein, including the PTP_{fru} activity, failed to be adsorbed to the column. Several attempts at acid precipitation were also attempted on the Sephadex G200 pool of PTP_{fru}. The pH of the protein sample was adjusted by addition of dilute acetic acid.

Figure 7. Polyacrylamide disc gel electrophoresis of PTP_{fru}.

A. 70 μg of protein from a DEAE cellulose pool.
B. 18 μg of protein from a second hydroxylapatite pool
C. Approximately 25 μg of PTP_{fru} from an overloaded disc gel of the hydroxylapatite pool.

Further details are given in the text.



A B C

A 1.8-fold purification of PTP_{fru} was attained in the pH 4.7 precipitate which was resuspended in 0.02 M potassium phosphate buffer (pH 8.0) containing 0.001 M DTT and 10 percent glycerol; however, only 50 percent of the activity was recovered. Due to this loss in activity, this step was not utilized.

A calcium phosphate gel purification was attempted; however, the activity eluted in several successive stepwise fractions and thus, the more successful hydroxylapatite column was used to replace this step.

Another sample of the Sephadex G200 pool was heated at 50°C for varying periods of time. There was a loss of 50 percent of PTP_{fru} activity during a 10 minute period of time with no concomitant purification.

Characterization of D-Fructose Phosphoryl Transfer Protein

Stability of PTP_{fru}

It became apparent during purification that PTP_{fru} loses activity upon storage at low concentrations of buffer and at low protein concentrations. Experiments to stabilize the activity involved dialysis of aliquots of a Sephadex G200 pool of PTP_{fru} against various buffers including 0.02 M Tris-HCL, 0.02 M potassium phosphate, 0.02 M glycylglycine, 0.02 M HEPES, 0.02 M Tricine, 0.02 M TES, 0.02 M PIPES, and 0.02 M Bicine at different pH levels (7.0 to 9.0), and in the presence of various

combinations of 0.001 M DTT, 0.001 M EDTA, 0.028 M 2-mercaptoethanol, 0.02 M sodium chloride, 0.02 M potassium chloride, and 0.02 M ammonium sulfate. The best recovery of activity after 48-hour dialysis and storage at $4^{o}C$ for one week was achieved with 0.02 M potassium phosphate buffer (pH 8.0) and with this buffer at pH 7.5 in the presence of 0.001 M DTT (Table II).

In another experiment, a sample of PTP_{fru} eluted from a calcium phosphate gel with 0.015 M potassium phosphate containing 0.001 M DTT, lost 50 percent of its activity in three days at $0^{o}C$, whereas a duplicate sample which was stored at $-18^{o}C$, retained 95 percent of its activity.

Three aliquots from another Sephadex G200 pool were incubated for 2 days with 5.0 mg per ml of bovine serum albumin, 5 percent glycerol, and 1 mM D-fructose in an attempt to stabilize activity at $0^{o}C$. The sample incubated with BSA lost 80 percent of its activity; the sample stored in D-fructose lost 50 percent of its activity; and the sample stored in 10 percent glycerol did not lose any activity.

These data show that 0.02 M potassium phosphate, 0.001 M DTT, and 10 percent glycerol stabilize PTP_{fru} at $0^{o}C$. Thus, for all stages of purification following the first step, the protein was stored in 0.02 M potassium phosphate buffer (pH 8.0) containing 0.001 M

Table II. Recovery of lipase activity after 24-hour dialysis against water and various 0.02 M buffers and storage at 4°C and -18°C for 1 week. After dialysis one-half of the sample was frozen and one-half was stored at 4°C for 1 week. Activity prior to dialysis was 21.5 "fold increase" units

Table II. Recovery of PTP_{fru} activity after 48-hour dialysis against water and various 0.02 M buffers and storage at 4°C and -18°C for 1 week. After dialysis one-half of the sample was frozen and one-half was stored at 4°C for 1 week. Activity prior to dialysis was 21.5 "fold increase" units per mg.

Dialysis Solution	pH	Percent Recovery	
		Stored at 4°C	Stored at -18°C
Water		70.2[a]	61.4[b]
Tris	7.5	66.7	63.2
Tris	8.0	91.3	83.5
Potassium phosphate	7.5	79.4	73.1
Potassium phosphate	8.0	103	88.4
Tris, 28 mM 2-mercaptoethanol	7.5	79.4	3.1
Tris, 1 mM DTT	7.5	89.2	70.6
Tris, 1 mM EDTA	7.5	87.8	66.3
Tris, 2-mercaptoethanol, 1 mM EDTA	7.5	43.5	8.0
Tris, 1 mM DTT, 1 mM EDTA	7.5	79.4	78.0

Table II.--Continued.

Dialysis Solution	pH	Percent Recovery	
		Stored at 4°C	Stored at -18°C
Potassium phosphate, 28 mM 2-mercapto-ethanol	7.5	98.3	8.0
Potassium phosphate, 1 mM DTT	7.5	105	92.7
Potassium phosphate, 1 mM EDTA	7.5	89.9	90.9
Potassium phosphate, 28 mM 2-mercapto-ethanol, 1 mM EDTA	7.5	68.1	2.5
Potassium phosphate, 1 mM DTT, 1 mM EDTA	7.5	105	93.3
Potassium phosphate, 0.02 M NaCl	7.5	96.9	70.6
Glycylglycine	8.0	82.2	--[c]
HEPES	8.0	82.9	--[c]
Tricine	8.0	78.0	--[c]

Table II.--Continued.

Dialysis Solution	pH	Percent Recovery	
		Stored at 4°C	Stored at -18°C
PIPES	8.0	74.4	--[c]
Bicine	8.0	63.9	--[c]
TES	8.0	69.5	--[c]

[a]Actual activity: 15.1±.4 "fold increase" units per mg PTP_{fru}.

[b]Actual activity: 13.2±.6 "fold increase" units per mg PTP_{fru}.

[c]--Signifies not determined.

DTT and 10 percent glycerol. Other PTP_{fru} samples were stored at -18°C for 6 months with 50 percent loss of activity.

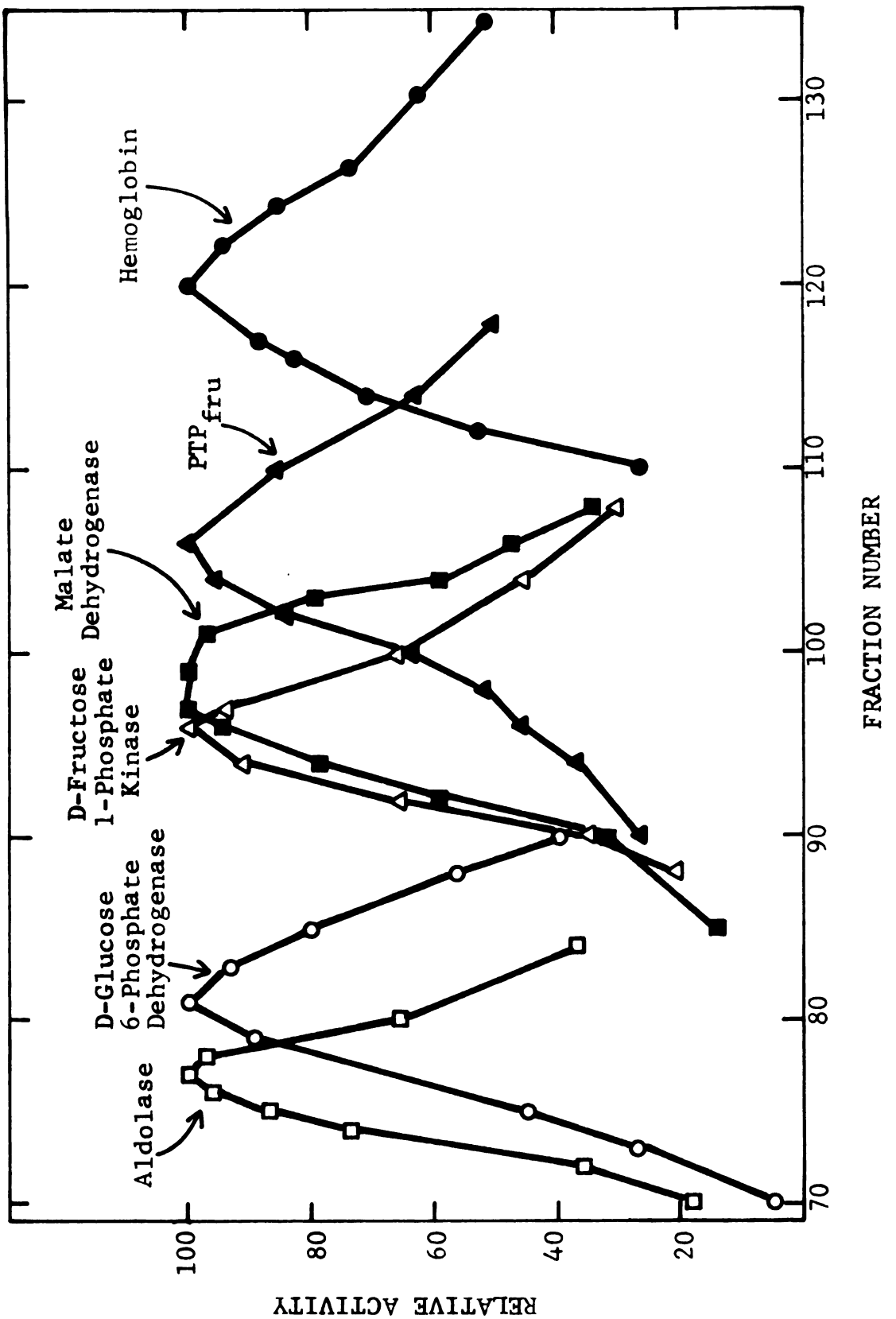
Molecular Weight Determination by Gel Filtration on Sephadex G100

The molecular weight of PTP_{fru} was determined by Sephadex G100 chromatography as described by Andrews (4). A sample of PTP_{fru} from a 45 to 65 percent ammonium sulfate step was mixed with the following proteins: aldolase (MW 149,000) (110), D-glucose 6-phosphate dehydrogenase (MW 128,000) (4), D-fructose 1-phosphate kinase (MW 75,000) (98), malate dehydrogenase (MW 70,000) (112), and hemoglobin (MW 32,000) (103). A total volume of 0.7 ml was carefully layered on a 2.5 x 42-cm Sephadex G100 column which was eluted with 0.02 M potassium phosphate buffer (pH 8.0) containing 0.001 M DTT and 10 percent glycerol. One-ml (30-drop) fractions were collected and assayed for the individual activities as described in Methods.

The elution profiles of relative activities are shown in Figure 8. From a plot of log molecular weight of the standards versus the fraction number of the center of each activity peak (Figure 9), the molecular weight of PTP_{fru} was estimated to be 52,000 daltons.

Figure 8. Elution profiles of aldolase, D-glucose 6-phosphate dehydrogenase, D-fructose 1-phosphate kinase, malate dehydrogenase, PTP_{fru}, and hemoglobin chromatographed on a Sephadex G100 column.

Figure 8


Hemoglobin
PTP fru
Malate Dehydrogenase
D-Fructose 1-Phosphate Kinase
D-Glucose 6-Phosphate Dehydrogenase
Aldolase
RELATIVE ACTIVITY
100
80
60
40
20
70
80
90
100
110
120
130
FRACTION NUMBER

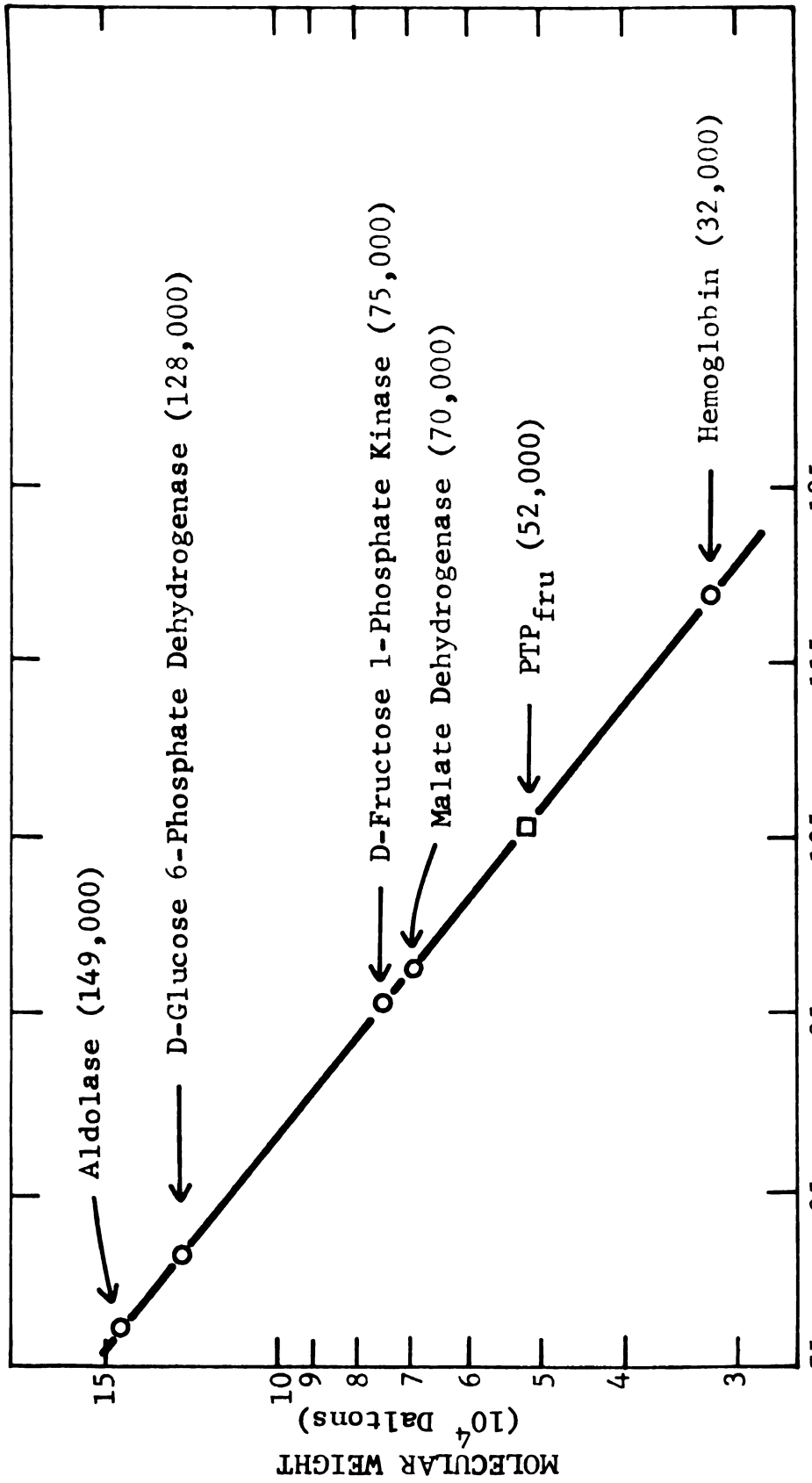


Figure 9. Molecular weight determination of PTP$_{fru}$ on standard Sephadex G100 column. Data are from Figure 8.

Subunit Molecular Weight Determination by SDS Polyacrylamide Disc Gel Electrophoresis

A sample of PTP_{fru} similar to that in Figure 7C was lyophilized to dryness and resuspended in the incubation mixture described in Methods. Six gels were run with different combinations of lactic dehydrogenase, carbonic anhydrase, trypsin, β-lactoglobulin, and lysozyme as standards (124). After staining and destaining, the gels were scanned at 550 nm and the relative migration distances, with respect to the methylene blue dye front, were calculated for the standards and PTP_{fru}. These R_f's were plotted against the log subunit molecular weight of the standards. The subunit molecular weight of PTP_{fru} was determined to be 26,000 daltons (Figure 10). Thus, native PTP_{fru} is a dimer with a molecular weight of 52,000 and consists of two monomers of equal molecular weight (26,000).

Role of D-Fructose Phosphoryl Transfer Protein

Requirement of PTP_{fru} for Growth on Low D-Fructose Concentrations

As indicated in the literature review, the PEP-dependent phosphotransferase system has been implicated in the vectorial phosphorylation of sugars in bacterial sugar metabolism (48). Mutants missing enzyme I, HPr, or enzyme II of this system have defective growth on one or several sugars. A mutant (strain QQ17) has been

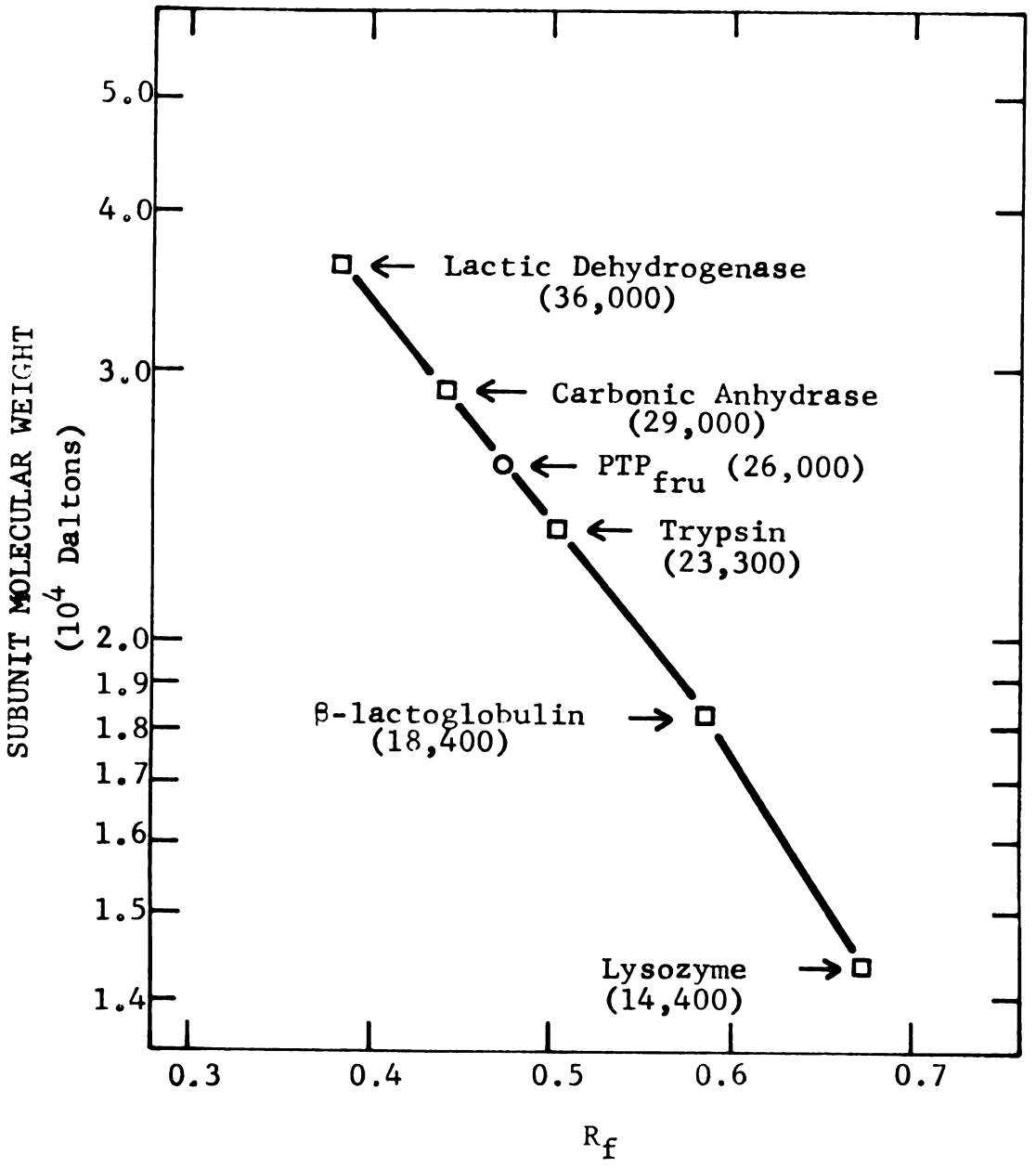


Figure 10. Molecular weight determination of subunits of PTP_{fru} by SDS polyacrylamide disc gel electrophoresis. Details are given in the text.

shown to be missing PTP_{fru} and has a slow growth rate only on D-fructose (35). To see if the growth rate of QQ17 was dependent on the concentration of D-fructose, QQ17, DD31, and PRL-R3 were grown on seven different concentrations of D-fructose and D-glucose (Figures 11 and 12).

Growth of QQ17 was dependent on D-fructose concentration (Figure 13A) and had an apparent K_m of 7.4 mM for D-fructose (Figure 13C). Strain DD31 (lacking D-fructose 1-phosphate kinase) had slow growth on D-fructose; however, increasing the D-fructose concentration had no effect on its rate of growth. By comparison, QQ17 and DD31 had higher growth rates than PRL-R3 on D-glucose (Figure 13B).

Thus, it appears as though growth on D-fructose is dependent on a constitutive PEP-dependent phosphotransferase system that is functional in QQ17 in the absence of PTP_{fru} and has an apparent K_m of 7.4 mM for D-fructose. Further evidence that D-fructose is phosphorylated by this system in QQ17 is the fact that growth on D-fructose does not induce high levels of D-fructokinase, whereas DD31, which cannot directly utilize the D-fructose 1-phosphate formed by its PEP system, induces a D-fructokinase to a level six times that of QQ17 or PRL-R3 (33, 53). If D-fructose was entering QQ17 by facillitated diffusion, it would have induced this enzyme to a higher level.

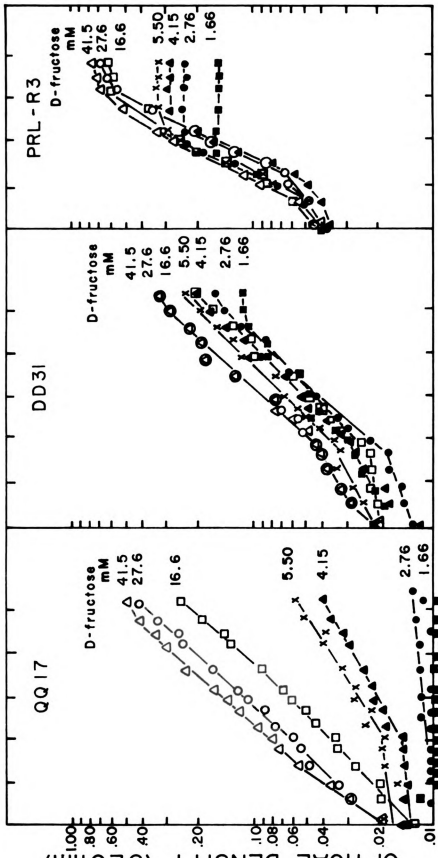


Figure 11. A comparison of the growth rates of QQ17, DD31, and PRL-R3 on seven different concentrations of D-fructose.

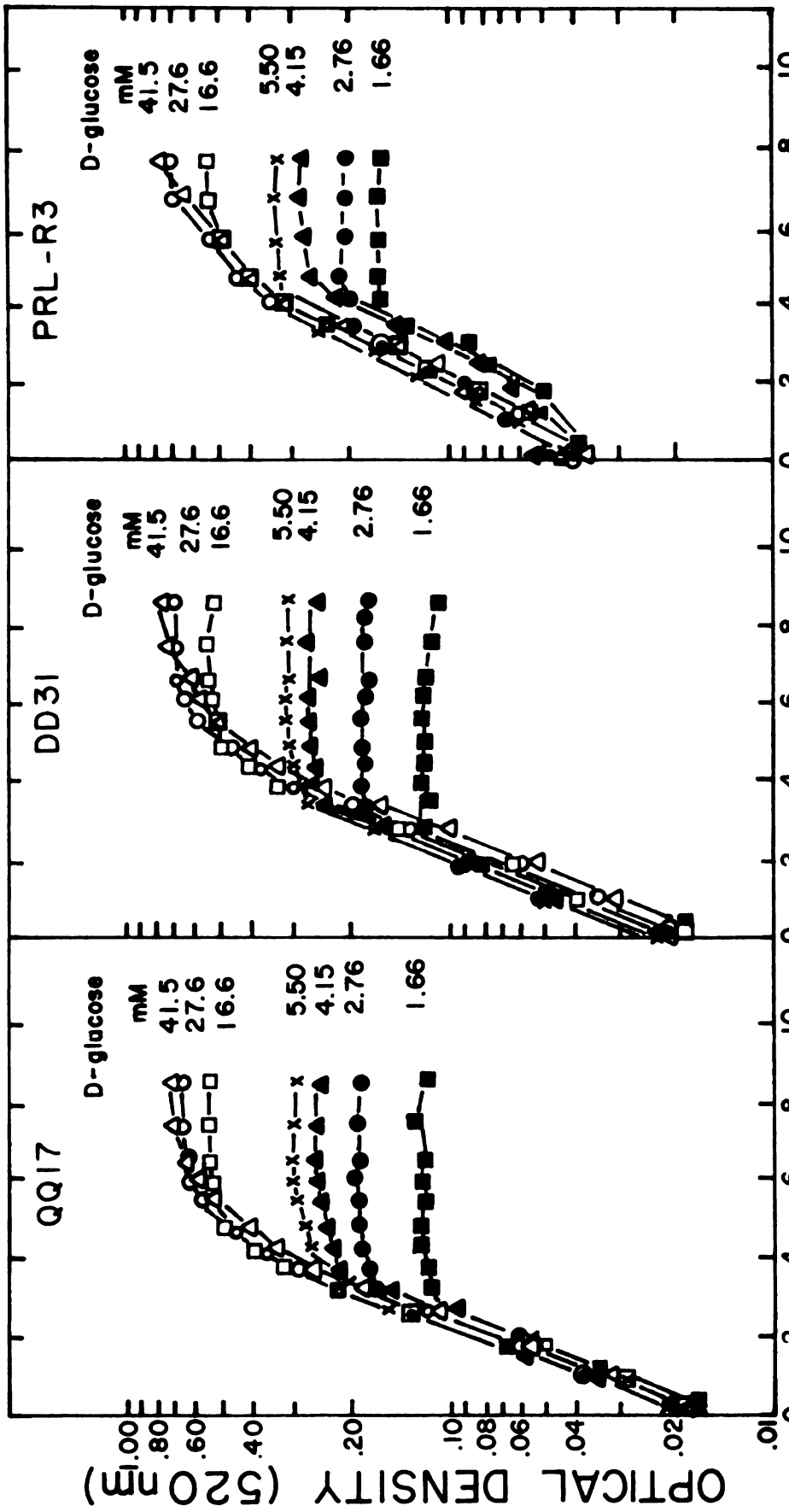


Figure 12. A comparison of the growth rates of CQ17, DD31 and PRL-R3 on seven different concentrations of D-glucose.

Figure 13. Substrate saturation and Lineweaver-Burk plots for growth of QQ17, DD31, and PRL-R3 on D-fructose and D-glucose.

A. D-fructose saturation curve.
B. D-glucose saturation curve.
C. D-fructose Lineweaver-Burk plot.
D. D-glucose Lineweaver-Burk plot.

Data are from Figures 11 and 12.

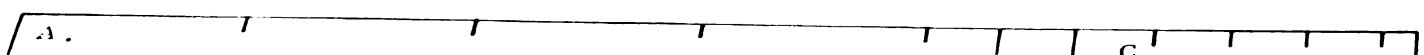
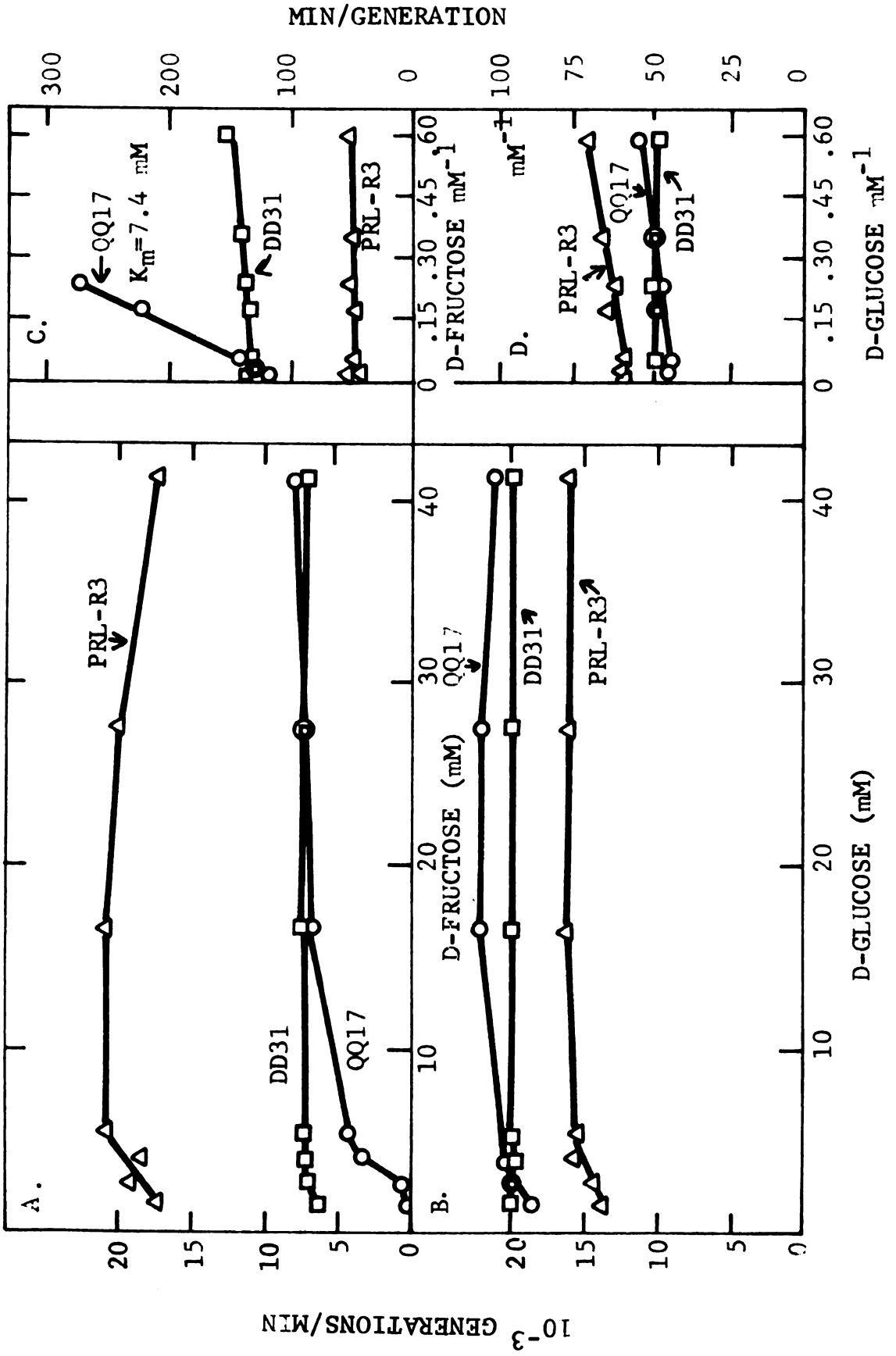


Figure 13


MIN/GENERATION
A.
B.
C.
D.
PRL-R3
DD31
QQ17
K_m=7.4 mM
D-FRUCTOSE (mM)
D-GLUCOSE (mM)
D-FRUCTOSE mM^{-1}
D-GLUCOSE mM^{-1}
10^{-3} GENERATIONS/MIN

Thus, QQ17 apparently metabolizes D-fructose by a phosphotransferase system that has a high K_m for D-fructose and does not require PTP_{fru} for activity and therefore is a constitutive system. For normal growth rates at low D-fructose concentrations, induction of active PTP_{fru} is required and thus, PTP_{fru} functions in an inducible phosphotransferase system.

Lack of Requirement of PTP_{fru} for D- [U-^{14}C] Fructose Binding

PTP_{fru} was shown to increase the apparent affinity of the phosphotransferase system for D-fructose (35). To determine if this effect was due to a PTP_{fru}-dependent change in affinity of enzyme II_{fru} for D-fructose, the amount of D-[U-^{14}C] fructose bound to a component(s) in crude extracts of Aerobacter aerogenes was measured. Crude extracts of PRL-R3 and QQ17 induced on D-fructose were incubated in 10^{-4} M D-[U-^{14}C] fructose at 30° C for 10 minutes and then chromatographed over Sephadex G25 to separate free D-[U-^{14}C] fructose from that which was bound to a high-molecular-weight component(s). QQ17 and PRL-R3 extracts had 1.16×10^{-5} and 1.06×10^{-5} μmoles of D-[U-^{14}C] fructose bound per mg of protein, respectively. Thus, the presence or absence of PTP_{fru} has no effect on the amount of D-fructose that becomes bound. When the bound radioactivity was chromatographed on Sephadex G200, the peaks of radioactivity eluted in the

void volume, indicating that it was bound to a high-molecular-weight particle (probably membrane vesicles).

Crude extracts isolated from PRL-R3 grown on D-glucose, when incubated at both 0°C and 30°C with D-[U-^{14}C] fructose, had less bound radioactivity than did extracts of cells grown on D-fructose (Table III).

Table III. D-[U-^{14}C] Fructose bound to crude extracts of PRL-R3 grown on D-fructose and D-glucose and incubated at 30°C and 0°C.

Growth Substrate	CPM[a]/mg Crude Extract Protein	
	30°C	0°C
D-fructose	560	477
D-glucose	167	207

[a]Counts per minute in the void volume of a Sephadex G25 column.

The data indicate that growth on D-fructose increases the capacity of extracts to bind D-fructose. The presence of active PTP_{fru} is not required for this binding; however, QQ17 could contain a defective PTP_{fru} that affects the binding of D-fructose but not its phosphorylation at low D-fructose concentrations. Another explanation is that a specific enzyme II is induced by growth on D-fructose that has a higher affinity for binding D-fructose.

Inducibility of Enzyme II by D-Fructose

Enzymes II (100,000 x g precipitates) obtained from crude extracts of PRL-R3 cells grown on D-fructose, D-galactose, D-glucose, D-mannitol, cellobiose, and sucrose contained various levels of PEP-dependent D-fructose phosphorylation activity (Table IV). The activities at high (200 mM) D-fructose concentrations, without added PTP_{fru}, ranged from 1.86 nmoles D-fructose 1-phosphate formed per minute per mg enzyme II_{glu} to 13.4 nmoles per minute per mg enzyme II_{mtl}. The activities at low (2 mM) D-fructose concentrations also varied; however, the D-fructose-induced enzyme II activity was 2.2 times greater than that of the next highest activity. Thus, the levels of D-fructose phosphorylation vary with the source of enzyme II. At high D-fructose concentrations the enzyme II_{fru} is in the middle range of activities. However, at lower D-fructose concentrations, it has a higher activity than the other enzymes II, and thus must have a higher affinity for D-fructose. This higher activity at 2 mM D-fructose is partially due to some PTP_{fru} that is trapped in the enzyme II_{fru} vesicles. This residual PTP_{fru} is removed from the enzyme II by Sephadex G200 chromatography as shown in the following section.

The results of these control experiments at high D-fructose concentrations show that D-fructose phosphorylation is catalyzed by a constitutive enzyme II. This

Table IV. Effect of different PEP concentrations on specific activities of 100,000 x g enzyme II precipitates obtained from cells grown on various substrates. Reactions were run as described for the reconstituted assay (general assay for PEP-dependent D-fructose 1-phosphate formation) in Methods at the D-fructose concentrations noted. Reactions contained 0.44 mg of enzyme I purified from D-mannitol-g[illegible]

Table IV. Effect of different PTP_{fru} fractions on specific activities of 100,000 x g enzyme II precipitates obtained from cells grown on various substrates. Reactions were run as described for the reconstituted assay (general assay for PEP-dependent D-fructose 1-phosphate formation) in Methods at the D-fructose concentrations noted. Reactions contained 0.44 mg of enzyme I purified from D-mannitol-grown QQ17 by Sephadex G200 and DEAE cellulose chromatography, and 0.01 mg of HPr purified from D-fructose-grown PL-122 by Sephadex G200 and DEAE cellulose chromatography. Where indicated, 0.21 mg of a Sephadex G200 fraction of PTP_{fru}, or 0.56 mg of a 35-55% ammonium sulfate fraction of the Sephadex G200 fraction of PTP_{fru} were added.

Enzyme II				PTP_{fru} Added			
				Sephadex G200 Fraction		35-55% Ammonium Sulfate Fraction	
Growth Substrate	Protein (mg/ml)	D-fructose (mM)	Control[a] SA[b]	SA[b]	ΔSA[c]	SA[b]	ΔSA[c]
D-fructose	11.0	2	2.90	7.55	4.65	9.61	6.71
		200	4.17	8.14	3.97	10.9	6.73
D-galactose	11.9	2	1.17	1.96	0.79	2.44	1.27
		200	6.57	4.93	-1.64	7.20	0.63
Cellobiose	11.3	2	0.71	1.16	0.45	1.61	0.90
		200	3.17	2.97	-0.20	3.87	0.70
Sucrose	18.0	2	0.48	0.94	0.46	1.28	0.80
		200	1.98	2.09	0.11	2.31	0.33

Table IV.--Continued.

Enzyme II				PTP_{fru} Added			
				Sephadex G200 Fraction		35-55% Ammonium Sulfate Fraction	
Growth Substrate	Protein (mg/ml)	D-fructose (mM)	Control[a] SA[b]	SA[b]	ΔSA[c]	SA[b]	ΔSA[c]
D-glucose	13.2	2	0.39	0.88	0.49	1.16	0.77
		200	1.86	1.83	-0.03	2.46	0.60
D-mannitol	8.3	2	1.31	2.05	0.74	1.94	0.63
		200	13.4	13.1	-0.30	14.4	1.00

[a]No PTP_{fru} added.

[b]Specific activity in terms of nmoles D-fructose 1-phosphate min^{-1} mg^{-1} enzyme II.

[c]Signifies difference between specific activity of control and specific activity in presence of added PTP_{fru}.

activity varies over a six-fold range and is present even in extracts obtained from cells grown on substrates not utilized by this phophotransferase system (D-galactose, cellobiose, and sucrose). When PTP_{fru} was added, the enzyme II specific activities at 2 mM and 200 mM D-fructose either increased equally or to a greater extent at low D-fructose than at high D-fructose concentrations. The increases, caused by addition of either PTP_{fru} sample, in the enzyme II_{fru} activity at both D-fructose concentrations were much greater than any of the other enzymes II. However, it is evident that PTP_{fru} did activate all of the enzymes II tested at 2 mM D-fructose. This was first thought to be an effect of lowering the K_m for D-fructose which would increase the activity at 2 mM D-fructose. Any increases in activity at 200 mM D-fructose were explained as a secondary role of PTP_{fru}, that of an activator (35). Another hypothesis which fits the data is the activation by PTP_{fru} of a separate system which has a low K_m for D-fructose, thus effecting equal increases in D-fructose 1-phosphate formed at both 2 and 200 mM. Since enzyme II_{fru} has a much greater increase in activity, D-fructose could be inducing a component of this separate system, namely a D-fructose enzyme II. The lower increases at 200 mM could be due to inhibition of some part of this complex system.

The high activity of the enzyme II_{mtl} at 200 mM

D-fructose may be due to an induced D-mannitol enzyme II (105) that also has a low affinity for D-fructose. D-Fructose phosphorylation catalyzed by enzyme II_{mtl} is inhibited by D-mannitol, whereas D-fructose phosphorylation by enzyme II_{fru} is not (35).

Effect of PTP_{fru} on Sephadex G200 Enzyme II Activities

The relationship between PTP_{fru} and enzyme II_{fru} was further studied with more purified enzyme II samples. Crude extracts were prepared from D-fructose- and D-mannitol-induced PRL-R3. They were chromatographed on Sephadex G200 and fractions containing maximal enzyme II activity were pooled and used for assaying the effect of (i) a Sephadex G200 pool of PTP_{fru} and (ii) the 35 to 55 percent saturated ammonium sulfate fraction of this pool on high (200 mM) and low (2 mM) D-fructose concentrations. Enzyme I and HPr which had been purified by Sephadex G200 and DEAE cellulose column chromatography were present at saturating levels.

The specific activities at 200 mM D-fructose of PRL-R3 enzyme II_{fru} and enzyme II_{mtl} were approximately equal (Table V). When the Sephadex G200 fraction of PTP_{fru} was added to the PRL-R3 enzyme II_{fru} the actual increase in activity was almost exactly the same as in Table IV. Thus, with equal additions of the same sample of PTP_{fru} to two different enzymes II_{fru} the total

Table V. Effect of different PTP_{fru} preparations on D-fructose phosphorylation catalyzed by enzymes II purified by Sephadex G200 column chromatography. The reactions are the same as Table IV except for the preparation of PRL-R3 enzymes II. Where indicated, 0.21 mg of a Sephadex G200 fraction of PTP_{fru}, or 1.12 mg of a 35-55% ammonium sulfate fraction of the Sephadex G200 fraction of PTP_{fru} were added.

Enzyme II				PTP_{fru} Added			
				Sephadex G200 Fraction		35-55% Ammonium Sulfate Fraction	
Growth Substrate	Protein (mg/ml)	D-fructose (mM)	Control[a] SA[b]	SA[b]	ΔSA[c]	SA[b]	ΔSA[c]
D-fructose	11.3	2	0.69	5.15	4.46	25.0	24.3
		200	2.83	6.76	3.93	25.2	22.4
D-mannitol	17.1	2	0.41	--[d]		1.33	0.92
		200	2.49	--[d]		3.44	0.95

[a]No PTP_{fru} added.

[b]Specific activity in terms of nmoles D-fructose 1-phosphate min^{-1} mg^{-1} enzyme II.

[c]Signifies difference between specific activity of control and specific activity in presence of added PTP_{fru}.

[d]Signifies not determined.

activity increases to an equal extent. The "fold increase" over the background was much greater, however, with the Sephadex G200 enzyme II_{fru}. This method of preparing enzyme II removes residual PTP_{fru}, whereas ultracentrifugation preparations of enzyme II retain varying levels of PTP_{fru}. This residual level of PTP_{fru} increases the activity at 2 mM D-fructose as seen in comparing the data in Tables IV and V.

When the ammonium sulfate fraction was added to the enzymes II described in Table V, the increase in activity of PRL-R3 enzyme II_{fru} was 24 times greater than the increase in activity of enzyme II_{mtl}; however, the increases at 2 and 200 mM D-fructose were equal for both enzymes II. These data, obtained with more purified enzymes II, fit the hypothesis that D-fructose induces to higher levels an enzyme II which is activated by addition of PTP_{fru} and has a low K_m for D-fructose. Thus, the net effect of activation is addition of equal amounts of D-fructose 1-phosphate-forming activity at both 2 and 200 mM D-fructose. The fact that the increase in PRL-R3 enzyme II_{fru} activity was greater at 2 mM than at 200 mM D-fructose could be due to the inhibition of one system, either the "constitutive" phosphotransferase system (measured in the absence of PTP_{fru}) or the "inducible" phosphotransferase system (activated by addition of PTP_{fru}), by the components required for the other system.

These data indicate that the role of PTP_{fru} is that of a specific requirement for a separate system with a low K_m for D-fructose. This system could involve an inducible enzyme II which specifically interacts with the PTP_{fru} in this low K_m system. The level of this enzyme II varies with the growth substrate. In these experiments the D-fructose-induced activity (the increase in 2 mM D-fructose phosphorylation affected by addition of PTP_{fru}) was 89.6 percent of the total activity (induced activity plus 200 mM constitutive activity measured in the absence of PTP_{fru}), while in D-mannitol-induced cells the level of this specific enzyme II was only 27 percent of the total enzyme II. Other data (discussed in a later section, Figure 19) have indicated that this inducible enzyme II activity is only 2 to 8 percent of the total enzyme II activity in enzymes II obtained from substrates other than D-fructose.

Induction of Both Enzyme II and PTP_{fru} by D-Fructose

The data in the previous two tables (IV and V) indicated that a D-fructose-specific enzyme II with a high affinity for D-fructose is induced by growth on D-fructose and that this enzyme II is activated by the addition of a fraction isolated from D-fructose-grown cells. The induction of both PTP_{fru} and enzyme II was examined by assaying combinations of 100,000 x g

supernatants and precipitates isolated from cells grown on different substrates. Crude extracts of PRL-R3 cells grown on nutrient broth, D-mannitol, D-mannose, D-glucitol, and D-fructose were centrifuged at 100,000 x g. The resulting pellets [resuspended in 0.02 M Tris-HCl buffer (pH 7.5) containing 0.028 M 2-mercaptoethanol] and the upper portions of the supernatants were recentrifuged at 100,000 x g for 2 hours. The second centrifugates were resuspended and adjusted to approximately equal protein concentrations as noted in Table VI. The supernatants were chromatographed over Sephadex G25 columns to remove endogenous low-molecular-weight compounds which might affect the activity and then were adjusted to approximately equal protein concentrations by dilution with 0.02 M Tris-HCl (pH 7.5) containing 0.028 M 2-mercaptoethanol. Assays were then run with saturating levels of HPr and enzyme I at 200 mM D-fructose and with limiting levels of enzyme II. Controls were run without adding the enzyme II fractions.

The only change in the activities of the enzymes II which was interpreted to be significant occurred when the D-fructose supernatant was combined with the enzyme II_{fru}, which resulted in a 160 percent increase in the specific activity of the enzyme II (Table VI). This supernatant had little effect on the activities of the other enzymes II. The only other noticeable effects were minor, namely (i) a 56 percent stimulation of

Table VI. Comparison of specific activities of different enzyme II 100,000 x g precipitates in the presence of different 100,000 x g supernatants. The assay (0.2 ml) contained 8.0 μmoles of Tris-HCl (pH 7.5), 0.06 μmole of 2-mercaptoethanol, 40 μmoles of D-fructose, 0.1 μmole of $MgCl_2$, 3.0 μmoles of NaF, 0.44 mg of enzyme I, 0.17 mg of HPr, 10 μl of the individual enzymes II (centrifuged twice at 100,000 x g), and 50 μl of the supernatants (centrifuged twice at 100,000 x g) and chromatographed on Sephadex G25. Protein was estimated by Lowry determination. Both HPr and enzyme I were purified by Sephadex G200 and DEAE cellulose column chromatography.

Table VI.

Enzyme II Precipate	Specific Activity[a]					
		100,000 x g Supernatant				
	Control[b]	D-fructose 5.93 mg/ml	D-mannose 5.71 mg/ml	D-glucitol 5.56 mg/ml	D-mannitol 5.28 mg/ml	Nutrient Broth 5.09 mg/ml
D-fructose 18.24 mg/ml	1.98	5.11	2.47	3.10	2.31	2.01
D-mannose 20.59 mg/ml	6.20	6.04	5.11	5.26	6.02	5.38
D-glucitol 22.06 mg/ml	4.02	4.49	4.18	4.29	4.07	4.23
D-mannitol 20.36 mg/ml	7.28	6.73	6.70	7.08	6.46	5.87
Nutrient Broth 20.42 mg/ml	1.34	1.50	1.24	1.62	1.48	1.99

[a]Defined as nmoles D-fructose 1-phosphate min^{-1} mg^{-1} enzyme II.

[b]No supernatant added.

enzyme II_{fru} by the D-glucitol supernatant, (ii) a 49 percent stimulation of enzyme II_{NB} by the nutrient broth supernatant, and (iii) a 19 percent inhibition of enzyme II_{mtl} by the nutrient broth supernatant.

The data in these experiments show that only growth on D-fructose induces a component (PTP_{fru}) in the 100,000 x g supernatant that significantly increases the PEP:D-fructose 1-phosphotransferase activity of enzyme II_{fru}. This supernatant had no effect on the activity of other enzymes II and the other supernatants had little effect on any of the enzyme II activities. Thus, the cytoplasmic component, PTP_{fru}, induced by D-fructose only interacts with the D-fructose-induced enzyme II. The sugar-specificity of these two inducible components has not been rigorously studied; however, preliminary data have shown that PTP_{fru} does not increase the rate of PEP-dependent phosphorylation of D-mannose, D-mannitol, or D-glucitol catalyzed by enzymes II isolated from extracts of cells induced on D-fructose, D-mannose, D-mannitol, and D-glucitol.

Additivity of Constitutive and Inducible Systems

If these two systems contain separate enzymes II, their activities should be additive. Data in Tables IV and V showed that addition of PTP_{fru} effected an increase in activity that appears to be additive. It was later discovered that the high affinity system

functions in the absence of HPr, and thus can be assayed in the absence of HPr. The constitutive activity can be assayed in the presence of HPr and absence of PTP_{fru}. These assays, which are described in Methods, were used to determine the effect of D-fructose concentration on reaction velocity (Figures 14-17). It was determined, using these assays and QQ17 enzyme II_{fru} obtained by 100,000 x g centrifugation of a pool of enzyme II activity from a Sephadex G200 column, that the V_{max} and K_m of the constitutive system were 5.34 nmoles D-fructose 1-phosphate formed per minute per mg and 7.14×10^{-3} M D-fructose, respectively (Figure 16). The V_{max} and K_m of the inducible system were determined to be 3.78 and 1.6×10^{-5} M D-fructose in the presence of HPr and 3.15 and 1.9×10^{-5} M D-fructose in the absence of HPr (Figure 17). The above six values were obtained by assaying D-fructose 1-phosphate formation at D-fructose concentrations from 0.01 to 90 mM in the presence and absence of HPr and PTP_{fru}. The activity of the reaction in the absence of both HPr and PTP_{fru} (0.2 to 0.3 nmoles D-fructose 1-phosphate min^{-1} mg^{-1} enzyme II) was subtracted from the activities found when (i) both HPr and PTP_{fru}, (ii) only HPr, or (iii) only PTP_{fru} were included. The three resulting values were termed (i) constitutive + inducible (+HPr), (ii) constitutive, and (iii) inducible (-HPr), respectively (Figures 14 and 15). The final D-fructose

Figure 14. Saturation curves of constitutive and inducible phosphotransferase activities. These reactions contained 0.135 mg of QQ17 and enzyme II_{fru} chromatographed on a Sephadex G200 column and 0.173 mg of enzyme I purified by DEAE cellulose and Sephadex G200 column chromatography. For inducible activities, 0.113 mg of PTP_{fru} purified through DEAE cellulose and Sephadex G200 columns was included. For constitutive activities, 0.155 mg of HPr purified through Sephadex G75 and DEAE cellulose columns was included. Further details are in the text. The Inducible (+HPr) curve was calculated by subtracting the Constitutive activity from the Constitutive + Inducible (+HPr) activity. Blank values in the absence of both HPr and PTP_{fru} ranged from 0.2 to 0.3 nmoles D-fructose 1-phosphate min^{-1} mg^{-1} enzyme II.

Figure 14

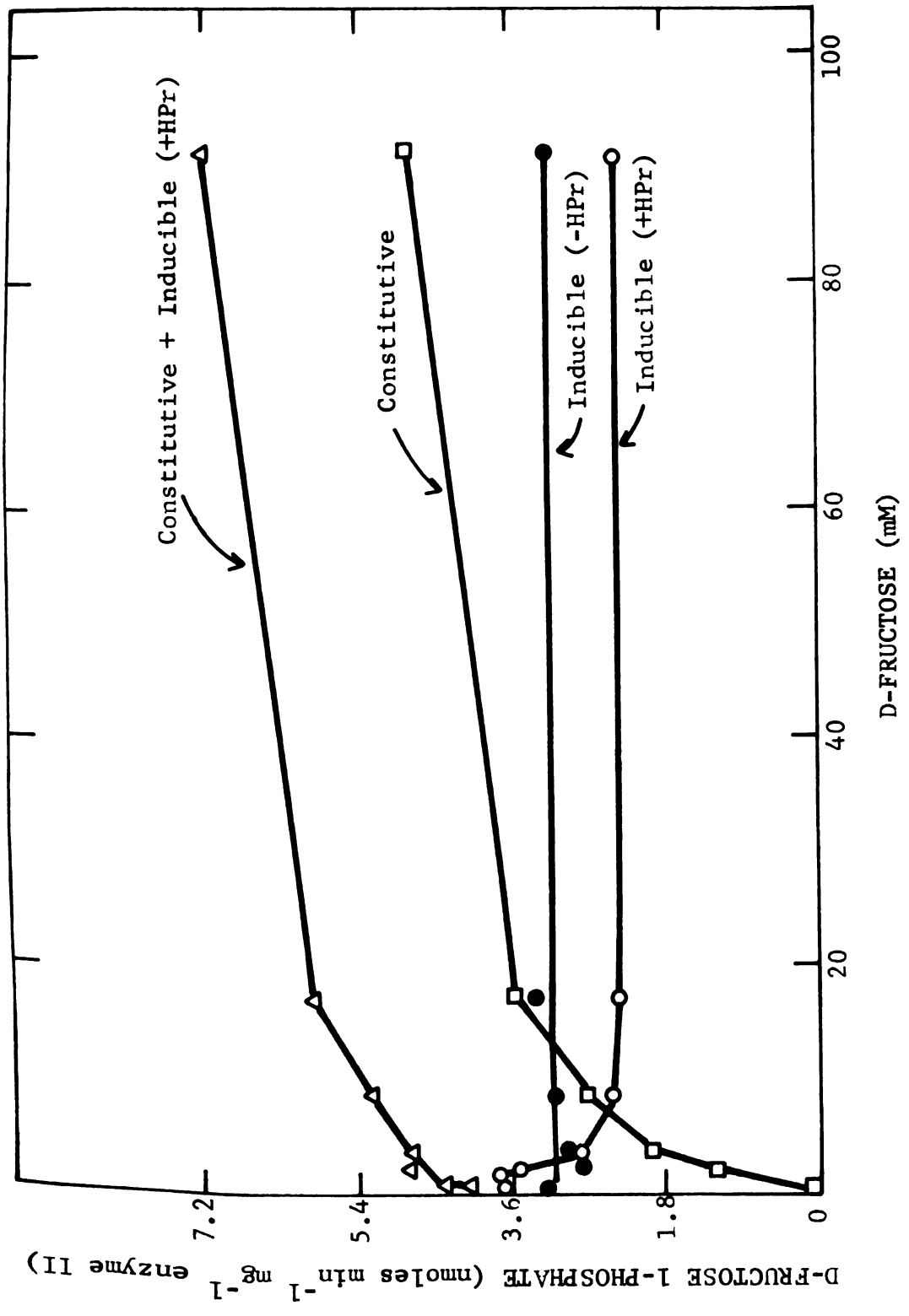

Constitutive + Inducible (+HPr)
Constitutive
Inducible (-HPr)
Inducible (+HPr)
D-FRUCTOSE (mM)
D-FRUCTOSE 1-PHOSPHATE (nmoles min-1 mg-1 enzyme II)
0
1.8
3.6
5.4
7.2
20
40
60
80
100

Figure 15. Saturation curves of constitutive and inducible phosphotransferase activities at low D-fructose concentrations. Components are as described in Figure 14. Further details are in the text.

Figure 15

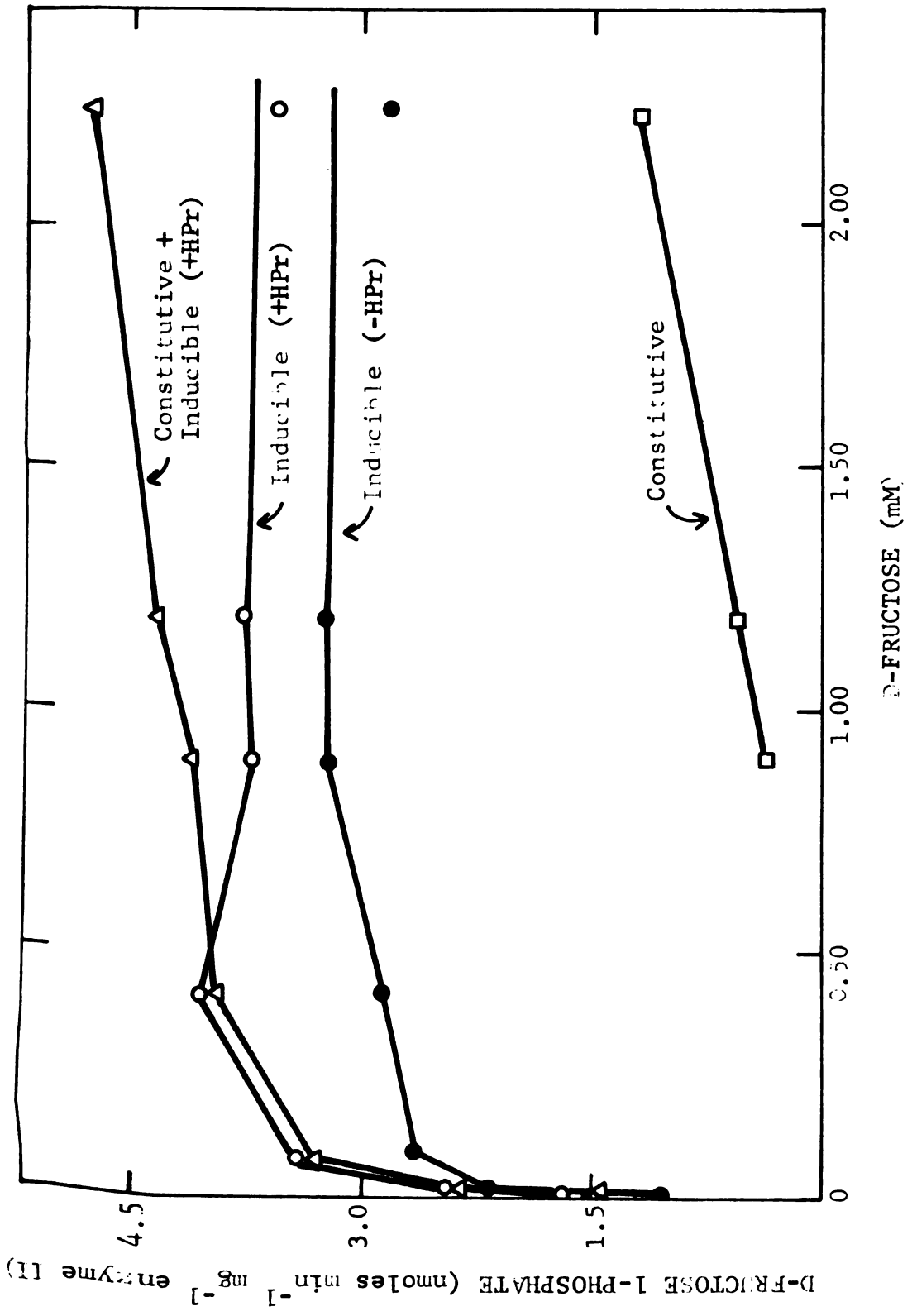

Constitutive +
Inducible (+HPr)
Inducible (+HPr)
Inducible (-HPr)
Constitutive
4.5
3.0
1.5
0
0.50
1.00
1.50
2.00
D-FRUCTOSE (mM)
D-FRUCTOSE 1-PHOSPHATE (nmoles min-1 mg-1 enzyme II)

Figure 16. Lineweaver-Burk plot of constitutive and inducible activities at high D-fructose concentrations. Data are from Figure 14. The open triangles (Δ) are data points for the Constitutive + Inducible (+HPr) activity; the solid line represents the calculated rates for Constitutive + Inducible (+HPr) as described in the text.

Figure 16

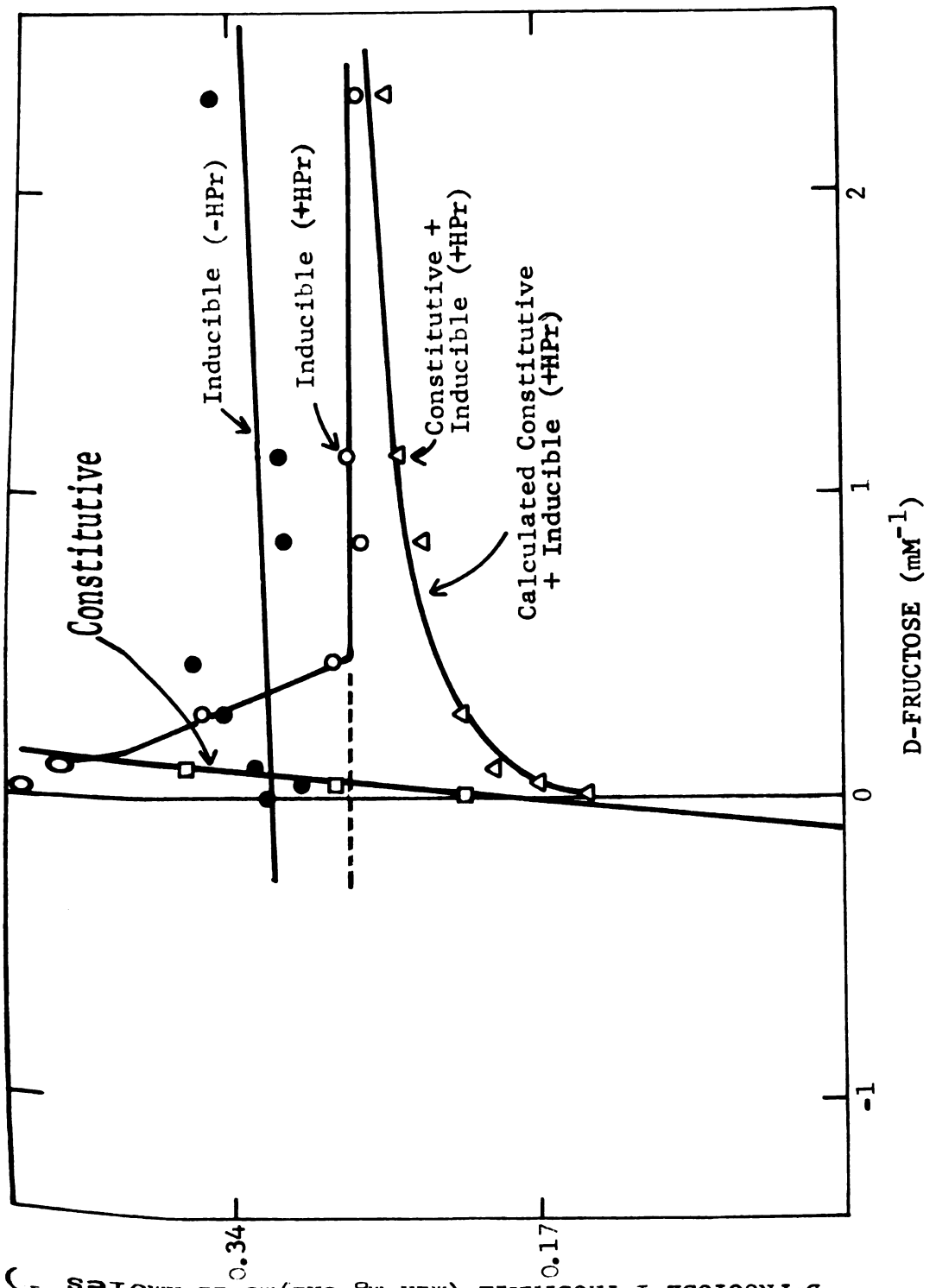
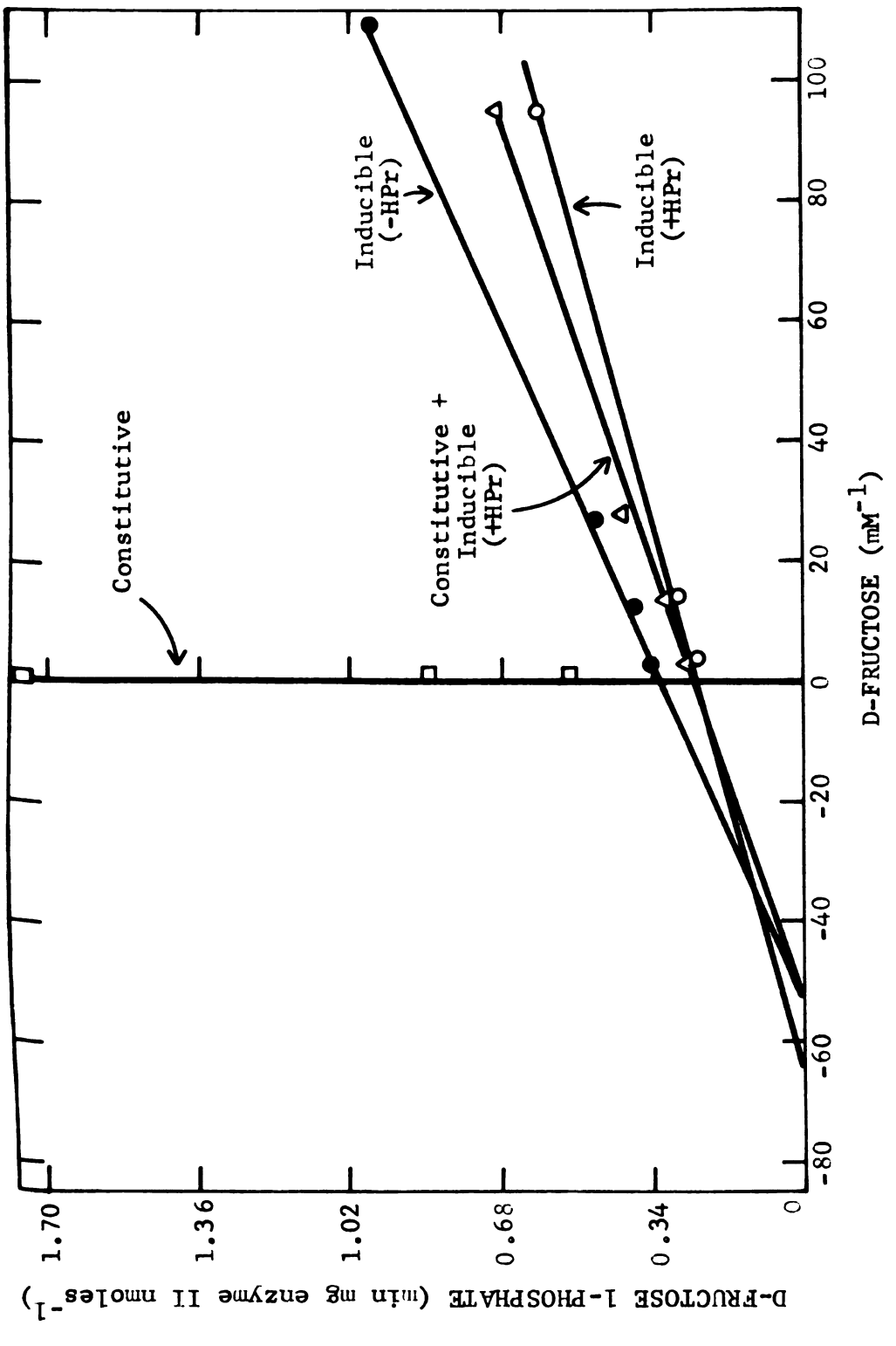

Inducible (-HPr)
Inducible (+HPr)
Constitutive + Inducible (+HPr)
Calculated Constitutive + Inducible (+HPr)
Constitutive
D-FRUCTOSE (mM^{-1})
D-FRUCTOSE 1-PHOSPHATE (min mg enzyme II nmoles^{-1})
0.34
0.17
-1
0
1
2

Figure 17. Lineweaver-Burk plot of constitutive and inducible activities at low D-fructose concentrations. Data are from Figure 15. Further details are in the text.

Figure 17


D-FRUCTOSE 1-PHOSPHATE (min mg enzyme II nmoles^{-1})
1.70
1.36
1.02
0.68
0.34
0
-80
-60
-40
-20
0
20
40
60
80
100
D-FRUCTOSE (mM^{-1})
Constitutive
Constitutive + Inducible (+HPr)
Inducible (-HPr)
Inducible (+HPr)

1-phosphate and D-fructose concentrations in each individual reaction were determined and the sum of these was used as the total initial D-fructose concentration. The initial concentration had to be measured because reactions without added D-fructose contained 6.4 nM D-fructose, which was most likely bound to the enzyme II vesicles. The average D-fructose concentration of the reaction was determined and used as the actual concentration in plotting the curves and calculating the data for this experiment (67). This procedure was necessary because at the lowest D-fructose concentration, 60 percent of the available D-fructose was converted to D-fructose 1-phosphate during the 10 minute reaction period (see Methods).

The activity of the inducible enzyme II_{fru} in the presence of HPr was determined by subtracting the constitutive activity from the constitutive + inducible (+HPr). An apparent inhibition of the calculated inducible (+HPr) activity was obtained at higher D-fructose concentrations (Figure 14); however, there was no inhibition of the inducible activity in the absence of HPr. Therefore, either HPr was inhibiting the inducible activity, or the PTP_{fru} was inhibiting the constitutive activity. Further experiments (to be described later and which are depicted in Figure 19) using enzyme II_{gly} isolated from QQ17 indicate that, in

fact, the PTP_{fru} does inhibit the constitutive enzyme II activity.

In the assays shown in Figures 14-17, the inhibition of the constitutive activity by PTP_{fru} is approximately 40 percent. To determine if the separate inducible and constitutive D-fructose 1-phosphotransferase activities were additive when corrected for this 40 percent inhibition, a theoretical curve was generated from the following equation:

$$v = \frac{V_{max_1}}{1 + \frac{K_{m_1}}{[S]}} + \frac{0.6\ V_{max_2}}{1 + \frac{K_{m_2}}{[S]}}$$

where v is the velocity, [S] is the D-fructose concentration, V_{max_1} and K_{m_1} are the kinetic constants for the inducible system in the presence of HPr, and V_{max_2} and K_{m_2} are the kinetic constants for the constitutive system. V_{max_2} is multiplied by 0.6 to correct for inhibition of the constitutive system by PTP_{fru}.

The resulting calculated velocities (Figure 16) superimposed over the actual data points. Thus, the separate inducible and constitutive systems are additive in their formation of D-fructose 1-phosphate when the inhibition of the constitutive system by PTP_{fru} is corrected for.

The K_m of 1.6×10^{-5} M D-fructose in the inducible

system is similar to the K_m (0.66 to 1.8 x 10^{-5}M, depending on source of enzyme II) for D-mannitol phosphorylation studied by this author (unpublished results), and lower than K_m values for other phosphotransferase systems reported in the literature (78, 92, 108). The K_m of the constitutive system (7.1 x 10^{-3} M D-fructose) is nearly equal to the apparent K_m for growth of QQ17 on D-fructose (7.4 x 10^{-3}, Figure 13C). This is consistent with the supposition that QQ17 utilizes the constitutive phosphotransferase system in its metabolism of D-fructose, that the rate of growth of QQ17 on D-fructose is limited by the rate of phosphorylation of D-fructose by enzyme II_{fru} (constitutive), and that the constitutive system does function when PTP_{fru} is absent. The fact that PRL-R3 has a higher rate of growth than that attained by QQ17 could be evidence for the *in vivo* additivity of the inducible system (which functions in PRL-R3) and the constitutive system (which functions in both PRL-R3 and QQ17).

Function of PTP_{fru} as a Substrate for Enzyme II_{fru}

When it was determined that there were two separate systems involved in PEP-dependent D-fructose phosphorylation and that PTP_{fru} is an absolute requirement for activity in the inducible system, a new assay had to be developed to replace the "fold increase" units of activity.

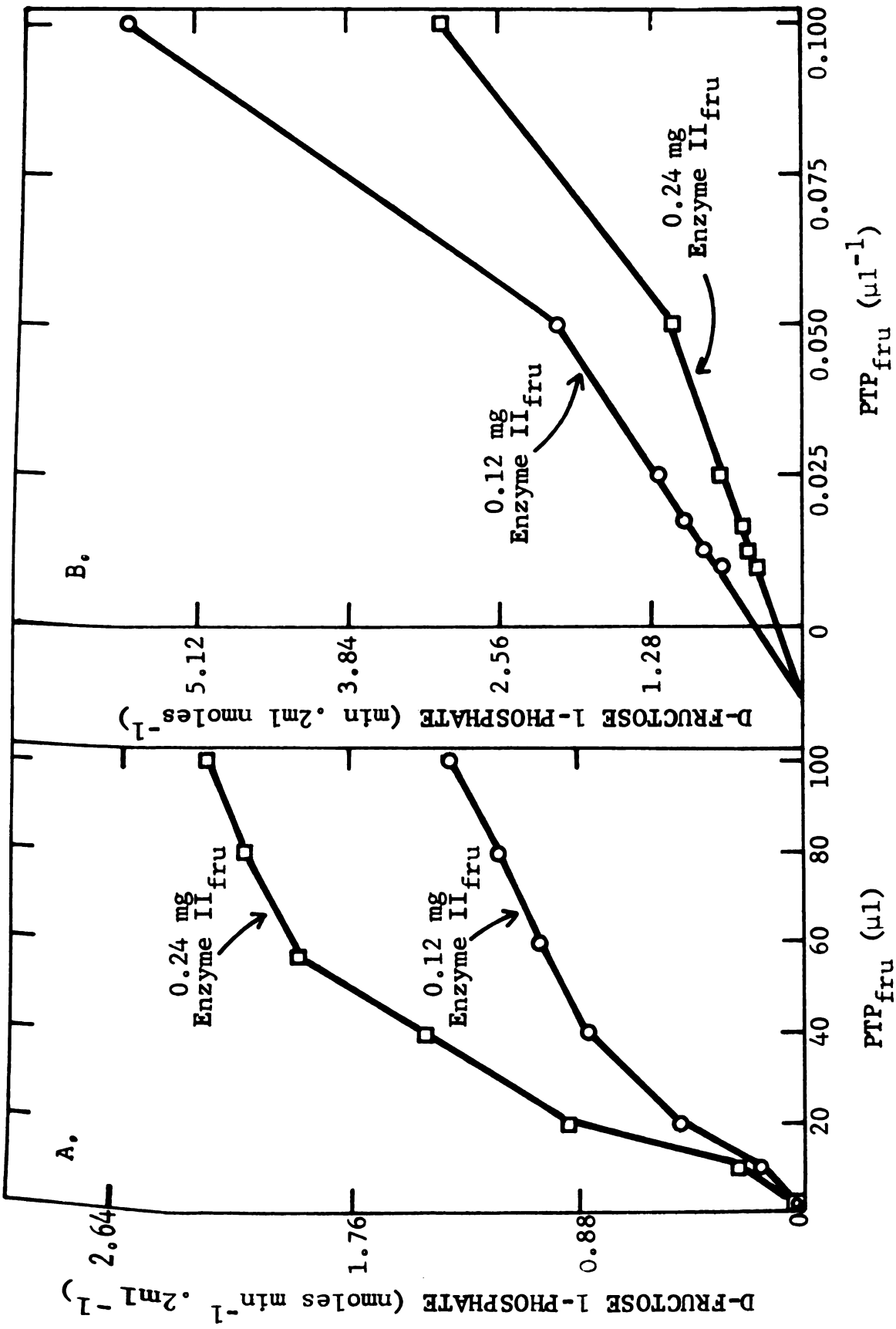
This new assay involves calculating the amount of PTP_{fru} that gives one-half the maximal activity of a given amount of enzyme II. The activity is defined by "half-maximal saturation" units as described in the PTP_{fru} assay in Methods. To demonstrate the validity of this assay, reactions were run at 0.5 mM D-fructose containing saturating enzyme I, a level of HPr which was half-saturating, QQ17 enzyme II_{fru}, and varying concentrations of a sample of PTP_{fru} purified through the second DEAE cellulose step. A slight sigmoidal characteristic seen at low PTP_{fru} concentrations (Figure 18A) has not yet been explained. For the following analysis, the sigmoidicity was ignored and the data treated in Lineweaver-Burk fashion. Double reciprocal plots of the data indicate that the apparent K_m for PTP_{fru} remained the same for two levels of enzyme II_{fru} (Figure 18B). Thus, the apparent K_m for PTP_{fru} is independent of the amount of enzyme II_{fru}, and PTP_{fru} acts as a substrate in the enzyme II-catalyzed phosphorylation of low concentrations of D-fructose.

If PTP_{fru} was an integral part of an enzyme II_{fru} complex, there would be a proportional relationship between PTP_{fru} and the enzyme II_{fru}. Doubling the level of enzyme II_{fru} should have doubled the amount of PTP_{fru} needed to saturate the system. This effect was not observed.

To determine if the data obtained with the "fold

Figure 18. Saturation and Lineweaver-Burk plots of inducible activity-dependence on PTP_{fru}. Reactions were run as described in Methods for the enzyme II inducible activity and contained 2.0 μg of HPr purified by Sephadex G75 and DEAE cellulose chromatography, 0.17 mg of enzyme I purified by chromatography on DEAE cellulose and Sephadex G200 columns, and either 0.12 or 0.24 mg of enzyme II_{fru}. PTP_{fru} (2.36 mg/ml) was isolated from a second DEAE cellulose column.

Figure 18


A.
B.
0.24 mg Enzyme II$_{fru}$
0.12 mg Enzyme II$_{fru}$
D-FRUCTOSE 1-PHOSPHATE (nmoles min^{-1} .2ml^{-1})
D-FRUCTOSE 1-PHOSPHATE (min .2ml nmoles^{-1})
PTP$_{fru}$ (µl)
PTP$_{fru}$ (µl^{-1})
0
0.88
1.76
2.64
20
40
60
80
100
1.28
2.56
3.84
5.12
0.025
0.050
0.075
0.100

increase" units correlates with the "half-maximal saturation" units, the D-fructose 1-phosphate formation catalyzed by QQ17 enzyme II_{fru} in the presence and absence of several purified PTP_{fru} fractions was measured and units of PTP_{fru} were calculated by both methods. The fold purification and percentage recovery obtained with both units were approximately the same.

The data in this experiment have shown that the apparent K_m for PTP_{fru} is not altered by changing the enzyme II concentration. A method of determining the "half-maximal saturation" units of PTP_{fru} was developed using this fact.

Inhibition of Constitutive Enzyme II Activity by PTP_{fru}

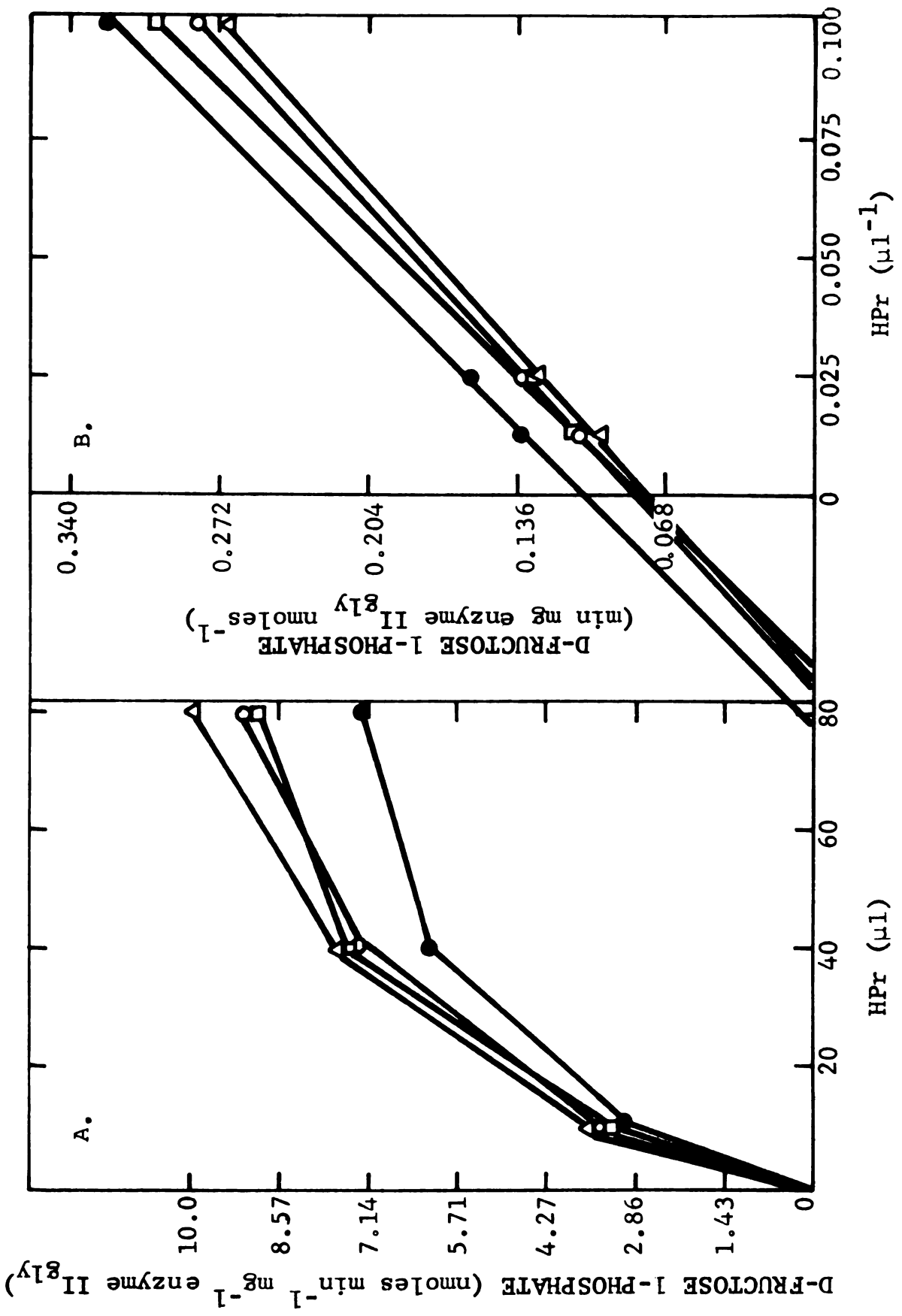
PTP_{fru} apparently inhibits the constitutive system as mentioned in the section on the additivity of the inducible and constitutive systems. This apparent inhibition of the constitutive activity was further studied using QQ17 enzyme II_{gly}, which has a relatively low level of inducible activity (3.5 percent of the total). Assays were run at 100 mM D-fructose with varying levels of HPr, obtained from D-fructose-grown PRL-R3, and PTP_{fru}. A slight increase (8 percent) in activity was observed when 5.0 µl of PTP_{fru} were added (Figure 19A). This was due to a low level of the inducible enzyme II in enzyme II_{gly} which was activated by levels of PTP_{fru} lower than those required for inhibition. Higher concentrations

Figure 19. Saturation and Lineweaver-Burk plots showing dependence on HPr concentration of D-fructose phosphorylation by the constitutive system. Assays were run using the HPr (D-fructose end-point) assay described in Methods and contained 0.146 mg of enzyme I and 0.272 mg of OQ17 enzyme II_{gly}. Amounts of PTP_{fru} (8.1 mg/ml) isolated from hydroxylapatite column chromatography were added as noted below. HPr isolated from D-fructose-grown cells contained 4.7 mg/ml.

Legend:

	PTP_{fru} (μl)
○	0
△	5.0
□	15
●	50

Figure 19


D-FRUCTOSE 1-PHOSPHATE (nmoles min^{-1} mg^{-1} enzyme II$_{gly}$)
10.0
8.57
7.14
5.71
4.27
2.86
1.43
0
A.
20
40
60
80
HPr (µl)
D-FRUCTOSE 1-PHOSPHATE
(min mg enzyme II$_{gly}$ nmoles^{-1})
0.340
0.272
0.204
0.136
0.068
0
B.
0.025
0.050
0.075
0.100
HPr (µl^{-1})

of PTP_{fru} decreased both the maximal velocity by 23 percent and the apparent K_m for HPr_{fru} from 0.113 mg to 0.095 mg (Figure 19B). The observed inhibition was most likely due to interaction of PTP_{fru} with the enzyme I; however, this cannot be determined without using separate uncoupled assays for enzyme I and enzyme II.

The data in this experiment show that PTP_{fru} inhibits the constitutive activity by 23 percent and decreases the apparent K_m for HPr_{fru} from 0.113 to 0.095 mg. The data also establish the definite requirement of HPr for activity of the constitutive system.

Requirement for HPr of the Constitutive and Inducible Systems

HPr was required for activity of the QQ17 enzyme II_{gly} constitutive system (Figure 19) and in the QQ17 enzyme II_{fru} activity termed "constitutive" in Figures 14-17. The inducible system functions in the absence of added HPr; however, its activity is increased 20 percent by addition of HPr (Figure 16).

To further determine the actual requirement for HPr of the constitutive and inducible activities, two enzymes II_{fru} were used to measure the apparent K_m's for HPr_{fru} of these activities, and enzyme II_{mtl} was used to measure the apparent K_m for HPr (obtained from both D-mannitol- and D-fructose-grown cells) of D-mannitol phosphorylation using the HPr (D-mannitol continuous) assay. (Both HPr's were purified

approximately 100-fold by the procedure described in Methods.)

Enzyme II_{mtl} had an apparent K_m for HPr_{mtl} of 26.3 µl (27 µg) and an apparent K_m for HPr_{fru} of 17 µl (80 µg) at two enzyme II concentrations (Figure 20). QQ17 enzyme II_{fru} assayed at 100 mM D-fructose had an apparent K_m for HPr_{fru} of 25 µl (13 µg); when assayed in the presence of PTP_{fru} at 0.5 mM D-fructose (Figure 21), addition of HPr had no affect.

Using another QQ17 enzyme II_{fru} preparation, there was an increase in activity at 0.5 mM D-fructose with the addition of HPr_{fru} (Figure 22A); however, the apparent K_A for HPr_{fru} of 0.4 µl (1.9 µg) (determined by subtracting activity in the absence of HPr prior to plotting the data, Figure 22B), is 1/50 of the K_m's for HPr_{fru} of the other enzymes II. Also, the level of activation is dependent on the level of PTP_{fru} present. HPr causes a 100 percent activation with limiting (0.25 "half-maximal saturation" units) PTP_{fru} and only a 20 percent activation at a four-fold higher level of PTP_{fru}. HPr actually decreases the apparent V_{max} at saturating PTP_{fru} (Figure 23). The different levels of PTP_{fru} had no effect on the K_A for HPr (Figure 22B); however, increasing HPr decreased the apparent K_m for PTP_{fru} (Figure 23).

These data show that a constitutive enzyme II_{gly} (Figure 19B), a constitutive enzyme II_{fru} (Figure 21B),

Figure 20. Saturation and Lineweaver-Burk plots showing dependence of D-mannitol phosphorylation on HPr concentration. Assays were run using the HPr (D-mannitol continuous) assay containing 0.073 mg of enzyme I_{fru} purified by DEAE cellulose and Sephadex G200 column chromatography and the indicated amounts of enzyme II_{mtl} and HPr (isolated from D-fructose- and D-mannitol-grown cells containing 4.7 and 1.04 mg/ml protein, respectively).

Figure 20

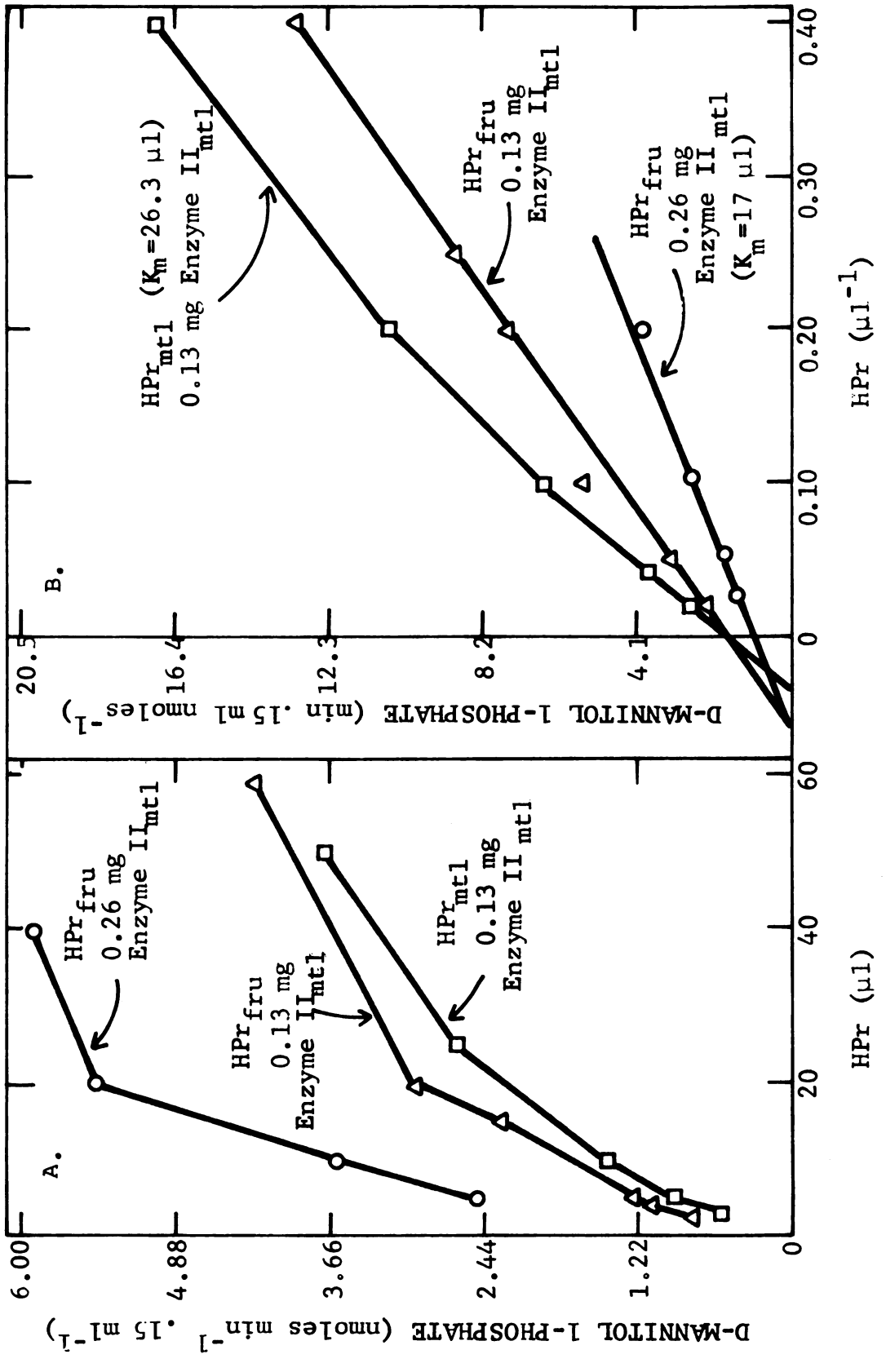

D-MANNITOL 1-PHOSPHATE (nmoles min^{-1} .15 ml^{-1})
6.00
4.88
3.66
2.44
1.22
0
A.
HPr$_{fru}$ 0.26 mg Enzyme II$_{mtl}$
HPr$_{fru}$ 0.13 mg Enzyme II$_{mtl}$
HPr$_{mtl}$ 0.13 mg Enzyme II mtl
20
40
60
HPr (µl)
D-MANNITOL 1-PHOSPHATE (min .15 ml nmoles^{-1})
20.5
16.4
12.3
8.2
4.1
B.
HPr$_{mtl}$ (K_m=26.3 µl) 0.13 mg Enzyme II$_{mtl}$
HPr$_{fru}$ 0.13 mg Enzyme II$_{mtl}$
HPr$_{fru}$ 0.26 mg Enzyme II mtl (K_m=17 µl)
0
0.10
0.20
0.30
0.40
HPr (µl^{-1})

Figure 21. Saturation and Lineweaver-Burk plots showing effect of HPr_{fru} on constitutive and inducible system activities. Assays contained the same components as Figure 19 except that 0.242 mg of QQ17 enzyme II_{fru} was used. The inducible system assays contained 0.405 mg of PTP_{fru} and 0.5 mM D-fructose.

Figure 21

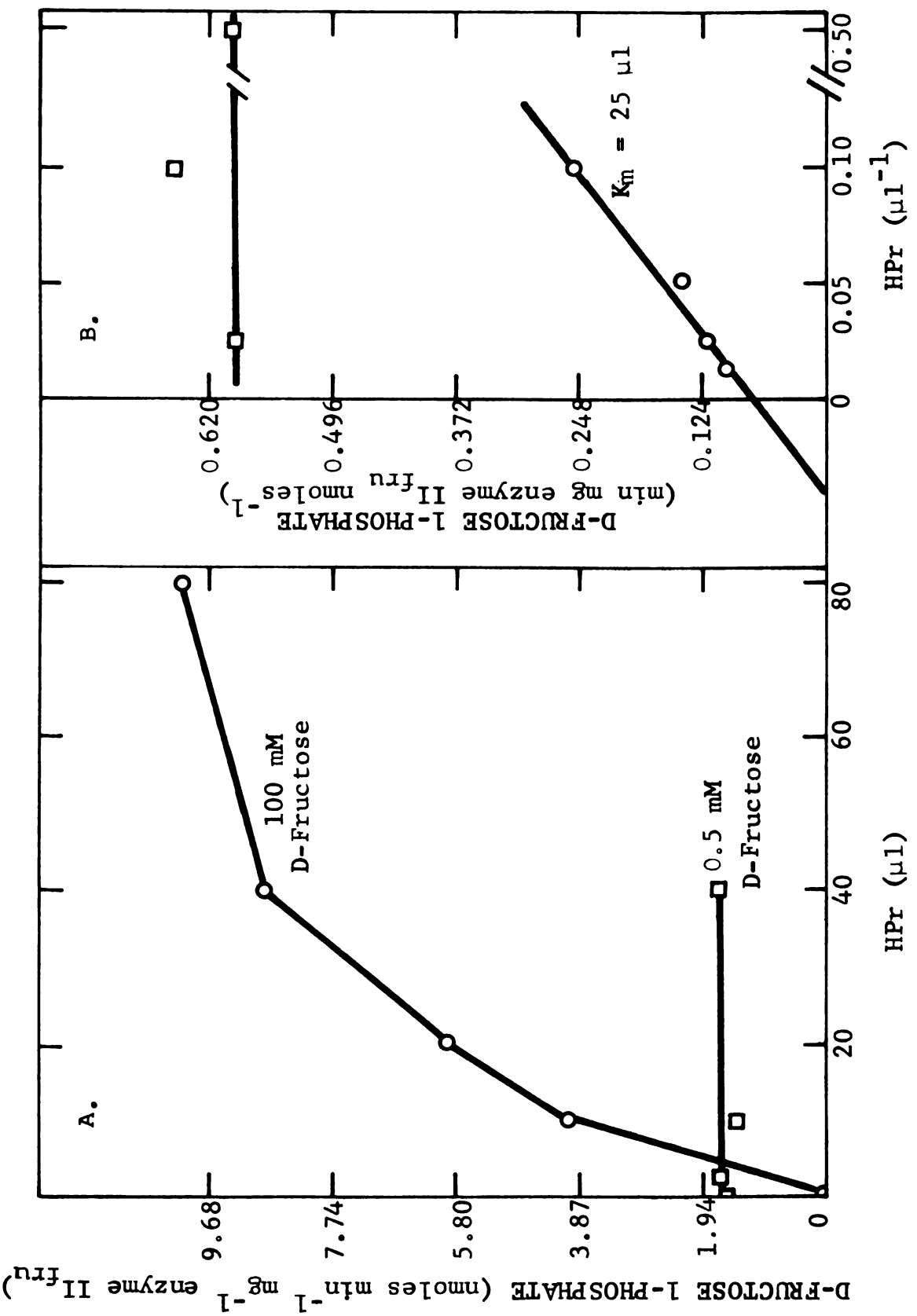
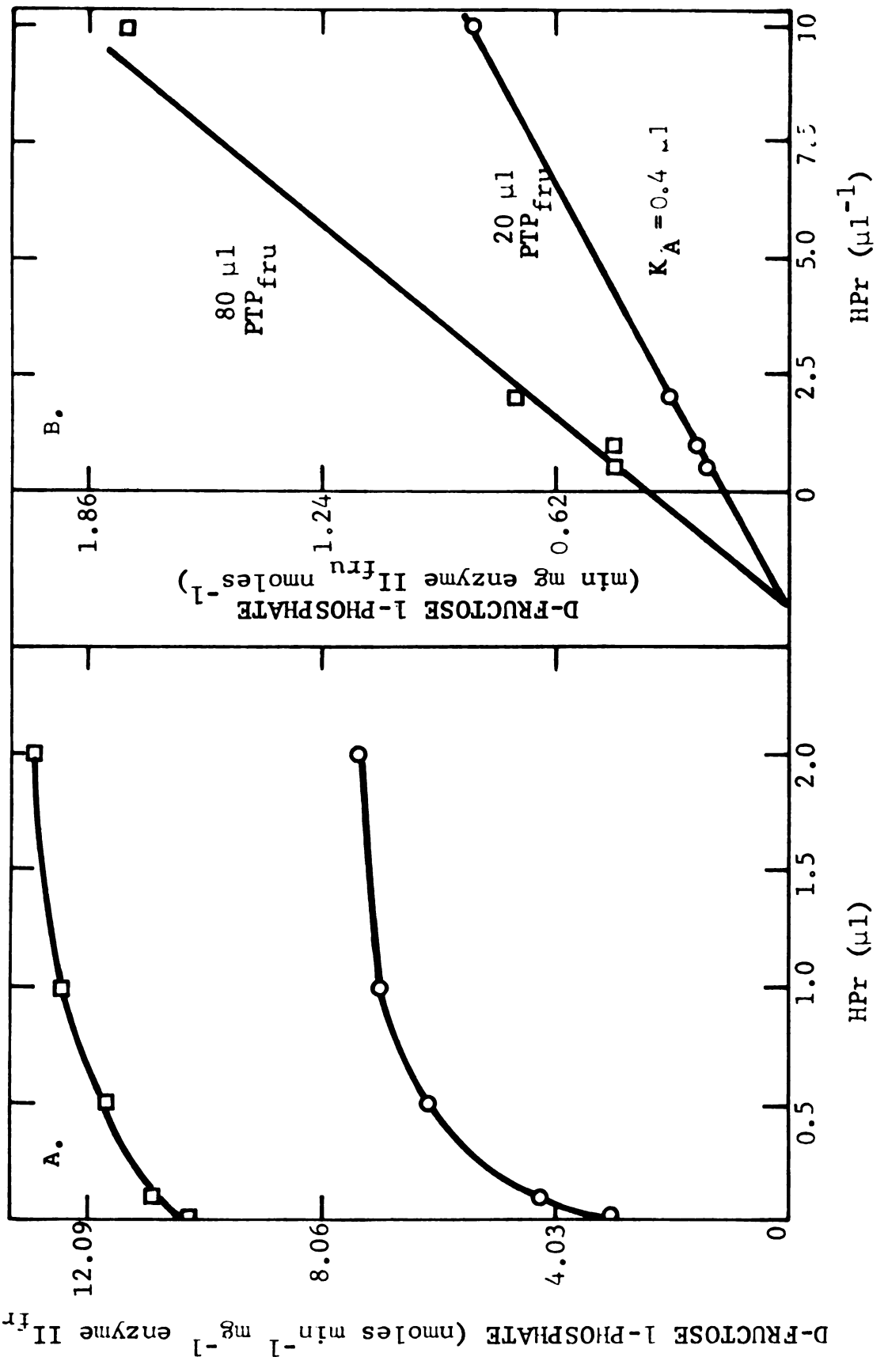

D-FRUCTOSE 1-PHOSPHATE (nmoles min^{-1} mg^{-1} enzyme II$_{fru}$)
9.68
7.74
5.80
3.87
1.94
0
A.
100 mM D-Fructose
0.5 mM D-Fructose
0
20
40
60
80
HPr (µl)
D-FRUCTOSE 1-PHOSPHATE (min mg enzyme II$_{fru}$ nmoles^{-1})
0.620
0.496
0.372
0.248
0.124
B.
K_m = 25 µl
0
0.05
0.10
0.50
HPr (µl^{-1})

Figure 22. Saturation and Lineweaver-Burk plots of dependence of inducible system on HPr. Assays contained the same components as Figure 18 except the amounts of HPr_{fru} (4.7 mg/ml) were varied and two levels of PTP_{fru} from a DEAE cellulose (II) fraction (0.019 mg and 0.076 mg) were used. Velocities for reciprocal curve had background activity subtracted.

Figure 22


D-FRUCTOSE 1-PHOSPHATE (nmoles min^{-1} mg^{-1} enzyme II$_{fru}$)
12.09
8.06
4.03
0
A.
0.5
1.0
1.5
2.0
HPr (µl)
D-FRUCTOSE 1-PHOSPHATE
(min mg enzyme II$_{fru}$ nmoles^{-1})
1.86
1.24
0.62
0
B.
80 µl
PTP$_{fru}$
20 µl
PTP$_{fru}$
K_A = 0.4 µl
2.5
5.0
7.5
10
HPr (µl^{-1})

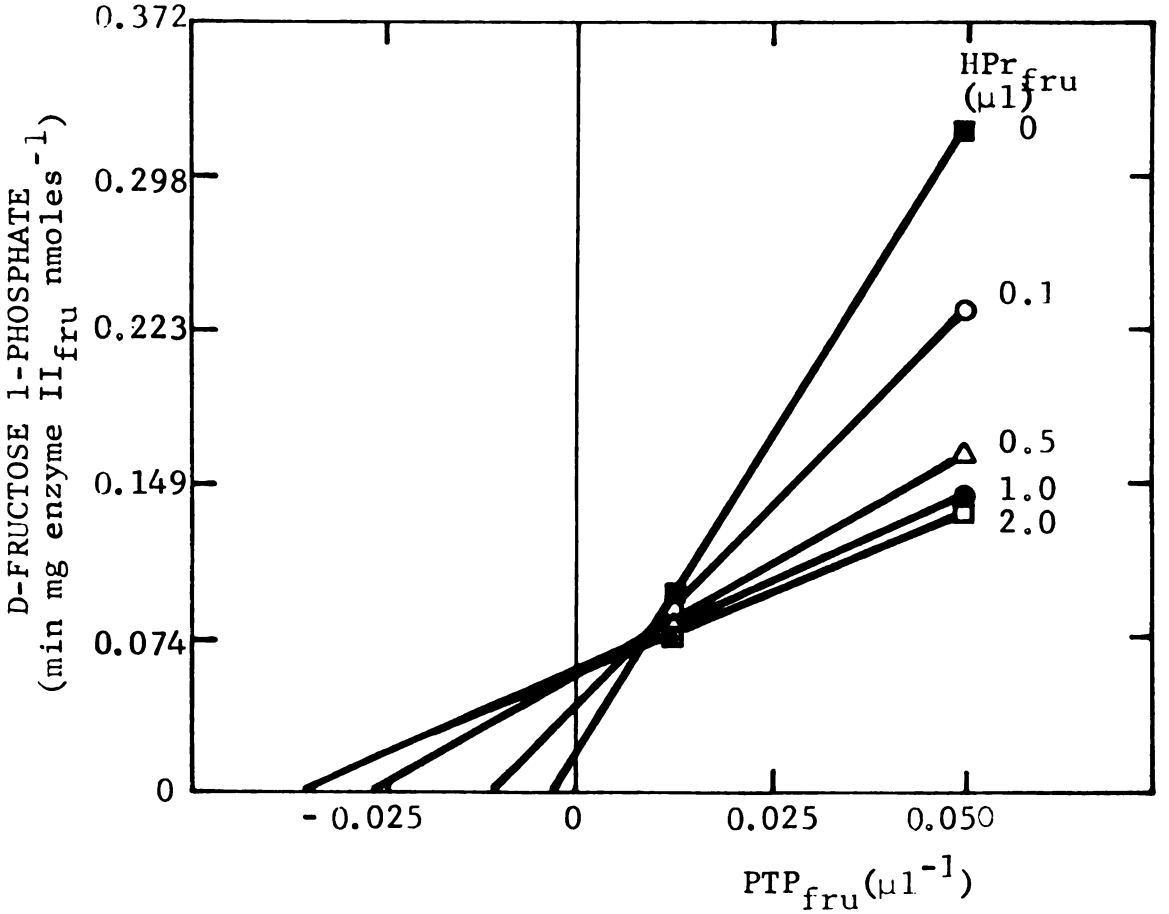


Figure 23. Lineweaver-Burk plot showing effect of HPr_{fru} on the apparent K_m for PTP_{fru}.

and an enzyme II_{mtl} used to catalyze D-mannitol phosphorylation (Figure 20B), all had similar affinities for HPr obtained from D-fructose-grown cells. Although the inducible system does not require HPr for activity, an enzyme II_{fru} assayed at 0.5 mM D-fructose had an affinity for this HPr 50 times greater than the other enzymes II. Because of this high affinity it is possible that very low levels of HPr which may be trapped in the enzyme II_{fru} vesicles could be enough to support activity of the inducible system.

Effect of 2-Mercaptoethanol on Enzyme II_{fru} Activity

The inducible and constitutive system as described thus far have different affinities for D-fructose and different requirements for HPr and PTP_{fru} for activity. Further evidence for two separate enzymes II for D-fructose phosphorylation was observed when a sample of QQ17 enzyme II_{fru} (100,000 x g precipitate of a Sephadex G200 pool), which had lost appreciable inducible activity, was incubated for 48 hours in fresh 28 mM 2-mercaptoethanol at 4°C. After this incubation, the inducible activity was four times greater than the activity of a control that was stored at 4°C without added 2-mercaptoethanol (Table VII). The constitutive activity was not affected by the 2-mercaptoethanol.

Table VII. Effect of 48-hour incubation of enzyme II_{fru} in fresh 2-mercaptoethanol. Assays are as described in Methods for the enzyme II reconstituted system and contained 20 μmoles of D-fructose, 0.26 mg of enzyme I, 0.11 mg of QQ17 enzyme II_{fru}, and the noted amounts of HPr and PTP_{fru}.

Enzyme II_{fru} Assayed	HPr (μg)	PTP_{fru} (μg)	Specific Activity[a]		
			Control[b]	Experimental[c]	Activity Increase (%)
Constitutive + Inducible	110	32	16.9	28.2	67
Constitutive	110	0	14.5	15.9	10
Inducible	0	32	2.1	10.3	400

[a]In terms of nmoles D-fructose 1-phosphate min^{-1} mg^{-1} enzyme II_{fru} at 100 mM D-fructose.

[b]Indicates no 48-hour incubation with 2-mercaptoethanol.

[c]Indicates enzyme II_{fru} was incubated for 48 hours with 2-mercaptoethanol.

Thus, the inducible enzyme II_{fru} is selectively reactivated by the addition of 2-mercaptoethanol.

Formation of Phosphorylated PTP_{fru}

To further determine the requirement for HPr of the inducible system and the actual role of PTP_{fru} as a substrate for the inducible enzyme II_{fru}, a series of experiments utilizing [^{32}P] PEP was designed.

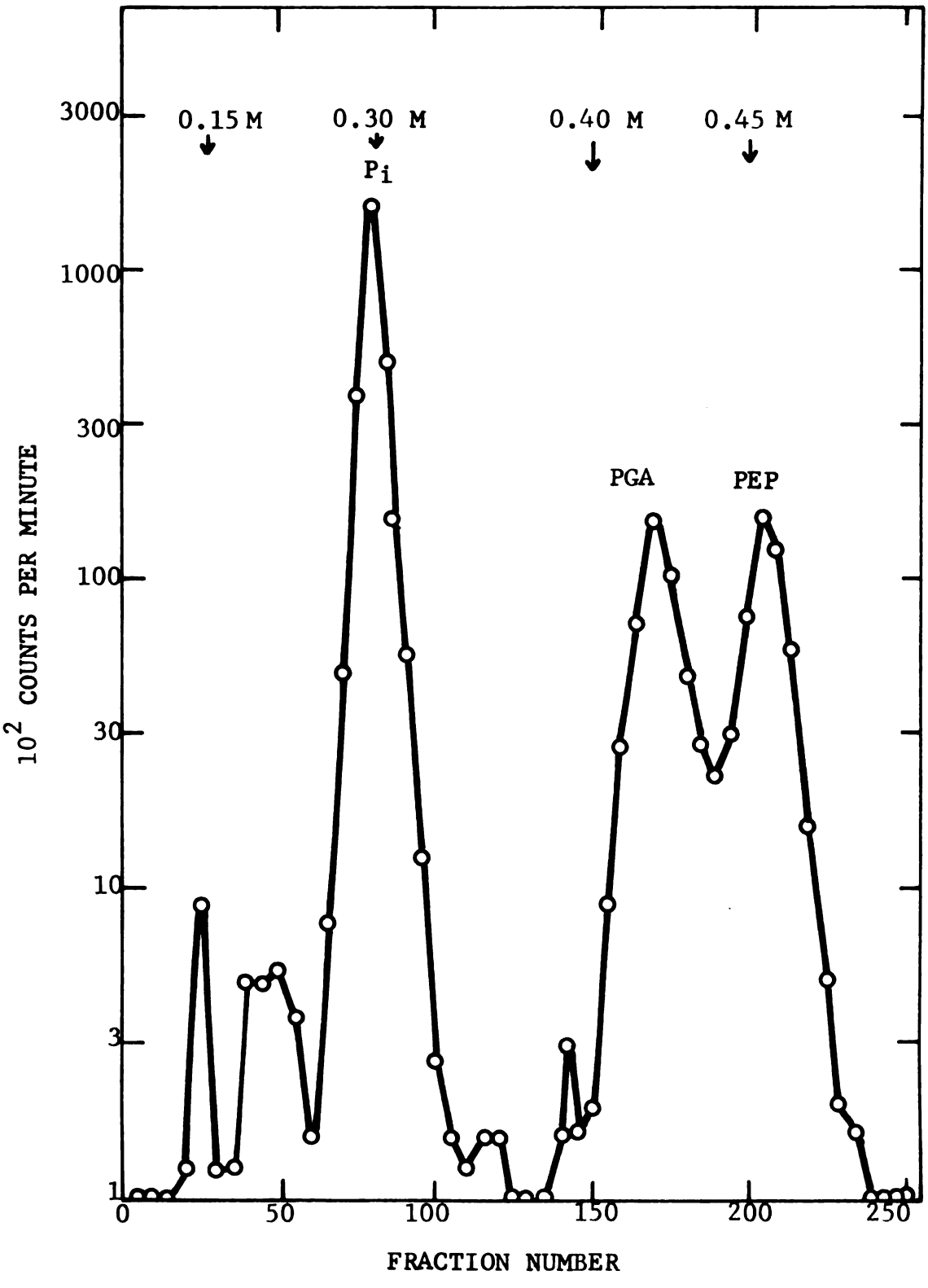
a. Preparation and Identification of [^{32}P] PEP

The products of a reaction containing $^{32}P_i$, L-malate, and mitochondria isolated from chicken livers, were separated by Dowex 1 chromatography (Figure 24) as described in Methods. The radioactivity that eluted with 0.45 M triethylammonium bicarbonate buffer was identified as [^{32}P] PEP by paper chromatography and its ability to form [^{32}P] ATP and D-fructose 1-[^{32}P] phosphate.

Formation of [^{32}P] ATP——Samples of radioactive fractions (170, 190, 206, and 215) from the Dowex 1 column were tested for their ability to form [^{32}P] ATP in the presence of pyruvate kinase and ADP in reactions as described in Methods. Acid-washed charcoal (Darco G-60) was added to the samples, filtered from the solution, and radioactivity bound to the charcoal was then measured by Čerenkov radiation. Only the charcoal from reactions containing samples of fractions 206 and 215 had bound

Figure 24. Elution of products of [^{32}P] PEP-formation reaction from Dowex 1-X10 column chromatography. Different concentrations of triethylammonium bicarbonate buffer were added as indicated. Further details are given in Methods.

Figure 24


0.15 M
0.30 M
Pi
0.40 M
0.45 M
PGA
PEP
3000
1000
300
100
30
10
3
1
0
50
100
150
200
250
10^2 COUNTS PER MINUTE
FRACTION NUMBER

radioactivity that was dependent on ADP (Table VIII). These data show that fraction 206 contained a compound that is utilized by pyruvate kinase to make [^{32}P] ATP.

Paper Chromatographic Identification of [^{32}P] PEP——Samples of fractions 80, 170, and a pool of fractions 200 through 221 from the Dowex 1 column were spotted on Whatman No. 1 filter paper and chromatographed with the alkaline solvent system. The chromatogram was dried and cut into strips which were then scanned for radioactivity (Figure 25). The sample from the pool of fractions 200 through 221 contained a radioactive compound that migrated with standard PEP; fraction 170 contained a major peak of radioactivity that corresponded to phosphoglyceric acid, and a minor peak that migrated with standard P_i. The radioactivity scan of fraction 80 had a single peak which migrated with P_i. The sections of the chromatogram of the pool (fractions 200 through 221) that corresponded to standard P_i and PEP were cut out and counted in glass scintillation vials using Čerenkov radiation. The section corresponding to PEP contained 95 percent of the total radioactivity in the two sections. These data show that 95 percent of the material in the pool of fractions 200 through 221 from a Dowex 1 column is similar in its migration in the alkaline solvent to standard PEP.

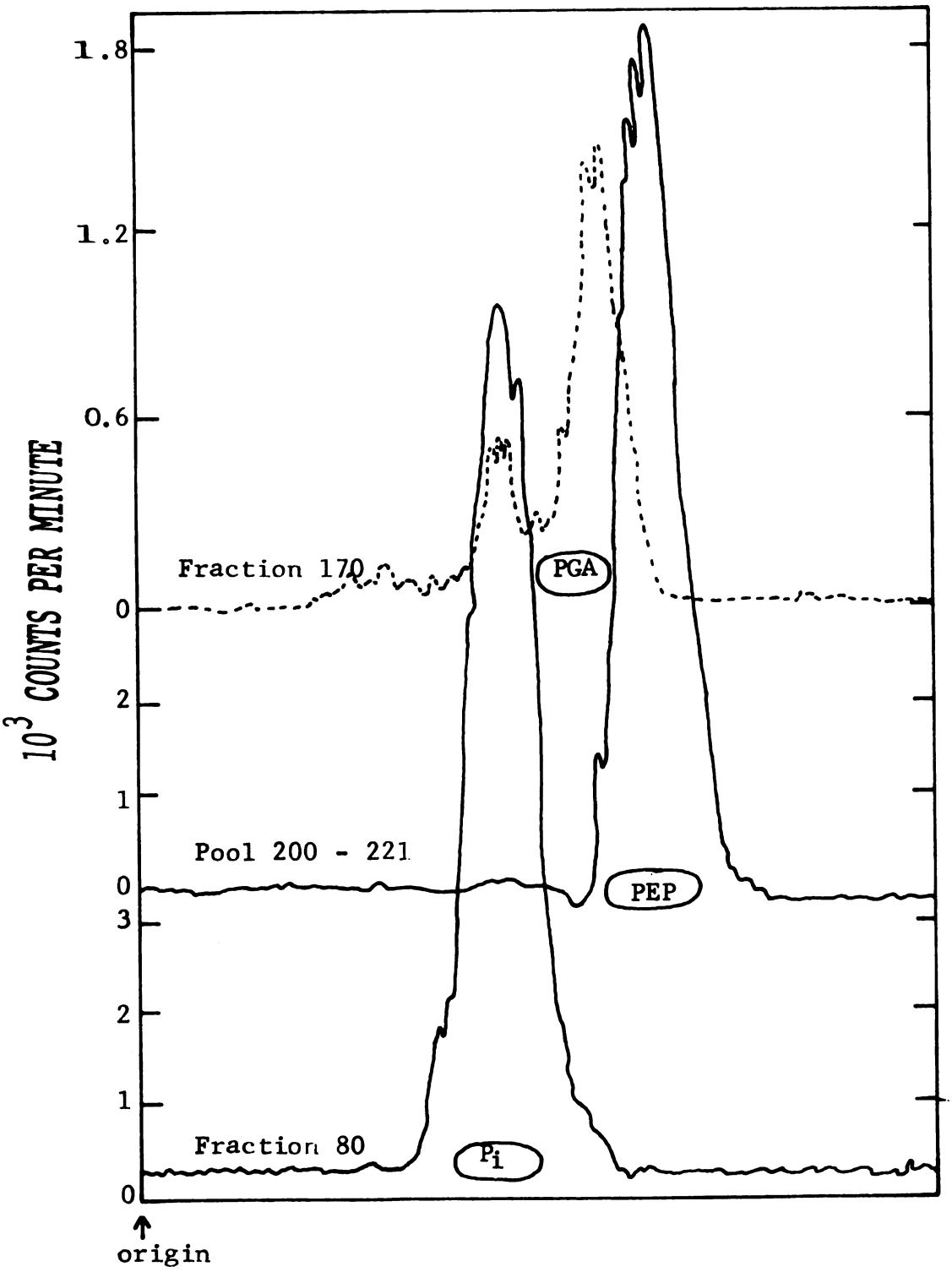
Table VIII. Radioactivity bound to charcoal dependent on ADP and fractions from Dowex 1-X10 column chromatography containing [^{32}P] PEP. Procedure is described in Methods.

Fraction Number	Radioactivity Bound to Charcoal[a]	
	+ADP	- ADP
170	4,252	5,606
190	3,401	3,619
206	10,784	4,506
215	5,748	3,871

[a]Counts per minute in total sample of charcoal.

Figure 25. Scan of radioactivity on paper chromatogram of fractions 80, 170, and pool of 200 through 221 from Dowex 1-X10 column chromatography. Standards were detected with acid molybdate spray. [Note: Scale for fraction 170 is different from scales for pooled fractions (200 through 221) and fraction 80. Extension of each peak remains on its own scale.]

Figure 25


1.8
1.2
0.6
0
2
1
0
3
2
1
0
10^3 COUNTS PER MINUTE
Fraction 170
PGA
Pool 200 - 221
PEP
Fraction 80
P_i
origin

Formation of D-Fructose 1-[^{32}P] Phosphate—To further determine that the pool of fractions 200 through 221 contained [^{32}P] PEP, samples of it were added to D-fructose 1-phosphate-forming reactions. These reactions were run at 100 mM D-fructose and are described in Table IX. After the heat-denatured protein was removed by centrifugation at 6,000 x g, 50 µl amounts from each supernatant were assayed for D-fructose 1-phosphate using the D-fructose 1-phosphate end-point assay. The relative level of radioactivity in each reaction was determined by counting 10 µl amounts of this supernatant in a liquid scintillation counter. Another 10 µl amount was chromatographed on paper using the acid solvent. Authentic D-fructose 1-phosphate and phosphoenolpyruvate were detected with acid molybdate spray and the radioactive spots were detected with a strip scanner (Figure 26). These spots were then cut out and Čerenkov radiation was measured in a liquid scintillation counter.

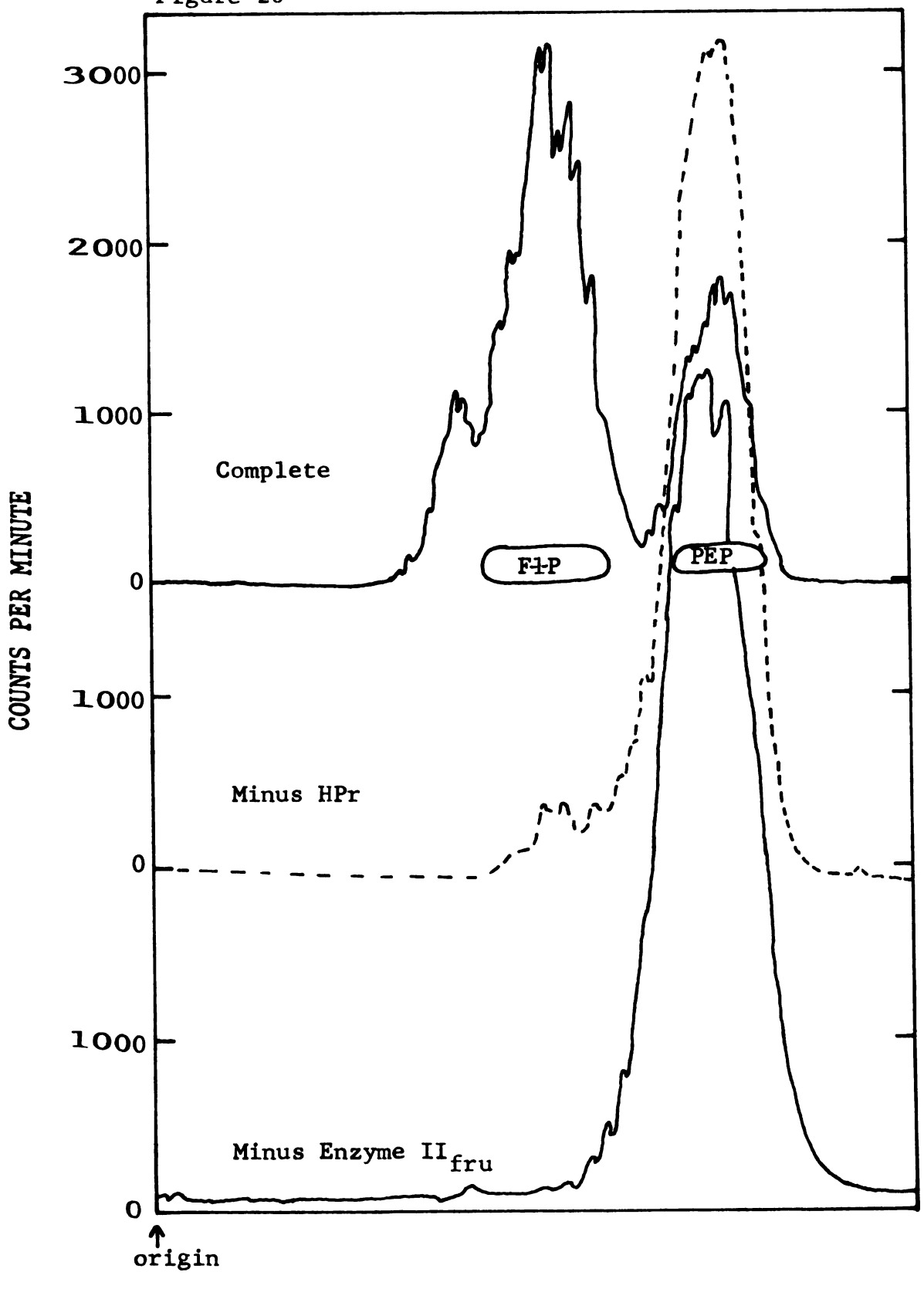
Reactions that contained enzyme I, enzyme II, and HPr formed D-fructose 1-phosphate as well as a radioactive material that co-chromatographed with standard D-fructose 1-phosphate on the paper chromatogram (Figure 26), whereas reactions lacking HPr or enzyme II did not form D-fructose 1-phosphate and only had a radioactive peak that corresponded to PEP. Addition of PTP_{fru} doubled the level of D-fructose 1-phosphate formed; however, the level of radioactivity in the spot

Table IX. *Formation of D-fructose 1-[^{32}P] phosphate from [^{32}P] PEP pool. Reactions utilizing the constitutive enzyme II_{fru} assay described in Figure 21 also included 235 µg of HPr, 0.2 µmole of unlabeled PEP and 10 µl of the pool* of fractions 200 through 221 from the Dowex 1 column. Where noted, 162 µg of PTP_{fru} was added. Reactions were stopped after 15 or 30 minutes by placing in a 95°C bath for 7 minutes. D-fructose 1-phosphate was measured using the D-fructose 1-phosphate end-point assay. 10 µl aliquots were assayed for total radioactivity and chromatographed to separate D-fructose 1-phosphate and PEP. The areas corresponding to D-fructose 1-phosphate and PEP were cut out and Cerenkov radiation was measured.

Reaction	PTP_{fru}	Time (Min)	µmoles D-Fructose 1-Phosphate	Counts Per Minute		
			Reaction	10 µl Supernatant	D-Fructose 1-Phosphate	PEP
Complete	-	30	0.0396	9,160	4,320	2,380
"	-	15	0.0365	9,720	4,850	1,940
"	+	30	0.0691	8,160	4,910	1,900
" Minus HPr	-	30	0.0003	9,130	430	8,100
" Minus Enzyme II	-	30	0.0009	8,310	210	7,920

Figure 26. Radioactivity scan of paper chromatogram showing formation of D-fructose 1- [^{32}P] phosphate dependent on enzyme II and HPr. Standards were detected with acid molybdate spray.

Figure 26


3000
2000
1000
0
1000
0
1000
0
COUNTS PER MINUTE
Complete
F1P
PEP
Minus HPr
Minus Enzyme II fru
origin

corresponding to D-fructose 1-phosphate did not increase. This could be due to the fact that the radioactive [^{32}P] PEP was added to the reactions before they were started by the addition of 0.2 μmole of unlabeled PEP. This time lag could have allowed a majority of the radioactivity to be utilized prior to addition of the unlabeled PEP. In the complete reaction, over 60 percent of the ^{32}P was transferred to D-fructose 1- [^{32}P] phosphate, whereas the total amount of D-fructose 1-phosphate formed was only 20 to 35 percent of the amount of unlabeled PEP added.

The data in the previous three experiments show that a compound that eluted with 0.45 M triethylammonium bicarbonate buffer (pH 7.5) (i) formed a radioactive compound that binds to charcoal [charcoal binds ATP (15)] when incubated with ADP and pyruvate kinase (Table VIII), (ii) migrated with standard PEP on both an alkaline chromatogram (Figure 25) and an acid chromatogram (Figure 26), and (iii) was utilized as a phosphoryl donor in enzyme I- and HPr-dependent phosphorylation of D-fructose (Figure 26, Table IX). This evidence confirms that 95 percent of the radioactive compounds in the pool of fractions 200 through 221 was [^{32}P] PEP. This [^{32}P] PEP pool was concentrated and was stored at -10°C in the carrier free form. Dilutions with unlabeled PEP were made immediately prior to the individual experiments in which the [^{32}P] PEP was used and specific activities

were noted as cpm (measured by Čerenkov radiation) per nmole PEP. The amount of PEP in the diluted sample was measured using the PEP end-point assay and was determined to be equal to the amount of unlabeled PEP added. It was concluded that if the [^{32}P] PEP contained any carrier PEP (due to contamination of the mitochondria by P_i) it was below the level of detection (4 percent of the amount of carrier PEP added) and considered insignificant.

b. Requirements for Phosphoryl Transfer From [^{32}P] PEP to PTP_{fru}

A sample of PTP_{fru} eluted from a native polyacrylamide disc gel similar to that shown in Figure 7C was used to prepare [^{32}P] phospho-PTP_{fru} in the reactions described in Table X. This homogeneous PTP_{fru} preparation was estimated by 210 nm absorbance to contain approximately 0.075 mg protein per ml. This value was obtained by the difference in absorbance of the sample containing the protein and a solution which it had been dialyzed and concentrated against. The background level absorbance was equivalent to a protein concentration of 0.29 mg per ml, and thus the 0.075 mg per ml could be in error.

The reactions described in Table X were incubated for 30 minutes at 30°C, cooled on ice, and chromatographed over a 0.75 x 56-cm Sephadex G25 (course) column to separate the [^{32}P] phospho-protein from the unbound [^{32}P]

Table X. Formation of [^{32}P] phospho-PTP_{fru} from [^{32}P] PEP, enzyme I, and PTP_{fru}. The reactions were run for 30 minutes at 30°C and consisted of 20 μmoles of Tris-HCl (pH 7.5), 0.1 μmole of $MgCl_2$, 0.003 μmole of [^{32}P] PEP (119,883 cpm), 86 μg of enzyme I, 1.03 μg of HPr, and 3.75 μg of PTP_{fru} in a total volume of 76 μl.

Reaction Components	Bound Radioactivity		^{32}P (nmoles)	moles ^{32}P / moles PTP_{fru}
	Total[a] (cpm)	PTP_{fru}[b] (cpm)		
Complete	5,710	2,420	0.061	0.85
" Minus HPr	5,580	2,290	0.057	0.79
" Minus PTP_{fru}	3,290	0	0	0
" Minus enzyme I	450	--[c]	--[c]	--[c]
" 1/10 PEP	2,970	--[c]	--[c]	--[c]

[a]Counts per minute in fractions 11 through 15.

[b]Total counts per minute of reaction minus total counts per minute of "Complete minus PTP_{fru}" reaction.

[c]-- Signifies not determined.

PEP. The elution profile of the complete reaction (Figure 27) was similar to others, all of which had some radioactivity in the void volume. The pool of fractions 11 through 15 was defined as the total radioactivity bound to protein (Table X). In the assay without PTP_{fru}, radioactivity was bound to enzyme I and was subtracted from the values of the complete reaction and the reaction in the absence of HPr to determine the amount of bound radioactivity which is dependent on PTP_{fru}. Omitting HPr from the reaction had little effect on the radioactivity bound to PTP_{fru}. Thus, HPr is apparently not required for phosphorylation of PTP_{fru}, whereas enzyme I is definitely required.

The amount of radioactivity bound to enzyme I was determined by incubating two concentrations of enzyme I with the [^{32}P] PEP pool in the presence and absence of HPr (Table XI). Doubling of enzyme I doubled the level of [^{32}P] phospho-protein that eluted in the void volume from the Sephadex G25 column. Addition of HPr had little effect on the level bound to protein, while increasing the incubation time slightly augmented the amount of bound radioactivity.

This series of experiments shows that both the enzyme I and homogeneous PTP_{fru} preparations are phosphorylated by [^{32}P] PEP in the absence of HPr. Enzyme I is required for the phosphoryl transfer from PEP to PTP_{fru} forming a phospho-PTP_{fru} with approximately 0.82 moles

Figure 27. Standard Sephadex G25 (0.75 x 56-cm) separation of bound radioactivity from unbound radioactivity. 20-drop fractions were collected. This particular elution is of the "Complete" reaction described in Table X.

Figure 27

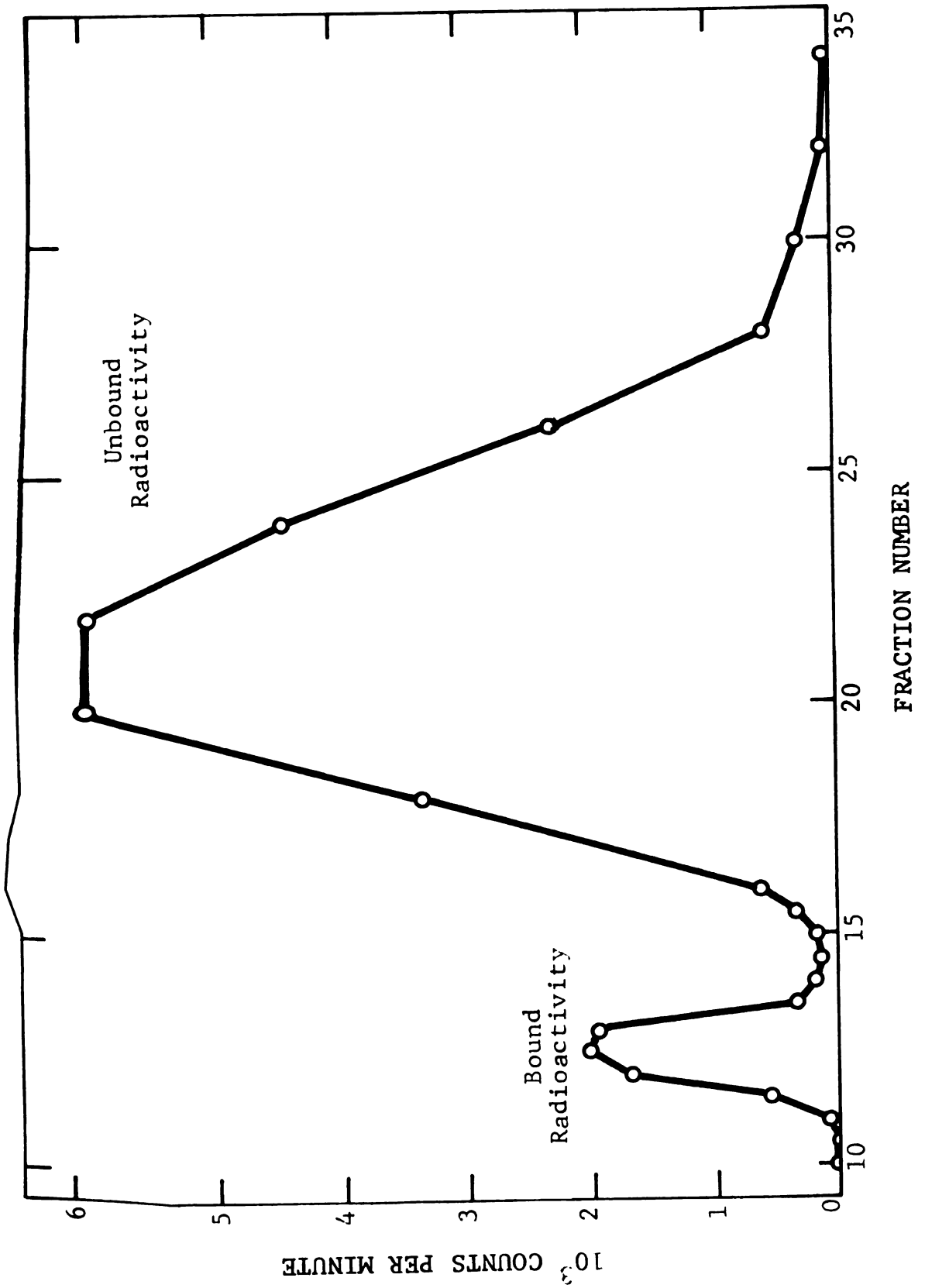

Unbound
Radioactivity
Bound
Radioactivity
FRACTION NUMBER
10³ COUNTS PER MINUTE
0
1
2
3
4
5
6
10
15
20
25
30
35

Table XI. Formation of [^{32}P] phospho-protein from enzyme I and [^{32}P] PEP. Reactions contained the same components as Table X except that PTP_{fru} was omitted.

Reaction Component		Time (Min)	Bound Radioactivity[a]
Enzyme I (μg)	HPr (μg)		
43	0	17	1,450
86	0	17	3,360
86	2.06	17	3,420
86	2.06	35	3,810

[a]Counts per minute in fractions 11 through 15 from Sephadex G25 column.

^{32}P bound per mole of protein of 52,000 molecular weight. This value could be in error due to estimation of protein concentration.

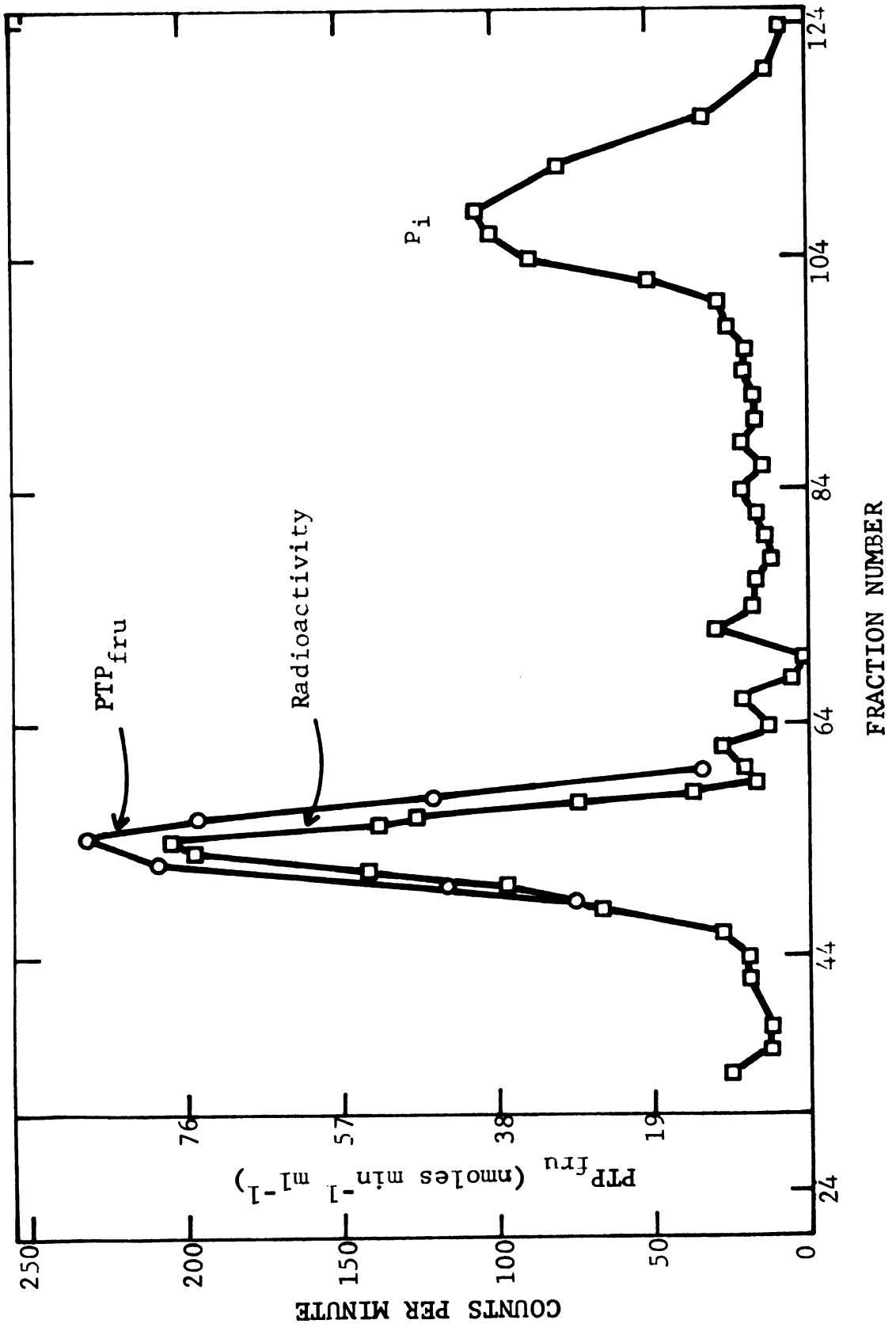
The protein-bound radioactivity from the complete reaction and the reaction minus HPr was pooled (Table X), concentrated by ultrafiltration, and chromatographed on Sephadex G100. The radioactivity in the fractions was measured and two peaks of radioactivity were detected (Figure 28). One peak superimposed with the PTP_{fru} activity assayed on a previous elution of this column. (The amount of PTP_{fru} in the radioactive fractions was too low to detect activity by kinetic assays). The second peak corresponded to the low-molecular-weight region, and is presumed to be $^{32}P_i$ which hydrolyzed from the PTP_{fru}.

Thus, in the 24-hour interim between ultrafiltration of the radioactive sample and elution of the column, approximately 50 percent of the phospho-PTP_{fru} was hydrolyzed. The phospho-histidine isolated by Anderson and co-workers (2) from HPr was found to hydrolyze to P_i in one-week, which would mean 50 percent hydrolysis in a 24-hour period (assuming logarithmic decay).

This Sephadex G100 column further documents that ^{32}P bound to protein was actually bound to PTP_{fru} and not to a protein in the enzyme I preparation which would have eluted close to fraction 40. It appears as though the ^{32}P bound to the enzyme I protein preparation is

Figure 28. Elution of [^{32}P] phospho-PTP_{fru} pool and activity of another sample of PTP_{fru} chromatographed separately on the same Sephadex G100 column. Details are described in the text. PTP_{fru} activity is in terms of nmoles D-fructose 1-phosphate formed per minute per ml fraction added.

Figure 28


COUNTS PER MINUTE
250
200
150
100
50
0
PTP$_{fru}$ (nmoles min^{-1} ml^{-1})
76
57
38
19
Radioactivity
PTP$_{fru}$
P_i
24
44
64
84
104
124
FRACTION NUMBER

more unstable than the [^{32}P] phospho-PTP_{fru} since enzyme I originally had approximately the same amount of ^{32}P bound as did PTP_{fru}, and there is no peak where the enzyme I would have eluted. The fact that the hydrolysis rate is approximately the same as the phosphohistidine reported by Anderson (2) implies that this D-fructose phosphoryl transfer protein could involve a histidine residue in its active site.

c. Determination of Moles ^{32}P Bound Per Mole PTP_{fru}

A second set of experiments was run to form a larger quantity of phospho-protein by using [^{32}P] PEP with a higher specific activity and a sample of protein eluted from a hydroxylapatite column. This PTP_{fru} fraction was judged to be 24 percent pure by running a native polyacrylamide gel, staining with Coomassie blue, and scanning the destained gel at 550 nm. The area under the peak of PTP_{fru} was 24 percent of the total area representing the sum of the total protein peak. An SDS gel was run on the same sample of protein and the area under a peak that corresponded to a molecular weight of 26,000 was 23 percent of the area representing the total protein. The relative staining of proteins by Coomassie blue is accurate at the low concentrations (5 to 10 μg total protein) of protein used in both of these experiments (29a). The protein concentration of

this PTP_{fru} sample was estimated to be 0.93 mg per ml by both 210 nm absorption and the Lowry method.

Each assay contained 18.6 μg of protein from the hydroxylapatite pool, which was equivalent to 4.46 μg or 0.085 nmole of PTP_{fru}, assuming a molecular weight of 52,000 daltons. The other components were as described in Table XII except for [^{32}P] PEP as noted. The assays were run for the times listed, then immediately chromatographed on a standard Sephadex G25 column (same column as Figure 27), and 20-drop fractions were eluted with 0.02 M potassium phosphate buffer (pH 8.0) containing 0.001 M DTT and 10 percent glycerol. The pool of fractions 11 through 15 was defined as the bound radioactivity fraction. Fractions 16 through 37 were pooled and contained the remainder of the radioactivity that was not bound to protein.

The data again indicate that formation of a phosphorylated PTP_{fru} is dependent on the presence of enzyme I. Adding HPr actually decreased the amount of radioactivity bound by 5 percent, and thus was not required for PTP_{fru} phosphorylation. Radioactivity was again bound to enzyme I in the absence of PTP_{fru}. The complete reaction run for 24 minutes shows that the formation of [^{32}P] phospho-PTP_{fru} was complete after 15 minutes. The control with 1/6 the level of [^{32}P] PEP shows that PTP_{fru} phosphorylation has a high affinity for [^{32}P] PEP and actually utilized 50 percent of the available [^{32}P]

Table XII. Requirements for formation of [^{32}P] phospho-PTP_{fru}. The reactions contained 2.0 μmoles of Tris-HCl (pH 7.5), 0.1 μmole of $MgCl_2$, 43 μg of enzyme I, 1.03 μg of HPr, 1.95 nmoles of [^{32}P] PEP (120,510 cpm), and 18.6 μg of PTP_{fru} pool from hydroxylapatite column chromatography, in a total volume of 66 μl.

Table XII.

Reaction	Time (Min)	Radioactivity: Total[a] (cpm)	Radioactivity: Bound[b] (cpm)	Radioactivity: PTP_{fru}-Specific[c] (cpm)	^{32}P (nmoles)	moles ^{32}P / moles PTP_{fru}
1. Complete	15	123,000	13,300	9,600	0.156	1.77
2. " Minus PTP_{fru}	15	133,000	3,700	0	0	0
3. " Minus HPr	17	128,000	13,800	10,100	0.164	1.93
4. " Minus Enzyme I	16	117,000	880	--[d]	--[d]	--[d]
5. " Minus 1.625 nmoles [^{32}P] PEP	19	18,800	9,380	5,680	0.092	1.08
6. Complete	34	114,000	13,000	9,300	0.150	1.77

[a]Total counts measured in all the fractions from Sephadex G25 column.

[b]Counts that eluted in the void volume (fractions 11 through 15).

[c]Bound counts found in each reaction minus the counts in the "Complete minus PTP_{fru}" reaction.

[d]--Signifies not determined.

PEP, although the amount of ^{32}P bound did not attain the same level as with more saturating [^{32}P] PEP.

The nmoles of radioactivity bound, dependent on PTP_{fru}, were calculated and the ratio of moles of ^{32}P to moles of PTP_{fru} was determined. This ratio ranged from 1.77 for the complete system to 1.93 for the complete system minus HPr. The reaction with 1/6 the level of [^{32}P] PEP had a ratio of 1.08. Thus, it becomes apparent that under completely saturating conditions and with no hydrolysis of the phospho-protein, the PTP_{fru} binds 2 moles of ^{32}P for each mole of protein with a molecular weight of 52,000 daltons. This is equivalent to 1 mole ^{32}P bound per subunit of molecular weight 26,000.

Reactions 1, 3, 5, and 6 (Table XII) were pooled, concentrated by ultrafiltration, applied to the standard Sephadex G100 column (same column as Figure 28), and 60-drop (2.0-ml) fractions were eluted with 0.02 M potassium phosphate buffer (pH 8.0) containing 0.001 M DTT and 10 percent glycerol. Fractions 46 through 60 were pooled, concentrated, and dialyzed against 0.01 M potassium phosphate (pH 8.0) containing 0.001 M DTT. Radioactivity was again found in the dialysates, ultrafiltrates, and the low-molecular-weight fractions eluted from the Sephadex G100 column, thus showing hydrolysis of ^{32}P from the [^{32}P] phospho-protein.

The concentrated [^{32}P] phospho-PTP_{fru} fraction was then incubated with enzyme II_{fru} and D-fructose, and

in some cases HPr, as described in Table XIII. The reactions were stopped by layering them on a 0.75 x 56-cm Sephadex G25 column (same column as Figure 27) and 20-drop fractions were eluted with water. Fractions 11 through 15 were pooled and contained radioactivity that remained bound to protein. The pool of fractions 16 through 26 would contain any D-fructose 1-phosphate formed as well as hydrolyzed ^{32}P.

Omitting enzyme II, adding HPr, or increasing the D-fructose concentration 10-fold, as well as altering the reaction times, had little effect on the ratio of unbound radioactivity (fractions 16 through 26) with respect to radioactivity bound (fractions 11 through 15) (Table XIII). The variability in the total radioactivity (sum of bound and unbound, Table XIII) in reactions 1 through 7 was due to addition of non-uniform amounts of [^{32}P] phospho-PTP_{fru} to the reactions.

The unbound radioactivity from reactions 1, 4, 5, 7, and 8 in Table XIII was pooled, treated with Dowex 50, lyophilized, dissolved in water, and chromatographed with standard D-fructose 1-phosphate and P_i in the acid solvent system. A scan of the radioactivity indicated that greater than 95 percent of the radioactivity in these pools was in the form of $^{32}P_i$ (Figure 29). The bound radioactivity from the same fractions was pooled and boiled to denature the protein which was then removed by centrifugation at 40,000 x g. This sample was then

Table XIII. Formation of D-fructose 1-[^{32}P] phosphate from [^{32}P] phospho-PTP$_{fru}$. Reactions contained 8.0 μmoles of Tris-HCl (pH 7.5), 0.2 μmole of D-fructose, 1.0 μmole of $MgCl_2$, 0.2 ml of [^{32}P] phospho-PTP$_{fru}$, 20 μl of enzyme II, and 1.0 μl of HPr (where noted). After 15 minutes (with exceptions noted) at 30°C, reactions were cooled on ice and chromatographed on a Sephadex G25 column equilibrated with water.

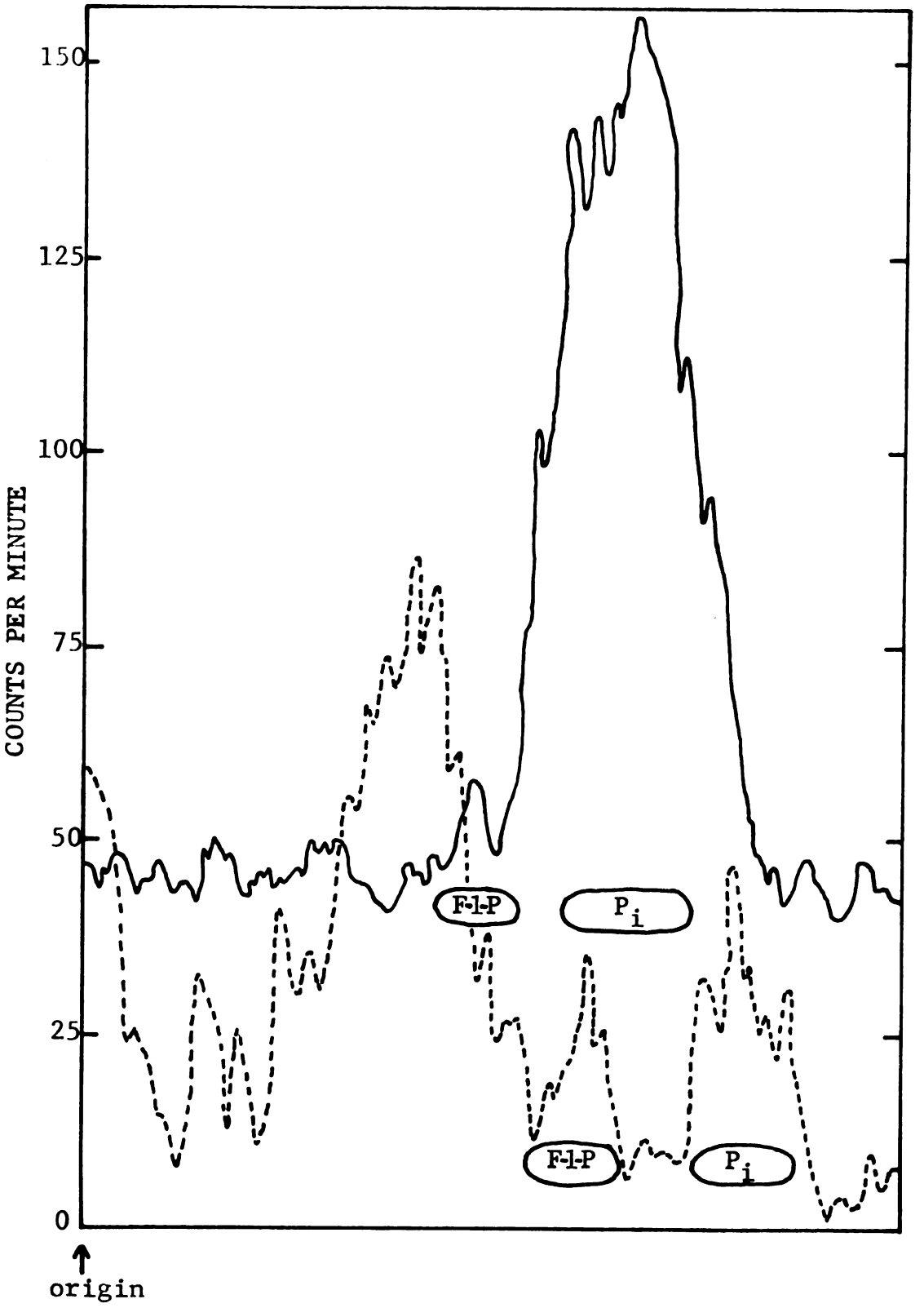
Reaction	Time (Min)	Radioactivity		
		Bound[a] (cpm)	Unbound[b] (cpm)	Unbound / Bound
1. Complete	15	434	487	1.12
2. " Plus HPr	15	344	399	1.16
3. " Plus HPr	15	579	749	1.29
4. Complete	2	348	425	1.22
5. "	30	327	434	1.33
6. " Minus Enzyme II	15	313	370	1.18
7. " 2.0 μmoles D-fructose	15	377	391	1.03
8. " 1/2 [^{32}P] phospho-PTP$_{fru}$	2	216	163	.75

[a]Counts per minute in pool of fractions 11 through 15.

[b]Counts per minute in pool of fractions 16 through 26.

Figure 29. Radioactivity scans of acid chromatograms of pooled bound and unbound radioactivity. The pool of unbound radioactivity from reactions 1, 4, 5, 7, and 8 in Table XIII was treated with Dowex 50, concentrated by lyophilization, and chromatographed (————). The pool of bound radioactivity from the same reactions in Table XIII was boiled, centrifuged to remove protein, treated with Dowex 50, concentrated by lyophilization, and chromatographed (---------). Standards were chromatographed with the readioactive samples and detected with Hanes-Isherwood spray reagent.

Figure 29



treated in a similar fashion as the unbound sample discussed above. The radioactivity scan had small peaks that co-chromatographed with P_i and D-fructose 1-phosphate, and a larger peak that migrated closer to the origin. Identification of this radioactive compound was not determined. The D-fructose 1-phosphate present in this pool of high-molecular-weight components was possibly trapped inside the membrane vesicles, and thus did not separate on the Sephadex G25 column.

d. Formation of D-Fructose 1-[^{32}P] Phosphate From [^{32}P] Phospho-PTP_{fru}

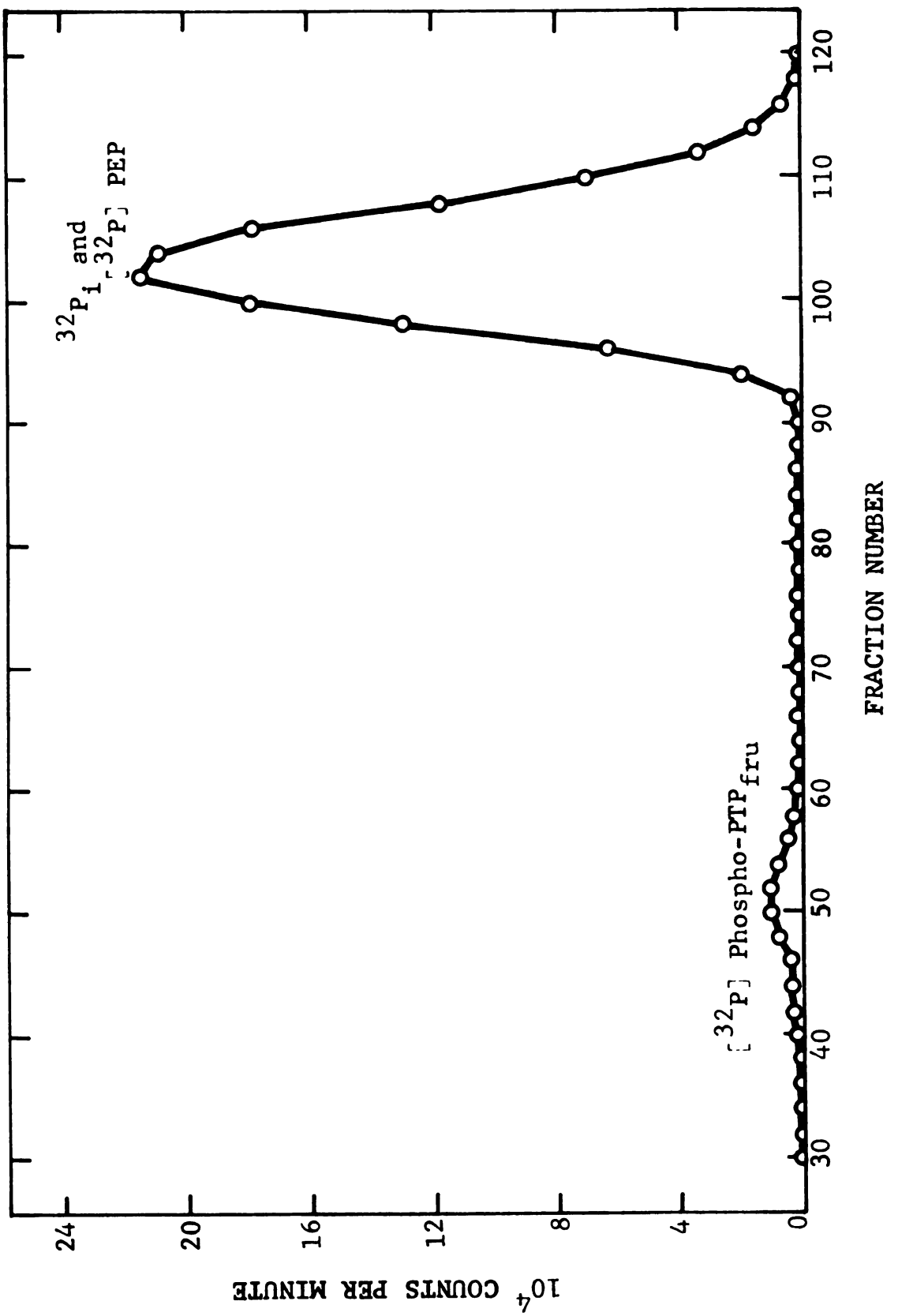
In the previous experiment the low levels of ^{32}P that were transferred to D-fructose 1-[^{32}P] phosphate may have been due to inactivation of the PTP_{fru} as a result of manipulation. A majority (75 percent) of the ^{32}P had hydrolyzed from the original [^{32}P] phospho-PTP_{fru} formed during these manipulations.

In this experiment the Sephadex G25 column and subsequent dialysis were omitted, and an enzyme I, [^{32}P] PEP, PTP_{fru} reaction was layered directly on the Sephadex G100 column (Figure 30). Fractions 44 through 66 were pooled and concentrated to 2.0 ml. This [^{32}P] phospho-PTP_{fru} preparation containing 68,000 cpm was obtained in one day rather than the two days required to prepare the sample in the previous experiment.

This preparation was used in four small reactions

Figure 30. Sephadex G100 chromatography of [^{32}P] phospho-PTP$_{fru}$ reaction. Fractions (2.0-ml) were eluted with 0.02 M potassium phosphate (pH 8.0) containing 0.001 M DTT and 10 percent glycerol.

Figure 30

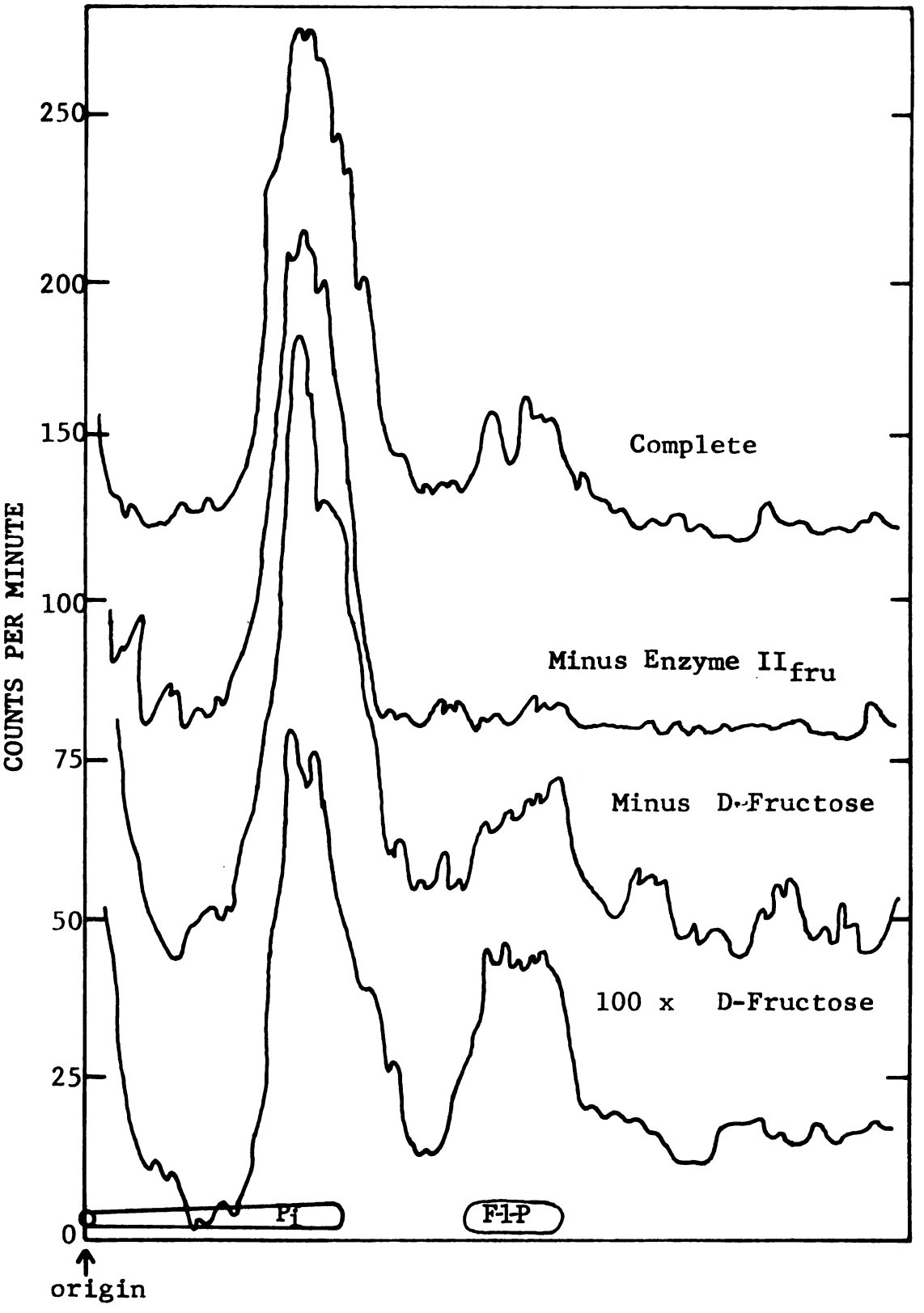

10^4 COUNTS PER MINUTE
FRACTION NUMBER
$^{32}P_i$ and [^{32}P] PEP
[^{32}P] Phospho-PTP$_{fru}$
0
4
8
12
16
20
24
30
40
50
60
70
80
90
100
110
120

and one large reaction to determine the extent of ^{32}P transfer from [^{32}P] phospho-PTP_{fru} to D-fructose. The small reactions contained 8.0 μmoles of Tris-HCl buffer (pH 7.5), 2.5 μmoles of $MgCl_2$, 0.15 μmole of D-fructose, 1.01 mg of enzyme II_{fru}, and 100 μl of [^{32}P] phosphoprotein (3,400 cpm) in a total volume of 140 μl. After 20 minutes at 30°C, the reactions were stopped by placing the tubes in a 95°C bath for 7 minutes. The denatured protein was removed by centrifugation at 6,000 x g and 25 μl aliquots were chromatographed in the alkaline solvent system (Figure 31). The radioactivity scans of the complete reaction and a reaction containing 15 μmoles of D-fructose exhibited radioactive peaks that corresponded to D-fructose 1-phosphate and P_i. The reaction without enzyme II had a single radioactive compound that chromatographed with $^{32}P_i$, whereas the reaction without D-fructose had a radioactive product that migrated with D-fructose 1-phosphate and P_i. Since enzyme II_{fru} prepared as the 100,000 x g precipitate contained up to 0.05 mM D-fructose, a low level of D-fructose was present and phosphorylated in the absence of added D-fructose. Increasing the concentration from 1 mM to 100 mM D-fructose increased the amount of D-fructose 1-[^{32}P] phosphate formed; however, this level is still low compared to the level of free $^{32}P_i$.

In another experiment, the complete reaction discussed above was scaled up ten fold and was incubated

Figure 31. Radioactivity scans of alkaline chromatograms of D-fructose 1-[^{32}P] phosphate-forming reactions. Standard D-fructose 1-phosphate and P_i were chromatographed with the reaction and detected with Hanes-Isherwood spray reagent. [Note: Each baseline had approximately 15 counts per minute; however, each was offset in order to separate the individual scans.]

Figure 31

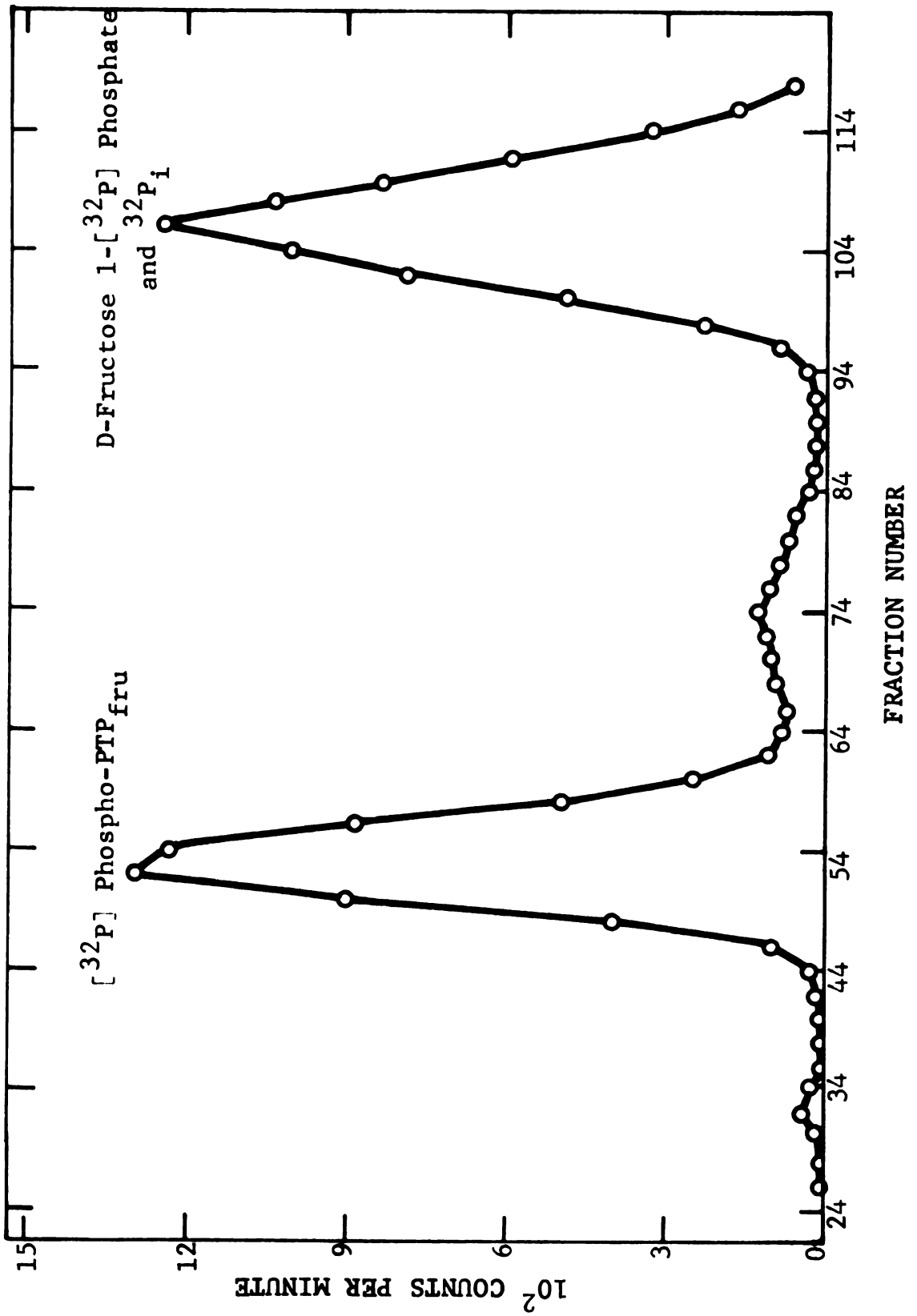

COUNTS PER MINUTE
250
200
150
100
75
50
25
0
origin
Complete
Minus Enzyme II fru
Minus D-Fructose
100 x D-Fructose
Pi
F-1-P

for 20 minutes at 30°C, stopped by cooling on ice, and layered on the Sephadex G100 column. Fractions (60-drop) were eluted with 0.02 M potassium phosphate buffer (pH 8.0) containing 0.001 M DTT and 10 percent glycerol. Radioactivity in the fractions was measured in a liquid scintillation counter (Figure 32). Two peaks were observed, each with approximately one-half of the total radioactivity. One peak corresponds to the location where PTP_{fru} elutes from this column (fractions 45 through 60) and the other peak corresponds to low-molecular-weight compounds such as $^{32}P_i$ and D-fructose 1-[^{32}P] phosphate. A very small peak of radioactivity eluted in the void volume where the enzyme II vesicles would be expected to elute. Thus, D-fructose 1-phosphate was not trapped in these vesicles. Another peak is centered at fraction 75. This could be ^{32}P bound to a low-molecular-weight protein that was in the enzyme II preparation (centrifuged at 100,000 x g), or radioactivity released from [^{32}P] phospho-PTP_{fru} by hydrolysis after it had been applied to the Sephadex G100 column.

From the results of this large reaction, it is apparent that 50 percent of the ^{32}P remained bound to PTP_{fru}, very little was trapped inside the membrane vesicles, and the other 50 percent eluted in the low-molecular-weight region of the column. The paper chromatograms of the small reactions (Figure 31) show that only 18 to 30 percent of radioactivity that migrated

Figure 32. Sephadex G100 column chromatography of large reaction of enzyme II and [^{32}P] phospho-PTP$_{fru}$.

Figure 32


[32P] Phospho-PTPfru
D-Fructose 1-[32P] Phosphate and 32Pi
10² COUNTS PER MINUTE
FRACTION NUMBER
0
3
6
9
12
15
24
34
44
54
64
74
84
94
104
114

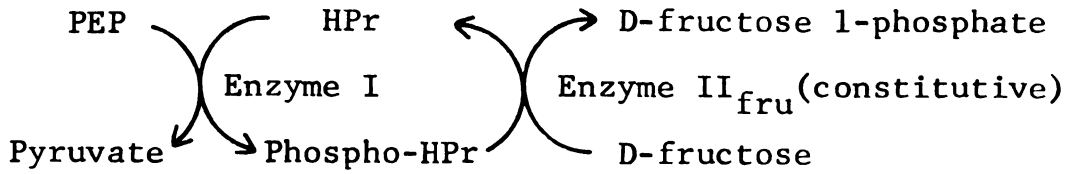
from the origin was D-fructose 1-[^{32}P] phosphate and the rest was $^{32}P_i$. Thus, only 9 to 15 percent of the [^{32}P] phospho-PTP_{fru} was converted to D-fructose 1-[^{32}P] phosphate. This low level of transfer of ^{32}P from [^{32}P] phospho-PTP_{fru} to D-fructose may be due to inactive phospho-PTP_{fru} or to incorrect conditions.

There are many unanswered questions about this system; however, the data definitely show that (i) the phosphoryl transfer from PEP to PTP_{fru} is dependent on enzyme I and does not require HPr, (ii) that the phospho-PTP_{fru} contains 1 mole P_i bound per mole of 26,000 molecular weight monomer, and (iii) that this phosphoprotein serves as the phosphoryl donor in an enzyme II_{fru}-catalyzed phosphorylation of D-fructose.

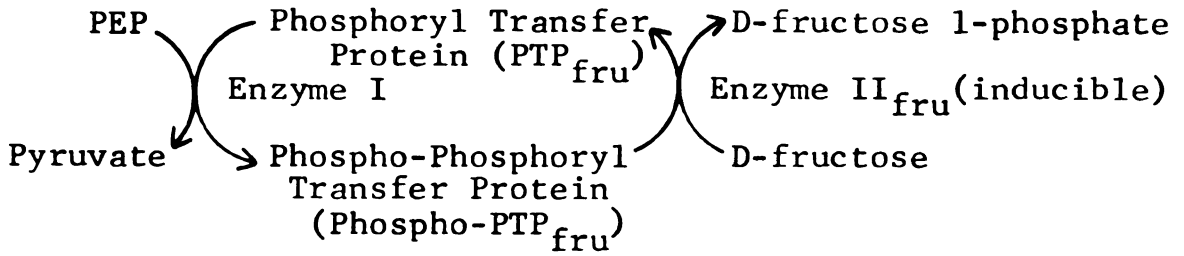
DISCUSSION

The data described in this thesis established that there are two separate phosphoenolpyruvate:D-fructose 1-phosphotransferase systems that function in Aerobacter aerogenes. One of these systems is constitutive, has a high K_m (7.1 mM) for D-fructose, and has an absolute requirement for HPr; it is thus similar to other reported systems (61, 65, 66) involving three constitutive components (HPr, enzyme I, and enzyme II). The second system is inducible, has a low K_m (0.016 mM) for D-fructose, and requires in place of HPr a protein component that is induced by D-fructose. The independence of the system from HPr makes it fundamentally different from all other PEP-dependent phosphotransferase systems that have been described (47, 78, 92). The two systems described in this thesis may be diagrammed as follows:

Constitutive:



Inducible:



These two phosphotransferase systems are comprised of five protein components: enzyme I, HPr, PTP_{fru}, enzyme II_{fru} (constitutive), and enzyme II_{fru}(inducible). PTP_{fru} has been obtained in homogeneous form for the first time and was shown to have a molecular weight of 52,000 and to be comprised of two 26,000 molecular weight subunits. It was completely separated from the other four components. Enzyme I and HPr have also been separated from each other and from the other three components. Enzyme II_{fru}, which presumably occurs as small membrane vesicles (49, 93), was freed from detectable amounts of enzyme I, HPr, and PTP_{fru}; however, it should be emphasized that enzyme II_{fru}(inducible) has not been separated from enzyme II_{fru}(constitutive).

The presence of the inducible form in addition to the constitutive form of enzyme II in preparations from D-fructose-grown cells may be inferred from the following. (i) Enzyme II obtained from D-fructose-grown cells is activated by PTP_{fru} whereas enzymes II active on D-fructose obtained from cells grown on any other substrates tested are not. (ii) Enzyme II_{fru}-dependent

phosphorylation of a low concentration (0.5 mM) of D-fructose does not require HPr; rather, it has an absolute requirement for PTP_{fru}. (iii) Enzyme II_{fru}-dependent phosphorylation of a high concentration (100 mM) of D-fructose in the absence of PTP_{fru} has an absolute requirement for HPr. (iv) The enzyme II_{fru} that requires PTP_{fru} for activity has a 450 times greater affinity for D-fructose than does the enzyme II_{fru} that requires HPr for activity. (v) The enzyme II_{fru} that requires PTP_{fru} is activated four-fold by 2-mercaptoethanol, whereas the enzyme II_{fru} that requires HPr is not activated by 2-mercaptoethanol. (vi) The individual inducible and constitutive activities dependent on PTP_{fru} and HPr, respectively, are additive when measured together, if corrected for the demonstrated inhibition of the constitutive enzyme II activity by PTP_{fru}.

Evidence that PTP_{fru} substitutes mechanistically for HPr in the inducible system is as follows. (i) Enzyme I catalyzes the transfer of ^{32}P from [^{32}P] PEP to purified PTP_{fru} in the absence of HPr and enzyme II_{fru}. (ii) Enzyme II_{fru} catalyzes ^{32}P transfer from [^{32}P] phospho-PTP_{fru} to D-fructose, forming D-fructose 1-[^{32}P] phosphate in the absence of HPr and enzyme I.

Enzyme I and HPr obtained from either D-fructose- or D-mannitol-grown cells have similar activities in both the constitutive and inducible systems and thus are not specifically induced by growth on D-fructose.

The above data are interpreted to mean that there are two separate PEP-dependent phosphotransferase systems in Aerobacter aerogenes for the formation of D-fructose 1-phosphate. The constitutive system for D-fructose phosphorylation is similar to the constitutive system examined in Escherichia coli (65, 66) in that both require HPr for activity and both have a constitutive level of enzyme II that phosphorylates D-fructose. However, the Escherichia coli system apparently has a lower K_m (since it is assayed at 5 mM D-fructose) than the Aerobacter aerogenes system. Also, the Escherichia coli system has been solubilized into two protein components (II-A and II-B) and a lipid (phosphatidylglycerol) (65, 66), whereas preliminary experiments by J. Markwell in this laboratory have shown that the enzyme II_{fru} is not susceptible to solubilization by identical procedures. Whether D-fructose is phosphorylated at C-1 or C-6 by the constitutive system of Escherichia coli has not been reported.

The inducible D-fructose system in Aerobacter aerogenes and the lactose system in Staphylococcus aureus (37, 40, 78, 105) both have inducible enzymes II and soluble protein components. The soluble protein components of both organisms bind two moles of ^{32}P per mole of protein and serve as phosphoryl donors to their respective substrates. However, the lactose system requires HPr for the enzyme I-catalyzed transfer of ^{32}P

from [^{32}P] PEP to Factor III^{lac}, whereas PTP$_{fru}$ is directly phosphorylated by PEP in the presence of enzyme I and does not require HPr. Also, Factor III^{lac} is a 36,000 molecular weight protein and contains three or four subunits of 9,000 to 12,000 molecular weight (78), whereas PTP$_{fru}$ is a 52,000 molecular weight protein with two monomers of 26,000 molecular weight and binds one mole of ^{32}P per mole of monomer. Thus, these systems differ both in mechanism of action and physical characteristics of the respective phosphoryl transfer proteins. Both induced components of the four-component lactose system are specific for lactose phosphorylation (105). In preliminary experiments, I showed that PTP$_{fru}$ does not affect D-mannitol, D-mannose, or D-glucitol phosphorylation catalyzed by enzymes II isolated from extracts of cells induced on D-fructose, D-mannitol, D-mannose, and D-glucitol; however, this substrate specificity has not been rigorously studied. Further examination of the sugar-specificity of the inducible system will require the separation of the inducible and constitutive enzymes II$_{fru}$.

The data in this thesis have further elucidated the PEP:D-fructose 1-phosphotransferase system originally described by Hanson (33). Its properties remain similar; however, by using purified components I have been able to detect and assay the separate inducible and constitutive activities and account for their slight nonadditivity

by the inhibition of the constitutive activity by PTP_{fru}. In detecting the separate activities it has been possible to determine the absolute requirements for each system. These systems function independently rather than as a single system that can be converted from a high K_m to a low K_m.

The inhibition of the constitutive system by PTP_{fru} and activation of the inducible system by HPr are not fully understood. PTP_{fru} apparently decreases both the K_m for HPr and V_{max} of the constitutive system, while HPr decreases the apparent K_m for PTP_{fru} and increase the velocity of the inducible system when PTP_{fru} is limiting; however, at saturating PTP_{fru} the velocity of the inducible system is apparently inhibited by HPr. PTP_{fru} had no effect on the K_m for HPr in the inducible system (which is 1/50 the K_m for HPr of the constitutive system); however, PTP_{fru} decreased the HPr-dependent activity of the inducible system. The slight sigmoidicity of the PTP_{fru} saturation curve may be involved in this unexplained effect of HPr. In these studies, enzyme II was limiting and thus, the apparent affinity for phospho-HPr of enzyme II was being measured. To further explain these effects a similar series of experiments with saturating enzyme II and limiting enzyme I are necessary. The individual enzyme I- and enzymes II-catalyzed reactions could also be studied independently with [^{32}P] PEP, [^{32}P] phospho HPr, and [^{32}P] phospho

PTP_{fru}. This would avoid any interaction between the enzyme I- and enzyme II-catalyzed reactions of this system which may be present when they are assayed as a coupled system and could lead to spurious results.

The apparent high affinity of the inducible system for HPr suggests that HPr somehow functions in this system even though there is no absolute requirement. Conceivably, low levels of HPr could be trapped in the vesicles (48, 49) and thus support phosphorylation of D-fructose without added HPr; however, this low level of contamination would not explain the phosphorylation of PTP_{fru} catalyzed by enzyme I in the absence of both HPr and enzyme II. If HPr were required for the enzyme II-catalyzed reaction (transfer of phosphoryl from PTP_{fru} to D-fructose), an increase in D-fructose 1-[^{32}P] phosphate production would be expected in the presence of HPr acting as a phosphoryl transfer protein from phospho-PTP_{fru} to D-fructose and thus, PTP_{fru} would not be required since HPr is also directly phosphorylated by enzyme I.

In summary, PTP_{fru} is required by Aerobacter aerogenes for growth on low concentrations of D-fructose and functions as a phosphoryl transfer protein in an inducible PEP:D-fructose 1-phosphotransferase system that has a low K_m for D-fructose and does not require HPr for activity. Aerobacter aerogenes also has a constitutive

PEP:D-fructose 1-phosphotransferase system that has a high K_m for D-fructose and requires HPr for activity.

SUMMARY

The constitutive and inducible PEP:D-fructose 1-phosphotransferase systems in Aerobacter aerogenes have been elucidated and an inducible soluble protein component (PTP_{fru}) of the inducible system has been purified to homogeneity. The inducible system has a low K_m for D-fructose, requires enzyme I, an inducible enzyme II_{fru}, and the D-fructose-induced phosphoryl transfer protein (PTP_{fru}) for activity. The presence of HPr is not required for activity; however, HPr activates the inducible system at limiting levels of PTP_{fru} and apparently decreases the V_{max} of the system at saturating PTP_{fru}. The constitutive system has a high K_m for D-fructose and requires enzyme I, HPr, and enzyme II for activity. The activities of these two systems are additive when corrected for the PTP_{fru} inhibition of the constitutive system. The inducible enzyme II_{fru} is activated by 2-mercaptoethanol, whereas the constitutive enzyme II is not. The inducible enzyme II also has a very low apparent K_A for HPr which is 1/50 the K_m for HPr of the constitutive enzyme II. In the inducible system, enzyme I catalyzes a phosphoryl transfer from PEP to PTP_{fru}, forming phospho-PTP_{fru} which then serves as the donor in

the inducible enzyme II_{fru}-catalyzed phosphoryl transfer to D-fructose. [^{32}P] Phospho-PTP_{fru} binds 2 moles ^{32}P per mole of 52,000 molecular weight protein, or 1 mole ^{32}P per mole 26,000 molecular weight monomer.

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