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SOME CHARACTERISTICS OF FRUCTOSE 1,6-DIPHOSPHATASE ACTIVITY IN RAT LIVER*

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ABSTRACT

This investigation is concerned with the establishment of a reliable assay for hepatic fructose 1,6-diphosphatase in the rat. It was found that the greatest enzymic activity and highest protein levels were eluted from the colored portion of the homogenate. When the substrate concentration was 0.01M, the enzyme had optimal activity when incubated with 0.01M $MgSO_4$ for 10 min. at 37° C in 0.05M Tris-HCl buffer, pH 7.5. Specificity for the substrate, fructose 1,6-diphosphate, was obtained at substrate concentration of 0.01M.

INTRODUCTION

D-Fructose 1,6-diphosphate phosphohydrolase (E.C. 3.1.3.11) is one of the four key gluconeogenic enzymes in the mammalian liver (Weber, Singhal & Srivastava, 1965). These four enzymes are glucose-6-phosphatase, fructose 1,6-diphosphatase (FDPase), phosphoenolpyruvate carboxykinase, and pyruvate carboxylase. These enzymes exert a rate-limiting role in the production of glucose from lactate and other gluconeogenic precursors. These conclusions are based on the fact that these enzymes govern one-way reactions, have low activity, are involved in circumventing thermodynamic barriers, and are localized in organs capable of gluconeogenesis (Weber et al., 1965).

Furthermore, the hormonal regulatory factors for rat hepatic glucose-6-phosphatase (G-6-Pase) and FDPase are well-known (Weber et al., 1955; Weber & Cantero, 1957; Ashmore et al., 1956; Weber, 1963; and Weber et al., 1964). These workers found that these enzymes are increased by the corticosteroids, but are suppressed *in vivo* by insulin.

Since many enzymes and hormones are known to undergo changes from a low level to a high level during a 24-hr period, it is the objective of this laboratory to compare the 24-hr patterns of all the four key gluconeogenic enzymes with each other and with the 24-hr pattern of the regulatory hormones (i.e. corticosteroids).

In this investigation, we have developed a reliable assay for rat hepatic FDPase to be used in this laboratory. In addition, we report some of the characteristics of hepatic FDPase in the partially inbred Holtzman rat.

MATERIALS AND METHODS

Male rats, purchased from Holtzman Co., Madison, Wis., were fed Purina rat chow and tap water *ad libitum*. The animals were killed by chloroform and weighed. The livers were removed, wrapped in aluminum foil and frozen on dry ice in acetone and stored at -70° C until assay.

One-half gram of frozen rat liver was minced and suspended in 2 ml of cold 0.05 Tris-(2-amino-2-(hydroxymethyl)-1,3-propanediol)-HCl buffer (pH 7.5) and homogenized with a ground glass homogenizer to obtain a uniform suspension. This homogenate was centrifuged at 16,000 g for 10 min. in a refrigerated International centrifuge. The pellet was discarded while the remaining 2 mls of supernatant were placed over a P-60 polyacrylamide gel column. After elution from the P-60 polyacrylamide gel column, aliquots of the supernatant fraction of the normal rat liver homogenates were incubated 10 min. (unless otherwise stated) at 37° C in 0.01M fructose 1,6-diphosphate (Sigma). The reaction was stopped by addition of 2 mls of 10% Trichloracetic acid. After centrifugation at 16,000 g, the pellet was discarded and the deproteinized supernatant was assayed for inorganic phosphorous by the colorimetric determination of Fiske and Subbarow (1925). Results were expressed as umoles of Pi released. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard protein.

(1) Elution Pattern of FDPase

After the 2 ml sample of the prepared liver homogenate was centrifuged, the supernatant was placed over the top of a P-60 gel column and eluted with the 0.05M Tris-HCl buffer. Two ml fractions were collected. Each fraction was assayed for FDPase (Fiske & Subbarow, 1925) and for protein (Lowry et al., 1951). It was determined that all of the enzymic activity and protein were in the reddish portion of the eluate. The red color was probably due to hemoglobin in the liver.

(2) Metal Ion Requirement

The chloride salts of various metal ions were incubated with normal liver homogenates at pH 7.5 using fructose 1,6-diphosphate as substrate. The metal ions included magnesium chloride, manganese chloride, zinc chloride, cupric chloride, calcium chloride, and ferrous chloride. Concentration for the metal ions was 0.01M.

(3) Time Requirement

Using fructose 1,6-diphosphatase as substrate, Tris-HCl as buffer, the supernatant fractions were incubated at 37° C from 5 - 35 minutes. The reaction was stopped with 10% Trichloracetic acid at the indicated times.

(4) Temperature Optimum

The effect of temperature on FDPase activity was determined by incubation for 10 min. at temperatures ranging from 0° C to 70° C.

(5) pH Optimum

The soluble fractions of rat livers were prepared by homogenization in 1.5% KCl and were put over a P-60 polyacrylamide gel column and eluted with 1.5% KCl (Mills, Sogandares-Bernal & Seed, 1966). The active fractions were pooled and assayed at different hydrogen ion concentrations for FDPase activity by incubation for 10 min. at 37° C with fructose 1,6-diphosphate. Tris-HCl buffer (0.05M) was prepared to give a range of pH from 3.0 to 9.4.

(6) Substrate Concentration

The effects of substrate concentration on FDPase activity were determined by incubating the liver fractions with fructose 1,6-diphosphate at concentrations varying from .001M to .025M at 37° C for 10 min. at a pH of 7.5.

(7) Enzyme Concentration

Once the saturating substrate concentration was established, the effect of enzyme concentration on FDPase activity was determined.

The enzyme preparations used were from the active fractions eluted from the P-60 gel column. The enzyme concentrations were zero dilution, 1:2 and 1:4 dilutions.

(8) Substrate Specificity

The preferred substrate for the phosphate-hydrolyzing enzymes from rat livers was determined by incubation of liver homogenates at pH 7.5 with several possible substrates at a concentration of 0.01M. These included sodium glycerolphosphate, adenosine monophosphate, adenosine diphosphate, adenosine triphosphate, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate and fructose 1,6-diphosphate.

RESULTS

Figure 1 shows the elution pattern of soluble protein and fructose 1,6-diphosphatase activity from a P-60 gel column. Two-ml fractions were collected and each fraction was assayed for fructose 1,6-diphosphatase activity and protein. The highest enzyme activity and highest protein levels were eluted in the fractions 7 through 9 which were the reddish portions of the eluate. The highest enzyme activity and protein levels are always found in the reddish portion of the eluate. This colored portion (probably due to hemoglobin) allows us to simplify the collecting of many samples because only the colored portion of the eluate needs to be collected.

Figure 2 shows the results for the metal ion requirement. The chloride salts of various metals were incubated with the rat liver fractions. Copper ion inhibited the enzyme while zinc and calcium had no effect. Magnesium, manganese and iron ions activated the enzyme. Magnesium ion was used in all the subsequent assays.

The time course for rat liver FDPase is shown in Figure 3. Enzyme activity was linear with respect to time for approximately 15 minutes.

The effect of temperature on FDPase activity is given in Figure 4. All enzyme preparations were preincubated until the preparations reached the temperatures shown in Figure 4. Highest enzyme activities were found from 40° C to 60° C.

The pH activity profile for rat hepatic FDPase is given in Figure 5. Optimim activity is found at a pH of 9.4. Since a pH higher than 9.4 was not tried, declining values of enzyme activity were not found. A secondary peak was demonstrated at neutrality.

The effect of substrate concentration on rat liver FDPase is shown in Figure 6. The activity increased with increasing substrate concentration up to approximately 0.007M - 0.010M where enzyme is saturated with substrate, and the reaction is zero-order with respect to substrate. The substrate concentration used for all future assays was therefore 0.010M.

Figure 7 demonstrates the effect of enzyme concentration on the reaction using substrate concentrations of 0.007M, 0.010M, 0.012M, and 0.015M. For these substrate concentrations, the reaction rate appeared to be dependent on the enzyme concentration alone.

Figure 8 demonstrates the results obtained from incubating rat liver homogenates with the variuos substrates at a concentration of 0.01M. Fructose 1,6-diphosphate was the preferred substrate.

DISCUSSION

Hepatic FDPase catalyzes the following reaction:

$$\text{fructose 1,6-diphosphate} + H_2O \xrightarrow{Mg^{++}} \text{Fructose-6-P} + \text{Pi}.$$

Since FDPase is a soluble enzyme, found in the cytoplasm of the liver, it is easily purified and has been studied extensively (For review, see McGilvery & Pogell, 1964; Taketa & Pogell, 1965; Pontremoli _et al_., 1965a, b, and c; Pogell _et al_., 1968 and Sarngadharan _et al_., 1970). This enzyme is an allosteric protein (Taketa & Pogell, 1965) with an allosteric site for adenosine 5'-monophosphate which is inhibitory to enzyme activity. The interation between FDPase and AMP is believed to be of importance in the physiological control of gluconeogenesis and glyconeogenesis (Taketa & Pogell, 1965). This fact is an important consideration for our current studies, since we do not purify this enzyme from each liver sample for assay. Rather, we use liver homogenates passed through a P-60 polyacrylamide gel column (to remove inorganic phosphate). Because of the large number of liver samples used for our studies, purifying the enzyme for each sample would be impractical. We therefore need a reliable assay for FDPase.

Figure 1 demonstrates that the greatest enzyme activity and highest protein levels were in the same fractions. In addition, both enzyme and protein were in the reddish portion of the eluate. This visual aid allows the simple collection of enzyme from many samples at the same time.

Our results show that magnesium, manganese and iron ions activated FDPase while zinc and calcium ions had very little effect. Copper ion inhibited rat liver FDPase (Figure 2).

Since 10 minutes is in the linear portion of the curve (Figure 3) this time was chosen for all subsequent assays.

The temperature optimum for rat hepatic FDPase ranged from 40° C to 60° C (Figure 4). The assay mixtures (substrate, metal ion and buffer) and the enzyme preparations were preincubated separately until the specified temperature was reached. These were then mixed together and allowed to react for 10 minutes. The higher enzyme activities at 50° C and 60° C could be due to the fact that the enzyme preparations were not preincubated for any length of time after they reached the 50° C and 60° C temperatures. Lustig and Kellen (1972) demonstrated for mammary gland FDPase, that when the enzyme preparation was preincubated at time intervals up to 60 minutes at 56° C, enzyme activity decreased with increasing time. Evidently, just reaching the 60° C is not enough time to denature the enzyme.

Hepatic FDPase is an enzyme that appears to have both neutral pH optimum and alkaline optimum depaending on the buffer used, Mg ion concentration and substrate concentration. (McGilvery, 1964). Figure 5 demonstrates for these experiments, using a substrate concentration of 0.01M, that optimum activity is found at almost any pH from 6.8 to 9.4. This is in agreement with the findings of others (McGilvery, 1964; Lustig & Kellen, 1972). In other experiments, we found that using a substrate concentration of 0.08M, there was decreased activity at higher pH (9.4).

At the physiological pH of 7.5, hepatic FDPase is inhibited by excess substrate (FDP). This supports the findings of Weber (1964). At concentrations of 0.08M, FDP inhibited enzyme activity. Figure 6 shows at substrate concentrations of approximately 0.01M, FDPase was saturated with respect to its substrate, but is not inhibited (see Figure 8 also).

In order to confirm that enzyme activity is dependent on enzyme concentration alone, the reaction was allowed to proceed with no dilution of the enzyme preparation, with a 1:2 dilution, and a 1:4 dilution of enzyme. Figure 7 demonstrates that at 4 saturating (but not inhibitory) substrate concentrations, the amount of enzyme activity is proportional to the enzyme concentration.

When the enzyme preparation was incubated with several substrates at 0.01M concentrations, FDP was the preferred substrate (Figure 8). At a much higher substrate concentration (0.08M), however, FDPase was inhibited, and there was no specificity for FDP.

For assay of rat liver FDPase the above results are summarized below:

(1) Only the reddish portion(fractions) of the eluate need to be collected since this portion contains the greatest enzyme activity and the highest protein levels.

(2) A metal ion, $MgSO_4$ (0.01M) is required for maximal enzyme activity.

(3) All incubations are carried out at physiological temperature of 37° C, at the physiological pH (7.5) for 10 minutes when using a substrate concentration of 0.01M.

(4) The reaction mixture consisted of:

0.2 ml of 0.05M Tris-HCl buffer (pH 7.5)
0.1 ml of 0.01M $MgSO_4$ (prepared in the buffer)
0.3 ml of 0.01M FDP
0.2 ml of enzyme preparation (liver homogenate passed through a P-60 gel column)

The reaction was allowed to proceed for 10 minutes and then was stopped with 2 ml of 10% Trichloracetic acid. Inorganic phosphorous was then assayed by the colorimetric method of Fiske and Subbarow (1925).

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Figure 1. Elution pattern of fructose 1,6-diphosphatase (●———●) and protein (▲- - -▲) from normal rat livers from a P-60 polyacrylamide gel column. Fructose 1,6-diphosphatase is expressed as umoles Pi released and protein is expressed Mg protein/ml.

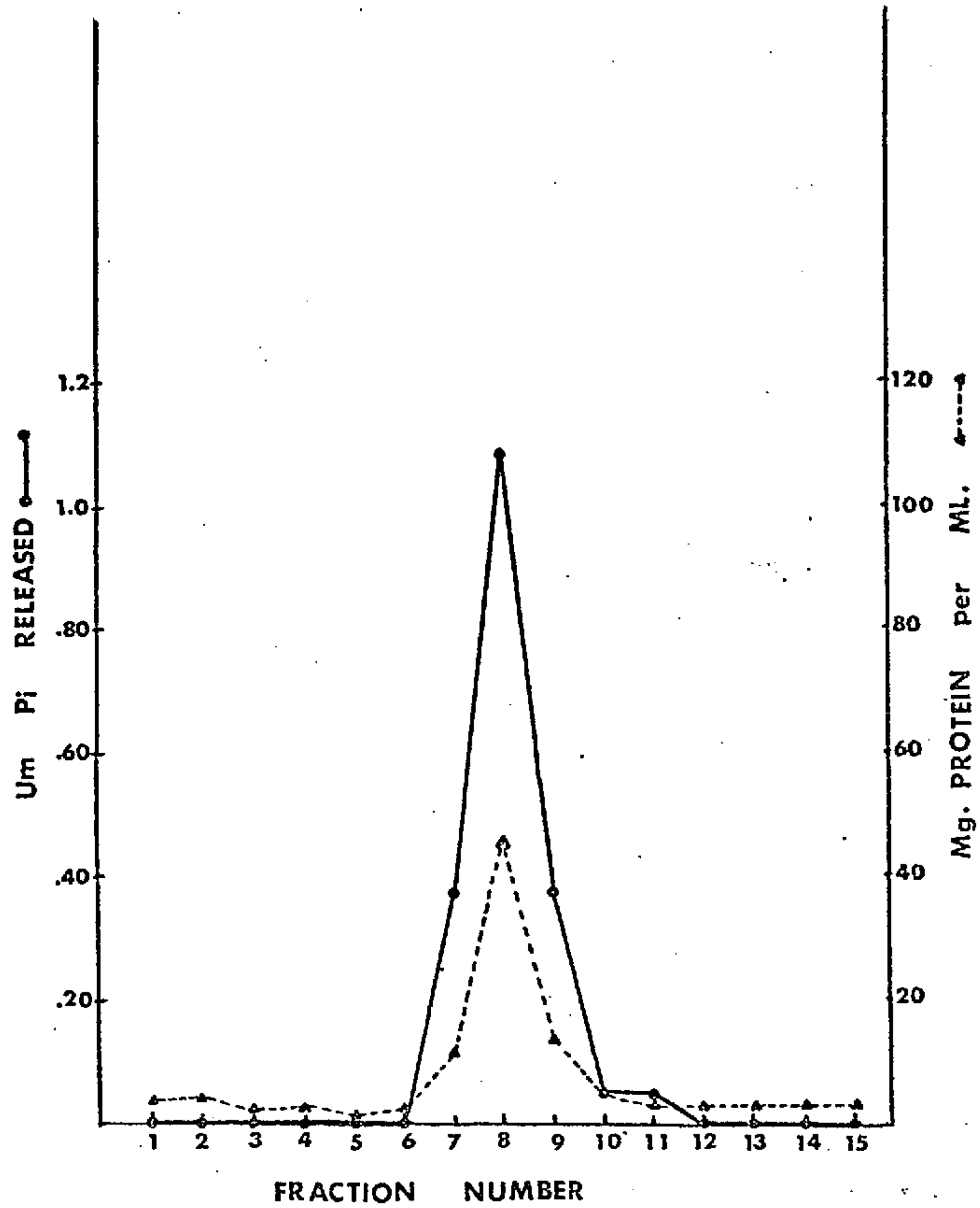

1.2
1.0
.80
.60
.40
.20
Um Pi RELEASED
120
100
80
60
40
20
Mg. PROTEIN per ML.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
FRACTION NUMBER

Figure 1

Figure 2. The effect of metal ions on fructose 1,6-diphosphatase activity from normal rat liver homogenates. Enzyme activity was assayed at pH 7.5, 37° C for 10 min. with fructose 1,6-diphosphate as substrate. Activity was expressed as umoles Pi released. The solid bars represent incubation with the metal ion and the cross-hatched bars represent incubation without the metal ion. Metal ion concentration was 0.01M.

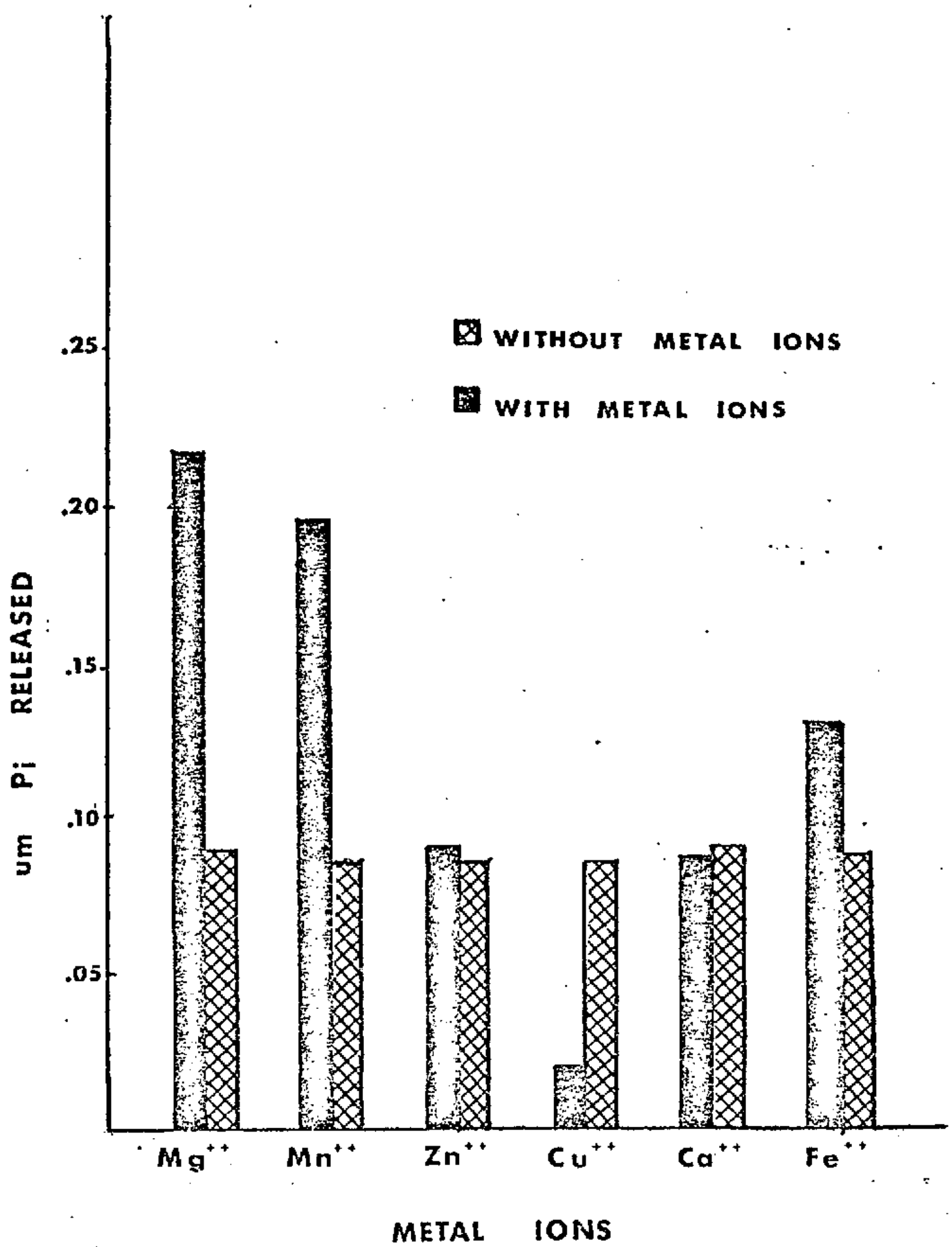

WITHOUT METAL IONS
WITH METAL IONS
.25
.20
.15
.10
.05
um Pi RELEASED
Mg++
Mn++
Zn++
Cu++
Ca++
Fe++
METAL IONS

Figure 2

Figure 3. Time course of normal rat liver fructose 1,6-diphosphatase. The reaction was stopped with 10% Trichloracetic acid at the indicated times. Enzyme activity was expressed as umoles Pi released. Assays were carried out at pH 7.5, 37° C, with 0.01M $MgSO_4$ and with fructose 1,6-diphosphate for the substrate.

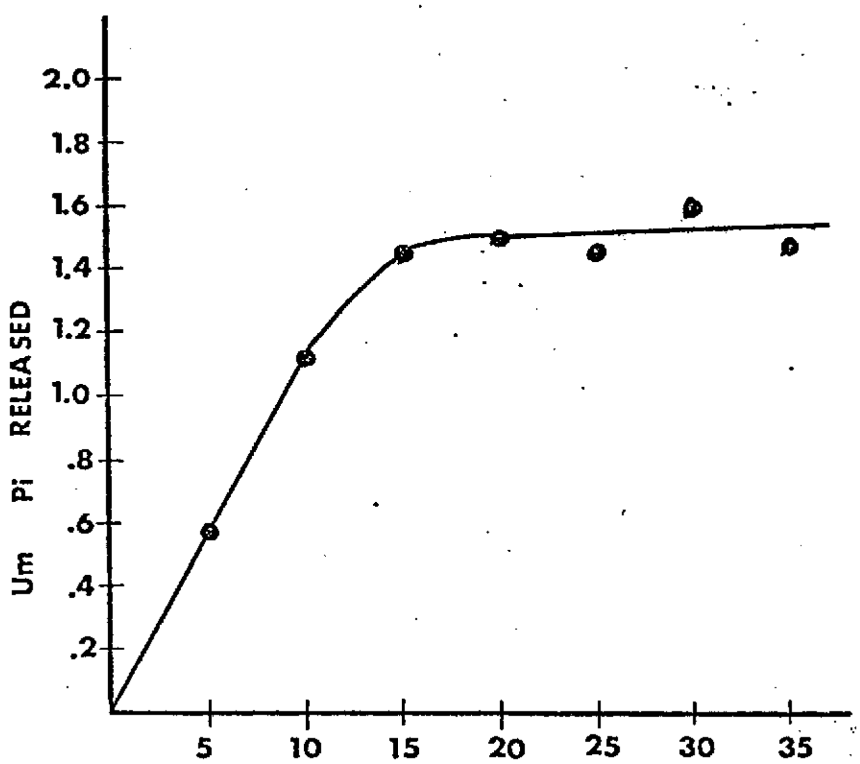


Figure 3

Figure 4. Temperature optimum of rat hepatic furctose 1,6-diphosphatase. Enzyme activity was assayed at pH 7.5 with 0.01M $MgSO_4$ for 10 min. at temperatures ranging from 0° C to 70° C, using fructose 1,6-diphosphate as substrate. Enzyme activity is expressed as umoles Pi released.

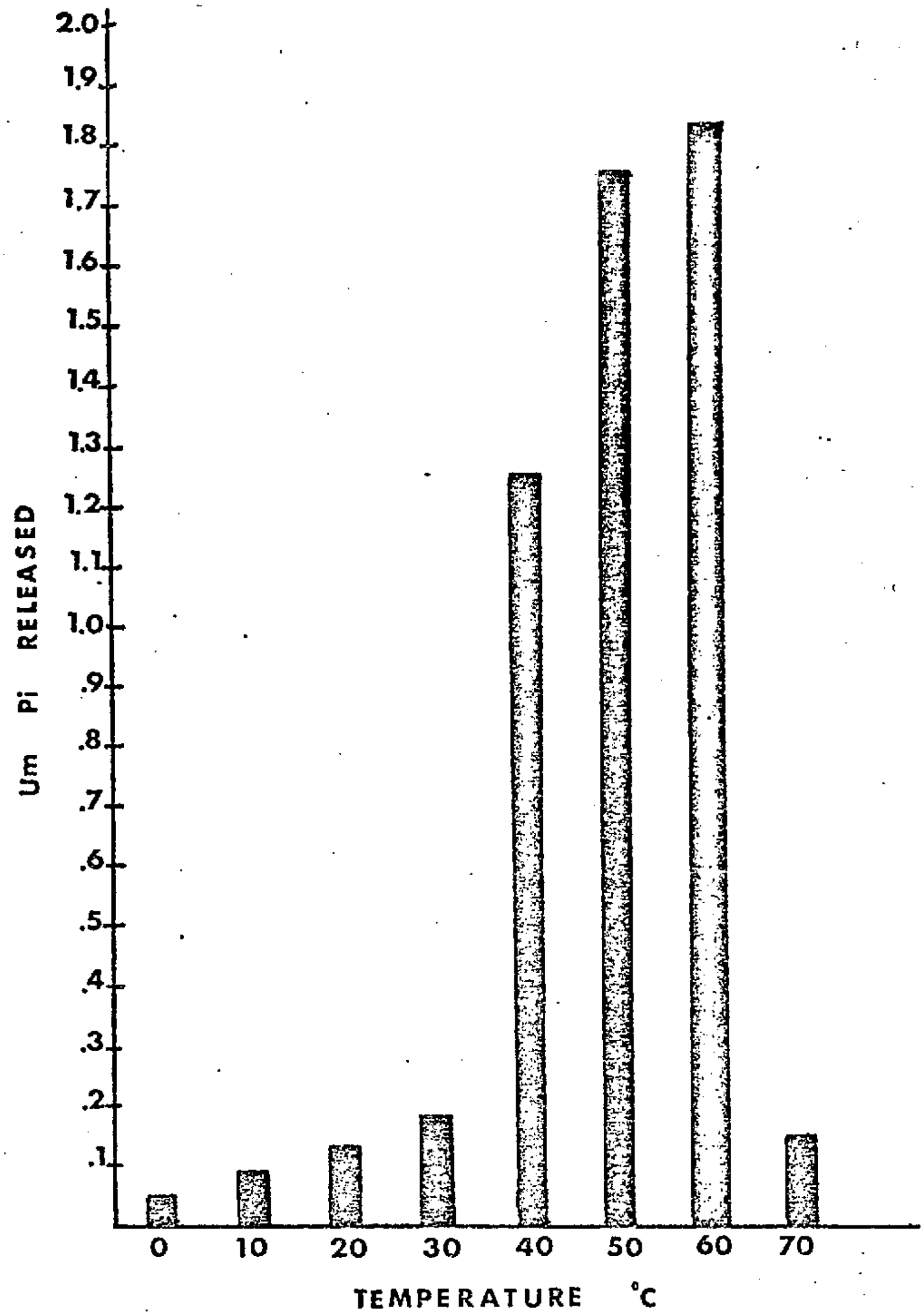
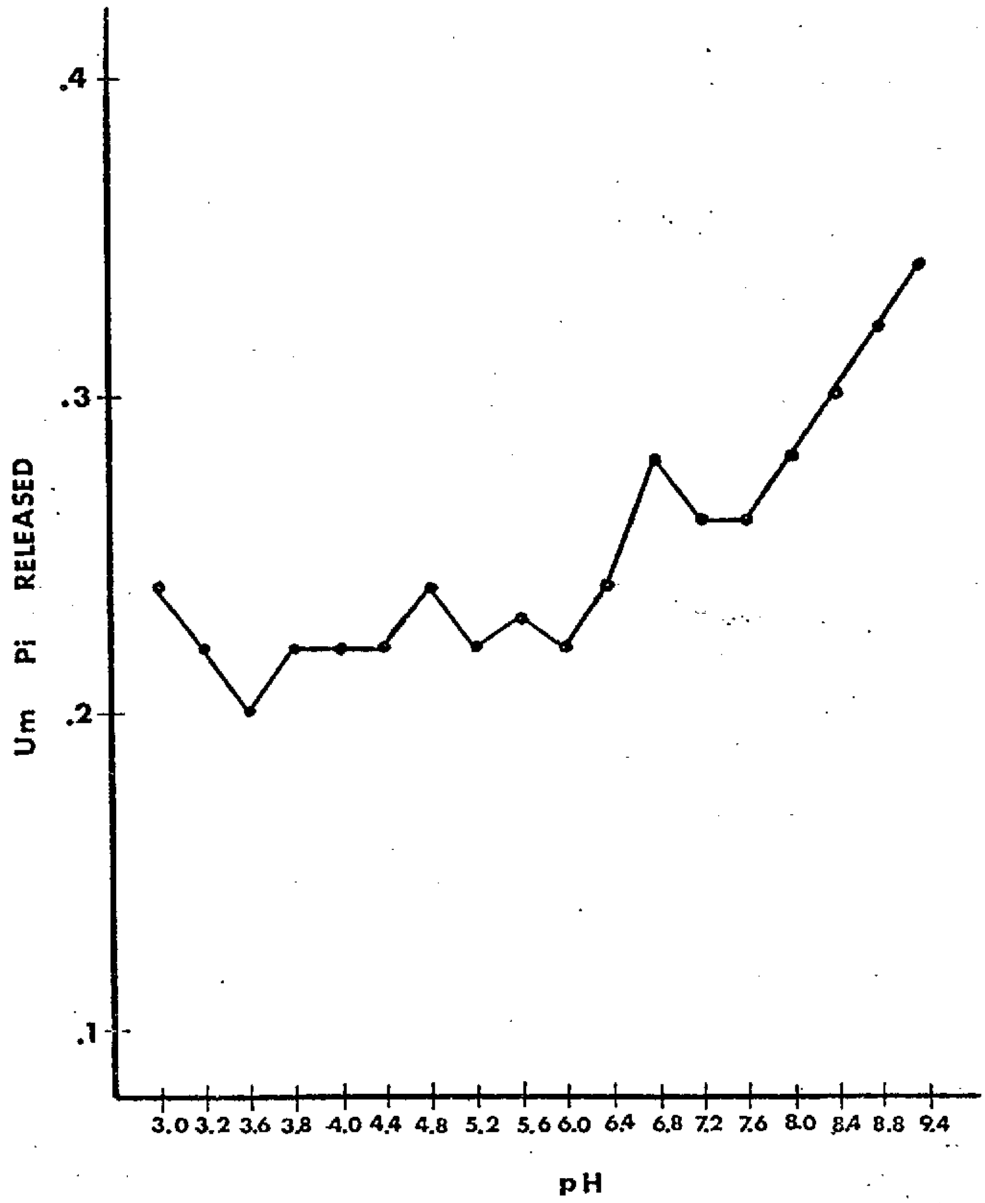

2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
Um Pi RELEASED
0
10
20
30
40
50
60
70
TEMPERATURE °C

Figure 4

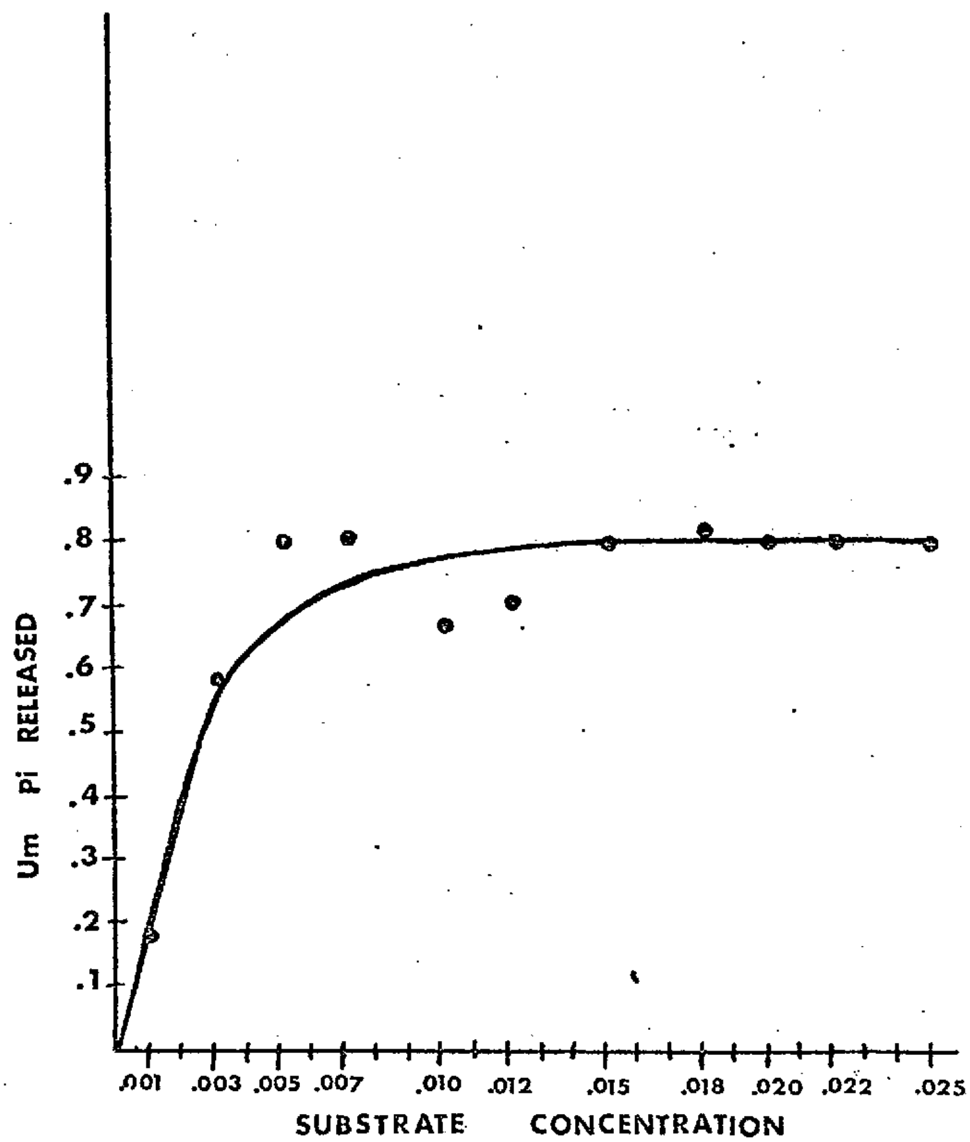
Figure 5. Effect of pH on the activity of rat hepatic fructose 1,6-diphosphatate activity. Enzyme activity was assayed over a range of pH 3.0 to 9.4 using Tris-HCl (0.05M) buffer. Assays were carried out at 37° C for 10 min. with 0.01M $MgSO_4$ with fructose 1,6-diphosphate for substrate. Enzyme activity is expressed as umoles Pi released.



.4
.3
.2
.1
Um Pi RELEASED
3.0 3.2 3.6 3.8 4.0 4.4 4.8 5.2 5.6 6.0 6.4 6.8 7.2 7.6 8.0 8.4 8.8 9.4
pH

Figure 5

Figure 6. Effect of substrate concentration on normal rat liver fructose 1,6-diphosphatase activity. Enzyme activity is expressed a umoles Pi released. Assays were performed using substrate concentrations varying from .001M to .025M at 37° C for 10 min. at pH 7.5.



Um Pi RELEASED
.9
.8
.7
.6
.5
.4
.3
.2
.1
.001 .003 .005 .007 .010 .012 .015 .018 .020 .022 .025
SUBSTRATE CONCENTRATION

Figure 6

Figure 7. Effect of substrate concentration (0.007M, 0.010M, 0.012M and 0.015M) and enzyme concentration (0 dilution, 1:2, and 1:4) on fructose 1,6-diphosphatase activity. Enzyme activity is expressed as umoles Pi released. Assays were carried out at 37° C for 10 min. at pH 7.5.

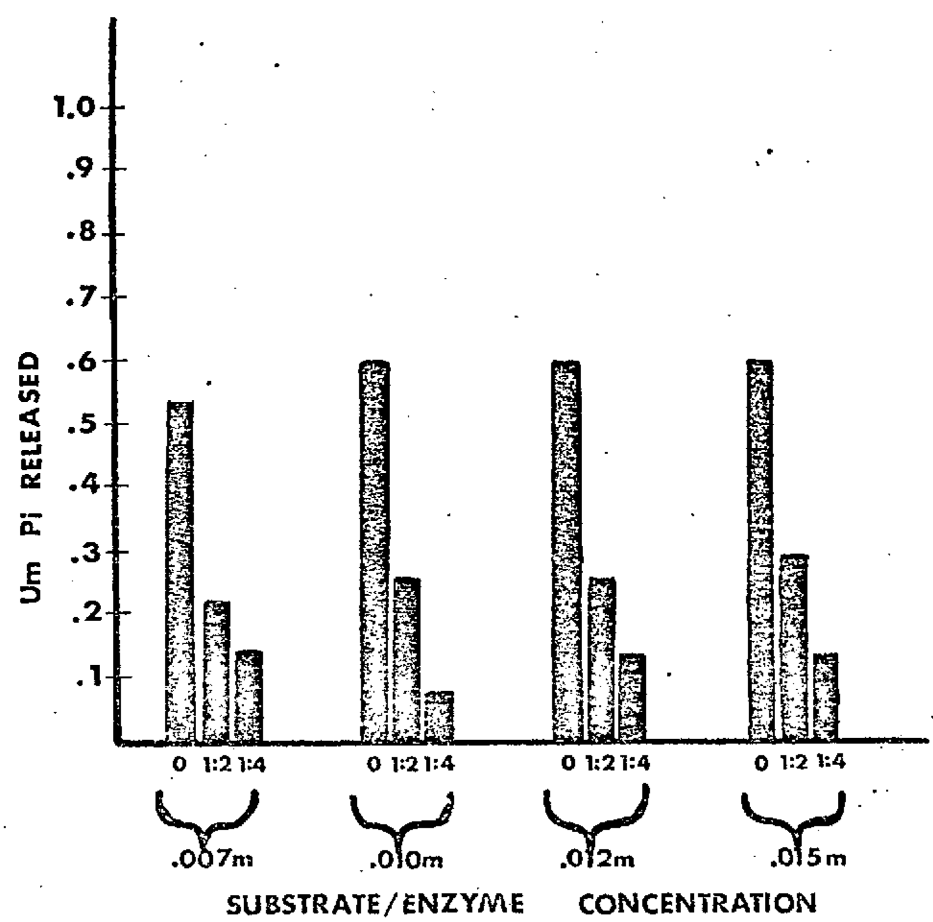

1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
Um Pi RELEASED
0 1:2 1:4
0 1:2 1:4
0 1:2 1:4
0 1:2 1:4
.007m
.010m
.012m
.015m
SUBSTRATE/ENZYME CONCENTRATION

Figure 7

Figure 8. Substrate specificity. The results obtained from incubating normal rat liver homogenates with the various substrates at 0.01M. The substrates included sodium glycerol-phosphate, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), glucose-1-phosphate (G-1-P), glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P), and fructose 1,6-diphosphate (F 1,6-di-P). The enzyme activity was assayed at pH 7.5, at 37° C for 10 min. Activity is expressed as umoles Pi released.

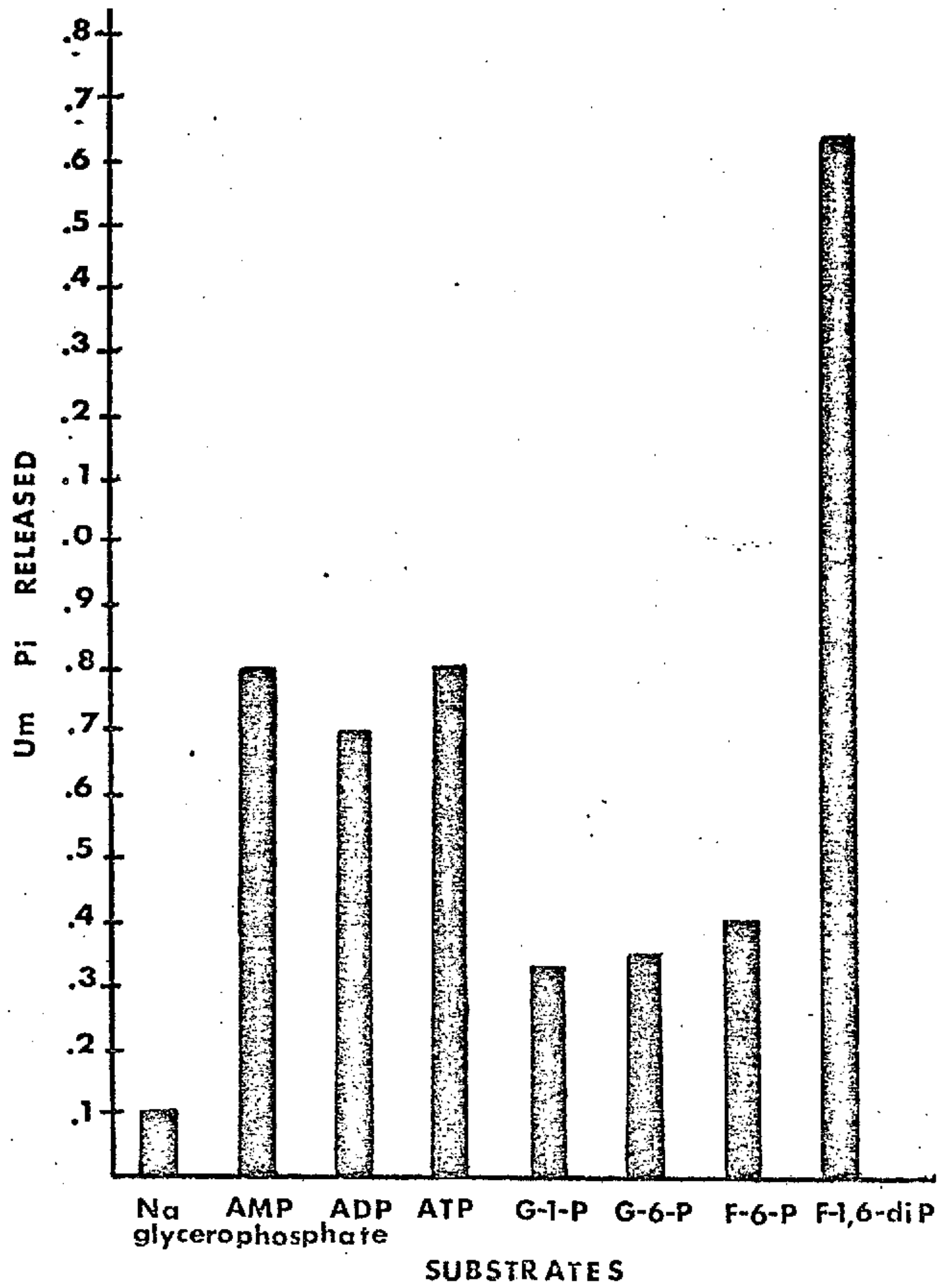

.8
.7
.6
.5
.4
.3
.2
.1
.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
Um Pi RELEASED
Na glycerophosphate
AMP
ADP
ATP
G-1-P
G-6-P
F-6-P
F-1,6-diP
SUBSTRATES

Figure 8