STUDIES ON THE PURIFICATION AND PHOSPHORYLATION OF PHOSPHOFRUCTOKINASE FROM ASCARIS SUUM

THESIS

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By

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A new procedure has been developed to concentrate the phosphofructokinase from muscle of Ascaris suum with minimum loss of activity. By utilizing this method, 50 ml fraction was concentrated to a final volume of 3 ml in about 1.5 h without loss in enzyme activity. The concentrated enzyme had a specific activity of 64 units per mg.

Ascaris muscle-cuticle was incubated in 50 μM solutions of either acetylcholine, serotonin, γ-aminobutyric acid, levamisole, or saline alone. Phosphate analysis of the isolated phosphofructokinase from each incubation revealed that the enzyme contained the following moles of phosphate per subunit: 2.9 (acetylcholine), 2.2 (serotonin), 2.0 (γ-aminobutyric acid), 1.5 (levamisole), and 3.4 (saline alone). The present study did not establish a direct correlation between degree of phosphorylation and phosphofructokinase activity.

Phosphofructokinase from muscle of Ascaris suum appears to contain several phosphorylation sites, and one of these sites is required to be phosphorylated in order for the enzyme to exhibit maximum activity under physiological conditions.

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CHAPTER I

INTRODUCTION

Phosphofructokinase (ATP: D-fructose-6-P-1-phosphotransferase, EC 2.7.1.11) catalyzes the transfer of the terminal phosphate of ATP to the C-1 hydroxyl of fructose 6-phosphate (F6P) to produce fructose 1,6-bisphosphate (FDP) (Equation 1).

$$F6P + ATP \xrightarrow{Mg^{2+}} FDP + ADP \qquad (1)$$

The reaction was first discovered in red blood cells in 1935 and in muscle in 1936 (Uyeda, 1979). The first phosphofructokinase preparation was obtained from yeast (Uyeda, 1979). A possible rate-limiting role of phosphofructokinase in muscle glycolysis was first suggested by Cori (1941), when he interpreted observations as showing administration of epinephrine to frog muscle causes an increase of the hexosemonophosphate level due to glycogen degradation with only a small increase of lactic acid.

Lardy and Parks (1959) observed that ATP strongly inhibited muscle phosphofructokinase and suggested that this inhibition may play an important regulatory role in carbohydrate metabolism. In the years since this discovery, an enormous number of studies have been done on structure,

catalytic properties and regulation of phosphofructokinase activity in a variety of sources (Uyeda, 1979).

Among prokaryotes, phosphofructokinase from Clostridium pasteurianum (Uyeda and Karooka, 1970), Escherichia coli (Blangy, 1968), and Bacillus stearothermophilus (Hengartner et al., 1975) have been purified, and the minimum molecular weights were found to be 144,000. Little is known about the molecular parameters and regulatory mechanisms of plant phosphofructokinase from the marine green algae, Dunaliella salina, other than the molecular weight of 320,000.

Phosphofructokinase is considered a cytosolic enzyme and is found in the soluble fraction of most tissues. Rabbit muscle phosphofructokinase, first purified by Ling et al. (1965), is a tetramer, has a minimum molecular weight of 320,000-360,000 and is the most well-studied phosphofructokinase. The kinetic properties of phosphofructokinase have been the subject of much investigation, since these properties are the basis for the concept that this enzyme catalyzes a key step in the regulation of glycolysis (Uyeda, 1979).

Citrate is an important inhibitor of phosphofructokinase. Garland et al. (1936) found citrate inhibition in rabbit muscle phosphofructokinase. Tsai and Kemp (1974) noticed that citrate inhibition, like ATP inhibition, varies depending upon the source of phosphofructokinase. AMP, cyclic AMP, ADP, Pi, fructose-6-P, and fructose-1,6-bisphosphate relieve or counteract the inhibition of phosphofructokinase by ATP and citrate in rabbit muscle (Uyeda, 1979).

Quite recently, evidence has been reported for the regulation of the rabbit muscle phosphofructokinase by phosphorylation. Brand and Söling (1975) have reported that phosphofructokinase precipitated by antibodies after the incubation of acid-fractionated liver extracts with [^{32}P]ATP contained ^{32}P. Hofer and Fürst (1976) have reported that phosphofructokinase isolated from the muscle of mice injected with ^{32}P, contained covalently bound ^{32}P. Brand and Söling (1975) presented evidence which suggested that rat liver phosphofructokinase was phosphorylated *in vitro* and that phosphorylation was involved in activation of the enzyme. In contrast, Uyeda and coworkers (1978) observed no direct correlation between the activity of rabbit muscle phosphofructokinase and its phosphate content. However, Foe and Kemp (1982) reported *in vitro* phosphorylated phosphofructokinase as being more inhibited with regard to its allosteric regulatory properties.

The site of *in vitro* phosphorylation in rabbit muscle phosphofructokinase is a serine residue located six amino acid residues from the carboxyl terminus (Riquelme and Kemp, 1980; Kemp et al., 1981). This site also appears to be the site of *in vivo* phosphorylation (Kemp et al., 1981). Sorenson-Ziganke and Hofer (1979), using the tryptic peptide-mapping technique, found that mouse muscle phosphofructokinase may be phosphorylated at two different peptide sites *in vivo*, and these two sites can be phosphorylated by the cyclic AMP-dependent protein kinase. Hofer and Sorenson-Ziganke (1979)

demonstrated that the number of phosphorylated sites increased from one to two during muscle contraction.

The energy metabolism of the parasitic roundworm, Ascaris suum, is essentially anaerobic (Saz and Lescure, 1969; Saz, 1970; Fairbairn, 1970). The parasite derives all of its known ATP from anaerobic metabolism due to the fact that the citric acid cycle does not seem to be functional in the adult stages. Therefore, glycogen and glucose serve as the primary source of energy. In Ascaris muscle, glycogen is metabolized by glycogenolysis as far as phosphoenolpyruvate. Thus, for every mole of glucose 6-phosphate, 2 moles of phosphoenolpyruvate are generated along with two net ATP's. Phosphoenolpyruvate is then carboxylated to give oxalacetate through the action of the enzyme phosphoenolpyruvate carboxykinase, where two moles of ATP are generated (Saz and Lescure, 1969). The oxalacetate is reduced to malate by a cytoplasmic malate dehydrogenase. Malate then passes into the mitochondria where it undergoes a dismutation. Half of it is converted to pyruvate via a mitochondrial, NAD-linked malic enzyme, the remainder of the malate is in equilibrium with fumarate through the action of a mitochondrial fumarase (Saz and Lescure, 1969; Fodge et al., 1972). Fumarate is then reduced to succinate by the action of succinic dehydrogenase (Kmetec and Bueding, 1961; Bueding, 1963). Saz and Lescure (1969) found that the reduction of fumarate to succinate in the Ascaris mitochondria is accompanied by the phosphorylation of ADP to ATP. It should be noted that this is the only known source of ATP in the mitochondrion of the parasite (Rew and Saz, 1974).

Starling et al. (1982) purified phosphofructokinase from the muscle of Ascaris suum. Hofer and coworkers (1982) purified two forms of phosphofructokinase from the muscle of Ascaris differing only in phosphate content. The enzyme with more phosphate carried out catalysis two times faster than the enzyme with lower phosphate under "near-physiological" assay conditions.

Since phosphofructokinase is important in the regulation of glycolytic metabolism in the parasite, and since it may be regulated in a different manner from the host enzyme, it was important to study the enzyme in more detail. The present project describes an improved purification procedure for ascarid phosphofructokinase. Further study has been done on the effect of hormones and the anthelmintic drug levamisole on phosphorylation of enzyme in vivo.

CHAPTER II

MATERIALS AND METHODS

MATERIALS

Substrate and Enzymes

Fructose 6-phosphate, ATP, ADP, AMP and a mixture of α-glycerophosphate dehydrogenase (EC 1.1.1.8, specific activity 100 units/mg), and triose phosphate isomerase (EC 5.3.1.1, specific activity 2,500 units/mg), aldolase, dithiothreitol, Malachite Green, mercaptoethanol and triethanolamine were obtained from Sigma Chemical Co. The disodium salt of the reduced nicotinamide adenine dinucleotide (NADH) was obtained from P-L Biochemical Co.

Materials for Column Chromatography

Cellulose phosphate (Coarse, exchange capacity, 0.86 meq/mg) was purchased from Sigma Chemical Co. Diethylaminoethyl cellulose (DE-52) (Coarse, exchange capacity, 1 meq/mg) was obtained from Whatman Co.

Others

Immersible-cx ultra-filters were purchased from Millipore Corp. and Formic acid was obtained from Aldrich Chemical Co. Ammomium molybdate obtained from Mallinckrodt Chemical Co. All other chemicals were reagent grade.

Animals

Female Ascaris suum were collected at the slaughterhouse and transported to the laboratory in a salt solution (Donahue et al., 1981), where they were frozen in liquid nitrogen and stored at -80° C until use for purification purposes.

Purification of Ascaris Phosphofructokinase

All purification procedures were carried out at 4° C. Two hundred g of whole female A. suum were subjected to a purification procedure developed by Starling et al. (1982) for extraction of PFK. This consisted of the homogenization of whole worms in 10 mM Tris-potassium phosphate, pH 8.0, 1 mM EDTA, 10 mM sodium fluoride and 40 mM 2-mercaptoethanol (Buffer 1). The homogenate was centrifuged at 27,000 x g for two hours. The resultant supernatant solution was filtered through glass wool. The filtered solution was then titrated to pH 8.0 with cold 1 M Tris solution, mixed with 200 g of damp-cake DE-52 equilibrated in Buffer 1 and allowed to stir for 10 min. The DE-52 mixture was then washed with Buffer 1 (12-16 liters). The cellulose was then washed in the same manner with 8 l of Buffer 1 + 50 mM NH_4Cl (pH 8.0). After the absorbance of the filtrate had dropped to 0.05 at 280 nm, the DE-52 was mixed with 400 ml of Buffer 1 plus 150 mM NH_4Cl (pH 8.0) and allowed to stir 10 min. This suspension was vacuum filtered, and the filtrate was mixed with 30 g of damp-cake cellulose phosphate which had been equilibrated with Buffer 1 plus 150 mM NH_4Cl (pH 8.0). The mixture was allowed to stir for 10 min and then poured into a 10 cm

diameter Buchner funnel, filtered and washed with 4 l of Buffer 1 plus 150 mM NH_4Cl, followed by 2 l of Buffer 2 (50 mM Tris-potassium phosphate, pH 8.0, 10 mM sodium fluoride, 1 mM EDTA, 40 mM 2-mercaptoethanol). The cellulose phosphate was packed into a 2.5 cm diameter column and allowed to settle. The phosphofructokinase was eluted from the phosphocellulose by application of 5 mM ATP in Buffer 2. Fractions of 10 ml were collected, and tubes containing phosphofructokinase activity were pooled.

Enzyme Assay and Definition of Units of Activity

Phosphofructokinase was assayed spectrophotometrically at 340 nm, following the oxidation of NADH, using the aldolase-glycerol phosphate dehydrogenase-triose phosphate isomerase-coupled assay system (Racker, 1947). Cuvettes of 1.0 cm light path were used. Tris-HCl, 50 mM, pH 8.0, was used as a buffer. The routine reaction mixture consisted of 40 mM fru-6-P, 0.1 mM ATP, 0.2 mM NADH, 40 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 50 µgram of aldolase, 30 µgram of the mixture of α-glycerolphosphate dehydrogenase and triose phosphate isomerase. Enzyme was added to initiate the reaction, and initial velocities were measured. The reaction was carried out on a Gilford Recording Spectrophotometer equipped with a thermostatically-controlled cuvette chamber at 30° C. For kinetic studies the full scale of the recorder was set to 0.1 optical density unit. A chart speed of 2 cm per min was used for routine assay, while a speed of 5 cm per min was used for a more precise measurement of initial velocity in the kinetic studies. One unit of enzyme activity is defined

as the amount of enzyme catalyzing the reaction of one micromole of substrate per min under initial velocity conditions at 30° C. The specific activity is expressed as units of enzyme per milligram of protein.

Protein Determination

The dye-binding method of Bradford (1976) was employed on pure samples using cyrstalline bovine serum albumin as the standard. Protein concentration in eluted fractions from column chromatography and batch chromatography was estimated spectrophotometrically by measuring absorbance at 280 nm.

Ion Exchange Chromatography

Phosphocellulose was washed repeatedly with acid and base according to the procedure suggested by Peterson and Sober (1962) and equilibrated with the desired buffer prior to use. After the column was packed, it was washed with buffer in a cold box maintained at 0-4° C. The eluant was tested to ensure the desired pH had been attained before the sample was applied. Fractions were collected at the same temperature, with constant flow rates maintained by a peristaltic pump.

DE-52 was washed with acid and base, according to the manufacturer's procedures, equilibrated with the appropriate buffer and vacuum filtered to a damp-cake immediately before use.

Specimen Preparation for Incubation

The worms were taken from the holding vessel (37° C) and placed in a dissecting pan containing prewarmed Ascaris

saline (Donahue et al., 1982). The worms were then stunned by dropping onto the table, and the intestinal and reproductive tracts were removed by rapid dissection. The remaining muscle-cuticle preparations were transferred to a beaker containing Ascaris saline incubated at 37° C and saturated with N_2/CO_2 (95%/5%).

Specimen Incubation

Five hundred ml of a 50 µM solution of the desired hormones or drug prepared in the saline solution were incubated at 37° C in a shaking water bath with continuous bubbling of N_2/CO_2 (95%/5%). When the incubation media was saturated with N_2/CO_2, 50 g of muscle-cuticle preparation were transferred to the solution and incubated for 1 hr. At the end of the 1 hr, the muscle-cuticle sections were frozen in liquid nitrogen and stored at -80° C until used.

Protein Bound Phosphate Determination

Preparation of Proteins

The purified enzyme (200 µg) was precipitated in 10% trichloroacetic acid and centrifuged; the pellet was resuspended and then reprecipitated in 5% trichloroacetic acid. The trichloeoacetic acid was removed by diethylether extraction. The precipitated protein was dissolved in 50 µl of 98% formic acid. The protein was precipitated again from formic acid with 10% trichloroacetic acid and reprecipitated in 5% trichloroacetic acid, washed once with ether, dried and dissolved in 50 µl 98% formic acid.

Protein concentration in the formic acid was determined by the dye-binding method of Bradford (1976). Recovery of

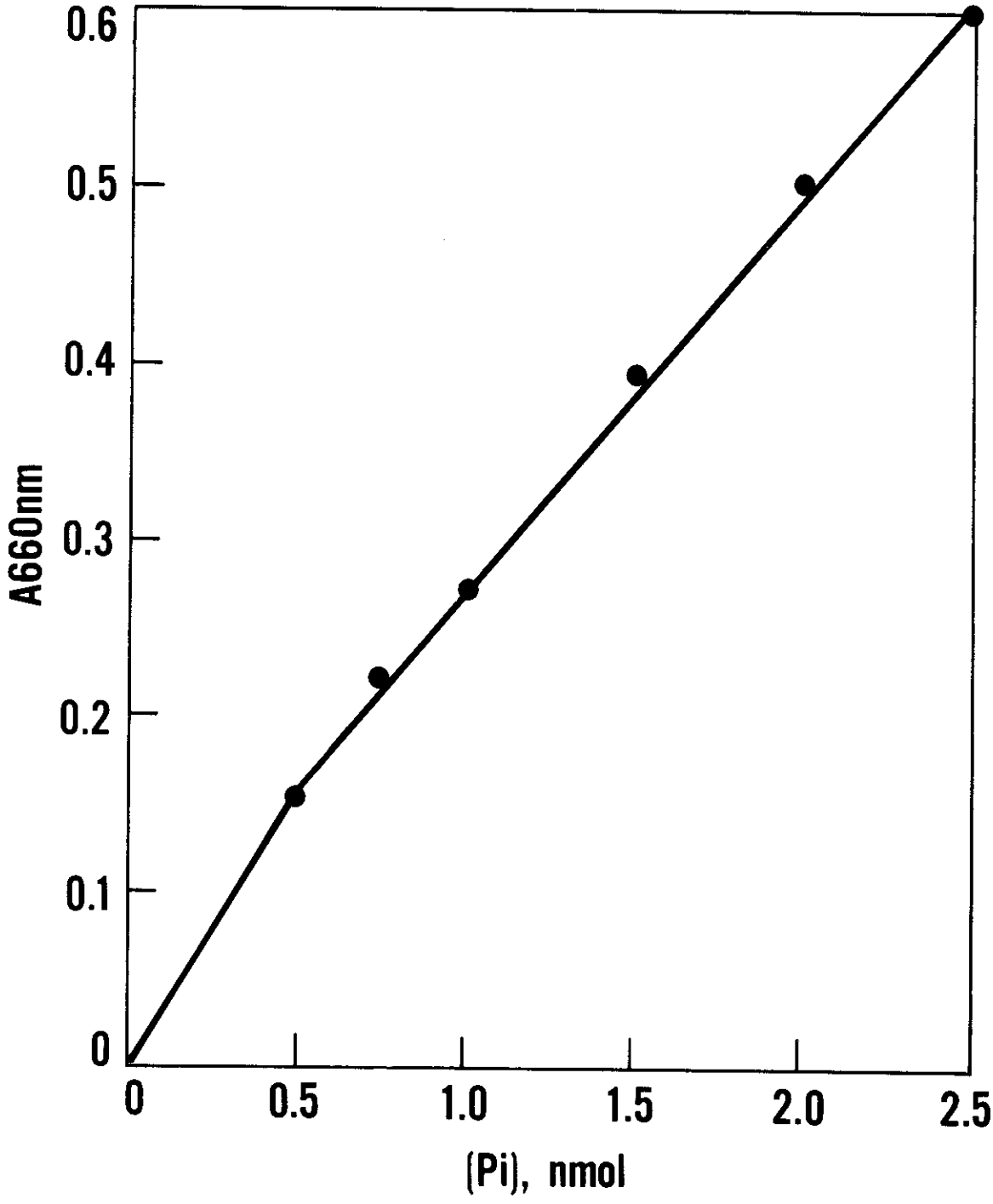
the protein after this washing procedure was essentially 100%, as measured by the addition of standard bovine serum albumin.

Phosphate Analysis

Phosphate analysis was carried out by the ashing method of Ames (1966) and phosphate assay method of Itaya and Michio (1966). Figure 1 shows the standard curve for this method.

Figure 1

Calibration plot for the determination of inorganic phosphate. In thick walled 6 x 50 mm borosilicate glass tubes 200 µgram of crystalline bovine serum albumin was added along with known amounts of KH_2PO_4, to which 25 µl of Ashing reagent (10% $MgNO_3.6H_XO$ in 95% ethanol) was added. The contents of the tube were then brought to dryness and then over an open flame to a white residue. At this point 150 µl of 1.2 HCl was added to dissolve the white residue. Finally by addition of 50 µl of phosphate reagent (1 vol 10% $(NH_4)_6MoO_{24}.4H_2O$ in 4N HCl with 0.2% Malachite Green). The tubes were then read at 660 nm.


0.6
0.5
0.4
0.3
0.2
0.1
0
A660nm
0
0.5
1.0
1.5
2.0
2.5
(Pi), nmol

CHAPTER III

RESULTS

According to the procedure of Starling et al. (1982) the eluant from phosphocellulose (see Methods) was dialyzed overnight aginst 3.94 M ammonium sulfate, 10 mM Tris-potassium phosphate, pH 8.0, and 1 mM EDTA. The precipitated protein was sedimented by centrifugation, resuspended and stored in storage buffer. During the above step an irreversible partial loss of phosphofructokinase activity was observed. In order to avoid loss of phosphofructokinase activity in this final step a new procedure was developed in which the sample was concentrated with an ultrafilter.

An immersible Cx 30 ultrafilter with 13 cm^2 membrane area and 1.6 ml filtrate hold-up volume with an average molecular weight cutoff of 30,000 was totally immersed in the phosphocellulose eluant. The solution was stirred with a magnetic stirrer. The filtrate outlet stem of the unit was connected to a silicone tubing (2.5 cm), and the other end of the silicone tubing was connected to a Luer taper-to-tubing adapter where it was connected to a rigid tubing (14.4 cm) and a vacuum trap. Care was taken not to touch the membrane surface which could damage the pellicon membrane.

In about 1.5 hr, a 50-ml fraction was concentrated to a final volume of 3 ml. As shown in Table I, this procedure using the ultrafilter resulted in a high overall recovery.

TABLE I

PURIFICATION OF PHOSPHOFRUCTOKINASE FROM MUSCLE OF *ASCARIS SUUM*

Procedure	Vol. (ml)	Total Protein (mg)	Total Activity (Units)	Specific Activity (Units/mg)	Purification	Recovery
Crude Supernatant	820	5412	690	0.12	-	100
DE-52	500	1040	654	0.6	5	94.7
Phosphocellulose	54	6.5	304	46.7	389	44
Immersible Cx 30 Ultrafilter	3	4.5	290	64.4	536	42

The concentrated protein was dialyzed overnight against 50 mM Tris-potassium phosphate, pH 8.0, 1 mM EDTA, 1.4 M $(NH_4)_2SO_4$, 1 mM ATP, 0.2 M KCl, and 10 mM dithiothreitol. The enzyme was stable for at least 6 months when stored at 4° C under N_2. The homogeneity of the enzyme preparation was checked by SDS-polyacrylamide gel electrophoresis, which indicated the enzyme was composed of only one species (data not shown).

Effect of Acetylcholine, Serotonin, γ-aminobutyric acid and Levamisole on Phosphorylation of Phosphofructokinase.

Donahue et al. (1981; 1982) have correlated muscle activity (contraction and relaxation) with glycogen regulatory enzymes in muscle of Ascaris suum. Their results indicated that perfusion of acetylcholine (ACh) through Ascaris muscle caused contraction, and the energy for this contraction was derived from endogeneous stores of glycogen. Whereas γ-aminobutyric acid (GABA) caused muscle relaxation and also glycogen utilization, both GABA and ACh have a direct effect on glycogen metabolism via a non-cAMP-mediated mechanism (Donahue et al., 1982). In Ascaris muscle; serotonin has an effect similar to that of epinephrine in mammalian skeletal muscle; i.e., it increases cAMP levels (Donahue et al., 1981). These increased levels of cAMP initiated glycogen breakdown through a series of phosphorylation reactions. Therefore, it was important to comprehend the effect of these neuromuscular effectors along with the anthelmintic levamisole on phosphorylation of phosphofructokinase.

Freshly-prepared Ascaris muscle-cuticle (50 grams) was used for each experiment. It was incubated for 1 hr in 50 μM solutions of acetylcholine, γ-aminobutyric acid, levamisole, and serotonin. Phosphofructokinase was isolated from the muscle-cuticle preparation as described above (see Methods). The phosphofructokinase from muscle-cuticle incubated with acetylcholine contained a total of 2.9 moles of phosphate covalently bound per subunit (Table III), while under similar conditions, the phosphofructokinase from serotonin-treated muscle contained 2.2 moles of phosphate per subunit. Phosphofructokinase isolated from the muscle-cuticle preparation that had been incubated with levamisole contained only 1.5 moles of phosphate per subunit. Phosphofructokinase from γ-aminobutyric acid-treated muscle had a total of 2.0 moles of phosphate per subunit. Finally, phosphofructokinase which had been isolated from muscle-cuticle preparation that had been incubated with perfusion buffer with no neuromuscular transmitter or drug as a control, contained 3.6 moles of phosphate per subunit (Table II).

Kinetics

The regulatory kinetic mechanisms of phosphofructokinases from several sources have been studied, but the task has not been easy due to the fact that the catalytic activity in the forward direction is a function of a number of factors (Pettigrew and Frieden, 1979; Bloxham and Lardy, 1973). Therefore, it was of great importance to examine the kinetic mechanism of Ascaris phosphofructokinase under different

physiological conditions and to determine the effect of phosphorylase on phosphofructokinase activity. The kinetic experiments described here (Figures 2-6) were all performed in the presence of physiological levels of substrates, products and known effectors under "near-physiological" conditions (Hofer et al., 1982). The fru-6-P saturation curve of phosphofructokinase that was purified following the incubation of muscle-cuticle with ACh exhibited a biphasic cooperativity (Fig. 2). The enzyme appears to have a similar activity at physiological levels of fru-6-P as that of the control preparation (Table III).

A biphasic cooperativity was also seen in the cases of the fru-6-P saturation curve of phosphofructokinase that had been purified from muscle-cuticle following its incubation with serotonin (Fig. 3) and γ-aminobutyric acid (Fig. 4). However, in the serotonin preparation, the enzyme had almost twice the activity in terms of percentage of the V_{max} as compared to control preparation. Kinetics of phosphofructokinase following incubation of muscle-cuticle with levamisole (Fig. 5) revealed that, even though the phosphate content was considerably lower than the control preparation (Table II), both enzymes exhibited similar activities under physiological conditions (Fig. 6, Table III).

TABLE II

PHOSPHATE ANALYSES AND SPECIFIC ACTIVITIES OF PHOSPHOFRUCTOKINASE ISOLATED FROM MUSCLE-CUTICLE OF *ASCARIS SUUM*

Experiment[1]	Phosphates/Subunit (moles)	Specific Activity (units/mg)
Acetylcholine	2.9	27
γ-aminobutyric acid	2.0	51
Levamisole	1.5	63
Serotonin	2.2	39
Control	3.4	84

[1] *Ascaris* muscle-cuticle (50 grams) was used for each experiment. Incubated for 1 hour in 500 μM solutions of acetylcholine; γ-aminobutyric acid; Levamisole; and Serotonin. Control muscle-cuticle sections were incubated for same period of time in perfusion buffer (see Methods).

Figure 2

Fructose 6-phosphate saturation curve of phosphofructokinase from acetylcholine-treated muscle. The assay contained 50 mM imidazole/HCl, pH 6.8, 5.25 mM potassium phosphate, 8 mM $MgCl_2$, 101.3 mM KCl, 3 mM ATP, 1 mM ADP, 0.36 mM AMP, 25 µM Glc-1,6-P_2, 0.2 mM NADH, 50 µg of aldolase, 50 µg of glycerol-phosphate dehydrogenase, and 10 µg of triose phosphate isomerase. The reaction was started by the addition of Ascaris phosphofructokinase. The fru-6-P was varied from 40 µM to 40 mM. Inset shows the curve obtained from 40 to 500 µM fru-6-P.

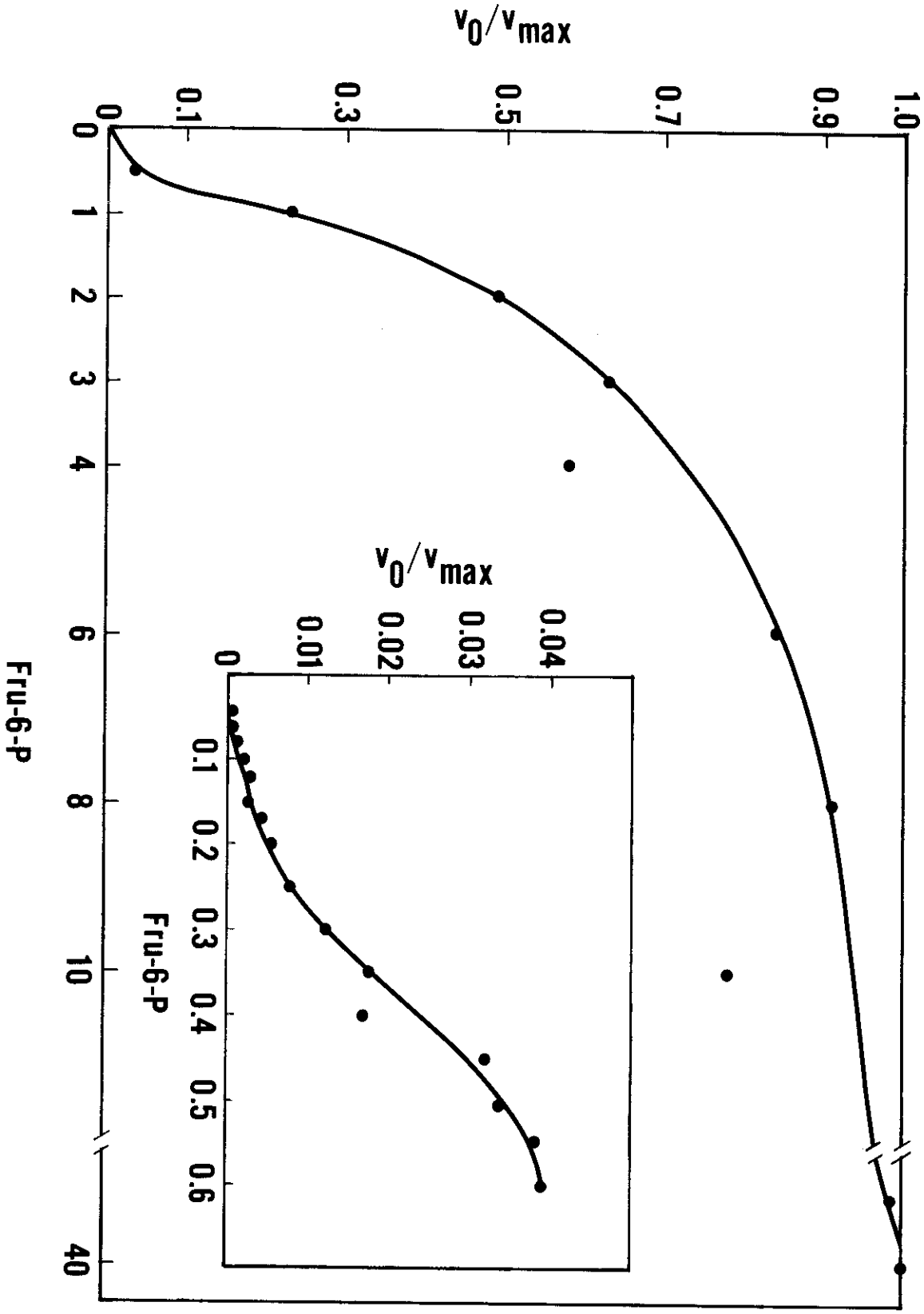

v_0/v_{max}
0
0.1
0.3
0.5
0.7
0.9
1.0
0
1
2
3
4
6
8
10
40
Fru-6-P
v_0/v_{max}
0
0.01
0.02
0.03
0.04
0.1
0.2
0.3
0.4
0.5
0.6
Fru-6-P

Figure 3

Fructose 6-phosphate saturation curve of phosphofructokinase from serotonin-treated muscle. For description see Fig. 2 and Methods.

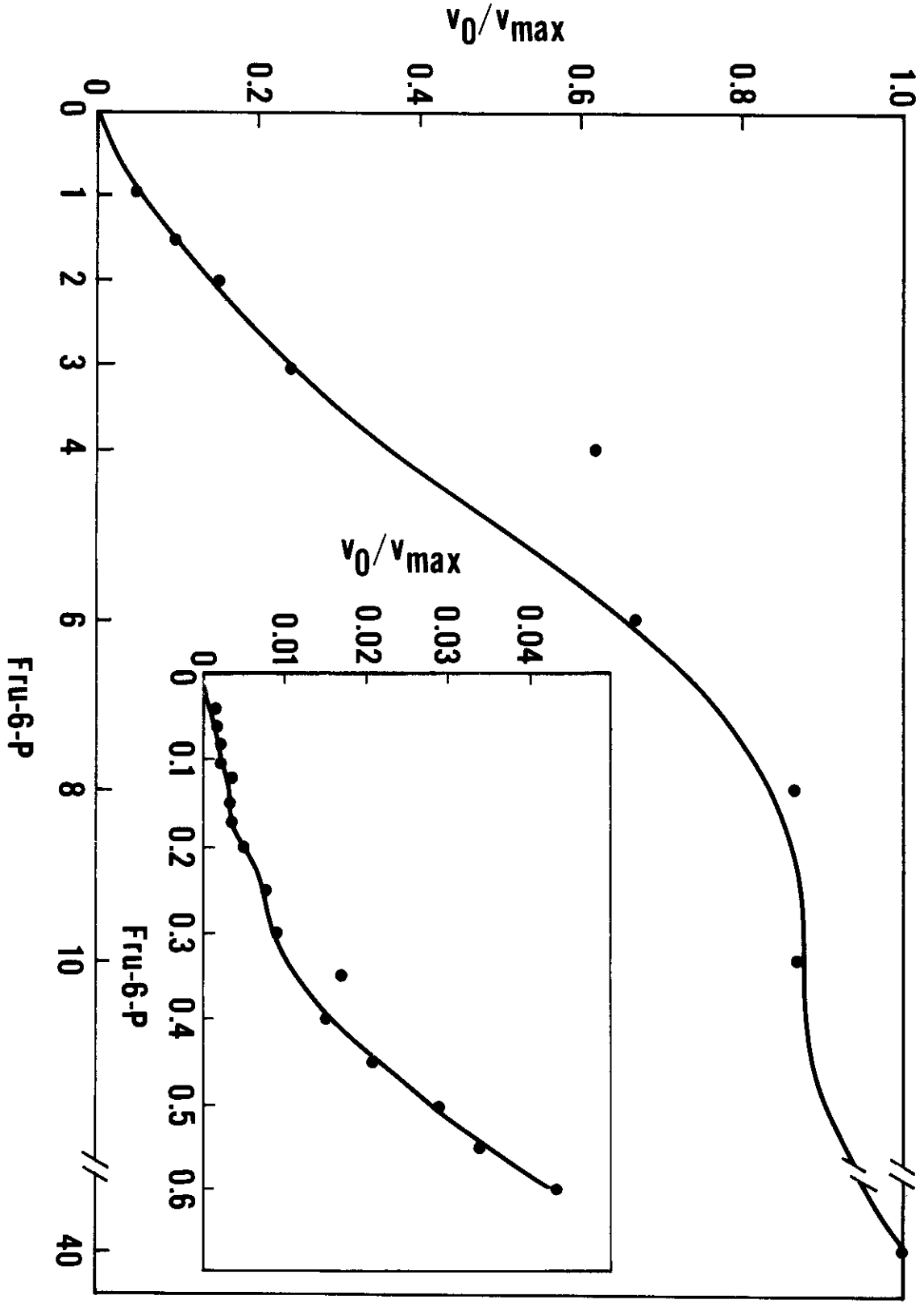

v_0/v_{max}
0
0.2
0.4
0.6
0.8
1.0
0
1
2
3
4
6
8
10
40
Fru-6-P
v_0/v_{max}
0
0.01
0.02
0.03
0.04
0
0.1
0.2
0.3
0.4
0.5
0.6
Fru-6-P

Figure 4

Fructose 6-phosphate saturation curve of phosphofructokinase from γ-aminobutyric acid-treated muscle. For description see Fig. 2 and Methods.

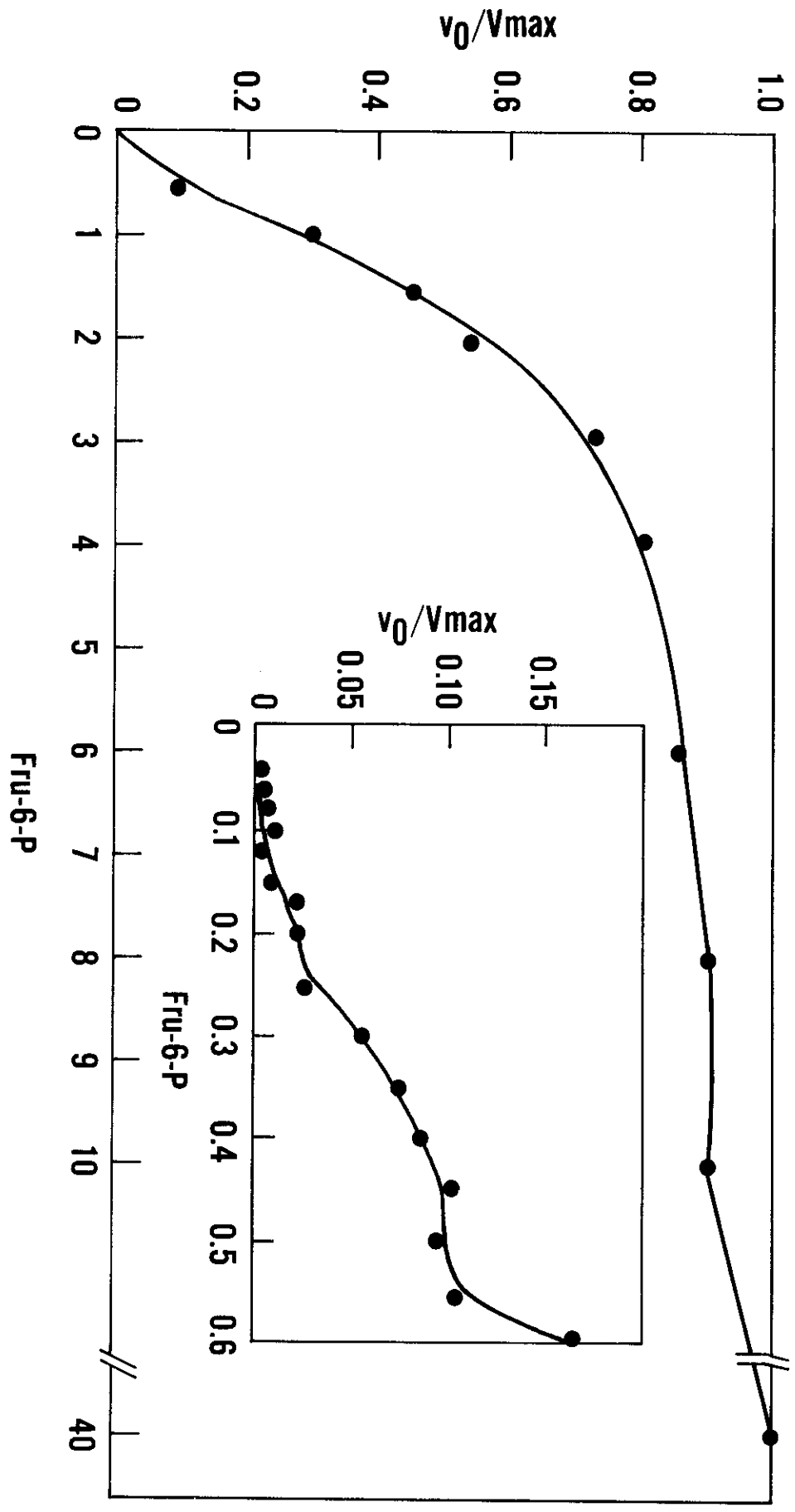

v_0/Vmax
0
0.2
0.4
0.6
0.8
1.0
0
1
2
3
4
5
6
7
8
9
10
40
Fru-6-P
v_0/Vmax
0
0.05
0.10
0.15
0
0.1
0.2
0.3
0.4
0.5
0.6
Fru-6-P

Figure 5

Fructose 6-phosphate saturation curve of phosphofructokinase from levamisole-treated muscle. For description see Fig. 2 and Methods.

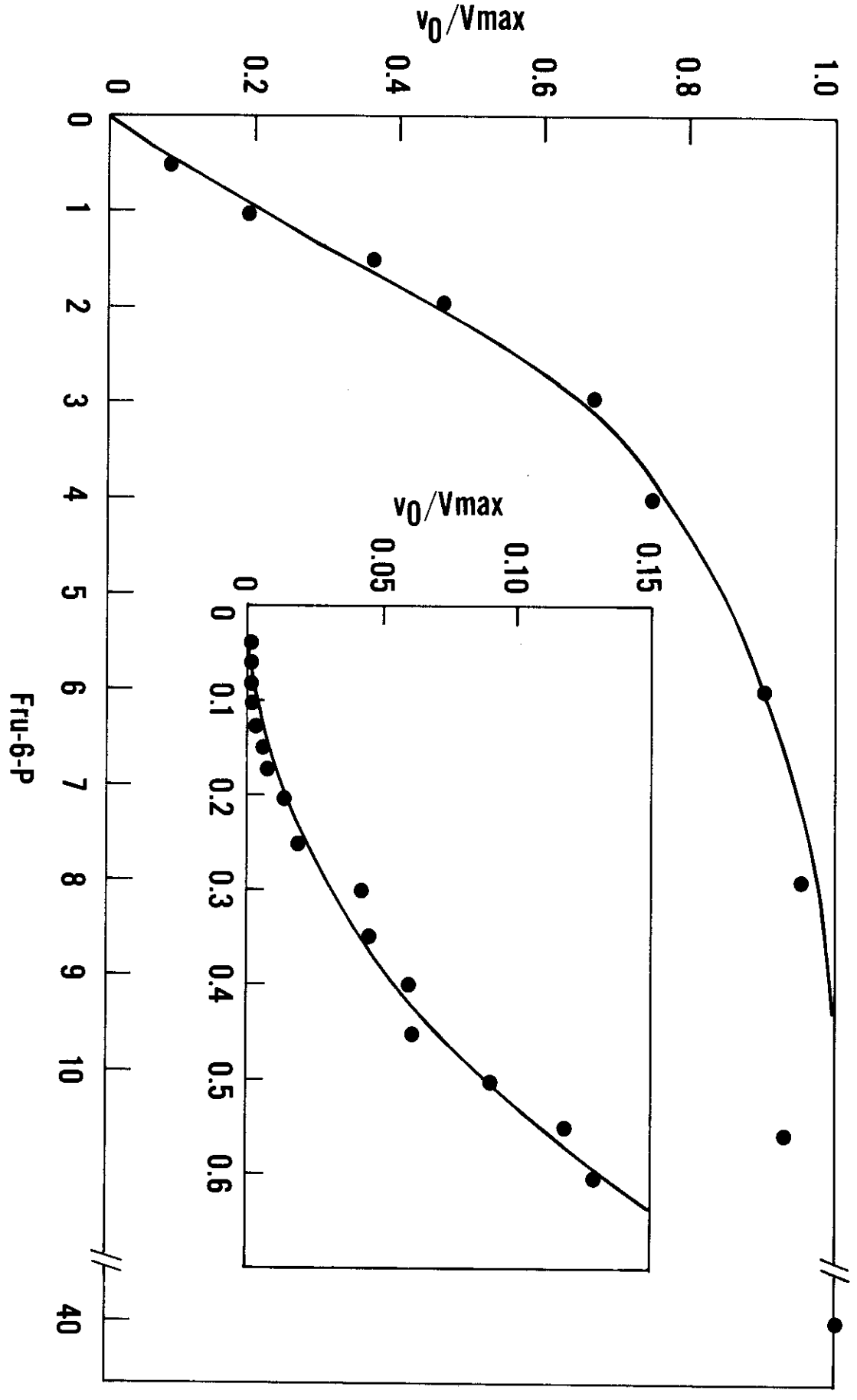

$v_0/Vmax$
0
0.2
0.4
0.6
0.8
1.0
0
1
2
3
4
5
6
7
8
9
10
40
Fru-6-P
$v_0/Vmax$
0
0.05
0.10
0.15
0
0.1
0.2
0.3
0.4
0.5
0.6

Figure 6

Fructose 6-phosphate saturation curve of phosphofructokinase from control-treated muscle. For description see Fig. 2 and Methods.

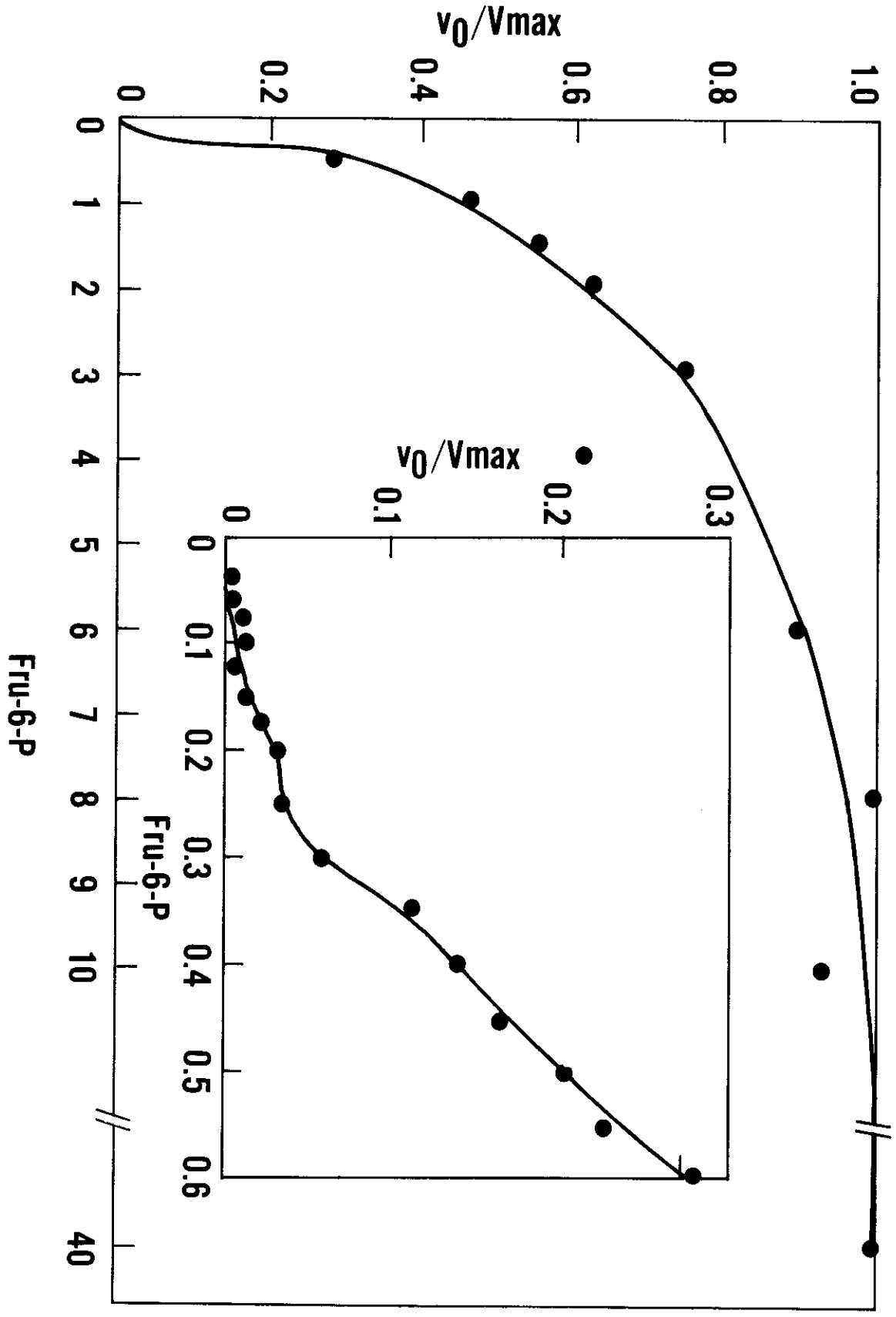

v_0/Vmax
0
0.2
0.4
0.6
0.8
1.0
0
1
2
3
4
5
6
7
8
9
10
40
Fru-6-P
v_0/Vmax
0
0.1
0.2
0.3
0
0.1
0.2
0.3
0.4
0.5
0.6
Fru-6-P

TABLE III

PERCENT V_{MAX} OF PHOSPHOFRUCTOKINASE ISOLATED FROM MUSCLE-CUTICLE OF ASCARIS SUUM AT 40, 100, 200 µM FRUCTOSE 6-PHOSPHATE[1]

Experiment[2]	Fru-6-P (µM)	% V_{max}
Acetylcholine	40	0.038
	100	0.15
	200	0.55
γ-aminobutyric acid	40	0.025
	100	0.12
	200	0.51
Levamisole	40	0.046
	100	0.13
	200	0.40
Serotonin	40	0.125
	100	0.23
	200	0.50
Control	40	0.06
	100	0.16
	200	0.43

[1]The assay contained 50 mM imidazole/HCl, pH 6.8, 5.25 mM potassium phosphate, 8 mM $MgCl_2$, 101.3 mM KCl, 3 mM ATP, 1 mM ADP, 0.36 mM AMP, 25 µM Glc-1,6-P_2, 0.2 mM NADH, 50 µg of aldolase, 30 µg of glycerolphosphate dehydrogenase, and 10 µg of triosephosphate isomerase. The reaction was started by addition of 4.5 µgram of Ascaris phosphofructokinase.

[2]Ascaris muscle-cuticle (50 grams) was used for each experiment Incubated for 1 hour in 50 µM solutions of acetylcholine; γ-aminobutyric acid; levamisole and serotonin. Control muscle-cuticle sections were incubated for the same period of time in perfusion buffer (see Methods).

CHAPTER IV

DISCUSSION

A new procedure has been developed to concentrate phosphofructokinase purified from the muscle of Ascaris suum according to the methods developed by Starling et al. (1982). When this method was employed for concentration of phosphofructokinase, a higher yield was obtained, and the enzyme exhibited a specific activity in the range of 120 to 150 units/mg. This value is at least two times higher than when ammonium sulfate was used to concentrate the enzyme (Starling et al., 1982). This method of concentration of the enzyme has been routinely carried out at least 20 times with similar results.

Hofer and Fürst (1976) reported that a maximum of one covalently-bound phosphate per subunit can be found in a phosphorylated form of the phosphofructokinase isolated from rabbit muscle. However, when the muscle was stimulated, purified phosphofructokinase contained two phosphates per subunit (Hofer and Sorenson-Ziganke, 1979). Hofer et al. (1982) reported that when Ascaris phosphofructokinase was isolated in a higher phosphorylated form (two phosphates/subunit) and assayed under physiological conditions, it carried out catalysis two times faster than the enzyme with

a lesser degree of phosphorylation (one phosphate/subunit). Previous work in this laboratory has demonstrated that there are at least two different methods of control of phosphorylation of carbohydrate metabolizing enzymes in Ascaris muscle. One is by a cAMP-mediated pathway. Donahue et al. (1981) have demonstrated that the putative hormone, serotonin, causes an increase in the level of cAMP, which results in an activation of phosphorylase and inactivation of glycogen synthase. The other pathway is a cAMP-independent pathway. This mechanism is mediated via the neurotransmitters, acetylcholine and γ-aminobutyric acid. This pathway has been shown indirectly to involve phosphorylation via glycogen synthase and phosphorylase (Donahue et al., 1982). In addition, it has been established that the anthelmintic drug levamisole causes dephosphorylation of the glycogen metabolizing enzymes, and this dephosphorylation has been shown to occur via a cyclic AMP-mediated pathway (Donahue et al., 1983). It was of importance to determine the in vivo effect of these compounds on the phosphorylation and kinetic activity of phosphofructokinase since this would be a possible method of correlating the mode of regulation of glycogenolysis and glycolysis. The results indicated that when endogenous phosphatase was inhibited during the purification procedure, phosphate analysis showed that phosphofructokinase contained 6.0-12.8 moles of phosphate/tetramer. The amount of phosphate covalently bound to the enzyme varied, depending on the manner the muscle was treated prior to purification;

the control preparation contained the highest phosphate, and the levamisole treated preparation the lowest.

As has been reported by Donahue et al. (1983), incubation of muscle-cuticle of Ascaris with levamisole caused a contraction followed by a flaccid paralysis. The results presented here showed that incubation of Ascaris muscle with levamisole did not increase the degree of phosphorylation, but instead, phosphofructokinase contained only half as much phosphate as compared to the control enzyme. Donahue et al. (1983) correlated flaccid paralysis in levamisole-treated Ascaris with an increase in activity in glycogen synthase, an inactivation of phosphorylase activity and concomitant decrease in cAMP levels and cAMP-dependent protein kinase activity. Therefore, incubation of Ascaris muscle with levamisole causes inhibition of the glycogenolytic cascade and the shunting of glucose into glycogen (Donahue et al., 1983).

Although the precise target of levamisole action in cAMP-mediated metabolism is not known at present, the results would certainly suggest that it might be in the area of response to serotonin. Serotonin has been identified as a neurotransmitter which mediates contraction in other invertebrate muscle (Mansour, 1979). In contrast, Donahue et al. (1981) found no muscle contraction or relaxation in an Ascaris muscle preparation perfused with serotonin. However, serotonin perfusion resulted in an increase in cAMP levels and activation of phosphorylase and inactivation of glycogen synthase.

The present results indicated that serotonin also increases phosphofructokinase activity, in spite of the fact that enzyme from serotonin-treated muscle contained 2.2 moles of phosphate/subunit as compared to 3.4 moles phosphate/subunit in the case of the control preparation. Under similar conditions, enzyme from serotonin-treated muscle had twice the activity as compared to a control. Furthermore, enzyme from levamisole-treated muscle with half the phosphate, as compared to control exhibited similar activity as the control. Therefore, there is reason to believe that the phosphofructokinase from Ascaris has several distinct phosphorylation sites.

The results indicate that there is one site of phosphorylation which somehow has been protected, since it was not possible to obtain a form of enzyme that contained less than 1.5 moles of phosphate/subunit. At this point, it appears that only one site is responsible for increased catalytic activity of the enzyme, and in order for Ascaris phosphofructokinase to exhibit its maximum activity, this particular site has to undergo phosphorylation. This was the case with serotonin-treated phosphofructokinase containing a lesser degree of phosphorylation as compared to the control preparation. The other phosphorylation site may function in self-association or dissociation for an alternative mode of enzyme regulation. Nevertheless, the role of phosphofructokinase phosphorylation in the regulation of carbohydrate metabolism of Ascaris suum muscle is apparent, and the modulators that regulate these reactions promise to be equally complex.

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