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PHOSPHOFRUCTOKINASE IN RAT JEJUNAL MUCOSA

SUBCELLULAR DISTRIBUTION, ISOLATION, AND CHARACTERIZATION

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SUMMARY

1. The subcellular distribution and certain characteristics of phosphofructokinase (ATP: D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) in rat jejunal mucosa were studied. 88% of the total phosphofructokinase activity was recovered in the 105 000 × *g* supernatant.

2. A 10-fold purification of jejunal phosphofructokinase was achieved by differential centrifugation, ammonium sulfate precipitation, and Sephadex gel filtration. Our studies of the gel-filtered enzyme suggest that the active enzyme may exist as a protein with a molecular weight of 190 000. The jejunal phosphofructokinase preparation demonstrates similar kinetic properties with respect to ATP, Mg^{2+}, and fructose-6-phosphate to those demonstrated for phosphofructokinase from other tissue sources.

3. Jejunal phosphofructokinase is stabilized by SO_4^{2-} and could be chromatographed in the absence of substrates, products, metal cations, or adenine nucleotides.

4. Na^+ concentrations of 10 mM or lower inhibit jejunal phosphofructokinase activity while K^+ has no apparent effect.

INTRODUCTION

Phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) represents an important control point in glycolysis because of its low activity, one-way reaction and allosteric properties[1,2]. Because of the large variety of compounds that can influence its activity, phosphofructokinase is one of the most complex enzymes that has been studied[2,3]. In addition to inhibition by ATP, citrate, and Mg^{2+}, phosphofructokinase activity is enhanced or deinhibited by NH_4^+, K^+, P_i, SO_4^{2-}, fructose 6-phosphate, fructose diphosphate, ADP, AMP, and cyclic AMP[1-4]. Of interest is the inhibition of skeletal muscle phosphofructokinase by Ca^{2+} (see ref. 5). These studies were undertaken because we were unable to obtain optimal activity of phosphofructokinase from jejunal mucosa using previously described homogenizing and assay media[6-8]. These results indicate that SO_4^{2-} is required to stabilize jejunal phosphofructokinase and that this enzyme is inhibited by Na^+.

MATERIALS AND METHODS

Tissue preparation

Male Sprague Dawley rats from BioScience Laboratories, Berkeley, California, weighing from 250 to 300 g were killed by decapitation. The abdominal cavity was incised and a 15-cm section of the jejunum, 15 cm below the pyloric sphincter, was removed, slit longitudinally, rinsed once with distilled water, and blotted dry. The mucosa was scraped off and weighed. Tissues from four animals were combined and homogenized, 50 mg/ml buffer, in a Duall glass tissue grinder. The homogenizing buffer consisted of 100 mM Tris–sulfate (pH 7.6), 5.0 mM dithiotheitol, 2.0 mM EDTA (tetrasodium salt). All procedures were conducted at 4°.

For the enzyme distribution study, the homogenate was first centrifuged at 750 × *g* for 10 min in a Sorvall Model RC 2-B refrigerated centrifuge. The sediment was resuspended in the original volume and the supernatant was centrifuged at 20 000 × *g* for 20 min. The 20 000 × *g* supernatant was again centrifuged at 105 000 × *g* for 30 min in a Spinco Model L centrifuge. From each fraction, 10-μl aliquots were taken for assay.

Ammonium sulfate precipitation of phosphofructokinase

Crystalline ammonium sulfate was slowly added to the 105 000 × *g* supernatant to 30% saturation. The solution was stirred slowly with a magnetic stirrer for 20 min and then centrifuged at 105 000 × *g* for 20 min. The supernatant was collected and sufficient ammonium sulfate was added to bring the solution to 50% saturation. Stirring and centrifugation were repeated. The sediment from the 50% saturated fluid was resuspended in homogenizing buffer at approximately 6–10 mg/ml. The protein was determined by the method of LOWRY *et al.*[9], using bovine serum albumin as standard.

Column chromatography

1 ml of the ammonium sulfate precipitated phosphofructokinase fraction was applied to a Sephadex G-200 column, 2.5 cm × 32 cm, and eluted with buffer consisting of 100 mM Tris–sulfate, 2.0 mM dithiothreitol, pH 7.6. The flow rate was adjusted to 0.4 ml/min and the fractions collected were monitored with a Gilford Model 2400 spectrophotometer at 280 mμ. The protein value was determined by comparison to known quantities of bovine serum albumin dissolved in column elution buffer. Fractions that had the highest phosphofructokinase activities were combined and used for the enzyme study. A periodic check was made on the characteristics of the column by chromatographing 1 ml of standards made up of 0.1% blue dextran, 25 μl (1.2 units) commercial phosphofructokinase from rabbit muscle (55 units per mg), 2.0 mg aldolase, 2.0 mg ATP (disodium salt). All biochemicals and enzymes were purchased from Sigma Chemical Co., St. Louis, Mo.

Phosphofructokinase assay

The basic medium consisted of 10.0 mM Tris–sulfate, 0.5 mM dithiothreitol, 0.25 mM NADH. The standard assay had, in addition, 1.0 mM fructose 6-phosphate, 0.5 mM ATP, 0.5 mM $MgSO_4$. Fructose 6-phosphate was omitted from the control curvette. In the study of cellular distribution of phosphofructokinase, 10-μl aliquots

of each fraction were used as the enzyme source for each assay. Before the start of the reaction, 10 μl of combined coupling enzymes was added to each assay. The coupling enzymes in each assay were equivalent to 1.3 units of α-glycerophosphate dehydrogenase, 0.3 units of aldolase, 12.4 units of triose phosphate isomerase. This enzyme mixture had previously been dialyzed against the homogenizing buffer for 15 h at 4°, 1 ml of enzyme per 1000 ml buffer. In the case of phosphofructokinase that had been eluted from the column, 50 μl of the column eluate was used per assay. The final volume was 1 ml. The reaction was monitored with a Gilford Model 2400 spectrophotometer at 340 mμ, 27°, by measuring the oxidation of NADH. Initial studies indicated that the optimal activity was obtained at pH 7.6, and all assays were performed at this pH. Spectrophotometric determination of phosphofructokinase in rabbit muscle utilizing our standard medium revealed 83% of the activity stated by the supplier.

RESULTS

Subcellular distribution of phosphofructokinase activity

The 105 000 × *g* supernatant had 88% of the jejunal mucosa phosphofructokinase activity (Table I); the remainder was distributed among the three centrifu-

TABLE I

ISOLATION OF PHOSPHOFRUCTOKINASE FROM RAT JEJUNAL MUCOSA

The standard medium was used for the assays of all the fractions. Phosphofructokinase activity is expressed as μmoles or nmoles substrate utilized as indicated.

Source of phosphofructokinase	*Protein (mg/g mucosa)*	*Phosphofructokinase activity*		
		μmoles/min per g mucosa	*% Homogenate activity*	*nmoles/min per mg protein*
Homogenate	159.0	12.60	—	79
750 × *g* sediment	76.5	0.88	7.0	11
20 000 × *g* sediment	15.5	0.10	0.8	6
105 000 × *g* sediment	7.6	0.37	2.9	48
105 000 × *g* supernatant	69.9	11.12	88.2	159
$(NH_4)_2SO_4$ precipitation fractions				
0–30% satn.	17.3	0.78	6.2	45
30–50% satn.	21.5	10.19	80.8	472
Gel filtration with Sephadex G-200				
Fraction 33	—	—	—	792

gation sediments. The activity (7%) in the 750 × *g* sediment may have been attributable to contamination or to incomplete cell breakage. Negligible activity was found in the 20 000 × *g* sediment. The 3% of the phosphofructokinase activity present in the 105 000 × *g* sediment may be attributable to volume entrapment. Of the total phosphofructokinase detected in the homogenate, 99% was recovered in the 4 subcellular fractions.

Ammonium sulfate precipitation of supernatant phosphofructokinase

Precipitation of the supernatant with ammonium sulfate yielded 31% of the

protein (Table I) which had 92% of the phosphofructokinase activity in the 30–50% saturation fraction. This fraction had 14% of the protein and 81% of the activity of the original homogenate, equivalent to 6-fold purification at this stage. Assay of the enzyme after it had been frozen for five weeks at −20° indicated no loss of activity. Similar ammonium sulfate precipitation results have been observed for primate spermatozoa[13]. Only 7% of the phosphofructokinase activity was detected in the 0–30% saturation sediment, and no further attempt was made to recover the phosphofructokinase in this fraction. An enzyme concentration study was made with the dialyzed ammonium sulfate-precipitated phosphofructokinase to determine the optimal quantity of enzyme required for the assay as well as the effect of dilution on enzyme activity. The precipitated enzyme concentration can be diluted as low as 68 μg/ml, 0.7 μg/assay, without losing its specific activity, indicating that the assay of phosphofructokinase subsequent to chromatography on Sephadex G-200 column should not be affected by dilution during elution from the column. All assays for phosphofructokinase were performed using an enzyme-protein concentration in the optimal range.

Sephadex G-200 column chromatography of phosphofructokinase

Chromatography of phosphofructokinase on a Sephadex G-200 column separated the 30–50% ammonium sulfate precipitate into two distinct zones of protein elution (Fig. 1). The first, a large sharp peak, was eluted in Fractions 20–26, followed by a minor peak at Fraction 27. The second zone consisted of a broad slope with half the absorbance of the first. The first peak coincided with the blue dextran, while the broad slope approximated the aldolase of the standards. The commercial phosphofructokinase prepared from rabbit muscle was eluted with the blue dextran. The jejunal mucosal phosphofructokinase, however, was situated between the two zones, overlapping into both areas. The highest activity was in Fractions 29–33. No detectable phosphofructokinase was found in other regions. The total recovery of all the

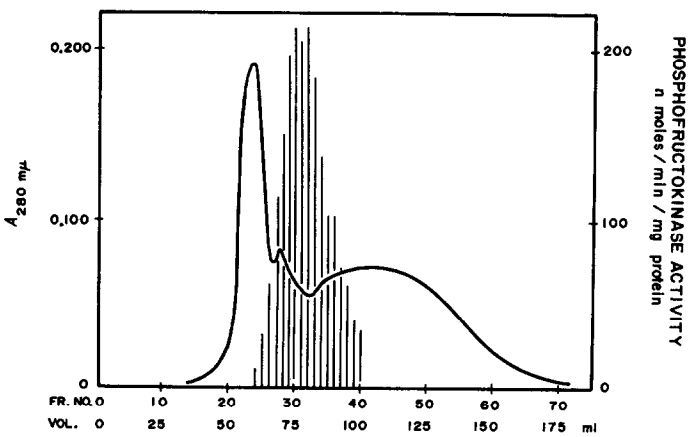


Fig. 1. Sephadex G-200 chromatography of jejunal mucosal phosphofructokinase. The elution buffer contained 100 mM Tris–sulfate (pH 7.6) and 2.0 mM dithiothreitol. Standard medium was used to assay the phosphofructokinase activity. The vertical bars represent the total phosphofructokinase activity as nmoles substrate metabolized per min per fraction and the solid line the protein concentration.

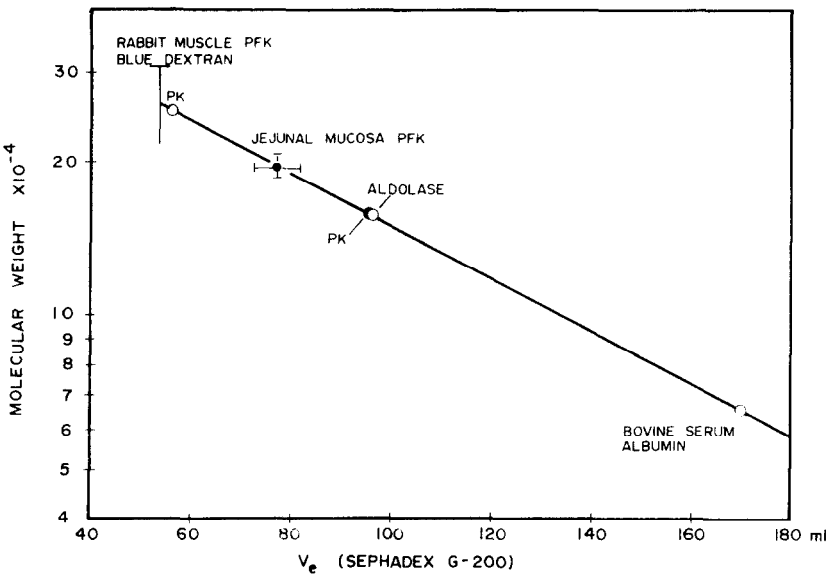
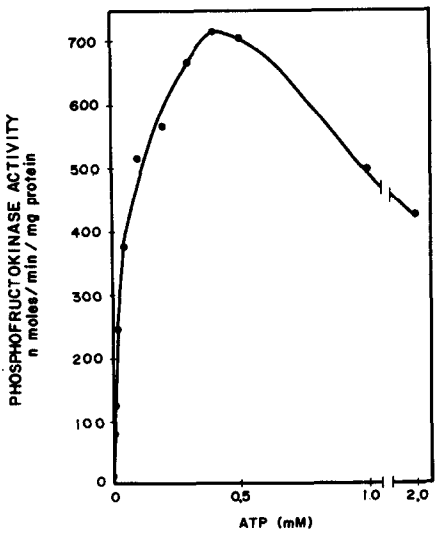


Fig. 2. Estimation of the mol. wt. of jejunal mucosal phosphofructokinase by Sephadex G-200 column chromatography. The elution buffer contained 100 mM Tris-sulfate (pH 7.6) and 2.0 mM dithiothreitol. The flow rate was adjusted to 0.4 ml/min. The pyruvate kinase (PK) and phosphofructokinase (PFK) peaks were determined by assaying for their activity. The elution patterns of rabbit muscle phosphofructokinase, blue dextran, aldolase, and bovine serum albumin represent the means of three or more studies.

fractions containing phosphofructokinase was 69% of the applied sample. The specific activity expressed as nmoles substrate metabolized per min per mg protein of the more active fraction ranged from 683 to 792, representing a 10-fold purification of the original homogenate. The specific activity of the combined fractions was 715 nmoles per min per mg protein.

ANDREWS[10] devised a method for estimating the mol. wt. of proteins by gel filtration. Using a similar technique, an estimation of the jejunal mucosa phosphofructokinase was made (Fig. 2). The standards were: pyruvate kinase, mol. wt. 250 000[11] and 166 000[12]; aldolase, 150 000; and bovine serum albumin, 66 000. Pyruvate kinase was detected by the assay method of BÜCHER AND PFLEIDERER[12]. The jejunal mucosa phosphofructokinase was chromatographed on the same column on 6 different occasions. The phosphofructokinase peak was eluted at volume 77.7 ± 5.2 ml. The estimated mol. wt. for this enzyme was 190 000 ± 20 000.

HOSKIN AND STEPHENS[13] found that phosphofructokinase was inactivated by passage through Sephadex G-200 column, necessitating the addition of ATP and $MgSO_4$ to the elution buffer to stabilize the enzyme. Using the methods reported here, the jejunal mucosa phosphofructokinase appeared to be stable without any additives, presumably due to the effects of SO_4^{2-}. The enzyme could be assayed directly, but for storage the column fractions were combined with the dialyzed coupling enzymes. When the dialyzed coupling enzymes were combined with the column eluate (1:5), the mixture could be kept frozen for at least a week with only 10% loss of activity. To avoid the addition of another protein such as albumin, these coupling enzymes



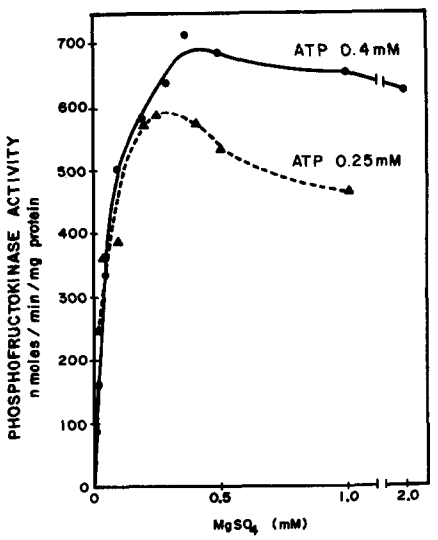


Fig. 3. Effect of ATP on gel-filtered jejunal mucosal phosphofructokinase. The assay medium contained 1.0 mM fructose 6-phosphate, 0.4 mM $MgSO_4$, and Tris–ATP in the indicated concentrations. Phosphofructokinase activity is expressed as nmoles substrate metabolized per min per mg protein.

Fig. 4. Effect of Mg^{2+} on gel-filtered jejunal mucosal phosphofructokinase. Fructose 6-phosphate, 1 mM, was used. Tris–ATP and Mg^{2+} were omitted from the medium and added independently back to the assay in the required concentrations. The ATP concentrations were 0.4 mM (●); and 0.25 mM (▲). Phosphofructokinase activity is expressed as nmoles substrate metabolized per min per mg protein.

were used. The stabilization of phosphofructokinase activity may, however, be attributable to the higher protein concentration in the solution rather than to a specific effect of the dialyzed coupling enzymes.

Effects of ATP, Mg^{2+}, and fructose 6-phosphate on phosphofructokinase activity

The kinetic properties of jejunal phosphofructokinase were studied using the

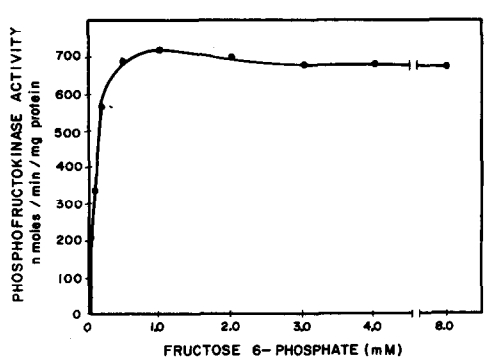


Fig. 5. Effect of fructose 6-phosphate on gel-filtered jejunal mucosal phosphofructokinase. ATP, 0.4 mM, and $MgSO_4$, 0.4 mM, were used. Fructose 6-phosphate was omitted from the medium and reintroduced into the assay independently in the required concentration. Phosphofructokinase activity is expressed as nmoles substrate metabolized per min per mg protein.

gel-filtered enzyme. 1.4 μg protein per assay. The optimal ATP concentration for this amount of enzyme protein was 0.4 mM and higher concentrations inhibited phosphofructokinase activity (Fig. 3). When the ATP concentration was maintained at this optimal level (0.4 mM), the optimal level of Mg^{2+} was found to be similar (Fig. 4). Reduction of ATP to 0.25 mM resulted in a corresponding decrease of Mg^{2+} requirements. These observations are similar to those of PASSONNEAU AND LOWRY[1] who reported that a Mg^{2+} to ATP ratio of 1.1 to 1.2 must be maintained for optimal activity for rat brain phosphofructokinase. When NaATP was used, a higher Mg^{2+} to ATP ratio was required for optimal activity for jejunal phosphofructokinase. Concentrations of Mg^{2+} above the optimal level inhibited jejunal phosphofructokinase. In addition to being a substrate for phosphofructokinase, fructose 6-phosphate acted as a deinhibitor of ATP and Mg^{2+}. However, at optimal levels of ATP and Mg^{2+}, increasing the fructose 6-phosphate concentration beyond the level of maximum activity produced only minimal inhibition (Fig. 5). These studies indicate that the kinetic properties of phosphofructokinase from jejunal mucosa are similar to those described for phosphofructokinase from other sources[1,2,13,14].

Effects of SO_4^{2-}, Na^+, and K^+ on phosphofructokinase activity

In preliminary studies we were unable to obtain optimal phosphofructokinase activity utilizing Cl^--containing, SO_4^{2-}-free homogenizing[7] and assay[6] media. This suggested that SO_4^{2-} might have a stabilizing effect on jejunal phosphofructokinase. Jejunal phosphofructokinase activity was decreased or increased by the removal or addition of SO_4^{2-} (Table II). When SO_4^{2-} was removed from the medium by substituting Tris–HCl for Tris–sulfate and $MgCl_2$ for $MgSO_4$, the phosphofructokinase activity of the gel-filtered enzyme was reduced to 40% of control activity. The addition of 1 mM Na_2SO_4 restored the phosphofructokinase activity to its original level. The gel-filtered enzyme was dialyzed to further remove SO_4^{2-} from the enzyme preparation,

TABLE II

EFFECT OF SO_4^{2-} ON GEL-FILTERED JEJUNAL PHOSPHOFRUCTOKINASE

Phosphofructokinase activity of the gel-filtered preparation was measured in the standard medium containing 34 mM SO_4^{2-} and in a SO_4^{2-} free medium in which Tris–HCl and $MgCl_2$ replaced Tris-sulphate and $MgSO_4$. $Na_2SO_4$was added to achieve the indicated concentrations. The dialyzed enzyme was dialyzed for 3 h in 100 mM Tris–HCl (pH 7.6), 2 mM dithiothreitol. Phosphofructokinase activity is expressed as nmoles substrate metabolized per min per mg protein.

Media	*Na_2SO_4 concn. (mM)*	*Gel-filtered phosphofructokinase activity*		*Dialyzed phosphofructokinase activity*	
		nmoles/min per mg	*% Control*	*nmoles/min per mg*	*% Control*
Standard medium	0	715	100	97	100
SO_4^{2-}-free medium	0	282	39.5	11	11.3
	0.01	333	46.6	—	—
	0.05	392	54.8	—	—
	0.20	403	56.4	—	—
	0.50	505	70.7	—	—
	1.00	712	99.6	97	98.9

TABLE III

EFFECTS OF Na^+ AND K^+ ON JEJUNAL MUCOSAL PHOSPHOFRUCTOKINASE

NaCl and KCl were added to achieve the indicated concentrations. The dialyzed enzyme was dialyzed for 3 h in 100 mM Tris–HCl (pH 7.6), 2 mM dithiothreitol. Phosphofructokinase activity is expressed as nmoles substrate metabolized per min per mg protein.

Salt	*Concn. (mM)*	*Gel-filtered phosphofructokinase activity*		*Dialyzed phosphofructokinase activity*	
		nmoles/min per mg	*% Control*	*nmoles/min per mg*	*% Control*
NaCl	0	712	100	96	100
	1.0	688	96.6	—	—
	4.0	—	—	92	95.8
	5.0	619	86.9	—	—
	10.0	607	85.2	61	63.5
	20.0	539	75.7	46	47.9
	40.0	—	—	24	25.0
KCl	1.0	718	100.8	113	117.7
	2.0	707	99.3	102	106.2
	5.0	718	100.8	98	102.1
	10.0	719	100.9	98	102.1

and after 3 h of dialysis the specific activity was reduced to 97 nmoles (Table II). When this dialyzed phosphofructokinase preparation was assayed in the absence of SO_4^{2-} only 11% of control activity was observed while the addition of 1 mM Na_2SO_4 restored activity to control values. Raising the Na_2SO_4 concentrations above 1 mM produced a reduction in phosphofructokinase activity. Since the standard assay medium contained a SO_4^{2-} concentration of 34 mM, this inhibition was attributed to Na^+.

The influence of Na^+ and K^+ on the gel-filtered and gel-filtered-dialyzed phosphofrunctokinase preparations was studied (Table III). Increasing the NaCl concentration produced a marked reduction in phosphofructokinase activity which was most striking in the dialyzed enzyme. Increasing the concentration of KCl to 10 mM produced no alteration in phosphofructokinase activity. The studies suggest that jejunal phosphofructokinase activity is inhibited by Na^+ but not by K^+.

DISCUSSION

These studies indicate that jejunal phosphofructokinase activity is essentially entirely supernatant. Subcellular fractionation of rat small intestinal mucosa is particularly difficult[15] because of the mucous content. Phosphofructokinase activity is largely supernatant in several tissues[14,16] but significant activity has been reported in the microsomal fractions of liver[16]. Our studies suggest that less than 3% of jejunal phosphofructokinase is associated with this fraction.

Our observations suggesting that jejunal phosphofructokinase may have its active form at a mol. wt. of 190 000 is of considerable interest. Extensive studies of heart and skeletal muscle phosphofructokinase have established a molecular weight of approx. 400 000 for this species[2,3,14,17]. Muscle phosphofructokinase can be dissoci-

ated into protomers with mol. wts. approximating 90 000 and 180 000, but these molecules have been less than 5% of the original activity[3,17]. Rabbit muscle phosphofructokinase has been dissociated into an inactive form of 192 000 with restoration of full activity with reaggregation[17]. These studies clearly demonstrate that the smallest active molecular species of muscle phosphofructokinase has a molecular weight of almost 400 000[3,17]. The mol. wt. estimation for jejunal phosphofructokinase must be considered tentative pending verification by other procedures. Our studies do not exclude the possibility that jejunal phosphofructokinase dissociates into smaller aggregates during gel filtration and reaggregates in the presence of ATP, fructose 6-phosphate, and Mg^{2+} in the assay media. Two facts, however, suggest that the active form of jejunal phosphofructokinase may exist as a 190 000 mol. wt. protein. Rabbit muscle phosphofructokinase chromatographed in the same manner was eluted as a larger mol. wt. protein, and no active form was found in any other fractions. Conversely, no jejunal phosphofructokinase activity was detected in fractions coinciding with the muscle phosphofructokinase.

Jejunal phosphofructokinase can be chromatographed without the addition of substrates, products, or Mg^{2+}. P_i and SO_4^{2-} have been demonstrated to deinhibit or activate various phosphofructokinase preparations[1,3,18] and the presence of 34 mM SO_4^{2-} stabilized jejunal phosphofructokinase. Primate spermatozoa phosphofructokinase isolated in a similar fashion was inactivated by gel-filtration and required the addition of ATP and $MgSO_4$ to the elution buffer to stabilize the enzyme[13]. AMP was required to stabilize muscle and erythrocyte phosphofructokinase during chromatography[14].

The inhibition of phosphofructokinase by Na^+ may be of physiologic significance in the control of glycolysis in intestinal mucosa. Glucose transport across the mucosal surface is dependent on Na^+ transport[19,20], and the entry of Na^+ and glucose may serve to modulate phosphofructokinase activity. The inhibition of yeast phosphofructokinase by sodium phosphate and sodium sulfate may be attributable to the Na^+ (Table III, ref. 4). Ca^{2+} is critical for skeletal muscle function. MARGRETH *et al.*[5] have demonstrated that Ca^{2+} inhibits frog skeletal muscle phosphofructokinase and suggested that this is of physiologic importance in regulating muscle glycolysis.

Jejunal phosphofructokinase activity is several-fold higher than we would have predicted from studies of other enzymes of carbohydrate metabolism in jejunal mucosa[6–8]. In tissues such as liver, brain, heart, and adipose tissue, the activity of phosphofructokinase and hexokinase are almost identical[21–23]. In jejunum, however, the phosphofructokinase activity of the homogenate is 3-fold higher than hexokinase activity while phosphofructokinase activity of the 105 000 $\times$ *g* supernatant is eight-fold higher than hexokinase activity (see ref. 7, 24). The studies of SRIVASTAVA *et al.*[24] suggest that hexokinase is the rate-limiting enzyme in glycolysis of intestinal mucosal preparations. The comparatively high level of jejunal phosphofructokinase that we observed also suggest that phosphofructokinase may not be as critical as hexokinase in the control of glycolysis in intestinal mucosa. Further investigation is necessary to clarify the physiologic importance of phosphofructokinase in the regulation of glycolysis in intestinal mucosa.

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