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The isolation and characterization of phosphofructokinase from the epithelial cells of rat small intestine

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1. Only a single phosphofructokinase isoenzyme is present in the mucosa of rat small intestine. 2. Mucosal phosphofructokinase was purified to yield a homogeneous preparation of specific activity 175 units/mg of protein. 3. The native enzyme is a tetramer, with monomer M_r 84500 ± 5000. 4. The native enzyme may be degraded by the action of endogenous proteinases to give two products with the same specific activity as the native enzyme: degradation occurs in the order native enzyme → proteolytic product 1 → proteolytic product 2. 5. Proteolytic product 1 has a greater mobility in cellulose acetate electrophoresis at pH 8 and binds more strongly to DEAE-cellulose than does native enzyme; the converse is true for proteolytic product 2. 6. Proteolytic product 1 is a tetramer with a monomer M_r about 74300; proteolytic product 2 is also a tetramer. 7. Native enzyme can only be prepared in the presence of proteinase inhibitors; partial purifications based on simple fractionation of crude mucosal extracts in the absence of proteinases inhibitors contain proteolytic product 2 as the main component and proteolytic product 1 together with little native enzyme. 8. Purified native mucosal phosphofructokinase displayed little co-operativity with respect to fructose 6-phosphate at pH 7.0 and was only weakly inhibited by ATP.

Simple preparations of rat small intestine *in vitro*, for example rings, slices, everted sacs and isolated loops perfused in non-segmented flow, are characterized by a high rate of aerobic glycolysis and also do not display a Pasteur effect, that is respiration does not control glycolysis (Ramaiah, 1974; Hanson & Parsons, 1976; Porteous, 1978). The locus of the Pasteur effect in other tissues is phosphofructokinase, and the lack of a Pasteur effect in simple intestinal preparations has been attributed to a relatively high ratio of activators to inhibitors present in epithelial cells, even under aerobic conditions, so that the activity of phosphofructokinase is unrestrained (Tejwani & Ramaiah, 1971; Tejwani *et al.*, 1974; Tejwani, 1978). This suggestion is in keeping with the conclusion drawn by Srivastava & Hübscher (1966) from studies on the rate of lactate production from different substrates by particle-free supernatants derived from homogenates of the mucosa of whole rat intestine that hexokinase is rate-limiting.

More recently, however, Jamal & Kellett (1983) have reported that under the experimental conditions chosen by Srivastava & Hübscher (1966), namely phosphate buffer (40mM, pH 7.8), phosphofructokinase is fully activated and does not display regulatory properties. Since the total activity of phosphofructokinase is 5–6-fold that of hexokinase, it was therefore inevitable that hexokinase should in those circumstances appear to be rate-limiting, for the experimental design permitted no other conclusion. Jamal & Kellett (1983) also reported that the susceptibility of mucosal phosphofructokinase to inhibition by ATP, when assayed in crude extracts under suboptimal conditions, is increased when rats have been starved for 48h. This observation suggests, though does not prove, that, contrary to earlier beliefs, phosphofructokinase does in fact play a significant role in the regulation of glycolysis in the epithelial cells of the rat small intestine. Further evidence for this idea is provided by the observation that vascularly perfused preparations of rat jejunum show far lower rates of glucose utilization than do simple intestinal preparations and also show a Pasteur-type effect in that glucose utilization is inversely related to the degree of oxygenation. Thus it appears that the lack of a Pasteur effect and the related favourable ratio of activators to inhibitors in simple intestinal preparations may have been an artefact caused by inadequate oxygenation (Lamers & Hülsmann, 1972; Hanson & Parsons, 1976; Porteous, 1978).

Detailed knowledge of the influence of effectors on phosphofructokinase activity is facilitated by the availability of purified, homogeneous enzyme. However, although the complete purification of phosphofructokinase has been described for many other tissues (Uyeda, 1979), this is not the case for intestine. Partial purifications of phosphofructokinase from rat jejunal mucosa have been described. However, these have resulted in preparations of very low specific activity, 0.8 unit/mg of protein, compared with values in the range 120–200 units/mg for highly purified enzyme from other tissues (Ho & Anderson, 1971; Tejwani & Ramaiah, 1971; Tejwani *et al.*, 1974). Moreover, the partial purification apparently did not include precautions to inhibit proteolysis during the isolation or storage, although the jejunum is the major site of peptide digestion in the gastrointestinal tract. Although the partially purified preparations have not been characterized in any detail, the report by Ho & Anderson (1971) suggests that mucosal phosphofructokinase is significantly different from other mammalian isoenzymes, since they found that it migrated on Sephadex G-200 as an active species of M_r 190000. All mammalian phosphofructokinases characterized to date have been found to be tetramers, of M_r about 320000; the tetramer is the minimal active unit, and dissociation to an inactive dimer occurs only below pH 7 (Goldhammer & Paradies, 1979). Present knowledge of the regulatory properties of mucosal phosphofructokinase is thus based solely on work with impure preparations, which apparently have very different characteristics from other mammalian phosphofructokinases.

The demonstration of a Pasteur-type effect in vascularly perfused intestine has prompted us to examine afresh the regulatory properties of mucosal phosphofructokinase, and as a prelude to kinetic studies we describe here the isolation and characterization of purified enzyme with a specific activity of 175 units/mg.

Materials and methods

All biochemicals were obtained from either Sigma or Boehringer–Mannheim and were used without further purification. All chemicals were AnalaR grade from BDH. Agarose for gel chromatography (Bio-Gel A-0.5m) was obtained from Bio-Rad Laboratories, ATP–agarose (N^6-amino-group linked) was from Sigma and DEAE-cellulose (DE52) from Whatman. Animals were female Wistar rats (250–300g) fed *ad lib* on standard laboratory diet (Oxoid, modified 41B).

Cellulose acetate electrophoresis

This was done at 4°C on strips (5.7cm × 14cm) of Whatman Cellogel in buffer (5mM-Tris/phosphate, pH 8.0) containing 5mM-$(NH_4)_2SO_4$, 5mM-2-mercaptoethanol, 0.1mM-EDTA and 0.1mM-ATP, at 300V (2mA/strip) for 2h. Enzyme activity was detected by use of the agar-gel staining technique described by Kemp (1971). In this case, however, because Cellogel was used, most of the enzyme remained on the cellulose acetate gel surface and the blue activity stains appeared on the cellulose acetate. If heavy loadings of enzyme were used, then staining bands also appeared in the activity gel.

Polyacrylamide-gradient slab-gel electrophoresis

This was done at room temperature in vertical slabs (170mm × 150mm × 1mm) of 4–12% acrylamide. The buffer system was the same as that used for cellulose acetate electrophoresis. Gels were first pre-equilibrated for 2h against the buffer, and then electrophoresis of protein samples (2–10μg each) was performed for 18h at 30mA/gel. Gels were stained for protein with Coomassie Blue.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

This was performed as described by Weber & Osborn (1969), except that samples were incubated with sodium dodecyl sulphate for 2min at 100°C in the presence of 1mM-phenylmethanesulphonyl fluoride to inhibit the action of endogenous proteinases (Pringle, 1970). Gels were stained with Coomassie Blue and scanned with a Joyce Loebl Chromscan 3 gel scanner.

Gel chromatography

Mucosal extracts were chromatographed on a column (38cm × 2.5cm) of Bio-Gel A-0.5m in extraction buffer (see below) and the peak of phosphofructokinase activity was determined. The M_r of phosphofructokinase was determined by reference to standards: ferritin, 444000; pyruvate kinase, 250000; aldolase, 158000; bovine serum albumin, 68000.

Enzyme assay

Phosphofructokinase activity under optimal conditions at pH 8.0 was measured at 27°C as described by Ling *et al.* (1965). Regulatory properties at pH 7.0 were determined as described by Hussey *et al.* (1977). One unit of activity is defined as the formation of 1μmol of fructose 1,6-bisphosphate/min.

Results and discussion

Purification of phosphofructokinase from rat jejunal mucosa

Storage and extraction of mucosa. Rats were killed by a blow on the head, followed by cervical fracture. The first 40cm of jejunum were quickly

removed, flushed with ice-cold extraction buffer (100mM-K_2HPO_4, pH8.0) containing 30mM-KF, 1mM-EDTA, 5mM-2-mercaptoethanol, 1mM-phenylmethanesulphonyl fluoride and 1mM-6-amino-n-hexanoic acid, opened by a lengthwise incision and gently blotted with tissue. The mucosa was then collected by gentle scraping of the luminal surface with a microscope slide and frozen immediately in liquid N_2. Mucosa was stored in a deep-freeze at −80°C until sufficient had been accumulated for a preparation, and then the frozen mucosa was homogenized directly without thawing in a Potter–Elvehjem homogenizer with 5vol. (v/w) of extraction buffer. The homogenate was then centrifuged at 75000*g* for 30min in an MSE HS25 centrifuge at 4°C, and the supernatant decanted through glass wool to remove lipid material. Less stringent procedures for collection, storage and extraction of mucosa resulted in large and often total loss of phosphofructokinase activity.

Ammonium sulphate fractionation. Solid $(NH_4)_2SO_4$ was added to the crude extract at 4°C with thorough stirring until 0.37 saturation and, after standing for 30min, the solution was centrifuged for 30min at 75000*g*. The precipitate was discarded and the fractional saturation of the supernatant increased to 0.57 with solid $(NH_4)_2SO_4$. After the solution had been left for 2h at 4°C, the precipitate was collected by centrifugation.

Chromatography on Sephadex G-100. The pellet from $(NH_4)_2SO_4$ fractionation was resuspended in extraction buffer and chromatographed on a column (2.5cm × 45cm) of Sephadex G-100 equilibrated with the same buffer. Phosphofructokinase was eluted at the void volume; fractions containing activity were pooled, precipitated at 0.6 saturation with $(NH_4)_2SO_4$ and the precipitate was collected again by centrifugation.

Affinity chromatography on ATP–agarose. This step was based on the method of Ramadoss *et al.* (1976), who reported the selective elution of muscle phosphofructokinase from ATP–agarose as the dead-end complex in the presence of fructose 6-phosphate and ADP. Because either fructose 6-phosphate or ADP independently caused slow elution of mucosal phosphofructokinase, the following procedure was adopted. The protein pellet was resuspended in extraction buffer and applied to a column (1.2cm × 5.0cm) of ATP–agarose equilibrated with extraction buffer. The column was first washed with 15 column vol. of extraction buffer and then with 20 column vol. of the same solution containing 1mM-fructose 6-phosphate and 1mM-ADP. Phosphofructokinase was quantitatively eluted as a sharp peak, and fractions containing activity were concentrated under N_2 in a pressurized ultrafiltration cell (Amicon) fitted with a Diaflo PM10 membrane with M_r cut-off limit 10000.

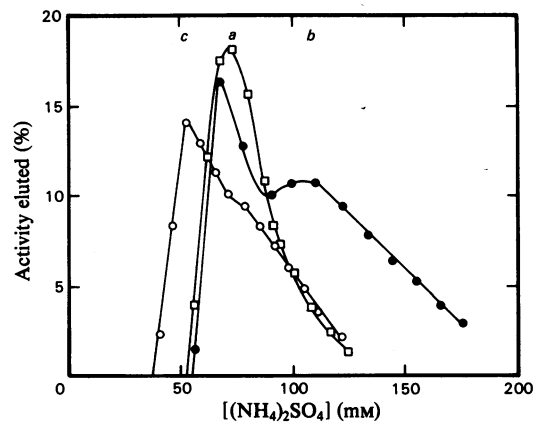

Fig. 1. *DEAE-cellulose chromatography of different mucosal phosphofructokinase fractions*

●, Separation of native enzyme and proteolytic product 1 in the last step of the purification of mucosal phosphofructokinase described in the text; □, crude mucosal extract prepared in the presence of proteinase inhibitors (1mM-phenylmethanesulphonyl fluoride, 0.5mg of soya-bean trypsin inhibitor/ml and 1mM-6-aminohexanoic acid); ○, crude mucosal extract prepared in the absence of proteinase inhibitors. Peak *a*, native enzyme eluted at 67mM-$(NH_4)_2SO_4$; peak *b*, proteolytic product 1 at 110mM-$(NH_4)_2SO_4$; peak *c*, proteolytic product 2 at 53mM-$(NH_4)_2SO_4$.

Chromatography on DEAE-cellulose. The column (1.5cm × 25cm) of Whatman DE-52 DEAE-cellulose was pre-equilibrated with buffer (25mM-Tris/phosphate, pH8.0) containing 5mM-$(NH_4)_2SO_4$, 1mM-EDTA, 5mM-2-mercaptoethanol and 1mM-phenylmethanesulphonyl fluoride. The enzyme sample after affinity chromatography was dialysed overnight at 4°C against pre-equilibration buffer and applied to the column. The column was washed with the same buffer until the A_{280} of the initial eluate had returned to zero; the sample was then eluted with a linear $(NH_4)_2SO_4$ gradient from 5 to 200mM established by two reservoirs each of 3 column vol. Fig. 1 shows a representative elution profile from the DEAE-cellulose column (solid circles), comprising two peaks of enzyme activity, the first (peak *a*) being eluted at about 67mM-$(NH_4)_2SO_4$ and the second (peak *b*) at about 110mM. The two peaks have the same specific activity, which, for different preparations, has ranged from 140 to 175 units/mg. A typical protocol for the preparation is given in Table 1.

Proteolysis of mucosal phosphofructokinase in different preparative steps

It was observed that the relative proportions of peaks *a* and *b* from DEAE-cellulose chromato-

Table 1. *Preparation of rat intestinal phosphofructokinase*
For this preparation 14.6 g of frozen mucosa was used.

Fractionation step	Volume (ml)	Activity (units)	Protein (mg)	Specific activity (units/mg)	Yield (%)
1. Extract	72	170	1214	0.14	100
2. $(NH_4)_2SO_4$ fractionation	8.5	163	345	0.47	96
3. Sephadex G-100	9.0	144	139	1.0	85
4. ATP–agarose	8.4	143	3.4	42	84
5. DEAE-cellulose (Fig. 1)					
Peak *a*	0.5	33	0.19	175	19
Peak *b*	1.0	32	0.18	177	19

graphy varied considerably from preparation to preparation. In some cases, peak *b* was a barely discernible shoulder on peak *a*, whereas in others peak *b* was about the same size as peak *a*. This suggested that one of the peaks was a proteolytic artifact of the preparation. Experiments were therefore performed to determine which peak corresponded to native enzyme.

Fresh mucosa (1g) was homogenized directly in the pre-equilibration buffer for DEAE-cellulose chromatography, which, in addition to the usual proteinase inhibitors, also contained soya-bean trypsin inhibitor (0.5 mg/ml). The sample was then immediately chromatographed on DEAE-cellulose as described above in the presence of inhibitors. About 95% of the initial activity of the crude extract was recovered, and only a single peak of activity was observed, which was eluted as the same concentration (67 mM) of $(NH_4)_2SO_4$ as peak *a* (squares, Fig. 1). Thus peak *a* corresponds to native enzyme and peak *b* is a proteolytic product derived from native enzyme during isolation.

This conclusion was confirmed by cellulose acetate electrophoresis (Fig. 2). Samples taken directly from DEAE-cellulose chromatography at the end of a preparation showed only a single band, the leading edge of peak *a* giving band *a* (channel v) and the trailing edge of peak *b* giving band *b* (channel vi). When crude extract was prepared by homogenization of mucosa directly in electrophoresis buffer containing proteinase inhibitors, only band *a* was observed (channel i), confirming that this corresponds to native enzyme. Band *b* is thus the proteolytic product, and the fact that it has a slightly greater mobility than the native enzyme is consistent with the fact that it binds to DEAE-cellulose more strongly. The native enzyme in crude extracts prepared in the presence of proteinase inhibitors is relatively stable. However, as the native enzyme is purified it becomes more susceptible to proteolysis; indeed, channel (iii) of Fig. 2 shows that significant conversion of native enzyme (band *a*) into proteolytic product (band *b*) takes place as soon as the extract is subjected to $(NH_4)_2SO_4$ fractionation.

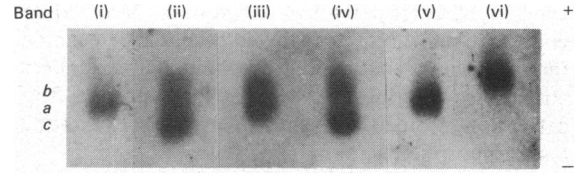

Fig. 2. *Cellulose acetate electrophoresis of different mucosal phosphofructokinase fractions*
Bands *a*, *b* and *c* have relative mobilities of 1.0, 1.05 and 0.95, and represent native enzyme, proteolytic product 1 and proteolytic product 2 respectively. Lanes: (i) crude mucosal extract prepared in the presence of proteinase inhibitors (1 mM-phenylmethanesulphonyl fluoride, 0.5 mg of soya-bean trypsin inhibitor/ml and 1 mM-6-aminohexanoic acid); (ii) crude mucosal extract prepared in the absence of proteinase inhibitors; (iii) $(NH_4)_2SO_4$ fraction of crude mucosal extract prepared in the presence of 1 mM-phenylmethanesulphonyl fluoride and 1 mM-6-aminohexanoic acid; (iv) $(NH_4)_2SO_4$ fraction of crude extract prepared in the absence of proteinase inhibitors; (v) peak *a* and (vi) peak *b* separated by DEAE-cellulose chromatography in the last step of the purification of mucosal phosphofructokinase (see Fig. 1).

Because of the increased susceptibility of mucosal phosphofructokinase to proteolysis even after limited purification, and because the partial purifications described previously in the literature did not include precautions to prevent proteolysis (Ho & Anderson, 1971; Tejwani & Ramaiah, 1971), the effect of omitting proteinase inhibitors altogether was investigated. Immediate electrophoresis of crude extract prepared in the absence of inhibitors revealed the presence of a second proteolytic product for, in addition to bands *a* and *b*, a third band, *c*, was observed that was less anodic than the native enzyme (channel ii). Bands *a* and *c* were of about equal intensity, and band *b* was rather weaker. When the same extract was subjected to $(NH_4)_2SO_4$ fractionation, further proteolysis occurred, for band *a* corresponding to the native enzyme was decreased

in intensity (channel iv). Under these conditions the major component was the second proteolytic product, together with a relatively small proportion of the first proteolytic product; the proportion of native enzyme left was usually, though not always, negligible. When this enzyme preparation was chromatographed directly on DEAE-cellulose, also in the absence of proteinase inhibitors, the elution profile showed a major peak of activity, which, as expected, was eluted before the native enzyme at about 53 mM-$(NH_4)_2SO_4$ (peak *c*), followed by a long trailing edge that stretched into the peak-*b* region (open circles, Fig. 1).

Three active components of phosphofructokinase could therefore be distinguished on the basis of their charge properties, either by chromatography on DEAE-cellulose or by electrophoresis on cellulose acetate. The latter was most convenient, although it should be emphasized that the relative mobilities of native enzyme, proteolytic product 1 and proteolytic product 2 were 1.0, 1.05 ± 0.01 and 0.95 ± 0.01, so that it was not always easy to obtain good resolution of the bands. This difficulty perhaps reflects the report by Gonzalez *et al.* (1975) that the differences in electrophoretic mobilities of phosphofructokinase isoenzymes from the rat are much less than for other animals, such as the rabbit, guinea pig and mouse.

The fact that only proteolytic product 1 is formed in the presence of proteinase inhibitors implies that the order of proteolytic degradation is native enzyme → proteolytic product 1 → proteolytic product 2. Table 1 shows that proteolytic product 1 has the same specific activity as native enzyme. Proteolytic product 2 was not purified to homogeneity. However, when a crude extract is prepared in the absence of proteinase inhibitors, the fact that there is no loss of activity for several hours implies that proteolytic product 2 also has the same specific activity as native enzyme.

Characterization of purified mucosal phosphofructokinase

All the protein in each of peaks *a* and *b* after DEAE-cellulose chromatography was separately concentrated by ultrafiltration and immediately subjects to electrophoresis in 4–12% polyacrylamide gels. By this criterion, native enzyme from peak *a* was found to be homogeneous (Fig. 3). Channels (iv)–(vii) contained native enzyme at different loadings, but even at the highest only a single band, *a*, was observed; no impurities could be detected. With ferritin [channels (iii) and (ix)], catalase [channels (i), (ii) and (x)] and bovine serum albumin (not shown) as standards, the M_r of phosphofructokinase was calculated to be 413000 ± 8000, just less than that of ferritin. This value is rather higher than that of 320000–340000 characteristic of mammalian phosphofructokinases

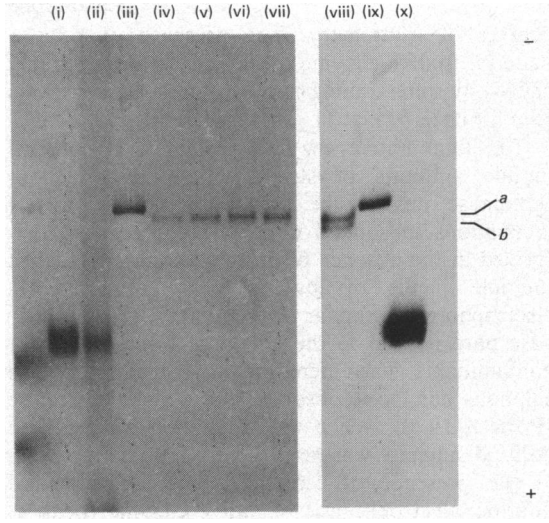

Fig. 3. *Polyacrylamide-slab-gradient-gel electrophoresis of purified mucosal phosphofructokinase fractions in 4–12% gels*

Channels (iv)–(vii), peak *a*, native enzyme, from DEAE-cellulose chromatography at loadings of 2, 4, 6 and 8 μg respectively; channel (viii), peak *b* enzyme. M_r markers: bovine serum albumin (68000) not shown; channels (i), (ii) and (x), catalase (232000); channels (iii) and (ix), ferritin (440000).

such as that from rabbit muscle (Walker *et al.*, 1976) or from human muscle (Kahn *et al.*, 1980). However, the behaviour of the rat mucosal isoenzyme on polyacrylamide gel is identical with that of the human muscle isoenzyme, for the latter also behaves as though it has an M_r just less than that of ferritin (see Fig. 3 of Kahn *et al.*, 1980). It is therefore apparent that rat mucosal phosphofructokinase has the same M_r as human muscle phosphofructokinase, about 320000–340000. The fact that both behave on gels as though they had a higher M_r probably reflects structural asymmetry; for example, the rabbit muscle tetramer has a value for f/f_0 of 1.4 (Goldhammer & Paradies, 1979).

Enzyme from peak *b* of DEAE-cellulose chromatography showed two distinct bands on the gel (channel viii), one of which corresponded to band *a* for the native enzyme. The second band, *b*, had a lower M_r and therefore corresponded to proteolytic product 1. Its M_r was determined by calibration with standards as described above and then calculated to be 305000 after correction by the factor (320000/413000). This calculation allows for the fact that the gradient gels give anomalously high values for phosphofructokinases and assumes that native enzyme and proteolytic products have similar

degrees of asymmetry. The presence of peak *a* material (native enzyme) in peak *b* was presumably caused by some trailing of peak *a* in the same way as seen for peak *b* (Fig. 1).

The final native enzyme product still contains minute amounts of strongly bound endogenous proteinase despite the precautions taken, for, if incubations in sodium dodecyl sulphate were performed in the absence of proteinase inhibitors, then multiple bands of low M_r were observed on electrophoresis. Similar susceptibility of an otherwise pure protein to the action of trace proteinase contaminants when denatured by sodium dodecyl sulphate has been observed for yeast hexokinase (Pringle, 1970). When the incubation with sodium dodecyl sulphate was performed at 100°C for 2min in the presence of 1mM-phenylmethanesulphonyl fluoride, electrophoresis of native enzyme (peak *a*) gave a band of M_r 84500 ± 5000. Rat mucosal phosphofructokinase is therefore a tetramer. On many, though not all, occasions, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of native enzyme also resulted in a minor band (about 10%), of M_r 74300 ± 5000. The latter corresponds well to the value expected for the subunit of proteolytic product 1 on the basis of the M_r of 305000 for the tetramer.

Ho & Anderson (1971) described a partial purification of mucosal phosphofructokinase in which extraction of jejunal mucosa was followed by $(NH_4)_2SO_4$ fractionation and chromatography on Sephadex G-200. The preparation was performed in the absence of proteinase inhibitors, the final product had a specific activity of 0.8 unit/mg and chromatographed on Sephadex G-200 as a particle of M_r 190000. Since both native enzyme and proteolytic product 1 are tetramers, we investigated whether the proteolytic product 2, which would have been the major component of the Ho & Anderson (1971) preparation (Fig. 2, channel iv), had a M_r of 190000.

Crude mucosa was therefore extracted, subjected to $(NH_4)_2SO_4$ fractionation and then chromatographed on Bio-Gel A-0.5m in extraction buffer at an initial concentration of about 30units/ml as described in the Materials and methods section. Within the experimental error, which is relatively large for this technique, no significant difference in M_r was observed whether the preparation was done in the presence ($n = 2$) or absence ($n = 3$) of proteinase inhibitors, or indeed whether crude extract prepared in the presence of proteinase inhibitors was chromatographed directly ($n = 2$, initial concentration 4 units/ml). The peaks of phosphofructokinase activity were all eluted between ferritin (M_r 440000) and pyruvate kinase (250000), and there was no suggestion of any component of M_r 190000. The average M_r of all the mucosal

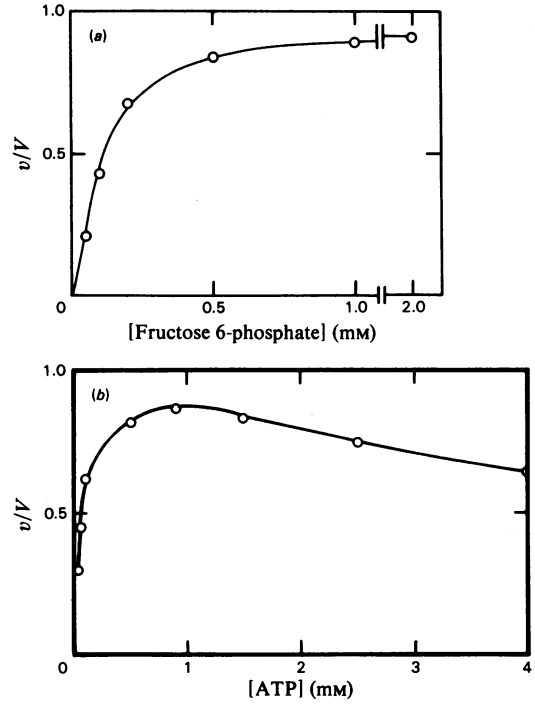

Fig. 4. *Regulatory properties of purified mucosal phosphofructokinase*

The enzyme was peak *a* material from DEAE-cellulose chromatography having specific activity of 175units/mg; assays were performed at pH7.0 as described by Hussey *et al.* (1977) and the activity at pH7.0, *v*, is expressed as a ratio to that at pH8.0, *V*. (*a*) Fructose 6-phosphate saturation curve determined at 2.5mM-ATP; (*b*) ATP inhibition curve determined at 0.5mM-fructose 6-phosphate.

phosphofructokinase samples chromatographed was calculated to be 380000 ± 50000. Again the value is higher than 320000, reflecting structural asymmetry of the tetramer. The error in M_r determination was similar to the difference between the tetramers of native enzyme and proteolytic product 1 determined by polyacrylamide gradient gels or calculated from the subunit M_r sodium dodecyl sulphate/polyacrylamide gels, so that this difference could not be detected readily by gel filtration. It is probable that the M_r of proteolytic product 2 is not much less than that of product 1. Were this to have been the case, then it would have been possible to distinguish between native enzyme and proteolytic product 2. We have no explanation for the discrepancy between our data and those of Ho & Anderson (1971).

Fig. 4 shows that the purified native enzyme from DEAE-cellulose chromatography (peak *a*) displays little co-operativity at pH7.0 with respect to fruc-

tose 6-phosphate and only a small degree of inhibition by ATP. This is in marked contrast with the properties of the enzyme when assayed directly in a crude mucosal extract prepared in the presence of proteinase inhibitors. In the latter case mucosal phosphofructokinase is quite markedly co-operative with respect to fructose 6-phosphate and is clearly inhibited by ATP (Jamal & Kellett, 1983). The possibility that purification removes a tightly bound inhibitor from the enzyme requires investigation. The maximum activity of the purified, native enzyme at pH 7.0 is 85–90% of that at pH 8.0, a value that has been repeatedly observed for the enzyme in crude mucosal extracts (Jamal & Kellett, 1983). This value is much greater than that of 55–60% characteristic of rabbit phosphofructokinase isoenzymes (Tsai & Kemp, 1974).

Although the present data apply only to phosphofructokinase, it seems inevitable that many other mucosal proteins must undergo proteolysis when mucosa is homogenized. The findings of the many papers in the literature, which are based on observations of enzyme properties in crude extracts prepared in the absence of proteinase inhibitors, must therefore be treated with reservation until the effect of endogenous proteinases on the particular enzymes are known [see, e.g., Srivastava & Hübscher (1966), Tejwani *et al.* (1974) and Budohoski *et al.* (1982)].

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