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IS HEXOKINASE PRESENT IN THE BASAL LATERAL MEMBRANES OF RAT KIDNEY PROXIMAL TUBULAR EPITHELIAL CELLS? *

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Summary

The possible presence of hexokinase in basal lateral membranes from rat kidney proximal tubules was investigated. Basal lateral membranes were obtained from homogenates of rat kidney cortex by differential centrifugation and free flow electrophoresis. They were further purified by density gradient centrifugation. Hexokinase activity was measured as the phosphorylation of D-[U-^{14}C]glucose. Throughout the purification of the membranes, the specific activity of hexokinase decreased while that of ($Na^+ + K^+$)-ATPase increased. Hexokinase activity in all fractions could be quantitatively accounted for in terms of cytosolic and mitochondrial enzyme contributions. It is concluded that there is no hexokinase activity in basal lateral membranes from rat kidney.

Introduction

According to the currently accepted view of glucose reabsorption in the proximal convoluted tubule of kidney, the sugar is accumulated across the brush border (luminal) membrane by a sodium cotransport system and diffuses through the cytosol to leave the cell via a Na^+-independent facilitated diffusion mechanism located in the antiluminal (basal lateral) membrane [1,2]. Active brush border transport of glucose can be shown to be driven by a Na^+ gradient alone, and hence is only secondarily dependent on cellular metabolism [3,4]. There is no evidence that sugar phosphorylation takes place in the microvillous membrane [5]. The outward transport across the antiluminal membrane is felt to resemble the system found in the human red cell to the extent that the latter is a Na^+-independent equilibrating system which is highly sensitive to phloretin

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[6]. There may be several such pathways of overlapping specificity in the renal tubular basal lateral membrane [7].

There is evidence that the glucose absorbed from the lumen contributes very little to renal metabolic pools [8—10]. This has been discussed as a separation of transport and metabolic pools, and a number of authors have raised the question of how this might occur [7,10,11]. If hexoses entering from the antiluminal face had a kinetic advantage for phosphorylation, metabolic pools might be saturated with sugar derived from the blood side of the cell, and luminally absorbed glucose would be spared. Evidence has been obtained that a number of hexoses may be rapidly phosphorylated when they enter from the antiluminal face and the possibility has been raised that they are phosphorylated during their transport by a hexokinase at the basal lateral membrane [7,12]. This would presumably be a special pathway with the functions of supplying substrate to the cell and aiding secretory processes; it would not play a role in sugar exit.

Mammalian hexokinases (EC 2.7.1.1) have been described in both soluble and membrane-bound forms [13]. Mitochondrial binding of hexokinase has been well documented [14]. There are scattered reports in the literature of hexokinase activity in plasma membranes. The entire glycolytic system has been reported to be associated with the bovine red-cell membrane [15]. Emmelot and Bos [16] and Davidova et al. [17] reported the presence of hexokinase in membranes from hepatoma cells, and Kang and Coe [18] studied the binding of hexokinase to ascites tumor cell membranes. All of these studies, however, suffered from inadequate characterization of the membrane fractions; the last three were particularly marred by the failure to evaluate for contamination by mitochondria (an important source of membrane-bound hexokinase). In addition, no attempt was made to account for soluble enzyme trapped in membrane vesicles.

This study was undertaken to see whether there is a renal basal lateral membrane-bound hexokinase which might contribute to renal metabolism and/or transport of glucose. Hexokinase activity was found to segregate independently from intrinsic plasma membrane markers in all separation procedures employed. All activity measured in brush-border and basal lateral membrane fractions could be accounted for in terms of mitochondrial or soluble enzyme contamination.

Methods and Materials

Tissue fractionation

Partially purified plasma membranes were prepared from the kidneys of 160—220 g male Wistar rats in a preparative medium consisting of 250 mM sucrose/10 mM triethanolamine, pH 7.6 (1 M HCl) essentially as described by Heidrich et al. [19]. This was modified only to the extent that a fourth 16 000 × *g* centrifugation was introduced. A Sorvall RC-2B centrifuge with SS-34 rotor refrigerated at 4°C was used throughout. Prior to electrophoresis, the plasma membranes were suspended to about 1.0 mg protein per ml by homogenization with a tight fitting Teflon-glass homogenizer at 1380 rev./min, and aggregated membrane fragments were removed by four centrifuga-

tions at 1000 × *g* for 7 min in a Christ Universal Junior 15 000 centrifuge with 12 × 10 ml rotor.

Separation of brush border and basal lateral membranes by free flow electrophoresis was carried out as previously described [20] using a chamber buffer consisting of 280 mM sucrose/8.5 mM triethanolamine/8.5 mM acetate, pH 7.4 (2 M NaOH). Conditions of electrophoresis were: 97 ± 10% V per cm, 85—90 mA, 6°C, at a flow rate of 2 ml/h per fraction. Injection rate varied according to the concentration of the membrane suspension, but was approximately 3 ml per h. Fractions were used directly for enzyme analysis or they were pooled according to alkaline phosphatase or $(Na^+ + K^+)$-ATPase activities and the brush border and basal lateral membranes, respectively, were collected by centrifugation for 20 min at 50 000 × *g*. For re-electrophoresis, basal lateral membranes were resuspended in chamber buffer to a protein concentration of 0.5—1.0 mg/ml by homogenization as above, and electrophoresed under exactly the same conditions as the first time. For gradient purification, basal lateral membranes were suspended by homogenization in 1.5 ml chamber buffer and layered on top of a discontinuous gradient consisting of 1.0 ml each 40 and 45% sucrose. The gradient was centrifuged for 90 min at 100 000 × *g* using a Christ S40 swinging bucket rotor in a Christ Omega 70 000 centrifuge. Basal lateral membranes were found at the chamber buffer-40% sucrose interface (F1). A second layer (F2) at the 40—45% interface was found to have a largely mitochondrial content. There was no pellet.

Mitochondria were obtained from the first 16 000 × *g* pellet. After removal of supernate and plasma membranes, the pellet was gently rinsed with preparative medium. The brown mitochondrial layer was scraped into a small amount of preparative medium, leaving behind the black lysosomal pellet. The mitochondria were resuspended by homogenization as above.

Particulate free supernates were obtained by centrifugation at 150 000 × *g* for 90 min.

All pH measurements were made at 20°C; all manipulations were carried out at 4°C. Intermediate samples were stored on ice $(Na^+ + K^+)$-ATPase, alkaline phosphatase, and sugar kinase activities were measured the day of the preparation. Succinate dehydrogenase, lactate dehydrogenase and protein were measured after storage at −70°C for not longer than 1 week.

Enzyme assays and protein determination

$(Na^+ + K^+)$-ATPase (EC 3.6.1.3) was assayed using a LKB 8600 reaction rate analyzer with 2082 Kinetic Data Processor (LKB Produkter AB, Bromma, Sweden) by the method of Schoner et al. [21]. 50 µl of membranes suspended in preparative or chamber buffer were added to a mixture consisting of 5 µmol of $MgCl_2$/100 µmol NH_4Cl/150 µmol imidazole (pH 7.2)/0.4 µmol NADH/3 µmol disodium ATP/0.6 µmol phosphoenolpyruvate/2 µg lactate dehydrogenase/3 µg pyruvate kinase, plus or minus 2.4 µmol ouabain in a final volume of 1.2 ml. The formation of ADP was measured at 37°C as the disappearance of NADH at 340 nm.

Alkaline phosphatase (EC 3.1.3.1) activity was measured as the hydrolysis of *p*-nitrophenylphosphate using the LKB reaction rate analyzer and the Merckotest® kit.

Hexokinase was measured as phosphorylation of D-[U-^{14}C]glucose. Product formation was determined by ion exchange chromatography on AG1X2 anion exchange resin (Biorad, 100—200 mesh, Cl form) essentially as described by Kleinzeller and McAvoy [12]. The reaction was carried out at 25°C in a shaking water bath. 200 μl of enzyme in preparative or chamber buffer were added to a tube containing 3 μmol $MgSO_4$, 0.75 μmol α-D-glucose, 3 μmol ATP (disodium salt), 52.5 μmol Tris · HCl (pH 7.6), and 1.25 μCi D-[U-^{14}C]glucose (prepurified on the column described below) in a final volume of 1.5 ml. * At, 5, 10 and 15 min, 500-μl aliquots were removed and heated to 100°C for 5 min after which they were stored on ice. Samples were then centrifuged for 15 min at 4°C in a Hettich Mikro Rapid microcentrifuge at about 15 000 $\times g$. The supernates were placed on top of 2.0 × 0.5 cm columns in Pasteur pipettes (plugged with glass wool) of AG1X2 resin which had been soaked in 1 M HCl and washed with 20 vols. of deionized water. Final gel bed volume was approximately 0.5 ml.

The columns were eluted with 4 times 0.5 ml deionized water and the eluate, containing 99% of the free sugar, was collected in plastic scintillation vials to which 1.0 ml 3 M HCl and 10 ml scintillation fluid (Rotiszint 22, Carl Roth KG, Karlsruhe, G.F.R.) were added before counting. They were then washed with 6 times 0.5 ml deionized water to remove remaining traces of free sugar. This eluate was discarded. Finally, the columns were washed with 6 times 0.5 ml 1 M HCl and the eluate (containing 98% of the sugar phosphate) was collected in vials to which was added 10 ml scintillant. Radioactivity was measured with a Berthold Betaszint BF 5000 refrigerated liquid scintillation counter (Laboratorium, Prof. Dr. Berthold, 7547 Wildbad, G.F.R.). 98.5 ± 2.2% (n = 3) of the counts were recovered from the columns. Sugar phosphate was determined as per cent of total sugar recovered and converted to nanomoles.

The method registers only the conversion of glucose to an anionic product, (e.g. glucose-1-phosphate or glucose-6-phosphate), and it is, unlike the glucose-6-phosphate dehydrogenase-linked assay, relatively insensitive to further metabolic steps, in as much as the products (e.g. 6-phosphogluconate) of the next few steps are also anionic. The activity was completely inhibited by 5 mM glucose-6-phosphate but unaffected by 5 mM glucose-1-phosphate, indicating that it was indeed hexokinase (EC 2.7.1.1) that was measured.

Non-radioactive components of the incubation medium were filtered through Nucleopore filters (pore size 0.2 μm, Nucleopore Corporation, Pleasantan, Calif.) to minimize bacterial contamination. As a control, unfiltered preparative buffers without membranes were homogenized, centrifuged and electrophoresed exactly as for an ordinary preparation and there was no measurable hexokinase activity in any fraction. In other experiments, ATP was omitted from the incubation medium and no activity was observed. Both findings indicate that bacterial contamination did not contribute to our results.

Hexokinase after free-flow electrophoresis of supernatant was measured using a glucose-6-phosphate dehydrogenase linked assay essentially as described by Rose and Warms [14], adapted to the LKB reaction rate analyzer.

* D-[1-^{3}H(n)]glucose could not be used because boiling in incubation medium produced a labelled anionic product which raised the background and lowered the sensitivity of the assay. The blank was 20—30 times lower with D-[U-^{14}C]glucose.

Lactate dehydrogenase (EC 1.1.1.27) was measured in preparative buffer with 100 μg sodium pyruvate and 200 μg NADH in a final volume of 1.21 ml. Oxidation of NADH at 37°C was followed at 334 nm in an Eppendorf 1101 M photometer. Preincubation of membranes with 0.08% sodium deoxycholate in order to open up vesicular structures was found to increase activity by only about 10% and was not employed routinely. (This is somewhat at variance with the finding of Busse et al. [5] and probably reflects differences in tightness of membrane vesicles obtained by two different preparative methods.)

Succinate dehydrogenase (EC 1.3.99.1) was measured as reduction of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride by the method of Pennington [22] after 30 min incubation on ice with 0.08% sodium-deoxycholate.

Protein was determined by the method of Lowry et al. [23] after precipitation of protein by ice-cold 10% aq. trichloroacetic acid. Bovine serum albumin was used as standard.

Protein in fractions from the free flow electrophoresis was measured as A_{280nm} relative to chamber buffer. This method was more sensitive than that of Lowry at low concentrations and protein distributions so obtained were superimposable on those determined by the Lowry method in the range in which the Lowry was reproducible (0.08—1.0 mg/ml). One unit at 280 nm corresponded to a protein concentration of approximately 0.20 ± 0.02 (n = 8) mg/ml.

Materials

Lactate dehydrogenase, pyruvate kinase, glucose-6-phosphate dehydrogenase, phosphoenolpyruvate, adenosine triphosphate, NADH and NADP were obtained from Boehringer, Mannheim, G.F.R. ATP used in sugar kinase assays only was obtained from Sigma, Munich, G.F.R. 2-(*p*-Iodophenyl)-3-(*p*-nitrophenyl)5-phenyl tetrazolium chloride was obtained from Serva, Heidelberg, G.F.R. D-[U-^{14}C]Glucose (specific activity 250—310 mCi/mmol) was obtained from New England Nuclear, Boston, Mass. Ouabain was obtained from Merck, Darmstadt, G.F.R. All other compounds were of highest available quality and were obtained from Merck, Darmstadt, G.F.R. Deionized water of resistance greater than 10 MΩ/cm purified and filtered (0.22 μm pore size) by the Milli-Q Reagent Grade Water System (Millipore Corporation, Bedford, Massachusetts) was used throughout.

Results

Enzyme enrichments and recoveries measured during the preparation of partially purified plasma membranes for free-flow electrophoresis. are summarized in Table I. Total recoveries of enzymes were comparable to recoveries of protein, suggesting that the enzymes are not inactivated by the preparative procedure. The enrichments of ($Na^+ + K^+$)-ATPase and alkaline phosphatase activities are very similar to values reported previously from this laboratory [19]. The small quantity and decreased activity of hexokinase recovered in these membranes suggest that the plasma membrane is not the major subcellular locus of glucose phosphorylating activity, a predictable finding since soluble and mitochondrial forms of hexokinase have been

TABLE I

ENZYME RECOVERIES AND ENRICHMENTS * DURING PREPARATION OF PARTIALLY PURIFIED PLASMA MEMBRANES FROM RAT RENAL CORTEX

Enzyme	% Recovered **	% Recovered in partially purified plasma membranes	Enrichment of activity in partially purified plasma membranes *** (homogenate = 1.0)
$(Na^+ + K^+)$-ATPase	74 ± 20 (4)	9.5 ± 3 (3)	9.8 ± 3.6 (10)
Alkaline phosphatase	89 ± 13 (3)	11 ± 3.6 (3)	5.2 ± 0.7 (8)
Hexokinase	87 ± 2 (3)	0.4 ± 0.2 (3)	0.30 ± 0.05 (3)
Protein	87 ± 9 (4)	1.4 ± 0.6 (4)	—

* Data expressed as mean ± standard deviation. Number of experiments is given in parentheses.

** Activity recovered in all fractions as percent of total present in original homogenate.

*** Enrichments calculated from the recoveries do not agree with the enrichments given in this column, probably due to scatter.

described by other workers [13]. A plasma membrane-bound hexokinase would contribute only a fraction to the total measured activity, and copurification with membrane markers might not be detectable until after mitochondrial and cytoplasmic contamination have been greatly reduced.

Partially purified plasma membranes were separated into brush-border and basal lateral membrane fractions by free-flow electrophoresis. Activities of $(Na^+ + K^+)$-ATPase, alkaline phosphatase, and hexokinase were measured in the various samples collected from the electrophoresis. An important consideration

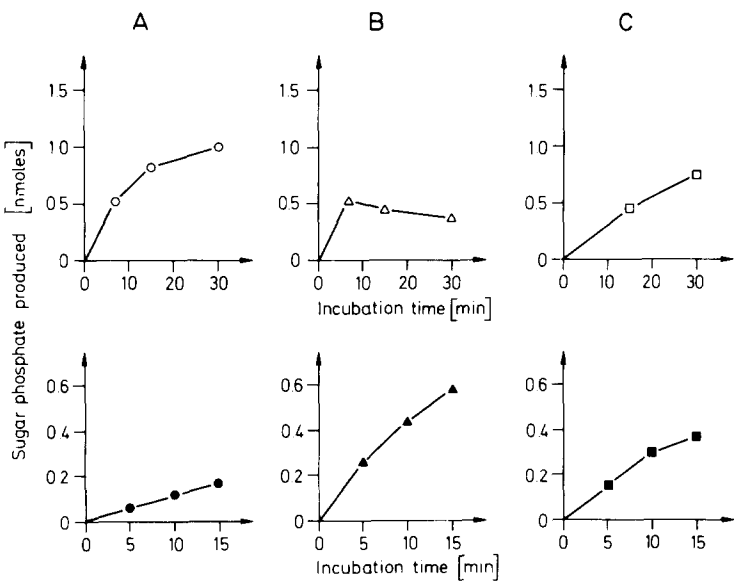

Fig. 1. Time course of hexokinase reaction in various fractions after free-flow electrophoresis of partially purified plasma membranes. The time courses in column A are from fractions with very high $(Na^+ + K^+)$-ATPase activity (e.g. fraction 31, Fig. 2). The time courses in column C are from fractions with high alkaline phosphatase activity (e.g. fraction 38, Fig. 2). The middle time courses (column B) are from fractions in the middle (e.g. fraction 35, Fig. 2). The samples shown in the upper panel (○, △, □) were assayed in a volume of 0.15 ml over 30 min with 0.025 mg protein per tube. The samples in the lower panel (●, ▲, ■) were assayed in a volume of 1.5 ml over 15 min with 0.020 mg protein per tube. Assay conditions: 25°C, 0.5 mM glucose, 2.0 mM ATP.

in such measurements is the influence hydrolytic enzymes might have on the obtained distribution pattern of glucose phosphorylation, especially since these enzymes vary in type and activity from fraction to fraction. The measured hexokinase activity might be affected by ATP depletion by ATPases and/or by glucose phosphate hydrolysis by glucose-6-phosphatase or alkaline phosphatase. Indeed, as is shown in the upper panel of Fig. 1, such interference was found in preliminary experiments in which 0.15 ml incubation volumes were used to maximize specific activity and minimize isotope usage. Saturating time courses were observed in fractions containing highly purified $(Na^+ + K^+)$-ATPase (column A, upper), and reversing time courses (column B, upper) were observed in regions known to contain some glucose-6-phosphatase activity [19]. The effect of high alkaline phosphatase activity (column C, upper) was less evident. The incubation volume was increased 10-fold to increase the ATP supply and decrease the product concentration. Enzyme added per incubation was reduced to minimize phosphatase activity. Time courses were then obtained which were linear for at least 10 min. (The lower panel shows time courses obtained in this manner for fractions corresponding to those shown in the upper panel.) Subsequent measurements always included 5-, 10-, and 15-min time points; occasionally the 15 min point had to be discarded. The larger volume assay is described in the methods section.

Fig. 2 shows the distribution of activities of $(Na^+ + K^+)$-ATPase, alkaline

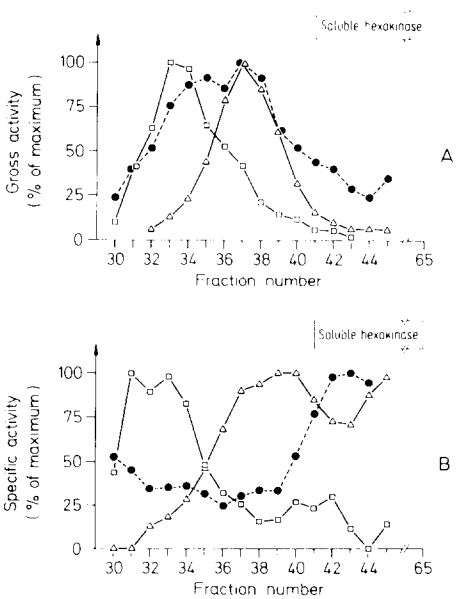

Fig. 2. Distribution of enzyme activities after free-flow electrophoresis. Results are expressed as per cent of maximum gross activity in the upper panel. △———△, Alkaline phosphatase (100% = 1800 munit/ml); □———□, $(Na^+ + K^+)$-ATPase (100% = 240 munit/ml); ●— — —●, hexokinase (100% = 0.24 munit/ml). Results as per cent of maximum specific activity, munit/mg protein, are given in the lower panel. △———△, Alkaline phosphatase (100% = 15 600 munit/mg); □———□, $(Na^+ + K^+)$-ATPase (100% = 3000 munit/mg); ●— — —●, hexokinase (100% = 6.30 munit/mg). The distribution of soluble hexokinase from electrophoresis under identical conditions of a 150 000 × g 90 min supernatant is indicated. Data are from a representative experiment.

phosphatase, and hexokinase in partially purified plasma membranes subjected to free flow electrophoresis. The upper panel (A) shows gross activities (activity per ml), lower panel (B) displays specific activities. The bars indicate the distribution of cytoplasmic hexokinase obtained upon electrophoresis under identical conditions of a 150 000 × *g* 90 min supernate. Gross activity of hexokinase shows no strong association with either brush border or basal lateral membranes. Specific activity of the enzyme is lowest in the region of high alkaline phosphatase activity. The peak to the right of the alkaline phosphatase peak is most likely soluble hexokinase. As is shown in Fig. 3A, this pattern was quite reproducible. Hexokinase activity (Table II) in the brush border fractions (35, 36, 37 in Fig. 3A) is 3-fold reduced relative to partially purified membranes, while alkaline phosphatase activity increased 20%. This separation of alkaline phosphatase activity from hexokinase activity strongly suggests that there is no ATP-dependent glucose phosphorylating activity in the brush border membrane.

To evaluate whether the hexokinase measured in the left fractions reflected enzyme associated with the basal lateral membranes, these membranes were electrophoresed a second time. Fig. 3A shows the pattern for the first electrophoresis. Fig. 3B presents the enzyme distribution after re-electrophoresis of the basal lateral membranes. The maximal hexokinase activity is lower than in the first electrophoresis, and its distribution is clearly not superimposable on

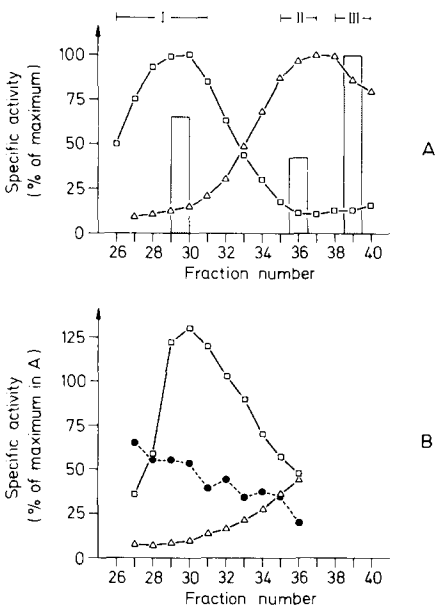

Fig. 3. Distribution of enzyme activities after re-electrophoresis of basal lateral membranes. A. Electrophoresis 1. Distribution of enzymes as percent maximum specific activity. △———△, Alkaline phosphatase (100% = 14 600 munit/mg); □———□, $(Na^+ + K^+)$-ATPase (100% = 3500 munit/mg). Bars represent hexokinase activity in fractions pooled as indicated (100% = 3.4 munit/mg). B. Enzyme distribution on re-electrophoresis of basal lateral membranes. The activities are plotted as per cent of the maximum specific activity obtained in electrophoresis 1. △———△, Alkaline phosphatase; □— — —□, $(Na^+ + K^+)$-ATPase; ●— — —●, hexokinase. Data in both plots represent the mean of 3 experiments. For fraction 30 $(Na^+ + K^+)$-ATPase = 4280 ± 650 munit/mg and hexokinase = 0.8 ± 0.1 munit/mg.

TABLE II

ENZYME ACTIVITIES IN VARIOUS FRACTIONS OF RAT KIDNEY CORTEX *

	$(Na^+ + K^+)$-ATPase (munit/mg)	Alkaline phosphatase (munit/mg)	Lactate dehydrogenase (munit/mg)	Succinate dehydrogenase (munit/mg)	Predicted hexokinase (munit/mg)	Measured hexokinase (munit/mg)
Homogenate	180 ± 70 (14)	1810 ± 460 (13)	1440 ± 180 (6)	32 ± 3 (3)	14	10 ± 2 (6)
Mitochondria	190 ± 90 (4)	1310 (2)	160 ± 30 (4)	76 ± 14 (4)	—	17 ± 4 (4)
Supernatant	13 ± 26 (4)	380 (2)	3400 ± 370 (4)	0.11 ± 0.06 (3)	—	15 ± 4 (5)
Partially purified plasma membranes	1500 ± 330 (10)	9300 ± 1500 (8)	160 ± 50 (6)	11 ± 2 (5)	3.3	3.1 ± 1.1 (7)
Brush-border membranes	50 ± 37**(3)	11500 ± 1300 ** (3)		4.6 (1)	1.0 ***	1.0 ± 0.4 (4) **
Basal lateral membranes	3600 ± 600 (4)	2840 ± 260 (3)	120 ± 30 (4)	13 ± 4 (5)	3.5	2.6 ± 1.4 (5)
F1 Mean:	3650 (2)	2040 (2)	85 (1)	6.2 (2)	1.8	1.3 (2)
F1 Values:	3590, 3720	1905, 2170		4.2, 8.2		0.6, 2.0
F2 Mean:	710 (2)	1500 (2)	580 (1)	24 (2)	8.3	4.2 (2)
F2 Values:	480, 940	1700, 1300		27, 22		5.8, 2.7

* Activities given as mean ± S.D. munits per mg protein, where 1 unit = 1 μmol substrate converted per min. (Number of experiments in parentheses.)

** Protein converted from ΔA_{280nm} by factor of 0.2 ± 0.02 mg/ml per unit A_{280nm}.

*** On the basis of succinate dehydrogenase only.

the $Na^+ + K^+$-ATPase distribution. The dissociation of glucose phosphorylating activity from $(Na^+ + K^+)$-ATPase activity is perhaps more clearly shown by direct comparison of the starting material for the re-electrophoresis experiments with fraction 30 containing the highest specific activity of $(Na^+ + K^+)$-ATPase. In this fraction $(Na^+ + K^+)$-ATPase specific activity increased by 30%, whereas hexokinase specific activity decreased by 50%.

Further purification of basal lateral membranes obtained by free-flow electrophoresis was also accomplished by density gradient centrifugation (see Methods). The results are given in Table II. The upper band (F1) showed a 1.02-fold increase in $(Na^+ + K^+)$-ATPase activity, while hexokinase specific activity decreased by half. More striking was the 5-fold decreased specific activity of $(Na^+ + K^+)$-ATPase noted in the lower layer (F2) where hexokinase activity increased 1.65 times.

The results clearly show that with each increase in specific activity of $(Na^+ + K^+)$-ATPase, there is a decrease in the specific activity of hexokinase and vice versa. This dissociation of the two activities strongly suggests that there is no glucose-phosphorylating enzyme localized in the antiluminal membrane. The possibility that the basal lateral membranes interfered in some manner with the hexokinase determination was ruled out by demonstrating that the addition of highly purified basal lateral membranes had no effect on the hexokinase activity measured in either a 150 000 $\times g$ supernate or in purified mitochondria (data not shown).

Experiments were then designed to determine whether the low, but measurable, hexokinase activity in antiluminal membranes could be entirely due to contamination by the mitochondrial and soluble enzymes. Mitochondria obtained from pellets usually discarded during the membrane preparation were used to determine the ratio of hexokinase activity to succinate dehydrogenase (a membrane-bound mitochondrial enzyme) activity in a mitochondrial fraction. Supernatants centrifuged for 90 min at 150 000 $\times g$ were used to determine the ratio of hexokinase to lactate dehydrogenase in the soluble fraction. Using the measured hexokinase/succinate dehydrogenase ratio of 0.224 ± 0.061 ($n = 4$) and hexokinase/lactate dehydrogenase ratio of 4.96 ± 0.64 ($n = 4$), hexokinase activities were predicted for each fraction from its lactate dehydrogenase and succinate dehydrogenase activities. As is shown in Table II, the measured hexokinase activity can always be explained by the contamination. In addition, variations in the hexokinase measured in individual preparations were paralleled by variations in the predicted activities for those particular preparations (data not shown). The predictability of residual glucose phosphorylating activity from measured contamination by mitochondrial and cytosolic enzymes further supports the view that there is no ATP-dependent glucose phosphorylating enzyme in the antiluminal membrane.

The enzyme recoveries from the free-flow electrophoresis can also be analyzed in terms of soluble and mitochondrial enzymes. In the usual fractions collected for analysis, the recovery of alkaline phosphatase was 81%, for $(Na^+ + K^+)$-ATPase was 74%, and for hexokinase was 22%. Extension of the collected fractions to include also the region in which the hexokinase was found increased the recovery of that enzyme from 22 to 50%, without changing the recovery of plasma membrane markers. Mitochondrial recovery in the latter

case was 40%, suggesting that the remainder of the hexokinase may have migrated with some of the mitochondria away from the collected fractions (possibly to the anode (Kinne, R., unpublished observations)).

Discussion

Of the four known types of hexokinases [13], those of low K_m (i.e., types, I, II, and III) have been shown to be present in the kidney [24], type I being by far the predominant form. There is a large intraorgan variation in the activity. Hexokinase activity is lowest in the proximal convoluted tubule and highest in the thick ascending loop of Henle and distal convolution [25]. Whether the intrarenal distribution varies among the three different types of hexokinase is unknown.

Types I and II have been shown to occur in both soluble and mitochondrially-bound forms [14]. Our membranes were isolated in media of low ionic strength at slightly alkaline pH, conditions unfavorable for solubilization of bound enzyme. Neither EDTA nor glucose-6-phosphate which might have eluted the enzyme was used in the buffers. Magnesium, which might have induced non-specific binding was not added. Mitochondria isolated in this medium retained hexokinase. During the gradient centrifugation, hexokinase moved with succinate dehydrogenase, suggesting that at least this binding was not disrupted. If these forces could affect binding to plasma membranes as well, there is no reason to assume that the conditions used for membrane isolation in the present report would have altered binding already established.

An enzyme which is potentially soluble, bound to subcellular organelles, and bound to plasma membranes presents the most complex problems in identifying its subcellular localization. For such a multilocular enzyme, a decrease in activity in plasma membranes relative to homogenate does not, when taken alone, rule out a plasma membrane location. If an enzyme is present in significant quantity in a membrane, however, whether bound to the membrane or trapped inside it, at some point during the isolation of that membrane, the enzyme should show copurification with intrinsic marker enzymes for the membrane. At every stage in the isolation of highly purified brush border and basal lateral membranes in the present study, hexokinase activity decreased, while alkaline phosphatase and $(Na^+ + K^+)$-ATPase activity, respectively, increased. Hexokinase did not appear to be in any way linked with intrinsic plasma membrane enzymes. Furthermore, because hexokinase is known to exist in soluble and mitochondrial forms, by the use of marker enzymes, an upper limit could be set on the activity which would have been expected on the basis of contamination by cytosol and mitochondria. (This estimation assumed that soluble hexokinase did not respond differently to isolation procedures than did lactate dehydrogenase and that both succinate dehydrogenase and mitochondrial hexokinase remained bound to mitochondrial membranes during the isolation.) Measured hexokinase activity could at all steps be accounted for in terms of these contaminants. This strongly suggests that there is no intrinsic hexokinase activity in either the luminal or the anti-luminal membrane of rat kidney proximal convoluted tubule.

The conclusion that there is no hexokinase activity in the brush border

membrane is consistent with previous observations [5,26–28].

To our knowledge, this is the first reported investigation into the presence or absence of hexokinase in the basal lateral membrane. Our finding that there is no hexokinase in the basal lateral membrane suggestes that ATP-supported phosphorylation of glucose does not occur as part of entry at the antiluminal face. *

This study suggests that glucose transport across the plasma membrane of renal proximal tubular epithelial cells is only indirectly related to metabolism. The apparent metabolic sparing of luminally absorbed glucose remains an intriguing and unexplained experimental observation. It is possible that, under physiological conditions, renal glucose-6-phosphate levels are high and hexokinase is inhibited [11]. Under such circumstances, the transepithelial flux of glucose could be very much higher than the metabolic flux of glucose, and the failure of the two pools to mix might be more apparent than real. In view of our finding of no hexokinase in the antiluminal membrane, phosphorylation of glucose during entry from that side of the cell seems unlikely. After passage through the membrane a preferential phosphorylation might, however, occur due to the high number of mitochondria present in the basal infoldings of the cell. The high concentration of the mitochondrial hexokinase and a high ATP/ADP ratio would clearly favour the formation of glucose-6-phosphate.

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* Preliminary experiments (Keljo, D.J. and Kinne, R., unpublished observations) indicate that while while phosphoenolpyruvate-supported glucose phosphorylating activity [28] is also present in rat kidney cortex, essentially all of it is in soluble form.

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