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Separation of Membrane-Bound γ-Glutamyl Transpeptidase from Brush Border Transport and Enzyme Activities†

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ABSTRACT: A new population of membranes from rat renal cortex containing γ-glutamyl transpeptidase (γ-GTP) has been found. Membranes with γ-GTP can be separated from brush border as well as basal-lateral transport and enzyme activities. Free-flow or liquid curtain electrophoresis was used to separate membranes with γ-GTP from brush border membranes containing alkaline phosphatase (AP), 5′-nucleotidase, and the Na^+-dependent D-glucose carrier. The electrophoretic mobility of membranes with γ-GTP was almost identical with that of basal-lateral infoldings containing NaK ATPase. Separation of two membrane populations containing γ-GTP and NaK ATPase was accomplished by using high-resolution density gradient centrifugation with a modified colloidal silica medium (Percoll). Mixtures of renal membranes containing AP in addition to NaK ATPase and γ-GTP were resolved by Percoll density gradient centrifugation into three distinct populations with bouyant densities of 1.038, 1.030, and 1.058 g/cm^3, respectively. The purification of NaK ATPase was 7-fold, that of AP was 5-fold, and that of γ-GTP was 10-fold compared to that of the homogenate. Thus, centrifugation in fixed-angle rotors of renal membranes in reorienting Percoll density gradients not only has defined a unique population of membranes with γ-GTP but also is capable of separating brush borders from basal-lateral infoldings. The rapidity and ease of this centrifugation method make it suitable for the preparative isolation of these three membrane fractions. Although our studies do not establish the anatomical location of membrane-bound γ-GTP, they do define, on the basis of both net surface charge and bouyant density, a previously unrecognized population of membranes containing γ-GTP.

The vectorial movement of solutes across epithelial cells is a result of the polarity of the cellular surface membranes (Ussing & Thorn, 1973). For example, Na^+ cotransport of D-glucose occurs via a phlorizin-inhibitable carrier at the brush border surface of the renal proximal tubule (Kinne et al., 1975; Kinne, 1976). The Na^+ ions which accumulate intracellularly are removed by the NaK ATPase on the basal-lateral surface (Skou, 1972). The glucose exits from the interior of the cell by facilitated diffusion via a phloretin-inhibitable carrier also located on the basal-lateral surface (Novikoff, 1960). With the development of subcellular fractionation procedures, it is now possible to isolate brush border and basal-lateral membrane vesicle populations which exhibit these two different glucose transport systems. Brush border vesicles are enriched for AP[1] while basal-lateral vesicles are enriched for the NaK ATPase (Kinne et al., 1975; Kinne, 1976). These copurifications of enzyme markers with transport systems correlate well with anatomical localization by histochemical and immunocytochemical techniques (Novikoff, 1960; Ashworth et al., 1963).

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[1] Abbreviations used: AP, alkaline phosphatase; γ-GTP, γ-glutamyl transpeptidase; FFE, free-flow electrophoresis.

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Similar studies have suggested that the membrane-bound enzyme γ-GTP is also a brush border enzyme like AP (Booth & Kenny, 1974; Goldman et al., 1976; Glossman & Neville, 1972; Wilfong & Neville, 1970). A brush border localization would be required for the proposed role of γ-GTP in the translocation of amino acids and oligopeptides across the epithelial cells of the renal proximal tubule (Meister, 1973; Prusiner et al., 1976; Orlowski & Meister, 1970). γ-GTP catalyzes the transfer of a γ-glutamyl moiety from a γ-glutamyl donor such as glutathione to an acceptor molecule such as an oligopeptide or amino acid (Meister, 1973). Since amino acid transport has been shown to occur at high levels in the renal proximal tubule and since the levels of γ-GTP in the kidney are higher than those in any other organ (Orlowski, 1963), the subcellular location of the renal enzyme has received much attention (Meister, 1973; Prusiner et al., 1976; Orlowski & Meister, 1970; Orlowski, 1963; Ball et al., 1953; Binkley, 1954).

We have found that the majority of membrane-bound γ-GTP can be readily separated from membranous structures which contain brush border transport and enzyme activities. Using free-flow electrophoresis (FFE), we can separate membranes prepared from rat renal cortex containing γ-GTP from those containing AP, 5′-nucleotidase, and Na^+-dependent D-glucose transport activities. The membranes with γ-GTP activity have an electrophoretic mobility similar to the basal-lateral membranes with NaK ATPase activity. Further fractionation of membranes containing γ-GTP and NaK ATPase prepared by free-flow electrophoresis was achieved by a new technique of high-resolution, density gradient centrifugation with a modified colloidal silica medium (Percoll). Membranes containing γ-GTP and NaK ATPase activities and possessing similar net surface charges could be separated into two distinct populations on the basis of density. In fact, Percoll density gradient centrifugation was capable of fractionating mixtures of renal membranes with the NaK ATPase, AP, and γ-GTP activities into three separate populations. These observations were unexpected in view of the presumed subcellular localization of γ-GTP on the brush border surface of the renal proximal tubule. Our findings force a reconsideration of the topography of γ-GTP in the renal tubule.

Materials and Methods

All chemicals used in these studies were of the purest grades commercially available. γ-Glutamyl-*p*-nitroanilide, *p*-nitrophenyl phosphate, and ouabain were obtained from Sigma; AMP and ATP were obtained from P-L Biochemicals. A modified colloidal silica (Percoll) for density gradient centrifugation was obtained from Pharmacia. A Sorvall SS34 rotor was used for all differential centrifugations.

Male Sprague-Dawley rats, weighing 150–180 g, were decapitated, and their kidneys were removed immediately. Homogenates of renal cortex were prepared as previously described (Hittelman et al., 1978) in a buffer containing 285 mM sucrose and 7.0 mM triethanolamine acetate, pH 7.4. All procedures were performed at 4 °C unless otherwise indicated. Membrane fractions were prepared according to the scheme depicted in Figure 1. The homogenate was centrifuged at 1000*g* for 10 min. The pellet was washed once, and the resulting supernatants (S_1) were centrifuged at 10000*g* for 10 min. The 10000*g* pellet was washed once, and the combined supernatants (S_2) were centrifuged either at 17000*g* for 20 min or at 48000*g* for 30 min, resulting in pellets P_3 and P_4, respectively. The pellet (P_3 or P_4) was suspended in a small amount of buffer and treated with 15 strokes of a tight fitting (clearance 0.10–0.15 mm) Teflon–glass homogenizer. The resulting suspension was aspirated 3 times through a 22-gauge needle and centrifuged at 5000*g* for 10 min to remove any remaining aggregates. The protein concentration of the supernatant was adjusted to 10 mg/mL.

The low-speed supernatants of P_3 and P_4 were fractionated by free-flow electrophoresis with a Model FF5 (Bender Hobein, Munich). The sample was introduced at a rate of 2.5 mL/h into the separation chamber buffer consisting of 285 mM sucrose and 7 mM triethanolamine acetate, pH 7.4. The chamber buffer was pumped at a rate of 200 mL/h. Throughout the electrophoresis a constant voltage of 1200 V/10 cm was applied and a current of 60–70 mA was maintained. The electrode buffer consisted of 100 mM triethanolamine acetate, pH 7.4.

A membrane preparation analogous to the low-speed supernatants of P_3 and P_4 was also prepared as described above except a buffer containing 250 mM sucrose and 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4, was substituted for the triethanolamine acetate buffer. A 3.5-mL amount of the membrane fractions was mixed with 8 mL of Percoll and 28.5 mL of buffer to give a final Percoll concentration of 20% (v/v). Thirty-eight milliliters of the membrane–Percoll mixture was centrifuged in a Beckman 60Ti rotor at 30000 rpm for 60 min. After centrifugation the density gradient was collected from the top with a Buchler AutoDensiflow apparatus into fractions containing 0.75 mL. The densities of the gradient fractions were calculated from the refractive indexes which were measured with a Bausch and Lomb refractometer.

A similar procedure was used to fractionate further the membranes collected from the free-flow electrophoresis. Several fractions, as indicated under Results, were pooled and then centrifuged at 48000*g* for 30 min. The pellet was suspended in the Tris-HCl-buffered sucrose and centrifuged at 17000*g* for 20 min. The resulting pellet was washed twice, resuspended, and homogenized in the manner described for P_3. As described above, aggregates were removed by centrifugation and the resulting supernatant was mixed with Percoll. Generation of Percoll density gradients was then performed as described above.

Glucose transport studies into membrane vesicles were performed as previously described (Hittelman et al., 1978) on freshly prepared membrane suspensions of the low-speed supernatants of P_3 and P_4. Fractions from the free-flow electrophoresis were pooled, as described under Results, and centrifuged at 17000*g* for 20 min. The pelleted membranes were washed twice in 100 mM mannitol and 10 mM triethanolamine–*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (–Hepes), pH 7.4, and then used for transport studies. The final protein concentration was adjusted to 25 mg/mL with the mannitol buffer for all transport studies. The concentration of D-glucose was 0.49 mM in the assay media.

γ-GTP (EC 2.3.2.2), AP (EC 3.1.3.1), and 5′-nucleotidase (EC 3.1.3.5) were measured as in previous studies (Kirk & Prusiner, 1977). NaK ATPase (EC 3.6.1.3) was measured by adding 0.3 mL of enzyme to 0.2 mL of reaction mix containing final concentrations of 4 mM ATP, 56 mM NaCl, 8 mM KCl, 1.2 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA), 2.2 mM $MgCl_2$, 1 mM sodium azide, and 5 mM Tris-HCl, pH 7.4. Samples were run in pairs; one sample in each pair contained 2 mM ouabain. Inorganic phosphate was determined spectrophotometrically after extraction with butyl acetate (Yoda & Hokin, 1970). One unit of AP or γ-GTP activity is equal to 1 μmol of substrate converted to product in 1 min. One unit of NaK ATPase

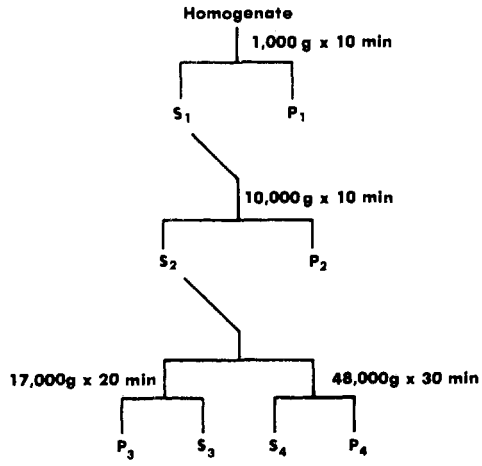

FIGURE 1: Preparation of rat renal cortical membranes by differential centrifugation. Rat renal cortices were homogenized with a polytron in either 285 mM sucrose and 7 mM triethanolamine acetate, pH 7.4, or 250 mM sucrose and 10 mM Tris-HCl, pH 7.4, as described in the text. Membrane fractions P_3 and P_4 were prepared by centrifugation in a Sorvall SS34 fixed-angle rotor. Fractions P_3 and P_4 were subjected to free-flow electrophoresis and/or density gradient centrifugation.

Table I: Enzyme and Protein Recoveries from Differential Centrifugation

fraction	% recovery of γ-GTP	AP	NaK ATPase	protein
homogenate	100[a]	100[b]	100[c]	100[d]
S_1	82 ± 5 (8)[e]	74 ± 7 (9)	57 ± 1 (7)	60 ± 3 (8)
P_1	17 ± 3 (7)	27 ± 4 (8)	43 ± 6 (6)	27 ± 3 (7)
S_2	65 ± 7 (8)	55 ± 8 (9)	35 ± 9 (7)	49 ± 3 (8)
P_2	16 ± 4 (8)	18 ± 3 (9)	18 ± 3 (7)	13 ± 1 (8)
S_3	30 ± 8 (4)	22 ± 6 (4)	6 ± 1 (3)	37 ± 4 (4)
P_3	14 ± 3 (4)	8 ± 2 (4)	11 ± 2 (3)	3 ± 1 (4)
S_4	5 ± 1 (5)	9 ± 2 (6)	2 ± <1 (5)	35 ± 3 (5)
P_4	48 ± 7 (5)	40 ± 7 (6)	32 ± 6 (5)	11 ± 2 (5)

[a] 22.6 units/mL. [b] 1.5 units/mL. [c] 1.05 units/mL. [d] 14.1 mg/mL. [e] Mean ± SE. The number of determinations is in parentheses.

activity is equal to 1 μmol of P_i produced in 1 min. Protein was determined by the biuret procedure (Gornall et al., 1949) or by the method of Lowry et al. (1951) after precipitation with 10% ice-cold trichloroacetic acid. Bovine serum albumin was used as a standard. All enzyme assays were linear over the range of protein concentrations and incubation times employed.

Results

A series of differential centrifugations was used to prepare two membrane fractions designated P_3 and P_4 as shown in Figure 1. Fraction P_3 is a heavy microsomal fraction similar to that used by other investigators in the preparation of membrane vesicles (Heidrich et al., 1972). Fraction P_3 contained 3% of the protein, 14% of the γ-GTP, 8% of the AP, and 11% of the NaK ATPase (Table I). The postmitochondrial supernatant (S_2) was centrifuged at 48000*g* for 30 min instead of at 17000*g* for 20 min in order to increase the recoveries of these membrane-bound enzyme activities. The fraction P_4 obtained by centrifugation at 48000*g* contained 11% of the protein, 48% of the γ-GTP, 40% of the AP, and 32% of the NaK ATPase.

The crude membrane fractions, P_3 and P_4, were further fractionated by free-flow electrophoresis. Prior to electrophoresis, large debris which could not be resuspended was removed from P_3 and P_4 by centrifugation at 5000*g* for 10 min. The pellets of these low-speed centrifugations contained less than 3% of the enzyme marker activities in all cases.

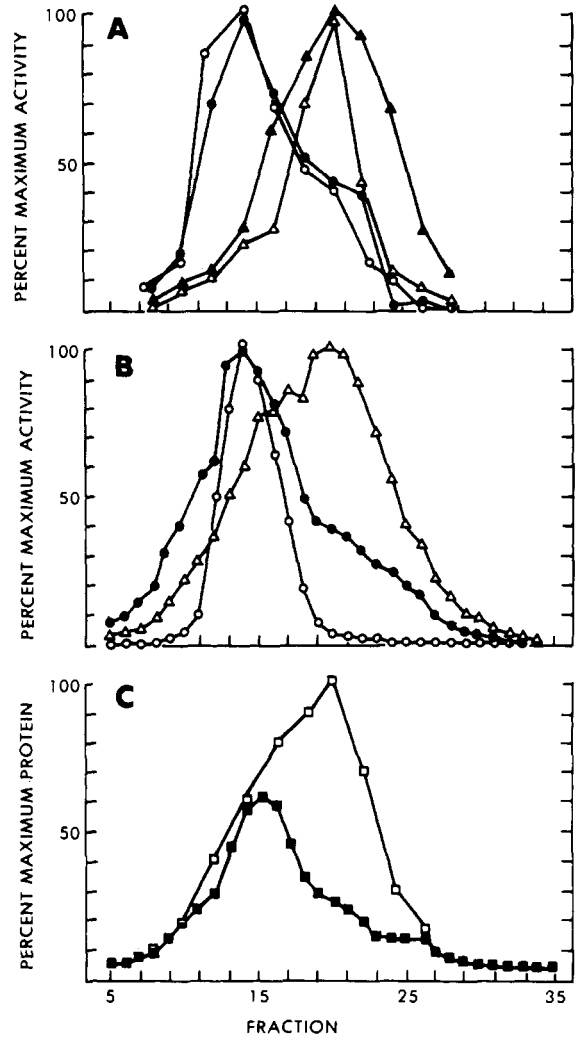

FIGURE 2: Free-flow electrophoresis of rat renal membranes. (A) Enzyme profiles from free-flow electrophoresis of P_3; (B) profiles of P_4; (C) protein profiles of P_3 and P_4. Membrane-bound enzyme activities: NaK ATPase (○), γ-GTP (●), AP (△) and 5′-nucleotidase (▲), and protein from P_3 (□) and P_4 (■). Conditions for electrophoresis are described in the text; anode is on the left.

Membranes in fraction P_3 containing AP and 5′-nucleotidase coelectrophoresed and were readily separable from membranes containing γ-GTP and those containing the NaK ATPase which had electrophoretic profiles of similar shape and mobility (Figure 2A). The net surface charge of the membrane containing γ-GTP and NaK ATPase activities was more negative than of those containing AP and 5′-nucleotidase. The activities in the peak fractions of AP, 5′-nucleotidase, γ-GTP, and NaK ATPase were 0.1, 0.2, 0.3, and 0.03 unit/mL, respectively. The recoveries of AP, γ-GTP, and NaK ATPase from P_3 in the free-flow electrophoretic fractions were 34, 41, and 42%, respectively (Table II). The specific activities of the enzymes were enriched from the homogenate to the electrophoretic fraction containing the respective peak activities by 5.9 times for AP and γ-GTP and 8.2 times for NaK ATPase.

Because of the relatively low yields of membrane-bound enzymes in P_3, we wanted to know whether the electrophoretic profiles of AP, γ-GTP, and NaK ATPase in P_3 were characteristic of the total population of these membrane-bound enzymes or whether they represented a unique subpopulation. As described above, increasing the centrifugal force applied

Table II: Fractionation of Membranes by Free-Flow Electrophoresis[a]

enzymatic marker	sp act. (units/mg of protein)			purifn[b] (x-fold)	recovery in electrophoretic fractions (%)[c]
	homogenate	P_3	FFE peak		
AP	0.12 ± 0.02 (14)	0.46 ± 0.12 (7)	0.71 ± 0.14 (8)	5.9	34
γ-GTP	1.71 ± 0.16 (14)	9.73 ± 1.69 (7)	10.13 ± 1.97 (7)	5.9	41
NaK ATPase	0.06 ± 0.01 (15)	0.24 ± 0.06 (7)	0.49 ± 0.16 (7)	8.2	42

[a] Mean ± SE. Number of determinations is in parentheses. [b] Purification is the specific activity in the peak fraction of the electrophoresis divided by that in the homogenate. [c] Recovery is the total enzymatic activity found in all electrophoresis fractions divided by the activity applied to the electrophoresis (×100).

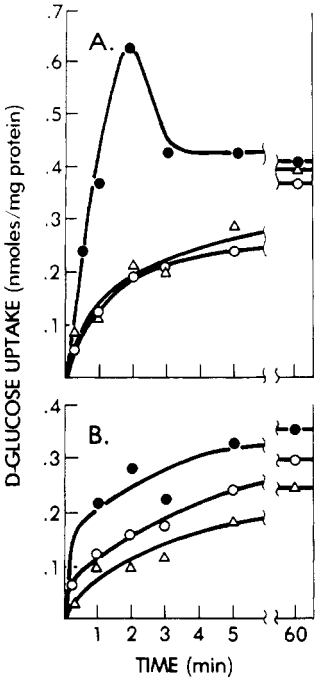

FIGURE 3: D-Glucose transport into membrane vesicles isolated by free-flow electrophoresis. Free-flow electrophoresis fractions (A) 20–28 and (B) 8–12 were pooled and membrane vesicles collected by centrifugation. D-Glucose accumulation as a function of time was measured in the presence of a 100 mM NaCl (●) or KCl (△) gradient or a 100 mM NaCl gradient with 0.2 mM phlorizin (○). See Materials and Methods for details.

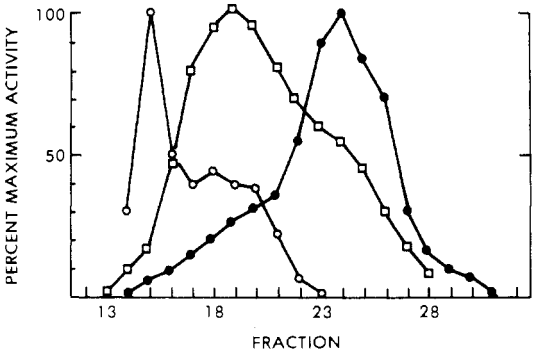

FIGURE 4: Density gradient centrifugation of membranes initially fractionated by free-flow electrophoresis. Membranes in fractions 8–14 shown in Figure 2A were fractionated in a density gradient of modified colloidal silica. The gradient, formed from 20% (v/v) Percoll in 250 mM sucrose and 10 mM Tris-HCl, pH 7.4, was centrifuged at 30 000 rpm for 60 min in a Beckman 60Ti rotor. Fractions were collected from the top (left) of the gradient. NaK ATPase (○); γ-GTP (●); protein (□).

to S_2 from 17000g to 48000g resulted in yields of membrane-bound enzymes (fraction P_4) that were increased three- to fivefold. Again, membranes containing γ-GTP and the NaK ATPase comigrated and were more acidic than those containing the majority of the AP (Figure 2B). The similarities of electrophoretic profiles of enzyme markers in fractions P_3 and P_4 indicate that the profiles for P_3 are representative and do not comprise a subpopulation. It is noteworthy that electrophoretic profiles of the membrane-bound enzyme activities in P_4 were broader than those observed for P_3. This is particularly apparent in the case of AP. The activities in the peak fractions were 0.2 unit/mL for AP, 7.8 units/mL for γ-GTP, and 0.58 unit/mL for NaK ATPase. Figure 2C compares the protein distributions obtained when fractions P_3 and P_4 were subjected to free-flow electrophoresis. A peak of protein was obtained from P_3 in fraction 20 (0.74 mg/mL) and from P_4 in fraction 15 (0.60 mg/mL).

Fractions 8–12 and 20–28 obtained from free-flow electrophoresis (Figure 2A) were pooled according to the profiles of enzyme marker activities, and studies of D-glucose transport were carried out in membrane vesicles harvested from the pooled fractions (Figure 3). Membranes enriched for AP and 5′-nucleotidase demonstrated Na^+-stimulated D-glucose uptake which could be abolished by phlorizin (Figure 2A). At 1 and 2 min the D-glucose uptake in the presence of 100 mM NaCl was 0.375 and 0.620 nmol/mg of protein, respectively. These values at corresponding time points were 3 times the uptake measured when the vesicles were incubated with 100 mM KCl. At 2 min the uptake in the presence of Na^+ exceeded the 60-min equilibrium value of 0.4 nmol/mg of protein. In the membranes enriched for NaK ATPase and γ-GTP, D-glucose uptake exhibited only minimal stimulation by a NaCl gradient (Figure 3B). After 15 s, the uptake rates were the same whether NaCl, KCl, or phlorizin was present in the incubating solution. No overshoot was observed in contrast to the brush border membranes where a prominent overshoot was observed.

To determine whether the NaK ATPase and γ-GTP were bound to the same membrane fragment, we developed a new method for fractionating renal membranes. Reorienting density gradient centrifugation in a modified colloidal silica medium (Percoll) gave sufficient resolution when extremely shallow gradients were formed in fixed-angle rotors. Membranes in fractions 8–14 (Figure 2A) obtained from free-flow electrophoresis were centrifuged in a self-generating gradient of Percoll. The NaK ATPase and γ-GTP activities were observed in two widely separated, distinct peaks with a protein peak between the enzyme activity peaks (Figure 4). The maximal activity of the NaK ATPase (0.08 unit/mL) was found at a density of 1.030 g/cm^3, while that of γ-GTP (9.7 units/mL) was found at 1.060 g/cm^3. The peak protein concentration was 0.58 mg/mL. The specific activity of NaK ATPase in fraction 15 was 0.95 unit/mg, and that for γ-GTP in fraction 24 was 24.7 units/mg. Thus, these two enzymatic activities are bound to different populations of membranes which have similar net surface charges but different bouyant densities.

We then asked whether Percoll density centrifugation was also capable of separating AP from the NaK ATPase and γ-GTP. When the free-flow electrophoresis procedure was

Table III: Fractionation of Membranes by Percoll Density Gradient Centrifugation[a]

enzymatic marker	sp act. (units/mg of protein) homogenate	P_3	gradient peak	purifn[b] (x-fold)	recovery in gradient fractions (%)[c]
AP	0.12 ± 0.02 (14)	0.46 ± 0.12 (7)	0.64 ± 0.19 (3)	5.3	56
γ-GTP	1.71 ± 0.16 (14)	9.73 ± 1.69 (7)	16.30 ± 1.05 (3)	9.5	55
NaK ATPase	0.06 ± 0.01 (15)	0.24 ± 0.06 (7)	0.40 ± 0.10 (3)	6.7	14

[a] Mean ± SE. Number of determinations is in parentheses. [b] Purification is the specific activity found in the peak fraction of the gradient divided by that in the homogenate. [c] Recovery is the total enzymatic activity found in all gradient fractions divided by the activity applied to the gradient (×100).

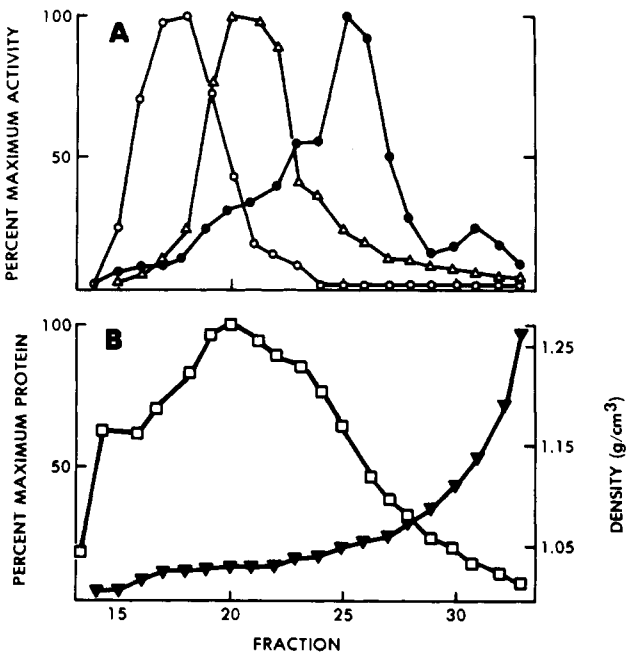

FIGURE 5: Density gradient centrifugation of membranes (P_3) prepared by differential centrifugation. (A) Enzyme profiles and (B) protein distribution patterns and the density of the modified colloidal silica gradient are shown. NaK ATPase (○); γ-GTP (●); AP (△); protein (□); density (▼).

omitted, fractionation of P_3 by density gradient centrifugation gave three separate peaks for activities of NaK ATPase (0.78 unit/mL), AP (5.8 units/mL), and γ-GTP (46.7 units/mL) at densities of 1.030, 1.038, and 1.058 g/cm^3, respectively (Figure 5). The protein was distributed widely over the entire gradient while the density of the gradient medium increased gradually in a nearly linear fashion in the region where the three membrane-bound enzymes were separated. The enzyme recoveries through the gradients were about 56% for AP, 55% for γ-GTP, and 14% for the NaK ATPase (Table III). The purifications of the enzymes from the homogenate to their respective peak Percoll fractions were 5.3-fold for AP, 9.5-fold for γ-GTP, and 6.7-fold for NaK ATPase (Table III). Similar separations of the three enzyme activities were obtained when fraction P_4 was used; however, the enzyme activities were widely distributed, giving broad peaks.

Discussion

Two fractionation methods based on different physical properties of membranes have been used to separate membranes containing γ-GTP from renal brush border and basal-lateral membranes. One fractionation method is based on the net surface charge of membrane fragments or vesicles while the other is based on bouyant density. While our observations do not establish a subcellular location for γ-GTP, they indicate that the majority of membrane-bound γ-GTP is not associated with renal brush border membrane vesicles as defined by AP, 5′-nucleotidase, and Na^+-dependent D-glucose transport.

Before attempting to reconcile our findings with those of others, let us first review the literature which suggests that γ-GTP is a renal brush border enzyme. The initial assignment of γ-GTP to a brush border location seems to be based mainly on histochemical studies which have shown that the apical portions of renal proximal tubule cells in guinea pig, mouse, rabbit, and rat preferentially stain for the enzyme (Glenner et al., 1962; Albert et al., 1961; Rutenburg et al., 1969). However, by both light and electron microscopic techniques, the staining for γ-GTP is not restricted to a defined membrane but extends well into the adjacent cytoplasm which is rich in endoplasmic reticulum (Rutenburg et al., 1969; Meister et al., 1976). Interestingly, an electron microscopic histochemical study of the pancreas of the rat has shown that γ-GTP is found associated with the endoplasmic reticulum (Rutenburg et al., 1969). Nevertheless, an immunocytochemical study of the convoluted portion of the rat renal proximal tubule has identified some γ-GTP on the brush border (Silbernagel et al., 1978).

A variety of subcellular fractionation studies have also been interpreted as evidence for a brush border location for γ-GTP. By sucrose gradient centrifugation AP and γ-GTP were found to copurify in a membrane fraction, whereas the NaK ATPase was diminished in the same fraction (Booth & Kenny, 1974; Goldman et al., 1976; Glossman & Neville, 1972; Wilfong & Neville, 1970). A decrease in specific activities of markers for mitochondria and endoplasmic reticulum was also noted, but a slight purification of lysosomal markers was found (Booth & Kenny, 1974). Goldman et al. (1976) found a copurification of AP and γ-GTP in membranes isolated from adult and newborn rat kidney. The specific activity of NaK ATPase was diminished compared to that of the renal homogenate in these membranes. The membrane preparation contained some enrichment of glucose-6-phosphatase, a microsomal marker. However, the observation that the degree of purification of membrane markers is the same for several enzymes is not sufficient to assign the enzymes to the same membrane fragment. The enzymes could be on separate particles with similar sedimentation or electrophoretic properties. In fact, another study of isolated membranes from rat kidney showed that AP and 5′-nucleotidase could be purified 16- and 17-fold, respectively, while γ-GTP was enriched 5-fold (Glossman & Neville, 1972). NaK ATPase was not measured, but in another study employing the same isolation procedure NaK ATPase activity increased 1.9 times compared to that of the homogenate (Wilfong & Neville, 1970). The difference in purification of γ-GTP from that of the other enzymes suggests that the cellular or intracellular distribution of γ-GTP is different from that of AP and 5′-nucleotidase.

Several interpretations of our findings are possible with respect to the subcellular as well as cellular localization of membrane-bound γ-GTP. First, the renal cortex contains many types of epithelial and endothelial cells. In the rat, two main populations of nephrons predominate: cortical nephrons

characterized by glomeruli in the outer cortex and by short loops of Henle and juxtamedullary nephrons characterized by glomeruli near the corticomedullary junction and by long loops of Henle (Tisher, 1976). Approximately 30% of the nephrons in the rat are of the juxtamedullary type (Schmidt-Nielsen & O'Dell, 1961). It is conceivable that γ-GTP is located on the brush border surface of the renal proximal tubule of one type of nephron and AP and 5′-nucleotidase are on the brush border of another type of nephron. A juxtamedullary nephron localization for the majority of γ-GTP would be consistent with enzyme measurements in coned sections of the kidney (Hughey et al., 1978). High levels of AP and minimal levels of γ-GTP were found in the outer cortex. At the corticomedullary junction both γ-GTP and AP were found to be present at high levels. A second explanation is that γ-GTP is present at high levels in a limited portion of the renal proximal tubule. Structural features of the epithelial cells distinguish three segments of the proximal tubule in rat kidney (Maunsbach, 1966). The first segment (S_1) of the proximal convoluted tubule is the major site of sugar and amino acid reabsorption (Burg, 1976) and presumably is the origin of membrane populations containing AP, 5′-nucleotidase, and the Na^+-dependent D-glucose carrier. The second segment (S_2) is a transitional zone between the convoluted and straight portions of the proximal tubule (Maunsbach, 1966). This segment has the least well developed brush border (Tisher, 1976). The third segment (S_3) corresponding to the straight portion of the proximal tubule has the most well developed brush borders but the least developed basal-lateral infoldings (Tisher, 1976). Even though microperfusion and immunocytochemical studies indicate that a portion of renal γ-GTP is found on the brush border surface of the convoluted proximal tubule (Silbernagel et al., 1978; Wendel et al., 1978), the majority of the membrane-bound enzyme may have an alternate location. In fact, the majority of γ-GTP might be bound to brush borders of the second and/or third segments of the proximal tubule. A third possibility is that γ-GTP exists within restricted domains of the same mosaic brush borders which contain AP and 5′-nucleotidase in other circumscribed domains. During homogenization, the brush borders might break up with γ-GTP on some membrane fragments and AP and 5′-nucleotidase as well as the Na^+-dependent D-glucose carrier on other fragments. The excellent reproducibility of our experiments would seem to argue against this alternative but certainly does not exclude it. A fourth explanation is that a minor portion of the γ-GTP is located on a brush border surface membrane, possibly the same membranes containing AP, but the majority of the enzyme has an intracellular location. The wide area of apical cytoplasm that reacts with histochemical stain for γ-GTP (Rutenburg et al., 1969; Meister et al., 1976) is consistent with an intracellular location for the enzyme. A fifth possibility is that γ-GTP is released from its native membrane-bound location during homogenization and associates with different populations of membranes. Against this alternative is the unique population of membranes on which the enzyme is found. If γ-GTP were released and then reinserted into membranes, the enzymatic activity would probably be more widely distributed. γ-GTP appears to be anchored firmly to the membrane by a hydrophobic polypeptide. Substantial concentrations of detergents are required to release the membrane-bound enzyme (Hughey & Curthoys, 1976). At present, we have insufficient information to permit us to choose among these five possible interpretations.

Our studies are of interest with respect to the hypothesis that γ-GTP functions as a carrier for amino acid reabsorption in the renal proximal tubule (Orlowski & Meister, 1970; Meister, 1973; Prusiner et al., 1976). Both Na^+-dependent D-glucose and L-amino acid transports occur in the brush border of the convoluted proximal tubule (Ussing & Thorn, 1973; Kinne et al., 1975; Kinne, 1976; Ullrich et al., 1973; Evers et al., 1976). If the separation of γ-GTP from brush border transport and enzyme activities is due to the fact that most of the γ-GTP in the renal cortex is not found in cells lining the convoluted portion of the renal proximal tubule, then our observations argue against a transport function for the enzyme. If, however, the majority of γ-GTP in the renal cortex is found on the surface of some cells lining the convoluted portion of the renal proximal tubule, then our data neither argue for nor against a transport role for γ-GTP. Recent studies in mice show that inhibition of γ-GTP leads to the development of a glutathionuria but not an associated amino aciduria (Griffith & Meister, 1979a,b). These experimental findings would seem to argue against the involvement of γ-GTP in the reabsorption of free amino acids from the renal tubule. Our studies are not inconsistent with a possible functional role of γ-GTP in the renal degradation of filtered glutathione.

Although gradient centrifugation procedures have proved useful in the simultaneous separation of brush border and basal-lateral membranes from rat jejunal epithelial cells (Murer et al., 1976), they have not been of value in the separation of these surface membranes from rat renal cortex. Our ability to separate simultaneously three membrane populations with Percoll density gradients is striking. Shallow gradients produced during centrifugation in fixed-angle rotors are required to achieve the high degree of resolution necessary to distinguish these three populations as defined by the enzyme markers NaK ATPase, AP, and γ-GTP (Figure 5). In addition, centrifugation with Percoll density gradients appears to be the first gradient technique capable of simultaneously resolving rat renal brush borders from basal-lateral infoldings. Previously, rat renal brush borders have been separated from basal-lateral infoldings by free-flow electrophoresis or divalent cation precipitation (Kinne et al., 1975; Kinne, 1976; Booth & Kenny, 1974). These techniques used in combination with Percoll gradient centrifugation may lead to the definition of other as yet unrecognized populations of membranes. The studies described in Figure 4 illustrate the high degree of separation which can be obtained by employing free-flow electrophoresis followed by Percoll gradient centrifugation.

Percoll density gradient centrifugation may also prove to be superior to free-flow electrophoresis and metal ion precipitation methods for the routine isolation of brush borders as well as basal-lateral infoldings. The rapid self-generation of stable density gradients makes Percoll an ideal medium for preparative procedures. In fact, preliminary studies indicate that Na^+-dependent D-glucose transport into membrane vesicles is unaltered by concentrations as high as 25% (v/v) of modified colloidal silica.

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