ANALYSIS OF THE PINOCYTIC PROCESS IN RAT KIDNEY

I. Isolation of Pinocytic Vesicles

from Rat Kidney Cortex

FOLKERT BODE, HANNELORE POCKRANDT-HEMSTEDT,
KARL BAUMANN, and ROLF KINNE

From the Max-Planck Institut für Biophysik, Frankfurt/Main, Germany

ABSTRACT

Pinocytosis was induced in rat kidney by exposure to horseradish peroxidase (HRP). Pinocytic vesicle preparations were enriched after homogenization of kidney cortex by differential centrifugation and free-flow electrophoresis with HRP as an exogenous marker. Vesicles were identified by enzymatic analysis and by electron microscopy, including specific staining procedures. Typical brush-border enzymes such as alkaline phosphatase, aminopeptidase, 5'-nucleotidase, lysosomal acid phosphatase, and mitochondrial succinic dehydrogenase were reduced in the vesicular fraction, compared to the kidney cortex homogenate. Glucose-6-phosphatase and Na^+-K^+-ATPase were only slightly increased in the fraction. These results indicate that preparations of pinocytic vesicles from rat kidney cortex can be enriched. They have biochemical characteristics that differ from those of the cell organelles and membranes previously purified from renal tissue.

Pinocytosis is defined as the vesicular uptake of macromolecules by living cells like amoebae or kidney proximal tubular cells (5, 6, 9, 28, 31, 48, 49). This process includes adsorption of macromolecules to the surface of the plasma membrane, followed by invagination and segregation of the endocytic vesicles. The reabsorbed substance is subsequently found in larger intracellular vacuoles ("apical absorption vacuoles"), and then transferred into lysosomes that contain hydrolytic enzymes (8). Because the primary state, the pinocytic vesicles, histochemically contains no acid phosphatase activity (42, 43), it is likely that the lysosomes are derived from fusion of pinocytic vesicles with enzyme-containing particles. This is supported by histochemical electron microscopy (33). In addition to their digestive activity (7, 32), the lysosomal vesicles may be involved in the transport of macromolecules through epithelial cells. In the mammalian kidney, molecules (up to a mol wt of 60,000), like albumin, enzymes, and drugs are filtered and then reabsorbed by the tubular cells by pinocytosis (10, 22, 29, 39, 41). Modification of macromolecules by enhancing their positive charges (22, 37) leads to a preferential pinocytic uptake. This may be due to an electrostatic interaction of the macromolecules with the negative surface charges of the sialic acid residues within the surface glycoproteins (50). Detailed information about the different steps involved in pinocytosis, the induction of invagination, the signal for pinching off into the cell, and the

transfer from pinocytic vesicles to lysosomes is still lacking. It is also unclear how far the digestion within the lysosomal vesicles of the renal tubular cells proceeds and whether unaltered or only partially disrupted macromolecules leave the cell at the interstitial cell site. Furthermore, the origin of the pinocytic membrane is unknown, as well as its reutilization.

The present paper deals with the isolation, and morphological and enzymatic characterization of pinocytic vesicles from rat kidney cortex. The results obtained show that no enzyme typical of kidney membranes or cellular organelles is present in the pinocytic vesicles, indicating that the vesicular membrane has a completely different composition.

MATERIALS AND METHODS

Isolation of Pinocytic Vesicles

For each experiment two male Wistar rats (150–220 g) were anesthetized with Inactin (Promonta, Hamburg, Germany) (100 mg/kg body wt i.p.). Polyethylenecatheters were inserted into the aorta in the region below the arteria renalis and into the vena jugularis. Horseradish peroxidase (HRP) (Boehringer & Söhne, Mannheim, Germany; 0.5 ml 0.10 g/ml dissolved in Ringer solution) was injected i.v. as a single dose. 3–5 min after injection, the aorta was ligated, the venae renales were opened, and the kidneys perfused for 3 min with Ringer solution (37°C, 145 cm H_2O pressure). Then the kidneys were removed and placed in ice-cold buffer (0.25 M sucrose, 0.07 M mannitol, 0.01 M Tris-HCl, pH 7.4). All further procedures were done at 4°C. The kidney cortex was dissected with a razor blade, minced, and gently homogenized in a glass homogenizer with 10 times its volume of sucrose buffer by 10 strokes with a loose-fitting Teflon pestle (45 rpm). The centrifugation steps used to obtain the microsomal pellet for free-flow electrophoresis (17, 18) are shown in Table I. The buffer used for the electrophoretic separation contained 280 mM/liter sucrose, 8.5 mM/liter triethanolamine, and 8.5 mM/liter acetic acid, adjusted to pH 7.4 with 2 N NaOH. The electrode buffer consisted of 0.1 M triethanolamine-acetate adjusted to pH 7.4 with 2 N NaOH. The conditions during free-flow electrophoresis were as follows: 90±9 V/cm, 85 mA, 5°C temperature, electrophoresis buffer flow 184 ml/h (2 ml/h per fraction). All the samples were spun down for 10 min at 500 g before separation to remove aggregates.

Quantitative Protein and Enzyme Assay

Alkaline phosphatase (EC 3.1.3.1) was determined by the Merckotest (Merck, Darmstadt, Germany) method in the presence of 0.5% bovine albumin. HRP was

measured by the method described by Pütter (36), with guaiacol as substrate. The activity of Mg^{++}-ATPase and Na^+-K^+-ATPase (EC 3.6.1.3) was measured in 75 mM Tris buffer, pH 7.6, with 3 mM Tris-ATP as substrate after treatment of the fractions with deoxycholate (21). The determination of acid phosphatase (EC 3.1.3.2) was carried out according to Lansing, Belkhode, Lynch, and Lieberman (26) with p-nitrophenylphosphate as substrate. The reaction mixture for glucose-6-phosphatase (EC 3.1.3.9) assay contained 6×10^{-2} M maleic acid, pH 6.7, and 20 mM glucose-6-phosphate, sodium salt. P_i was determined according to Fiske and SubbaRow (12). 5'-nucleotidase (EC 3.1.3.5) was estimated by the rate of P_i formation with a reaction mixture with 100 mM Tris, 200 mM KCl, 100 mM $MgCl_2$, and 50 mM 5'-AMP at pH 7.5. The assay for aminopeptidase (EC 3.4.1.2) contained leucin-β-naphthylamide as substrate and was performed with a micromodification of the method described by Goldbarg and Rutenburg (14). Succinic dehydrogenase (EC 1.3.99.1) was determined by the method of Gibbs and Reimers (13). Protein was determined according to Lowry, Rosebrough, Farr, and Randall, with bovine albumin as a standard, after precipitation of the membranes with 10% ice-cold TCA (27).

Electron Microscopy

3 min after infusion of 0.5 ml HRP into the vena jugularis of an anesthetized rat, fixation was started by

dripping 4% glutaraldehyde (buffered with 0.1 M caco-dylate, pH 7.2) onto the kidney for 20 min. Then the kidney was removed; small cortical tissue blocks were cut and fixed for an additional 40 min in the same medium at room temperature.

Subsequently, the tissue was reacted for peroxidase by the method of Karnovsky and Rice (23), postosmicated, and embedded in Epon (Shell Chemical Co., New York) of Vestopal W (Madame Martin Jaeger, Geneva, Swit-erland). The pellet containing the HRP-filled pinocytic vesicles was treated like the tissue; except that after osmication, the pellet was embedded in 2% agar for easier handling. Alkaline phosphatase was demonstrated by the method of Mayahara, Hirano, Saito, and Ogawa (30), with β-glycerophosphate as substrate.

Materials

Tris-ATP was obtained from Sigma Chemical Co., Inc., St. Louis, Mo. Glutaraldehyde, cacodylate, and leucin-β-naphthylamide were purchased from Schu-chardt, Munich, Germany. Glucose-6-phosphate, sodium salt, and 5'-AMP came from Boehringer & Söhne, Mannheim, Germany, while all other sub-stances were obtained from Merck, Darmstadt, Ger-many.

RESULTS

Labeling of Pinocytic Vesicles with HRP

To date, there is no specific enzyme known to be present in the pinocytic vesicles that can be used as a marker during an isolation procedure. Therefore pinocytic vesicles were labeled before the isolation with HRP, an enzyme reported to be pinocytized (16). Fig. 1 shows the distribution of HRP in the proximal tubule epithelial cell 3–5 min after intra-venous infusion. In the longitudinal section (Fig. 1 a) HRP activity can be detected at the microvilli surface and in the tubular system at the base of the brush border microvilli, where the pinocytic proc-ess is induced. Inside the cell, enzyme activity can be demonstrated either in small (0.1–0.5 μm diameter) pinocytic vesicles or in the larger apical absorption vacuoles (1 μm diameter). Lysosomes and the cytosolic compartment are devoid of HRP activity. The cross-section (Fig. 1 b) illustrates the localization of HRP: the microvilli adsorb HRP on the outside, while the tubular system or the pinocytic vesicles have a heavy deposition of the reaction product on their inside.

Enrichment of Pinocytic Vesicles

DIFFERENTIAL CENTRIFUGATION: By this method we tried to isolate pinocytic vesicles that

contained HRP. The amount of HRP and other marker enzymes in each fraction is given in Table II. Most of the HRP activity (70%) was in the final supernate (S4), which represents the total supernate from all pellets. Although the kidneys were rinsed before homogenization, most of HRP activity was present as soluble unbound enzyme.

The HRP activity in the first pellet (P1) repre-sents unbroken tissue and perhaps an unspecific binding of HRP to nuclei, which may have oc-curred during homogenization, as recently re-ported (44). The HRP binding to pellets 2 and 3 is probably due to glycocalyx binding of the brush-border microvilli (47). This correlates well with the electron microscopic picture (Fig. 1 a) and with a simultaneous enrichment of brush-border micro-villi marker enzymes (alkaline phosphatase, ami-nopeptidase, and 5'-nucleotidase) in these frac-tions. Other cellular structures found in these pellets were the acid phosphatase-containing lyso-somes and the mitochondria, represented by maxi-mal activity of succinic dehydrogenase. The high-est specific activity of HRP of all particulate fractions was found in the microsomal pellet (P4), but it was accompanied by enriched specific activi-ties of all other marker enzymes, although the amount of the latter was always less than $\frac{1}{3}$ of the total in the homogenate. Therefore, this pellet was used for further purification by free-flow electro-phoresis. The enzyme activities in the pinocytic vesicles purified by this procedure are given in the last column of Table II. In this typical experiment the specific activity of HRP was 0.24 U/mg protein, and the mean value ($\pm$ SEM) obtained in 10 different preparations was 0.34 $\pm$ 0.05. The amount of HRP recovered in the fraction 24 represents about 0.5% of the HRP content of the starting homogenate of rat kidney cortex. All other marker enzymes were decreased compared with the homogenate, with the exception of a slight enhancement in Na^+-K^+-ATPase and glucose-6-phosphatase.

FREE-FLOW ELECTROPHORESIS: In Fig. 2 the distribution of protein and HRP ob-tained after electrophoresis of the microsomal pellet is shown. The highest specific activity of HRP was found in tube 24. This enzyme activity most probably represents HRP engulfed by vesi-cles, because the distribution pattern of a pure en-zyme solution, as indicated in Fig. 2, differed markedly. This is also supported by the electron
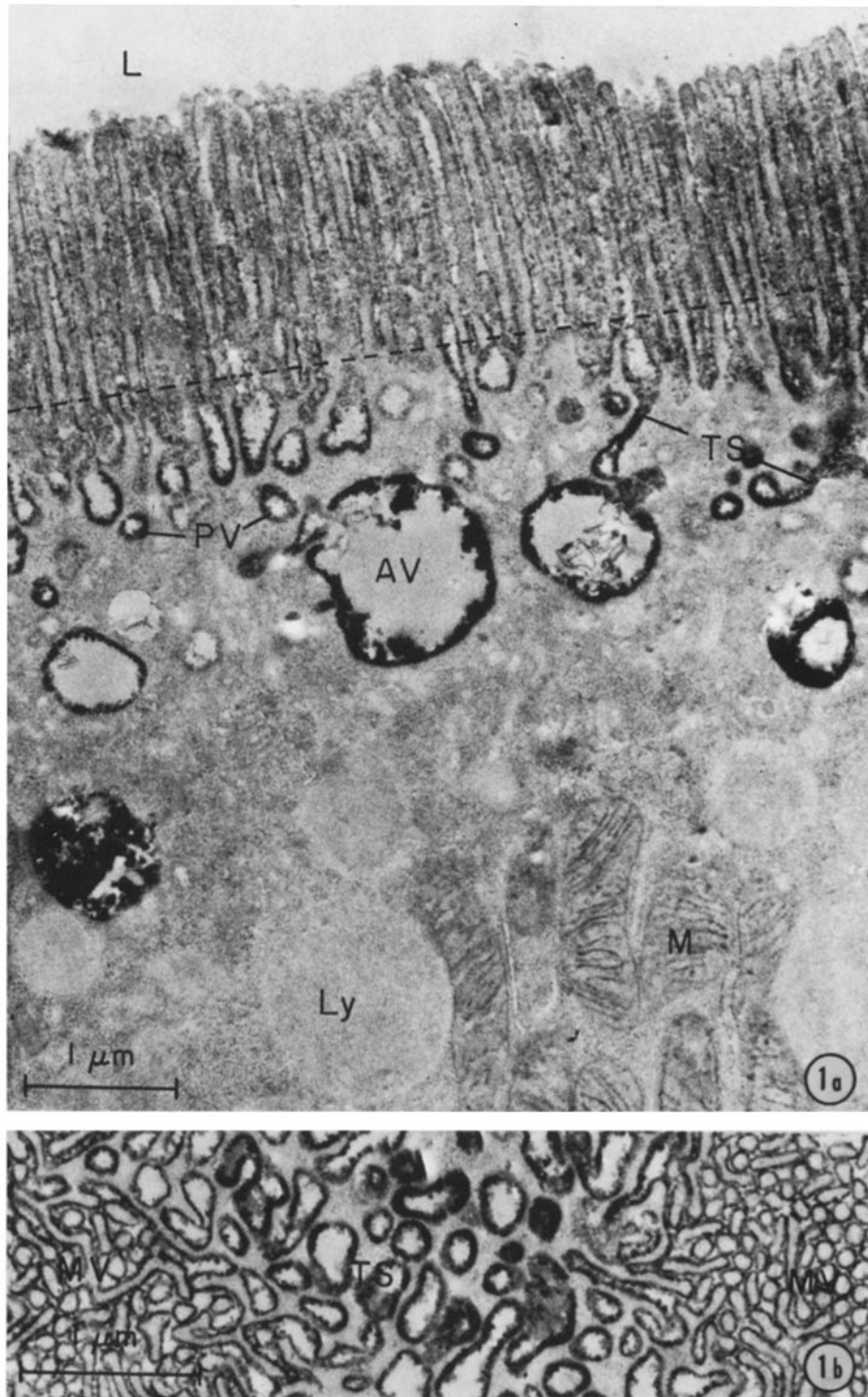
FIGURE 1   Fig. 1 a: Longitudinal section through a proximal tubule cell. The outside of the microvilli and the inside of the tubular system and of the pinocytic vesicles are outlined by the reaction product of peroxidase. L, lumen; Ly, lysosome; M, mitochondrion; AV, apical absorption vacuole; TS, tubular system; PV, pinocytic vesicle; ----, section plane of Fig. 1 b. × 21,000. Fig. 1 b: Cross-section of a proximal tubule cell. Section plane marked in Fig. 1 a by a dashed line. MV, microvilli; TS, tubular system. × 25,000.

TABLE II

*Enzyme Activity and Protein Content in Every Fraction during Isolation**

Structure	Enzyme	Homoge-nate	Pellet 1	Pellet 2	Pellet 3	Pellet 4	Super-nate 4	Re-covery‡	Frac-tion 24
Pinocytic vesicles	Peroxidase (HRP)	132.45	11.74	7.32	2.82	3.34	97.60	92.7	0.65
		0.49	0.19	0.10	0.23	0.27	1.71		0.24
Brush border micro-villi	Alkaline phospha-tase	4,372.80	689.85	1,062.00	738.00	350.10	185.36	69.2	30.78
		16.10	11.36	14.42	59.76	28.46	3.24		11.40
	5'-Nucleotidase	2,423.60	293.50	789.95	281.20	324.30	161.30	76.3	12.93
		8.92	4.83	10.73	22.77	26.37	2.82		4.79
	Aminopeptidase	955.40	122.44	411.55	196.47	78.48	66.28	91.6	4.13
		3.52	2.02	5.59	15.91	6.38	1.16		1.53
Lysosomes	Acid phosphatase	2,121.60	460.80	624.30	198.90	153.00	402.19	86.7	10.26
		7.82	7.59	8.48	16.11	12.44	7.03		3.80
Mitochondria	Succinic dehydrog-enase	304.00	93.20	196.20	7.40	16.80	0	103.0	0.05
		1.12	1.53	2.66	0.60	1.37	0		0.02
Endoplasmic reticulum	Glucose-6-phos-phatase	40.04	12.09	21.44	5.26	4.47	2.43	114.0	0.46
		0.14	0.20	0.30	0.43	0.36	0.04		0.17
Basal-lateral plasma membranes	Na^+-K^+-ATPase	696.80	106.20	153.20	79.90	99.80	33.10	67.8	13.53
		2.57	1.75	2.14	6.47	8.12	0.58		5.01
	Protein, mg	271.60	60.75	73.65	12.35	12.30	57.23	79.6	0.54

* In the upper line the amount of enzyme in μmol/h/fraction (for HRP in U) is given, in the lower line the activity in μmol/h/mg protein and U/mg protein, respectively.

‡ The amount of enzyme recovered in all fractions is expressed as percent of the amount present in the starting material.
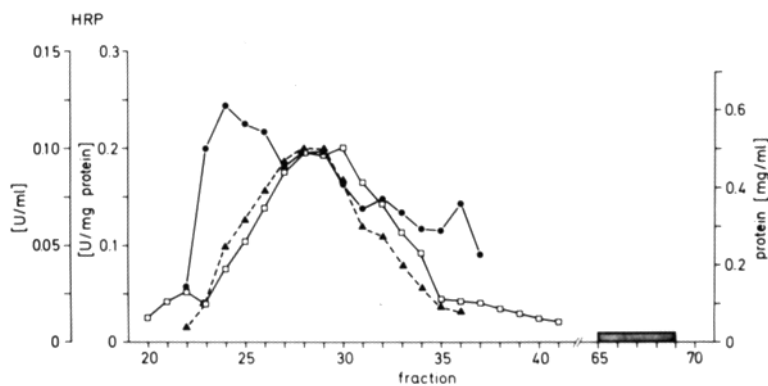

HRP

FIGURE 2 HRP and protein distribution after free-flow electrophoresis. ●———●, HRP-specific activity in U/mg protein; ○———○, protein (mg/ml); ▲———▲, HRP, amount in U/ml fraction; one typical experiment is given. The bar represents the fractions in which HRP activity is found if a solution of pure enzyme HRP is separated under the same conditions used for the microsomal pellet.

microscopic examination of tube 24 (Fig. 3), which shows HRP activity inside vesicular structures with a diameter of 0.1–0.5 μm, dimensions identical with those of pinocytic vesicles after in vivo fixation of the cell (see Fig. 1 a). The surface of the pinocytic vesicles appears to be smooth, and no specific surface structure can be detected when negative staining techniques are used (Fig. 4).

Since pinocytic vesicles originate from the apical cell membrane, which is characterized by a high content of alkaline phosphatase, it was of great interest to determine whether this enzyme could be
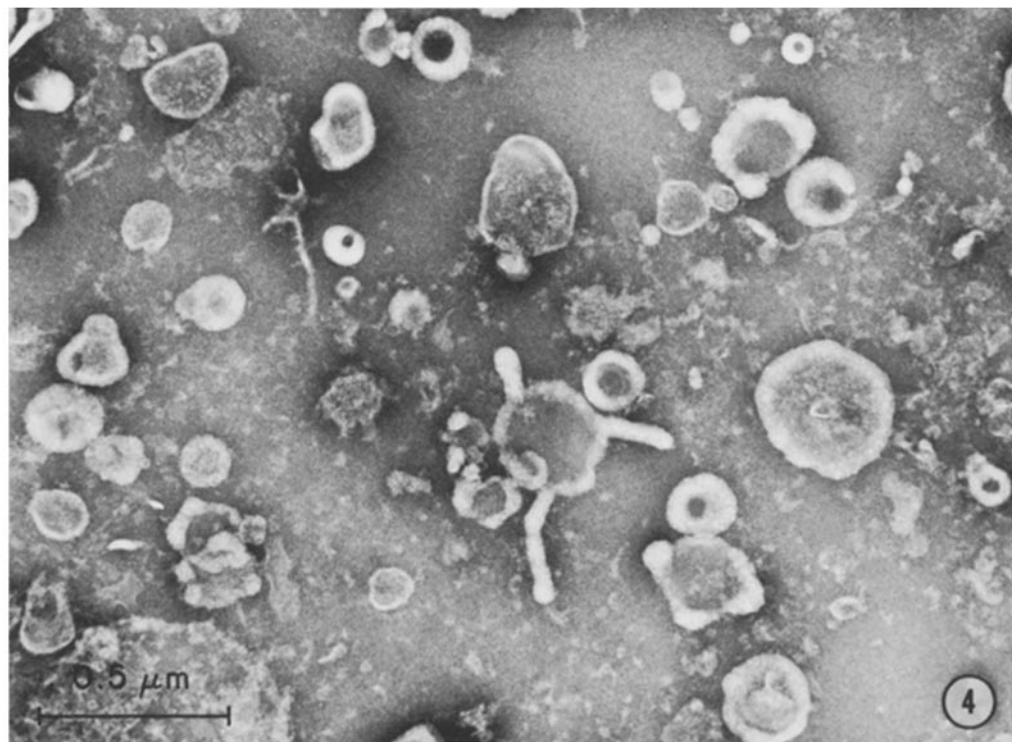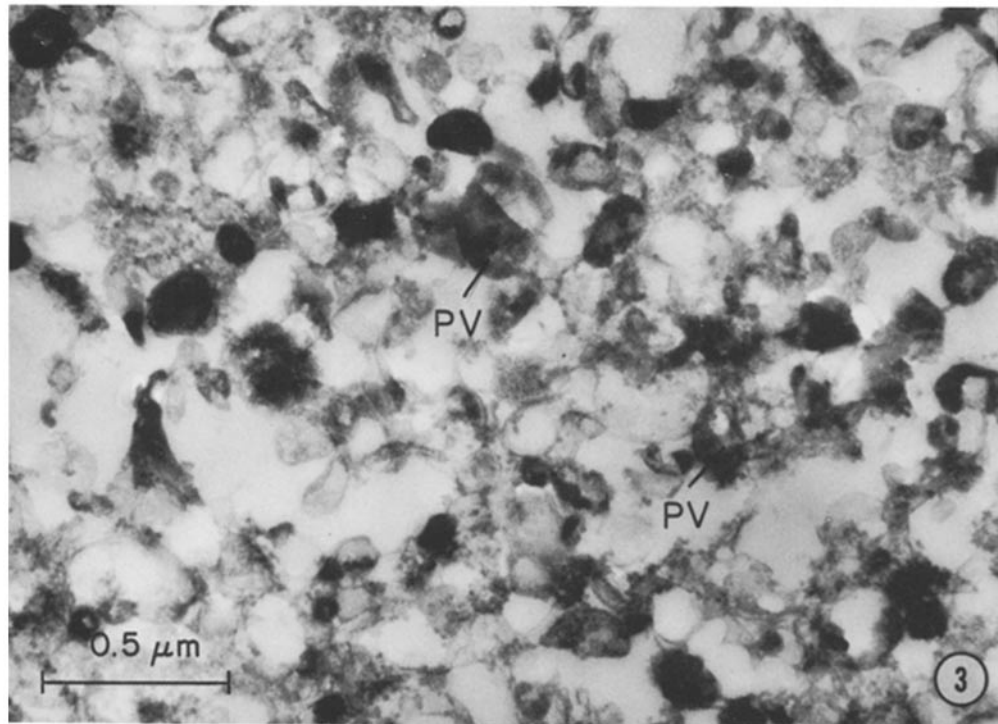
FIGURE 3 Section through pellet, tube 24. Pinocytic vesicles (*PV*) marked with peroxidase. Ultrathin section was stained with uranylacetate and lead citrate. × 50,000.

FIGURE 4 Pinocytic vesicles. Negative staining. × 50,000.

found in pinocytic vesicles, too. As shown in Fig. 5, in free-flow electrophoresis the distribution of alkaline phosphatase and HRP was different. Furthermore, in histochemical electron microscopy, only very slight alkaline phosphatase activity could be observed in pinocytic vesicles, in contrast to a high activity in the brush-border microvilli

FIGURE 5 Enzyme distribution after free-flow electrophoresis. ●——●, intravesicular HRP; △——△, alkaline phosphatase; O——O acid phosphatase. HRP (U/mg protein); others (μM/h/mg protein).

(Fig. 6). The vesicular HRP distribution was also different from that of the acid phosphatase (Fig. 5). The latter enzyme was investigated because pinocytic vesicles are a precursor element of acid phosphatase-containing lysosomes.

DISCUSSION

The existence of an endogenous marker enzyme is a useful tool for the isolation of a subcellular structure. However, if none is known, as in the case for pinocytic vesicles, the introduction of a pinocytized substance as an artificially introduced marker is useful. The advantages of using HRP are related to its enzymatic activity. Very low concentrations of this enzyme can be detected in vivo by a specific staining method (16), and the amount taken up can be measured quantitatively in small tissue samples by photometric methods. Since HRP has a mol wt of 40,000, it can be assumed to be filtrable in the kidneys (35). Therefore, since this enzyme is taken up by pinocytic vesicles and no peroxidase activity has been described in kidney
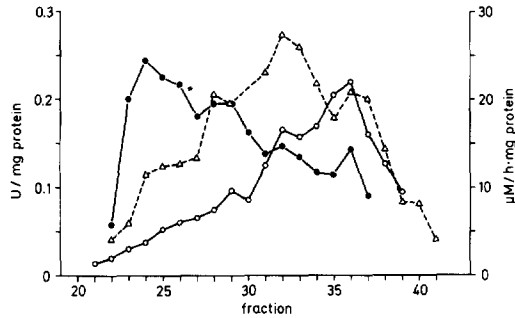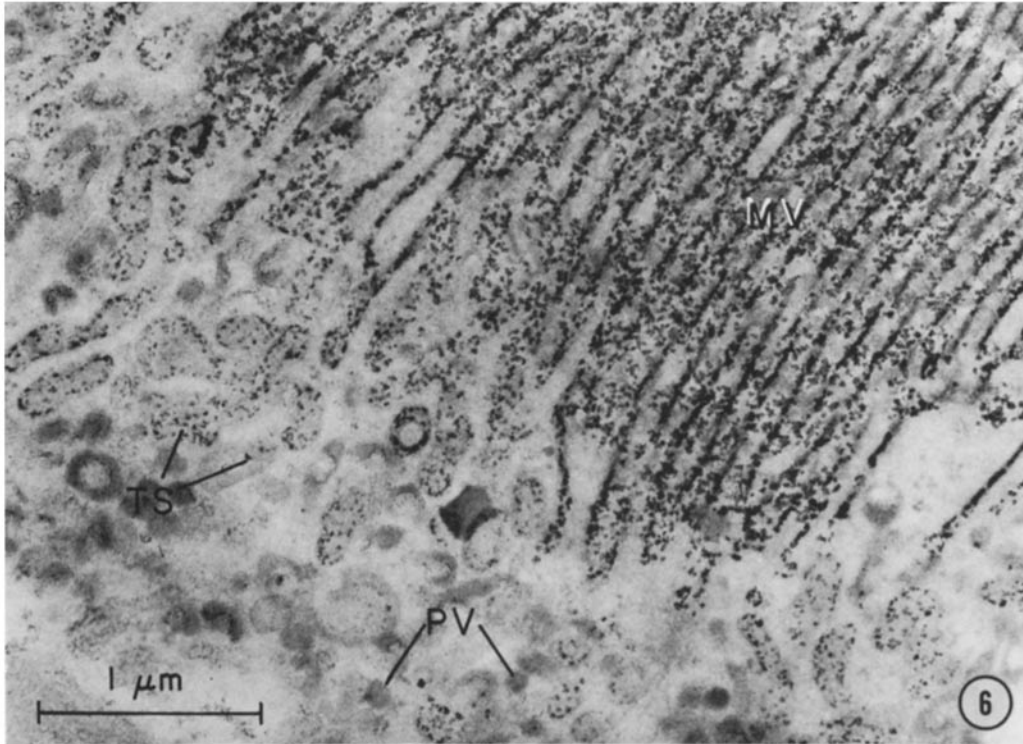

FIGURE 6 Proximal tubule cell. Alkaline phosphatase reaction. The reaction product is found along the microvilli, hardly any reaction in the pinocytic vesicles. MV, microvilli; TS, tubular system; PV, pinocytic vesicles. × 29,000.

tubule cells, its activity can be used as a marker (40).

Theoretically, this would be possible if all HRP were taken up by pinocytosis and bound exclusively in these vesicles. However, only a small portion of filtered HRP is pinocytized. Furthermore, morphological studies have shown that after the injection of HRP, not all proximal tubules contain the same amount of the enzyme. Since during the labeling period pinocytosis probably proceeds in all tubules, the population of pinocytic vesicles present in the starting material used for the enrichment procedure must be heterogeneous with respect to HRP content. This might explain why only a few of the vesicular structures found in fraction 24 after electrophoresis are filled with HRP (see Fig. 3). Due to this phenomenon, and for other reasons discussed below, a calculation of the recovery of pinocytic vesicles from the data obtained from the HRP distribution seems impossible. Table II demonstrates that more than 70% of the total HRP activity in the homogenate is soluble and therefore is recovered in the final supernate. We tried to minimize this portion by perfusion of the kidneys with Ringer solution after the injection of HRP before homogenization, but this proved inadequate. In addition, nonspecific binding to cellular structures may occur during homogenization. HRP is attached, for example, to the intraluminal glycocalyx of the proximal tubule (47). This is also confirmed in our experiments by electron microscopy (Fig. 1) and by the high HRP content in pellet 2 and 3, which coincides with an enrichment of alkaline phosphatase and leucinaminopeptidase, the marker enzymes for the luminal membrane of the proximal tubule, the brush border.

The fact that the isolated pinocytic vesicles are not identical with small brush-border fragments is emphasized by a comparison of the enzyme activities of isolated pinocytic vesicles and purified brush-border membrane (as shown in Table III) as well as by the different enzyme distribution in free-flow electrophoresis (Fig. 5). In addition, pinocytic vesicles do not show a sodium-dependent stereospecific glucose transport (unpublished results) as observed in brush-border vesicles (25).

Furthermore, basal-lateral plasma membranes, which could have taken up HRP during homogenization because of their well-known ability to form vesicular structures (for references, see 20), can be excluded as the source of the isolated structures

TABLE III

Enrichment Factors‡ of Marker Enzymes in Pinocytic Vesicles from Electrophoretic Fraction 24 and in Apical and Basal-Lateral Plasma Membranes of Rat Kidney Cortex Purified by the Same Procedure (18)

	Alkaline phosphatase	$Na^+ - K^+ -$ ATPase
Electrophoretic fraction 24	0.40 (6)* ± 0.07 SEM	5.77 (4)* ± 1.34 SEM
Apical membranes (brush border)	15	
Basal-lateral plasma membranes		16

* Number of experiments.
‡ Enrichment factors of enzymes are defined as the ratio of the specific activity (μmol/h/mg protein) found in the fraction compared to the specific activity measured in the starting material, the rat kidney cortex homogenate.

because of the low specific activity of Na^+-K^+-ATPase in the pinocytic vesicles as compared to isolated basal-lateral plasma membranes (Table III).

The microsomal pellet (P4) was used for further purification of the pinocytic vesicles because Straus has demonstrated that "protein absorption droplets", found in the early phase of protein reabsorption, are enriched in this fraction (38). The microsomal pellet is known to contain mainly endoplasmic reticulum, Golgi vesicles, and small fragments of plasma membranes. The quantity of the latter structures was reduced by the gentle homogenization procedure used in the present study. More than 90% of the respective marker enzymes were sedimented with lower centrifugal forces. The amount of endoplasmic reticulum was drastically reduced by electrophoresis, as indicated by the decrease of the specific activity of glucose-6-phosphatase. Histochemical evidence suggests that the Golgi vesicles contain acid phosphatase (11). Therefore, the acid phosphatase activity in fractions 30-39 of free-flow electrophoresis might represent Golgi vesicles and not lysosomes, which are known to sediment together with mitochondria (1); as a consequence of this behaviour, 34% of acid phosphatase was found in pellet 2, together with 66% of the mitochondrial marker, succinic dehydrogenase. Preliminary electron microscopic studies demonstrate typical Golgi vesicles in the above-

mentioned fractions, together with a high activity of thiaminpyrophosphatase (2, 15, 34).

Isolation of pinocytic vesicles makes it possible to compare different cellular structures involved in the pinocytic process in the proximal tubule cell. These results demonstrate that after the formation of pinocytic vesicles, the enzymatic content as well as the biochemical composition of the vesicular membrane (4) differ markedly from brush border microvilli. This might be due either to a basic difference in composition of the membrane regions where pinocytosis takes place or to a transformation of the membrane during the invagination and segregation of pinocytic vesicles from the apical cell surface. If lysosomes are compared with plasma membranes (19, 24, 45, 46), it is obvious that each step of the endocytic process has its own specified membrane equivalent with typical biochemical composition. The interactions between these cellular organelles and the pinocytized molecule are still unknown. The process consists of inducing the pinocytic process, attachment of the molecule to the membrane, invagination, segregation, migration of pinocytic vesicles, and finally transformation of endocytic vesicles into lysosomes. The question remains whether the latter are only digestive vacuoles or are involved in the transcellular transport of macromolecules or both.

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