Biochimica et Biophysica Acta, 433 (1976) 531–546

BBA 77286

ISOLATION OF THE BASAL AND LATERAL PLASMA MEMBRANES OF RAT KIDNEY TUBULE CELLS

H. EBEL, E. AULBERT and H. J. MERKER*

*Institut für Klinische Physiologie und *Institut für Anatomie, Freie Universität, Berlin (Germany)*

(Received October 29th, 1975)

SUMMARY

A method was developed to isolate renal basolateral membranes from cortical kidney tubule cells of single rats. The isolated membrane fraction was characterized by the measurement of marker enzyme activities and by electron microscopy.

1. After centrifugation of crude plasma membranes on a discontinuous sucrose density gradient the basolateral membranes accumulated at a sucrose density of ρ = 1.14–1.15 g/ml. The yield was 147 μg membrane protein/g kidney wet weight. Protein recovery was 0.1 %.

2. $(Na^+ + K^+)$-ATPase was enriched 22-fold from the homogenate. The recovery was 2.6 %. The $(Na^+ + K^+)/Mg^{2+}$-ATPase ratio was 4.1.

3. The contamination by brush borders was small. Alkaline phosphatase was 1.6-fold enriched and 0.2 % was recovered. Aminopeptidase was 1-fold enriched with a recovery of 0.1 %. The contamination by mitochondria, lysosomes and endoplasmic reticulum was negligible.

4. In electron micrographs the basolateral membranes showed a typical triple layered profile and were characterized by the presence of junctional complexes, gap junctions or tight junctions.

INTRODUCTION

Transepithelial sodium transport in the kidney involves at least two steps: passive uptake by the brush borders and active transport through the basal and lateral plasma membranes of the epithelial cell. Attempts to analyse the molecular mechanism of active sodium transport have been hindered by the difficulties involved in isolating purified basolateral membranes.

Preparations of renal plasma membranes usually contain both brush borders and basolateral membranes [1–5]. Since separation of renal plasma membrane subfractions has proved to be difficult, isolated brush border fractions have been considerably contaminated by basolateral membranes [2, 6–8]. The isolation of basolateral membranes is apparently even more difficult. Thus, no methods based on density gradient centrifugation have been reported in the literature. Only by free-flow electrophoresis of renal plasma membranes has a separation of brush borders

and basolateral membranes been obtained [7, 9]. However, this method is not suitable for a parallel isolation of the basolateral membranes from the kidneys of single rats since it is necessary to pool the plasma membranes of several animals for one electrophoresis run.

In this study a method is described to isolate basolateral membranes from cortical tubule cells of single rats. By centrifugation of crude plasma membranes on a discontinuous sucrose density gradient basolateral membranes with a minimal contamination by brush borders were obtained. A preliminary report has been published earlier [10].

METHODS

Isolation of basal lateral membranes

Male, pathogen-free Wistar rats of an inbred strain (180–220 g) were purchased from the Zentralinstitut für Versuchstierkunde, Hannover, Germany. The animals were allowed free access to food and water. They were killed by decapitation and were bled. The kidneys were removed, placed on ice and the capsule was stripped off. The cortex was dissected with a razor blade. All subsequent steps were carried out at 4 °C. To 1 g of minced tissue 5 ml of a homogenization medium was added, consisting of sucrose 250 mM, EDTA 1 mM, Tris 4 mM, pH 7.6. The tissue was poured into a Potter Elvehjem homogenizer with a Teflon pestle, stated clearance 0.15 mm (Potter type B, A. H. Thomas Company, Philadelphia, U.S.A.). Gentle but complete homogenization was performed with 5 strokes of a tight-fitting pestle at 45 rev./min and 25 strokes at 90 rev./min by using an automatic cell homogenizer (Colora, Lorch, Germany). The times employed for each stroke were 10 and 5 s, respectively. The homogenate was filtered through a fine-mesh nylon sieve into polyethylene tubes.

For differential centrifugation a Christ 15 000 centrifuge (Hereaus Christ, Osterode, Germany) with a special rate-meter was used which indicated the speed with an accuracy of 50 rev./min. Fig. 1 outlines the centrifugation procedure. To precipitate all of the nuclei the homogenate was centrifuged twice at $700 \times g_{av}$ for 10 min and the sediments were discarded. The supernatant was centrifuged at $1350 \times g_{av}$ for 10 min to spin down large mitochondria and the resulting sediment was discarded. The supernatant containing plasma membranes, small mitochondria and microsomes was centrifuged at $2500 \times g_{av}$ for 20 min. The resulting supernatant was carefully removed without the fluffy layer and discarded. The remaining sediment and the upper fluffy layer were gently homogenized by 5 strokes by hand in 5 ml of the homogenization medium. This suspension was recentrifuged twice at $2500 \times g_{av}$ for 20 min and the supernatants were discarded. The final pellet, a crude plasma membrane preparation contaminated by mitochondria and endoplasmic reticulum was suspended in 5 ml of homogenization medium by 10 hand strokes. The membrane suspension was loaded on top of a discontinuous sucrose gradient of the following densities: ρ = 1.14/1.15/1.16/1.17/1.18/1.20 g/ml. The volume of each layer was 5.5 ml. The sucrose solutions were prepared in water only at 20 ± 0.1 °C. The densities at 5 °C were calculated from the Handbook of Biochemistry [11]. Sucrose designed for density-gradient centrifugation was used. The density-gradient centrifugation was performed with a swinging bucket rotor SW 27 in a Beckmann ultracentrifuge Spinco

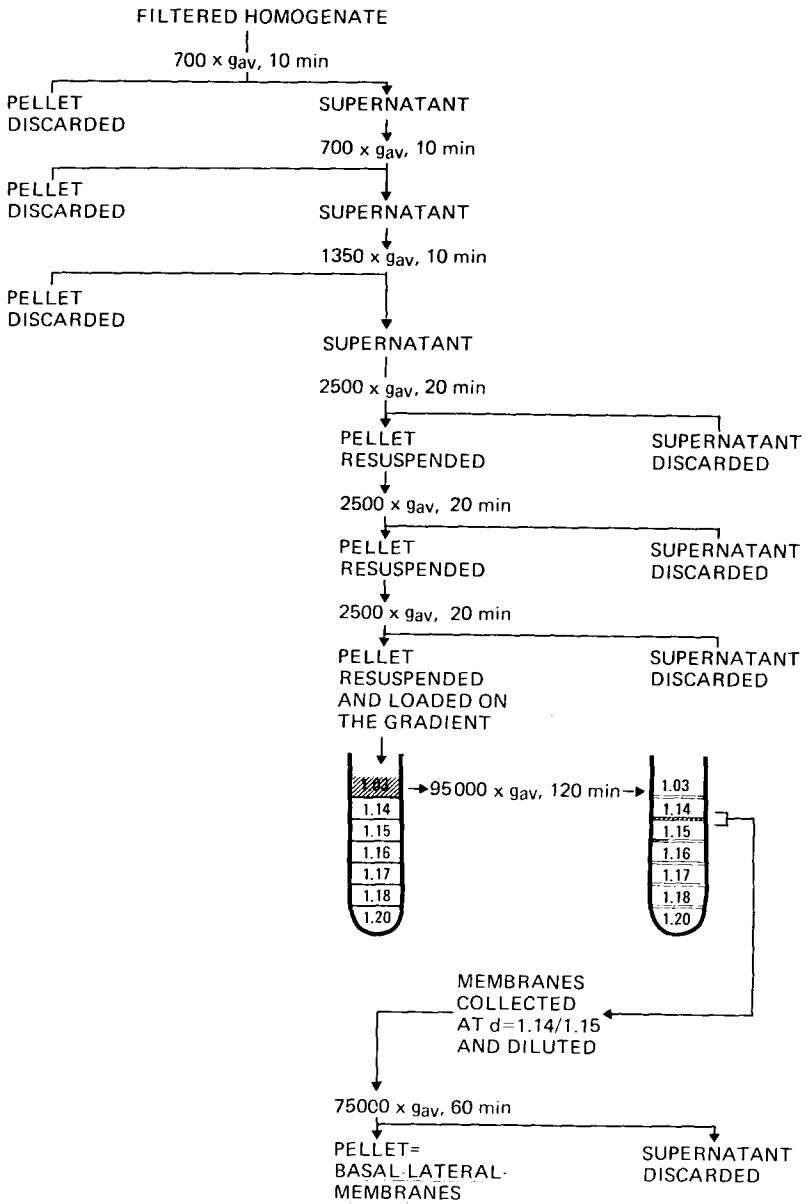

Fig. 1. Flow diagram for the isolation of basolateral membranes from rat kidney cortex tubule cells.

L2 65 B at 95 000 $\times g_{av}$ for 120 min. No brake was used. The basolateral membranes accumulated in a fine sharp band at the 1.14/1.15 g/ml density interface. Approximately 3 ml of the membrane material was collected. After dilution to isotonicity with ice-cold distilled water the membranes were precipitated at 75 000 $\times g_{av}$ for 60 min. The final pellet was suspended in 0.5 ml homogenization medium and stored at −25 °C.

TABLE I

Enzyme activities and recoveries in kidney cortex homogenate and subcellular fractions. Values are given as means ± S.E.M. Abbreviations: N + M sediment = Nuclear and mitochondrial sediment from the combined 0–700 *g* and 700–1350 *g* sediments. PM sediment = plasma membrane sediment of 1350–2500 *g*. SA = specific activity, defined as μmol product or substrate/mg protein · h, in case of succinate dehydrogenase and lactate dehydrogenase in μmol/mg protein · min. TA = total activity in μmol/g kidney · h or for succinate dehydrogenase and lactate dehydrogenase μmol/g · min. RSA = relative specific activity, defined as the ratio of specific activity of a fraction to that of the homogenate. Recovery % represents percentage of total activity of the homogenate recovered in the fraction.

		Homogenate			N + M Sediment (0–1350 *g*)			PM Sediment (1350–2500 *g*)			Supernatant (> 2500 *g*)			Total recovery %
Protein	mg/g	126.0	± 4.0	(37)	72.0	± 1.3	(19)	1.96	± 0.13	(23)	56.9	± 1.5	(25)	
	recovery %	100.0			57.1			1.6			45.2			103.9
Mg^{2+}-ATPase	SA	18.8	± 1.7	(27)	18.2	± 1.7	(12)	37.5	± 1.5	(12)	16.9	± 1.1	(11)	
	TA	2368.8	± 214.2		1310.4	± 122.0		73.5	± 2.9		961.9	± 63.7		
	RSA	1.0			1.0			2.0			0.9			
	recovery %	100.0			55.3			3.1			40.6			99.0
$(Na^+ + K^+)$-ATPase	SA	4.30	± 1.20	(27)	4.94	± 0.40	(12)	21.3	± 1.2	(12)	2.69	± 0.40	(11)	
	TA	541.8	± 151.2		355.7	± 28.8		41.7	± 2.4		153.1	± 22.8		
	RSA	1.0			1.1			5.0			0.6			
	recovery %	100.0			65.1			7.7			28.3			101.1
5′-Nucleotidase	SA	8.20	± 0.60	(17)	8.00	± 0.80	(12)	30.2	± 1.9	(16)	7.30	± 0.60	(12)	
	TA	1033.2	± 75.6		576.0	± 57.6		59.2	± 3.7		415.3	± 34.1		
	RSA	1.0			1.0			3.7			0.9			
	recovery %	100.0			55.7			5.7			40.2			101.6
Glucose-6-phosphatase	SA	10.0	± 0.7	(18)	5.35	± 0.06	(8)	19.9	± 1.8	(11)	15.1	± 1.44	(10)	
	TA	1260.0	± 88.2		385.2	± 42.8		39.0	± 3.5		859.2	± 82.0		
	RSA	1.0			0.5			2.0			1.5			
	recovery %	100.0			30.5			3.1			68.2			101.8

Alkaline phosphatase	SA	6.68 ± 0.90 (17)	7.93 ± 1.69 (12)	17.7 ± 1.8 (21)	4.39 ± 0.46 (22)	
	TA	841.7 ± 113.4	571.0 ± 121.7	34.7 ± 3.5	249.8 ± 26.2	
	RSA	1.0	1.2	2.7	0.7	
	recovery %	100.0	67.8	4.1	29.7	101.6
Aminopeptidase	SA	0.950 ± 0.100 (16)	0.774 ± 0.044 (10)	3.32 ± 0.33 (16)	0.854 ± 0.053 (11)	
	TA	119.7 ± 12.6	55.7 ± 3.2	6.51 ± 0.65	48.6 ± 3.0	
	RSA	1.0	0.8	3.5	0.9	
	recovery %	100.0	46.5	5.4	40.6	92.5
Succinate dehydrogenase	SA	0.216 ± 0.012 (11)	0.346 ± 0.024 (8)	0.126 ± 0.002 (14)	0.016 ± 0.003 (8)	
	TA	27.216 ± 1.449	24.890 ± 1.758	0.248 ± 0.004	0.927 ± 0.148	
	RSA	1.0	1.6	0.6	0.1	
	recovery %	100.0	91.6	0.9	3.4	95.9
Lactate dehydrogenase	SA	4.06 ± 0.38 (16)	1.48 ± 0.14 (9)	0.975 ± 0.793 (14)	7.01 ± 1.01 (10)	
	TA	511.6 ± 47.9	106.6 ± 10.1	1.91 ± 1.55	398.9 ± 57.5	
	RSA	1.0	0.4	0.2	1.7	
	recovery %	100.0	20.8	0.4	77.9	99.1

Determination of enzyme activities

Adenosine triphosphate phosphohydrolase (ATPase, EC 3.6.1.3) activity was determined at 37 °C by measuring the amount of inorganic phosphate liberated during incubation of the membranes with 3 mM sodium-free ATP and 3 mM $MgCl_2$ in a 70 mM imidazole/HCl buffer of pH 7.5. For measuring total ATPase 100 mM NaCl and 20 mM KCl were added. Inorganic phosphate was determined by the method of Fiske and SubbaRow [12]. $(Na^+ + K^+)$-ATPase was calculated as the difference of total ATPase minus basal Mg^+-ATPase. 5'-Nucleotidase (EC 3.1.3.5) was measured at 37 °C by a modification of the method of Heppel and Hilmoe [13] with 5 mM 5'-IMP as the substrate and 1 mM $CoCl_2$ for activation. Glucose-6-phosphatase (EC 3.1.3.9) was assayed at 37 °C by the method of Swanson [14] as modified by Hübscher and West [15]. Alkaline phosphatase (EC 3.1.3.1) was determined at 37 °C by the method of Ostrowski and Tsugita [16] with 5 mM *p*-nitrophenylphosphate as the substrate and 1 mM $MgCl_2$ in a 50 mM glycine/NaOH buffer of pH 9.5. *p*-Nitrophenol was used as the standard. Acid phosphatase (EC 3.1.3.2) was determined at 37 °C by the method of Ostrowski and Tsugita [16]. Aminopeptidase (EC 3.4.11.2) was determined at 25 °C as described by Tuppy et al. [17] with 5 mM leucine/*p*-nitroanilide as the substrate and 1 mM $MgCl_2$ for activation in a 50 mM triethanolamin buffer of pH 7.6. *p*-Nitroaniline was used as the standard. Succinate dehydrogenase (EC 1.3.99.1) activity was measured at 25 °C by the method of Green et al. [18] as modified by Earl and Korner [19] by recording the succinate dependent reduction of dichloroindophenol. Lactate dehydrogenase (EC 1.1.1.27) was measured as described earlier [3]. Protein was determined by the method of Lowry et al. [20] with crystalline bovine serum albumin (Behring Werke) as the standard.

Electron microscopy

The membrane pellets were fixed in centrifuge tubes with a solution of 2 % glutaraldehyde buffered to pH 7.2 with 100 mM of a phosphate buffer and were postfixed in a 1 % solution of osmium tetroxide in the same phosphate buffer. After dehydration in acetone, embedding was carried out in Vestopal. Thin sections were cut on a LKB and a Reichert microtome. The sections were double stained first with uranyl acetate and then with lead citrate. The sections were studied in a Siemens Elmiskop I at an accelerating voltage of 80 kV.

Materials

Glass-distilled deionized water was used in all experiments. Chemicals of analytical grade were obtained from E. Merck, Darmstadt. The sodium salts of adenosine triphosphate, glucose 6-phosphate, 5'-inosine monophosphate and succinate, the cyclohexylammonium salt of phosphoenolpyruvate and lactate dehydrogenase were purchased from Boehringer, Mannheim. Bovine serum albumin was from the Behringwerke AG., Marburg. Leucine/*p*-nitroanilide was from Serva, Heidelberg. *p*-Nitrophenyl phosphate was from Sigma, St. Louis, Mo., U.S.A.

RESULTS

Differential centrifugation

In the subcellular fraction obtained by differential centrifugation the activities

of several marker enzymes were measured and the relative specific activities were calculated. The results are presented in Table I. In the 1350–2500 *g* fraction the specific activities of plasma membrane marker enzymes were higher than in the homogenate. $(Na^+ + K^+)$-ATPase which is recognized as a marker for the basolateral plasma membranes [9, 21] was enriched by a factor of 5. Although this membrane fraction contained only 1.6 % of the total protein, the recovery of total $(Na^+ + K^+)$-ATPase was 7.7 %. 5'-Nucleotidase, a plasma membrane marker [22], was enriched 3.7-fold with a recovery of 5.7 %. Alkaline phosphatase and aminopeptidase, marker enzymes for the brush border membranes [23–26], were enriched 2.7-fold and 3.5-fold, respectively. The recoveries were 4.1 % and 5.4 %. A smaller enrichment was obtained for glucose-6-phosphatase, a marker for endoplasmic reticulum. The relative specific activity was only 2 and the recovery averaged 3 %. The other marker enzyme activities measured showed a decrease relative to the homogenate. The relative specific activity of succinate dehydrogenase which is located in the mitochondrial matrix as 0.6. The recovery was 0.9 %. Lactate dehydrogenase, a marker enzyme for the cytosol, showed a relative specific activity of 0.2 and a recovery of 0.4 %. Thus the marked enrichment of plasma membrane-bound enzymes and the loss of marker enzymes of other subcellular origin indicates that the 1350–2500 *g* fraction is a crude plasma membrane fraction with a contamination by endoplasmic reticulum and some mitochondria. Since enrichment and recoveries of $(Na^+ - K^+)$-ATPase were higher than that of the brush border enzymes alkaline phosphatase and aminopeptidase the plasma membranes may contain more basolateral membranes than brush borders.

The other subcellular fractions differed quantitatively from the plasma membrane sediment and they exhibited a rather heterogenous composition. The combined 700 *g* and 1350 *g* sediments were composed of nuclei, mitochondria, plasma membranes and even endoplasmic reticulum. As is indicated by a recovery of nearly 90 % of the mitochondrial succinate dehydrogenase, most of the mitochondria were sedimented in this fraction. The four plasma membrane enzymes, however, showed relative specific activities of only 0.8–1.2 but a recovery in the range of 47 to 68 %. On the other hand the supernatant contained about 68 % of the endoplasmic reticulum and 78 % of the cytosol as is calculated from the recovery of glucose-6-phosphatase and lactate dehydrogenase. Again the relative specific activities of the plasma membrane enzymes were rather low and in the range of 0.6–0.9 while a recovery of 28–40 % was obtained.

Density gradient centrifugation

After centrifugation of the plasma membranes on a discontinous sucrose gradient a separation of $(Na^+ + K^+)$-ATPase associated with the basolateral plasma membranes and of alkaline phosphatase and aminopeptidase located in the brush borders was achieved. The results are reported in Table II and Fig. 2. In the membranes accumulating at the sucrose density interface of $\rho = 1.14/1.15$ g/ml only 0.1 % of the total protein but 2.6 % of the total $(Na^+ + K^+)$-ATPase activity were recovered. With 95.6 ± 5.9 μmol P_i/mg protein · h the enrichment of $(Na^+ + K^+)$-ATPase from the homogenate was 22-fold. Since Mg^{2+}-ATPase was not significantly enriched the ratio of $(Na^+ + K^+)/Mg^{2+}$-ATPase increased from 0.3 in the homogenate over 0.6 in the plasma membranes to 4.1 in this fraction (not shown in Table II). This means that more than 80 % of the ATPase activity was $(Na^+ + K^+)$-ATPase.

TABLE II

Enzyme activities and recoveries of plasma membranes purified by discontinuous density gradient centrifugation. Basolateral membranes accumulated at the density interface of ρ = 1.14/1.15 g/ml. SA = specific activity, TA = total activity. For details see legend of Table I.

		Homogenate	Sucrose density gradient (g/ml)	
			1.03–1.14	1.14–1.15
Protein	mg/g	126.0 ± 4.0 (37)	0.136 ± 0.020 (33)	0.147 ± 0.024 (42)
	recovery %	100.0	0.1	0.1
Mg^{2+}-ATPase	SA	18.8 ± 1.7 (27)	57.3 ± 13.9 (11)	23.2 ± 1.5 (54)
	TA	2368.8 ± 214.2	7.8 ± 1.9	3.4 ± 0.2
	recovery %	100.0	0.3	0.1
$(Na^+ + K^+)$-ATPase	SA	4.30 ± 1.20 (27)	55.8 ± 5.4 (11)	95.6 ± 5.9 (54)
	TA	541.8 ± 151.2	7.6 ± 0.7	14.1 ± 0.9
	recovery %	100.0	1.4	2.6
5'-Nucleotidase	SA	8.20 ± 0.60 (17)	39.7 ± 2.9 (7)	40.1 ± 4.4 (12)
	TA	1033.2 ± 75.6	5.4 ± 0.4	5.9 ± 0.6
	recovery %	100.0	0.5	0.6
Glucose-6-phosphatase	SA	10.0 ± 0.7 (18)	22.3 ± 4.3 (10)	7.0 ± 1.3 (14)
	TA	1260.0 ± 88.2	3.0 ± 0.6	1.0 ± 0.2
	recovery %	100.0	0.2	0.1
Alkaline phosphatase	SA	6.68 ± 0.90 (17)	10.5 ± 1.6 (12)	10.5 ± 1.5 (30)
	TA	841.7 ± 113.4	1.4 ± 0.2	1.5 ± 0.2
	recovery %	100.0	0.2	0.2
Aminopeptidase	SA	0.95 ± 0.10 (16)	0.75 ± 0.13 (8)	0.97 ± 0.51 (14)
	TA	119.7 ± 12.6	0.10 ± 0.02	0.14 ± 0.07
	recovery %	100.0	0.1	0.1
Succinate dehydrogenase	SA	0.216 ± 0.012 (11)	0.058 ± 0.21 (9)	0.070 ± 0.012 (12)
	TA	27.216 ± 1.449	0.008 ± 0.003	0.010 ± 0.002
	recovery %	100.0	0.03	0.04
Lactate dehydrogenase	SA	4.06 ± 0.38 (16)	0.05 ± 0.03 (12)	0.06 ± 0.02 (16)
	TA	511.6 ± 47.9	0.010 ± 0.004	0.010 ± 0.003
	recovery %	100.0	0.002	0.002

A much lower enrichment was found for 5'-nucleotidase. The relative specific activity was 4.9 and the recovery only 0.6 %. In the same fraction relative low specific activities of alkaline phosphatase and of aminopeptidase were registered. The enrichment of these brush border enzymes was 1.6-fold and 1.0-fold, respectively and the recoveries were in the range of 0.2 and 0.1 %. The contamination by glucose-6-phosphatase derived from endoplasmic reticulum was minimal, as is indicated by the relative specific activity of 0.7 and the recovery of 0.1 %. Furthermore almost no mitochondria were present: the relative specific activity of succinate dehydrogenase was 0.3 % and the recovery averaged 0.04 %. In addition, the contamination by acid phosphatase, a lysosomal marker, was rather small. It was found (n = 9) that the relative specific activity averaged 0.5 and the recovery was 0.7 %. Cytosolic lactate dehydrogenase, possibly included into the membrane vesicles could be rarely detected before and after sonication. The relative specific activity was 0.01 and the recovery as small as 0.002 %.

1.15–1.16	1.16–1.17	1.17–1.18	1.18–1.20
0.114 ± 0.013 (42)	0.254 ± 0.037 (28)	0.391 ± 0.037 (24)	0.330 ± 0.036 (24)
0.1	0.2	0.3	0.3
26.7 ± 6.2 (30)	28.2 ± 7.3 (9)	26.4 ± 7.8 (8)	22.9 ± 6.9 (8)
3.0 ± 0.7	7.2 ± 1.9	10.3 ± 3.0	7.6 ± 2.3
0.1	0.3	0.4	0.4
48.3 ± 13.2 (30)	18.4 ± 5.6 (9)	14.9 ± 8.2 (8)	2.8 ± 1.4 (8)
5.5 ± 1.5	4.7 ± 1.4	5.8 ± 3.2	0.9 ± 0.5
1.0	0.9	1.1	0.2
79.5 ± 6.0 (8)	74.6 ± 7.0 (6)	38.4 ± 6.5 (6)	21.4 ± 3.4 (6)
9.1 ± 0.7	18.9 ± 1.8	15.0 ± 2.5	7.1 ± 1.1
0.9	1.8	1.5	0.7
6.6 ± 2.2 (8)	6.0 ± 2.0 (8)	7.1 ± 3.8 (8)	8.3 ± 4.3 (6)
0.8 ± 0.3	1.5 ± 0.5	2.8 ± 1.5	2.7 ± 1.4
0.1	0.1	0.2	0.2
31.4 ± 2.1 (11)	22.6 ± 2.5 (10)	13.1 ± 1.8 (9)	8.8 ± 1.3 (8)
3.6 ± 0.2	5.7 ± 0.6	5.1 ± 0.7	2.9 ± 0.4
0.4	0.7	0.6	0.3
2.29 ± 0.50 (7)	3.23 ± 0.44 (8)	3.94 ± 0.51 (8)	2.86 ± 0.60 (8)
0.26 ± 0.06	0.82 ± 0.11	1.54 ± 0.20	0.94 ± 0.2
0.2	0.7	1.3	0.8
0.066 ± 0.016 (7)	0.067 ± 0.024 (6)	0.066 ± 0.021 (6)	0.066 ± 0.022 (6)
0.008 ± 0.002	0.017 ± 0.006	0.026 ± 0.008	0.022 ± 0.007
0.03	0.06	0.1	0.08
0.09 ± 0.05 (12)	0.17 ± 0.09 (11)	0.42 ± 0.15 (11)	0.34 ± 0.12 (11)
0.010 ± 0.005	0.04 ± 0.02	0.16 ± 0.06	0.11 ± 0.04
0.002	0.01	0.03	0.02

At higher densities of the discontinuous sucrose gradient lower activities of $(Na^+ + K^+)$-ATPase were measured. At $\rho = 1.15/1.16$ g/ml and 1.16/1.17 g/ml the enrichment was 11.2 and 4.2, respectively. Also the recovery of $(Na^+ + K^+)$-ATPase decreased and ranged between 0.9 and 1.0 %. However, in these membrane fractions an increase of brush border enzyme activities was registered. At $\rho = 1.15/1.16$ g/ml the enrichment of alkaline phosphatase was 4.7 fold and that of aminopeptidase 2.4-fold. The recovery of both enzymes was also slightly increased. At the higher sucrose densities also a considerable increase of 5′-nucleotidase activity was observed, resulting in 9- to 10-fold enrichment with a recovery of 0.9 and 1.8 % respectively.

It should be noted that employing an isopynic density gradient centrifugation for 20 h, the same enzyme profile was obtained at the different densities. No higher purification of the membrane fraction collected at 1.14/1.15 g/ml was observed. Thus, for practical reasons the centrifugation was performed for 2 h only.

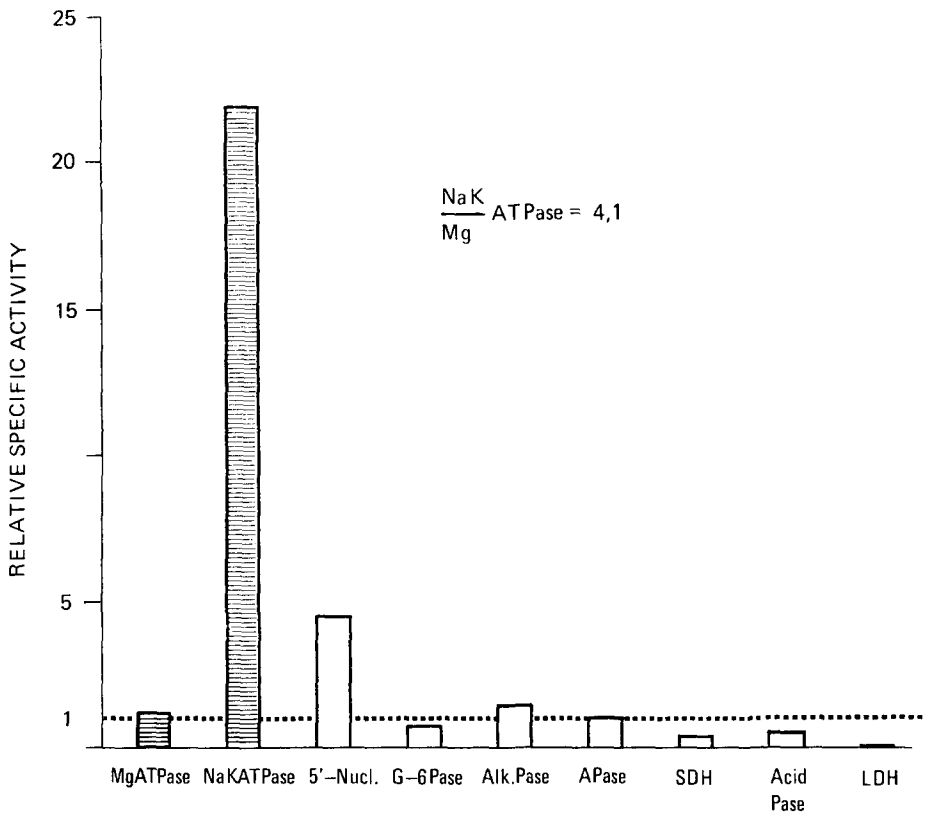

Fig. 2. Relative specific activities of marker enzymes in basolateral membranes prepared from rat kidney cortex tubule cells. Values higher than 1 indicate the enrichment of an enzyme from the homogenate. Abbreviations: 5'-Nucl = 5'-nucleotidase, G-6-Pase = glucose-6-phosphatase, Alk. Pase = alkaline phosphatase, APase = Aminopeptidase, SDH = succinate dehydrogenase, Acid Pase = acid phosphatase, LDH = lactate dehydrogenase, NaKATPase = $(Na^+ + K^+)$-ATPase. Values are calculated from Table II. For acid phosphatase calculation see in Results.

Electron micrographs of the membrane fraction collected at $\rho = 1.14/1.15$ g/ml are shown in Fig. 3 and 4. At low magnification a relatively homogenous membrane fraction was seen consisting of closed vesicles and open sheets of membranes. At higher magnification the membranes showed a trilayered structure, about 90–100 Å thick, as is typical for plasma membranes. Part of these membranes were forming membrane pairs with an interspace of 150–200 Å resembling to the infolded basolateral membranes in vivo. In addition, numerous junctional complexes, gap junctions or tight junctions were detected. The presence of adhering membranes indicates that a substantial portion of the membrane fraction is derived from the infolded basolateral membranes. The contamination by other cellular structures was low. Single vesicles or clusters of microvilli hanging together, resembling to brush border membranes, were only occasionally present. In addition, other cell particles, such as lysosomes and microbodies, mitochondria and endoplasmic reticulum were not detected.

Fig. 3. Electron micrograph of basolateral membranes prepared from rat kidney cortex tubule cells. Magnification 1:20000. The membranes appeared as closed vesicles (⊗) or open vesicles and stretched sheets (↗).

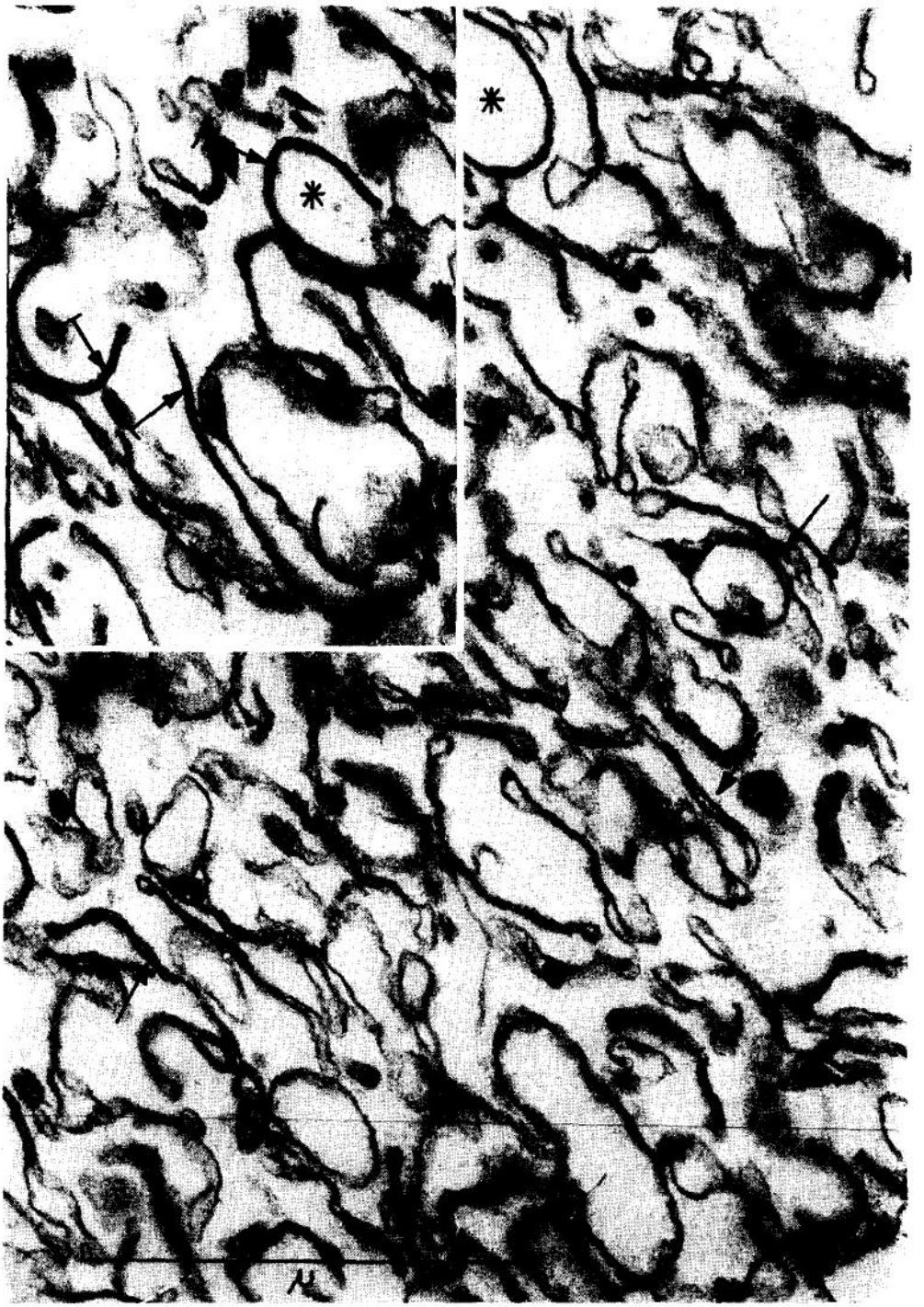

Fig. 4. Electron micrograph of basal lateral membranes from rat kidney cortex tubule cells. Magnification 1:62500. Symbols: ↙, typical triple layered membranes; *, round membrane profiles, identified at higher magnification as junctional complexes. Inset: ↙ gap junctions or tight junctions.

DISCUSSION

The object of the present study was to develop a simple rapid method for the isolation of basolateral membranes from tubule cells of single rats. Since both zonal centrifugation and free-flow electrophoresis are unsuitable for the parallel isolation of membranes of several individual rats a discontinuous sucrose gradient centrifugation in a swinging-bucket rotor was used. The membrane fractions were identified by marker enzymes and electron microscopy.

The main difficulty in the isolation of basolateral membranes is the overlap in density of brush borders [7]. The density of plasma membrane particles is critically dependent on the degree of homogenization, which is usually carried out by hand [9, 23]. Consequently, a wide variability in the density of renal plasma membranes has been observed, ranging from 1.13–1.14 [27] to 1.17–1.18 [23, 28, 29]. Thus, our strategy was to standardize the homogenization procedure and to find a plasma membrane fraction which contained more basolateral membranes than brush borders.

The homogenization procedure was carefully standardized by using an automated homogenizer. Since the use of a hypo-osmotic medium caused a contamination of renal plasma membranes by various damaged or broken subcellular elements [30–34] an isosmotic medium was used in the present study for homogenization and differential centrifugation. Empirically it was found that the 1350–2500 *g* sediment was a plasma membrane fraction most suitable for further purification. In this fraction obviously more basolateral membranes than brush borders were present. $(Na^+ + K^+)$-ATPase associated with the basolateral membranes was enriched 5-fold and 7.7 % recovered. The brush border enzymes alkaline phosphatase and aminopeptidase showed a significantly lower enrichment and recovery.

The basolateral membranes which accumulated at the sucrose density of ρ = 1.14/1.15 g/ml were characterized by a high enrichment of $(Na^+ + K^+)$-ATPase and a small enrichment of the brush-border enzymes alkaline phosphatase and aminopeptidase. The enrichment factor of $(Na^+ + K^+)$-ATPase was 22, that of the brush border enzymes 1.6 and 1.0, respectively. The same relationship was observed for the enzyme recoveries, that were 2.6 % for $(Na^+ + K^+)$-ATPase and 0.2–0.1 % for the brush-border enzymes. These results indicate that a basolateral membrane fraction with a low contamination by brush borders has been isolated. The $(Na^+ + K^+)$-/Mg^{2+}-ATPase ratio of 4.1 indicates that more than 80 % of ATPase is $(Na^+ + K^+)$-ATPase. The remaining activity is most likely to be due to a Ca^{2+}-ATPase which is also activated by Mg^{2+} [35] and is located in the basolateral membranes [36]. Electron micrographs confirm that the isolated membranes are, in fact, derived from the infolded basolateral membranes, showing trilayered plasma membranes, about 90–100 Å thick, plasma membrane pairs with an interspace of about 150–200 Å and several junctional complexes. Microvilli, however, were only rarely encountered and other subcellular structures were not apparent. Since rat kidney cortex is composed of 4 times more proximal than distal tubules [37] and since other structures such as the glomeruli and capillaries are lost during the first centrifugation steps it seems reasonable to suppose that most of the basolateral membranes originate from proximal tubule cells.

5′-Nucleotidase was the only enzyme other than $(Na^+ + K^+)$-ATPase which was also enriched in the basolateral membranes. However, compared to $(Na^+ + K^+)$-

ATPase a 4.9-fold enrichment and a 0.6 % recovery were low values. Similar results have been reported for a renal plasma membrane fraction. The enrichment factors for $(Na^+ + K^+)$-ATPase and 5'-nucleotidase were 11.4-fold and 3.7-fold, respectively, while alkaline phosphatase was enriched only 1.1-fold [4]. However, a 17- to 25-fold enrichment of 5'-nucleotidase has been measured in purified brush borders [24, 25], suggesting that 5'-nucleotidase is a brush border enzyme. Histochemical studies have shown that 5'-nucleotidase is present in the brush borders but that some activity is also located in the basolateral membranes [38]. Thus, the question as to whether 5'-nucleotidase is exclusively associated with the brush borders or is also present in the basolateral membranes remains to be clarified.

The contamination of the basolateral membranes by mitochondria, lysosomes and endoplasmatic reticulum was low. This is indicated by the low relative specific activities and small recoveries of the marker enzymes, succinate dehydrogenase, acid phosphatase and glucose-6-phosphatase. The enrichment factors of the enzymes were 0.3, 0.5 and 0.7 respectively, the recoveries 0.04, 0.7 and 0.1 %. Practically no cytosolic lactate dehydrogenase was present: the enrichment factor was 0.01, the recovery as small as 0.002 %. It should be kept in mind that the difficulty in estimating the purity of a membrane fraction is, that the marker enzymes are often present in more than one subcellular fraction. Alkaline phosphatase, most frequently used as a marker for brush borders is believed to be present also in basolateral membranes of the tubule cells [39, 40]. Furthermore the enzyme has also been detected in glomeruli [41]. Aminopeptidase is not only present in brush borders but also in glomeruli [42, 43] and endoplasmic reticulum [44]. Moreover, the enzyme is only partially particle bound. In our studies 20 % of the total activity remained in the cytosol. 5'-Nucleotidase, a plasma-membrane marker, has also been detected in purified microsomes [45, 46]. Glucose-6-phosphatase, the classical marker for endoplasmic reticulum, was also detected in the nuclear membranes [47]. For acid phosphatase, a lysosomal marker, evidence for the presence in the rough endoplasmic reticulum has been presented [48, 49]. In conclusion, it is clear that marker enzyme studies indicating the purity of a membrane fraction, must be interpreted with caution.

Comparing the basolateral membranes isolated by us with those purified by free flow electrophoresis by Kinne et al. it is evident that a smaller enrichment of $(Na^+ + K^+)$-ATPase was obtained by these authors. The enrichment factor ranged between 10.5 [36], approximately 12 [50] and 15.8 [7] compared with 22 in the present study. Accordingly the ratio of $(Na^+ + K^+)$-/Mg^{2+}-ATPase was also lower and nearly 2.3 (calculated from Fig. 2 of ref. 9) while 4.1 was obtained in the present study. The contamination by alkaline phosphatase was nearly the same in both studies, the enrichment factors were 1.3 after free-flow electrophoresis [36] and 1.6 in our study. The contamination by succinate dehydrogenase was the same in both methods. Unfortunately no values for the enrichment of other marker enzymes and no recoveries have been reported by the other authors.

It is an open question, if the membranes isolated by the procedure of Marx et al. [28] are in fact purified basolateral membranes, disintegrated parts of it or from another cellular region. The authors reported only a 2.7 fold enrichment of $(Na^+ + K^+)$-ATPase but a 7-fold enrichment of basal adenylate cyclase and a 25-fold enrichment of parathyroid hormone stimulated adenylate cyclase [28]. Adenylate cyclase is according to Kinne et al. [51] associated with basolateral membranes.

However, if the membranes of Marx et al. were really basolateral membranes, then $(Na^+ + K^+)$-ATPase would be enriched by a factor of 10–22.

Since the purity of the basolateral membranes was achieved at the expense of yield, the question of yield in membrane protein is of obvious importance. With the method presented in this study 147 μg membrane protein/g kidney cortex wet weight were obtained. This yield is high enough for further analysis of the membrane structure and function. In a first attempt, synthesis and turnover of single membrane proteins have been studied by us [52]. The yield for basolateral membranes described by us is not much lower than that of 170 μg/g reported for renal plasma membranes [32]. The yield for basolateral membranes purified by free flow electrophoresis has not been given by the authors, but must be low since one electrophoresis run required 10 mg/ml protein, necessitating the pooled kidneys of 10 rats [9].

ACKNOWLEDGEMENT

This work was supported in part by a grant of the Deutsche Forschungsgemeinschaft.

REFERENCES

1 Fitzpatrick, D. F., Davenport, G. R., Forte, L. and Landon, E. J. (1969) J. Biol. Chem. 244, 3561–3569
2 Kinne, R. and Kinne-Saffran, E. (1969) Pflügers Arch. 308, 1–15
3 Ebel, H., De Santo, N. G. and Hierholzer, K. (1971) Pflügers Arch. 324, 1–25
4 Kirschbaum, B. K. and Bosmann, H. B. (1973) Nephron 12, 211–218
5 Busse, D., Jahn, A. and Steinmaier, G. (1975) Biochim. Biophys. Acta 401, 231–243
6 Berger, S. J. and Sacktor, B. (1970) J. Cell Biol. 47, 637–645
7 Kinne, R., Schmitz, J. E. and Kinne-Saffran, E. (1971) Pflügers Arch. 329, 191–206
8 Quirk, S. J. and Robinson, G. B. (1972) Biochem. J. 128, 1319–1328.
9 Heidrich, H. G., Kinne, R., Kinne-Saffran, E. and Hannig, K. (1972) J. Cell Biol. 54, 232–245
10 Aulbert, E., Froese, P., Leipnitz, W. and Ebel, H. (1973) Pflügers Arch. 339, R50
11 Anderson, N. G. (1968) in Handbook of Biochemistry, (Sober, H. A. and Harte, R. A., eds.), J248–J252, The Chemical Rubber Co., Ohio
12 Fiske, C. H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375
13 Heppel, L. A. and Hilmoe, R. J. (1955) in Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds.), Vol. 2, 546–550, Academic Press Inc., New York
14 Swanson, M. A. (1950) J. Biol. Chem. 184, 647–659
15 Hübscher, G. and West, G. R. (1965) Nature 205, 799–800
16 Ostrowski, W. and Tsugita, A. (1961) Arch. Biochem. 94, 68–78
17 Tuppy, H., Wiesbauer, U. and Wintersberger, E. (1962) Hoppe Seyler's Z. Physiol. Chem. 329, 278–288
18 Green, D. E., Mii, S. and Kohout, P. M. (1955) J. Biol. Chem. 217, 551–567
19 Earl, D. C. N. and Korner, A. (1965) Biochem. J. 94, 721–734
20 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
21 Schmidt, U. and Dubach, U. C. (1971) Pflügers Arch. 330, 265–270
22 Emmelot, P., Bos, C. J., Benedetti, E. L. and Rühmke, P. (1964) Biochim. Biophys. Acta 90, 126–145
23 Wilfong, R. F. and Neville, Jr., D. M. (1970) J. Biol. Chem. 245, 6106–6112
24 Glossmann, H. and Neville, Jr., D. M. (1972) FEBS Lett. 19, 340–344
25 George, S. G. and Kenny, A. J. (1973) Biochem. J. 134, 43–57
26 Thomas, L. and Kinne, R. (1972) Biochim. Biophys. Acta 255, 114–125
27 Jørgensen, P. L., Skou, J. C. and Solomonson, L. P. (1971), Biochim. Biophys. Acta 233, 381–394

28 Marx, S. J., Fedak, S. A. and Aurbach, G. D. (1972) J. Biol. Chem. 6913–6918
29 Price, R. G., Taylor, D. G. and Robinson, D. (1972) Biochem. J. 129, 919–928
30 Coleman, R. and Finean, J. B. (1966) Biochim. Biophys. Acta 125, 197–206
31 Finean, J. B., Coleman, R., Green, W. A. (1966) Ann. N. Y. Acad. Sci. 137, 414–420
32 Manitius, A., Bensch, K. and Epstein, F. H. (1968) Biochim. Biophys. Acta 150, 563–571
33 Bockaert, J., Roy, C., Rajerison, R. and Jard, S. (1973) J. Biol. Chem. 248, 5922–5931
34 Sutcliffe, H. S., Martin, T. J., Eisman, J. A. and Pilczyk, R. (1973) Biochem. J. 134, 913–921
35 Parkinson, D. K. and Radde, I. C. (1971) Biochim. Biophys. Acta 242, 238–246
36 Kinne-Saffran, E. and Kinne, R. (1974) J. Membrane Biol. 17, 263–274
37 Rapp, J. P. (1967) Am. J. Physiol. 213, 947–953
38 Hardonk, M. J. (1968) Histochemie 12, 1–17
39 Reale, E. and Luciano, L. (1967) Histochemie 8, 302–314
40 Schmidt, U. and Dubach, U. C. (1972) Experientia 28, 385–386
41 Schmidt, U. and Dubach, C. (1969). Z. Gesamte Exp. Med. 151, 93–102
42 Marosvari, I. and Tanka, D. (1969) Klin. Wochenschr. 47, 1178–1179
43 Taylor, D. G., Price, R. G. and Robinson, D. (1971) Biochem. J. 122, 641–645
44 Seligman, A. M., Wasserkrug, H. L., Plapinger, R. E., Seito, T. and Hanker, J. S. (1970) J. Histochem. Cytochem. 18, 542–551
45 Song, C. S., Kappas, A., Bodansky, O. (1969) Ann. N. Y. Acad. Sci 166, 565–572
46 Widnell, C. C. (1972) J. Cell Biol. 52, 542–558
47 Kartenbeck, J., Jarasch, E. D. and Franke, W. W. (1973) Exp. Cell Res. 81, 175–194
48 Maunsbach, A. B. (1966) J. Ultrastruct. Res. 16, 197–238
49 Miyayama, H., Solomon, R., Sasaki, M., Lin, C. and Fishman, W. H. (1975) J. Histochem. Cytochem. 23, 439–451
50 Kinne, R. Murer, H., Kinne-Saffran, E., Thees, M. and G. Sachs (1975) J. Membrane Biol. 21, 375–395
51 Kinne, R. Shlatz, L. J., Kinne-Saffran, E. and Schwartz, J. L. (1973) in Proceedings of the 9th International Congress on Biochemistry, Abstract 5b4, p. 258
52 Ebel, H., Gebhardt, A. and Aulbert, E. (1975) 6th International Congress on Nephrology, Abstract 42, Poligrafici Luigi Pharma S.P.A., Bologna, Italy