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Further Evidence for the Existence of an Intrinsic Bicarbonate-Stimulated Mg^{2+}-ATPase in Brush Border Membranes Isolated from Rat Kidney Cortex

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Summary. The aim of this study was to provide further evidence for the existence of a nonmitochondrial bicarbonate-stimulated Mg^{2+}-ATPase in brush border membranes derived from rat kidney cortex. A plasma membrane fraction rich in brush border microvilli and a mitochondrial fraction were isolated by differential centrifugation. Both fractions contain a Mg^{2+}-ATPase activity which can be stimulated by bicarbonate. The two Mg^{2+}-ATPases are stimulated likewise by chloride, bicarbonate, and sulfite or inhibited by oligomycin and aurovertin, though to different degrees. In contrast to these similarities, only the Mg^{2+}-ATPase activity of the mitochondrial fraction is inhibited by atractyloside, a substance which blocks an adenine nucleotide translocator in the inner mitochondrial membrane. On the other hand, filipin, an antibiotic that complexes with cholesterol in the membranes inhibits exclusively the Mg^{2+}-ATPase of the cholesterol-rich brush border membranes. Furthermore it could be demonstrated by the use of bromotetramisole, an inhibitor of alkaline phosphatase activity, that the Mg^{2+}-ATPase activity in the membrane fraction is not due to the presence of the highly active alkaline phosphatase in these membranes. These results support the assumption that an intrinsic bicarbonate-stimulated Mg^{2+}-ATPase is present in rat kidney brush border membranes.

Several years ago we described the presence of a Mg^{2+}-ATPase activity in a brush border membrane fraction isolated by free flow electrophoresis, which is stimulated by oxyanions such as sulfite and bicarbonate [14]. One of the difficulties in studies on the distribution of this enzyme in the cells of the proximal tubule is the presence of a high bicarbonate stimulated Mg^{2+}-ATPase activity in the mitochondria. So the existence of a plasma membrane bound anion sensitive Mg^{2+}-ATPase in the kidney and also in various other tissues has been recently questioned and has been attributed to the mitochondrial contamination of the membrane fractions [1–3].

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We therefore reinvestigated the problem of a nonmitochondrial bicarbonate-stimulated Mg^{2+}-ATPase in rat kidney using "mitochondrial" inhibitors as well as substances which do not interact with the enzyme molecule itself but which interact with essential and more or less unique components of the membranes in which the enzymes are embedded.

Materials and Methods

Materials

Na_2-ATP and carboxy-atractyloside were obtained from Boehringer (Mannheim, Germany), oligomycin from Serva (Heidelberg, Germany), and D- and L-bromotetramisole from Aldrich (Milwaukee, Wisc.). The following items are generous gifts from several people to whom we want to express our gratitude herewith: aurovertin from Dr. H. Penefsky (New York, N.Y.), filipin from Dr. B. de Kruijff (Utrecht, The Netherlands) and Upjohn Company (USA), mitochondrial inhibitor protein from Dr. D.A. Harris (Oxford, England).

All other reagents are from Merck (Darmstadt, Germany) and are of analytical grade.

Preparation of Membrane Fractions

Rat kidney cortex of male wistar rats with a body weight of about 200 g was homogenized in 6 volumes (wt/vol) of 250 mM sucrose and 10 mM triethanolamine-HCl, pH 7.6, employing 10 strokes by hand and then 3 strokes at 340 rpm using a loose fitting Teflon-glass homogenizer. A crude brush border membrane fraction and a mitochondrial fraction were obtained by differential centrifugation in the above-mentioned sucrose medium. The centrifugation scheme is shown in Table 1.

Determination of the Bicarbonate-Stimulated Mg^{2+}-ATPase Activity

The ATPase activity in the mitochondrial fractions as well as in the brush border membrane fractions was determined according to Bornancin *et al.*[1] The incubation medium contained 2×10^{-3} M ouabain, 1×10^{-3} M $MgSO_4$, 1×10^{-3} M Na_2-ATP, 5×10^{-2} M glycine-Tris, pH 8.0, and HCO_3^- in a final concentration of 2.5×10^{-2} M.

The reaction was started by addition of ATP to 600 µl reaction medium that contained about 100 µg mitochondrial protein or about 20 µg brush border membrane protein, respectively. After incubation for 15 min at 37 °C, the reaction was stopped by heating the samples for 2 min in boiling water. The samples were then chilled and centrifuged. The inorganic phosphate released was measured in aliquots of the supernatants according to Bartlett and Fiske-Subbarow [4, 7]. All assays were performed in duplicate. Any influence of the inhibitors present in the incubation medium on the enzyme assay itself was checked by running the blank, substrate blank, and the P_i standards with and without inhibitor. The effect of bovine heart mitochondrial inhibitor protein on the ATPase activity was

[1] Bornancin, M., Renzis, G. de, Naon, R. 1978. A Cl^-/HCO_3^--ATPase in the gills of fresh water or sea water adapted *Salmo gairdneri*. Evidence of its microsomal localization (*submitted for publication*)

Table 1. Isolation scheme for the preparation of a mitochondrial fraction and a crude brush border membrane fraction by differential centrifugation

Renal cortex of rat kidneys

Homogenize in 6 volumes (wt/vol) of isotonic sucrose medium with a loose fitting Teflon-glass homogenizer. Ten strokes by hand and three strokes at 340 rpm. Centrifuge homogenate for 10 min at $700 \times g$ (S_1, P_1). Discard P_1.

Supernatant 1

Centrifuge for 10 min at $700 \times g$ (S_2, P_2). Discard P_2.

Supernatant 2

Centrifuge for 10 min at $2000 \times g$ (S_3, P_{31}, P_{32}). Discard P_{31} and P_{32}.

Supernatant 3

Centrifuge for 10 min at $10{,}000 \times g$ (S_4, P_{41}, P_{42}).

P_{42}	S_4+P_{41}
Resuspend in isotonic sucrose medium. Centrifuge for 10 min at $10{,}000 \times g$ (S_5, P_{51}, P_{52}). Discard S_5 and P_{51}.	Combine S_4 and P_{41}. Centrifuge for 20 min at $16{,}000 \times g$ (S_5, P_{51}, P_{52}). Discard S_5 and P_{52}.
P_{52}	P_{51}
Resuspend in isotonic sucrose medium. Centrifuge for 10 min at $10{,}000 \times g$ (S_6, P_{61}, P_{62}). Discard S_6 and P_{61}. P_{62} = mitochondrial fraction	Resuspend in isotonic sucrose medium. Centrifuge for 20 min at $16{,}000 \times g$ (S_6, P_{61}, P_{62}). Discard S_6 and P_{62}. P_{61} = brush border membrane fraction

tested after preincubation of mitochondria or brush border membranes with the inhibitor for 15 min at 37 °C before adding ATP. When it was necessary to dissolve substances in ethanol a control was run with the same ethanol concentration in the incubation medium. Depending on the inhibitors mechanism of action, the enzyme assay was performed with intact or with frozen and thawed mitochondria [19]. Brush border membranes were treated the same way. Pretreatment is indicated in the legends to the tables and figures.

Determination of other Enzymes, Protein and Cholesterol

The activity of alkaline phosphatase and (Na^+-K^+)-ATPase was determined with tests used in this laboratory as described in [13]. As (Na^+-K^+)-ATPase we define the activation of a ouabain-inhibited Mg^{2+}-sensitive ATP-phosphohydrolase by Na^+ and K^+. The assay for succinic dehydrogenase activity was performed after preincubation with 0.1% sodium-desoxycholate for 30 min at 0 °C with a method described by Gibbs and Reimer [9]. The protein content of the fractions was estimated after precipitation of the protein by 10% TCA in the cold and dissolution of the precipitate in 1 N NaOH according to Lowry *et al.* [17] with bovine serum albumin serving as standard.

For the determination of cholesterol, the lipids were resolved according to Pfleger *et al.* [20] and visualized on thin layers of Silica Gel G, 250 µm, according to Gluck *et al.* [10].

Results

Characterization of the Mitochondrial Fraction and the Brush Border Membrane Fraction

Table 2 gives the activities of some marker enzymes in the cortex homogenate, in the mitochondrial fraction, and in the brush border membrane fraction. Alkaline phosphatase as a marker enzyme for brush border membranes shows a slightly reduced activity in the mitochondrial fraction compared to the homogenate, whereas it is 10 to 11 times enriched in the brush border membrane fraction. $(Na^+ + K^+)$-ATPase, a marker enzyme for basal lateral plasma membranes in kidney cortex, remains also practically unchanged in the mitochondrial fraction and shows a 4 times higher activity in the brush border membrane fraction. In contrast to these two enzymes, the activity of succinic dehydrogenase which was used as a marker enzyme for mitochondria is enriched from 48 µmol/hr/mg protein in the homogenate to 212 µmol/hr/mg protein in the mitochondrial fraction, and is reduced to 10 µmol/hr/mg protein in the brush border membrane fraction.

Mg^{2+}-ATPase and $(Mg^{2+} + HCO_3^-)$-ATPase Activity in the Mitochondrial Fraction and in the Brush Border Membrane Fraction

During the isolation of a brush border membrane fraction from kidney cortex homogenate the Mg^{2+}-ATPase activity is enriched about

Table 2. Activities of some marker enzymes and of Mg^{2+}-ATPase and of $(Mg^{2+} + HCO_3^-)$-ATPase in the homogenate of rat kidney cortex, in the mitochondrial fraction, and in the crude brush border membrane fraction

	Alkaline phosphatase	$(Na^+ + K^+)$-ATPase	Succinic dehydrogenase	Mg^{2+}-ATPase	$(Mg^{2+} + HCO_3^-)$-ATPase[3]	ΔHCO_3^-
Homogenate	10.5±0.8	3.1±0.4	48.4±2.7	18.9±1.3	24.3±0.5	5.4±1.2
Mitochondrial fraction	8.3±0.7 (0.8)	4.2±0.4 (1.3)	211.6±7.0 (4.4)	15.3±0.4 (0.8)	28.9±1.0 (1.2)	13.6±0.7 (2.5)
Brush border membrane fraction	112.8±3.8 (10.7)	12.5±1.1 (4.0)	9.7±1.0 (0.2)	58.1±1.6 (3.1)	76.5±1.8 (3.1)	18.4±1.2 (3.4)

The enzyme activities are given in µmol/hr/mg protein. Mean values of five experiments ±SD are shown. Numbers in brackets give the enrichment factor, which represents the ratio between the activity in the fraction and the activity in the homogenate.

Table 3. Recovery of enzyme activities and protein during preparation of the mitochondrial and the brush border membrane fraction

	Alkaline phosphatase	Succinic dehydrogenase	$(Na^+ + K^+)$-ATPase	Mg^{2+}-ATPase	$(Mg^{2+} + HCO_3^-)$-ATPase	ΔHCO_3^-	Protein
Amount present in the initial homogenate	12,240	20,176	1,653	8,065	10,482	2,417	602
Amount found in the pooled discarded pellets and supernatants	9,835	14,729	736	7,489	9,264	1,775	561
% recovered	80.0	73.0	44.5	92.8	88.4	73.4	93.2
Amount found in the mitochondrial fraction	48	6,559	39	295	579	284	24
% recovered	0.4	32.5	2.4	3.6	5.5	11.7	3.9
Amount found in the brush border membrane fraction	2,401	276	155	590	825	235	13
% recovered	19.6	1.3	9.3	7.3	7.8	9.7	2.1
Recovery[a]	100.0	106.8	56.2	103.7	101.7	94.8	99.2

The enzyme activities are given in μmol/hr/mg fraction; the protein amount is given in mg/fraction. One typical experiment is shown.
[a] The amount of enzymes and protein recovered in all fractions is expressed as percent of the amount present in the initial homogenate.

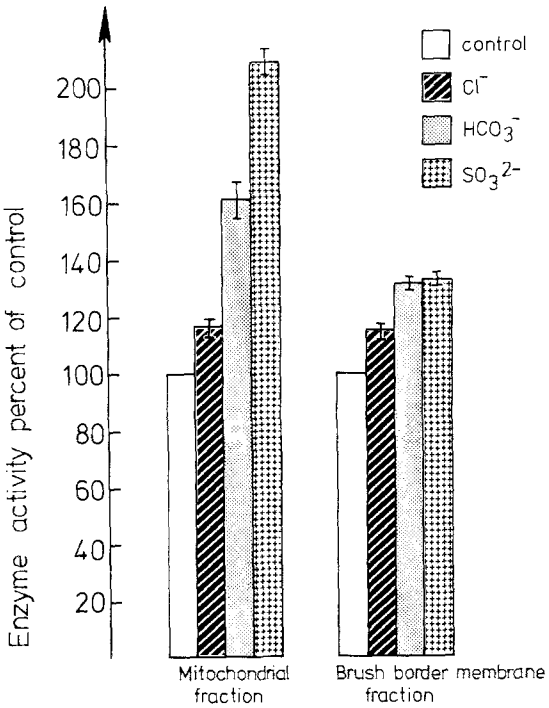

Fig. 1. Effect of different anions on the Mg^{2+}-ATPase activity in the mitochondrial fraction and in the brush border membrane fraction from kidney cortex. The values given represent mean values $\pm$ SD derived from four experiments and are expressed as percent stimulation of the enzyme activity in the absence of any anion in the incubation medium. 100% in the mitochondrial fraction equals a specific activity of 16.2 ± 1.8 µmol/hr/mg protein; 100% in the brush border membrane fraction equals a specific activity of 60.2 ± 3.6 µmol/hr/mg protein

3 times. The same holds for the activity of the $(Mg^{2+} + HCO_3^-)$-ATPase. In the mitochondrial fraction, however, the Mg^{2+}-ATPase activity is reduced, whereas the $(Mg^{2+} + HCO_3^-)$-ATPase activity rises compared to the homogenate (Table 2). The recovery of all enzyme activities and of protein was nearly 100% (Table 3), indicating that no gross inactivation or activation of the enzyme activities occurred during the preparation of the membrane fractions.

As shown in Fig. 1, the Mg^{2+}-ATPase in the mitochondrial fraction as well as in the brush border membrane fraction is stimulated increasingly by chloride, bicarbonate, and sulfite, tested at a final concentration of 25 mM. But unlike the mitochondrial enzyme the activity of the brush border membrane enzyme is increased by bicarbonate only up to maximally 40%, and sulfite is not more effective than bicarbonate.

Effect of Oligomycin, Aurovertin and the Mitochondrial Inhibitor Protein on the Mg^{2+}-ATPase and the ($Mg^{2+} + HCO_3^-$)-ATPase Activity in the Mitochondrial Fraction and in the Brush Border Membrane Fraction

Table 4 summarizes the effects of several so-called mitochondrial inhibitors. All miscellaneous agents inhibited the Mg^{2+}-ATPase activity as well as the ($Mg^{2+} + HCO_3^-$)-ATPase activity in the mitochondrial fraction so that the percent stimulation of the Mg^{2+}-ATPase by bicarbonate remained unaffected (last column in Table 4). Discussed in the sequence of their efficacy, aurovertin decreased the enzyme activity about 25% and the mitochondrial inhibitor protein about 50%. Oligomycin was the most potent inhibitor. The enzyme activity in the presence of the highest concentration of oligomycin was decreased to 25% of the untreated enzyme activity. Aurovertin and oligomycin also affected the Mg^{2+}-ATPase and the ($Mg^{2+} + HCO_3^-$)-ATPase activity in the brush border membrane fraction but to a lesser degree. Aurovertin inhibited about 5 to 10% and oligomycin about 10 to 20% of the enzyme activity, again leaving the percent stimulation by bicarbonate of the Mg^{2+}-ATPase unaffected. The mitochondrial inhibitor protein had no influence on the Mg^{2+}-ATPase but caused a slight inhibition of the ($Mg^{2+} + HCO_3^-$)-ATPase.

Effect of Carboxy-Atractyloside on the Mg^{2+}-ATPase and the ($Mg^{2+} + HCO_3^-$)-ATPase Activity in the Mitochondrial Fraction and in the Brush Border Membrane Fraction

Carboxy-atractyloside as an inhibitor of the mitochondrial adenine nucleotide translocator [15] lowered the Mg^{2+}-ATPase activity as well as the bicarbonate-stimulated enzyme activity in the mitochondrial fraction to about 50% of the untreated enzyme activity (Table 5). The inhibitory effect was – as expected – only seen with freshly prepared, intact mitochondria. If freeze-thawed mitochondria were used, the enzyme activity was no longer affected. The activity of the Mg^{2+}-ATPase and the ($Mg^{2+} + HCO_3^-$)-ATPase of the brush border membrane fraction was never influenced by the presence of carboxy-atractyloside in the incubation medium, indicating the absence of intact mitochondria in the membrane fraction.

Table 4. The effect of oligomycin, aurovertin, and the mitochondrial inhibitor protein on the Mg^{2+}-ATPase and the ($Mg^{2+}+HCO_3^-$)-ATPase activity in the mitochondrial fraction and in the brush border membrane fraction

	Added Ions						$Mg^{2+}+HCO_3^-$
	Mg^{2+}	% of control	$Mg^{2+}+HCO_3^-$	% of control	ΔHCO_3^-	% of control	Mg^{2+}
Mitochondrial fraction							
Control	20.2±2.0	100	38.9±1.8	100	18.7 ±0.5	100	1.93
40 μM oligomycin	6.1±0.4	30.2	11.8±0.6	30.3	5.7 ±0.6	30.5	1.93
80 μM oligomycin	5.4±0.3	26.7	10.4±0.3	26.7	5.0 ±0.3	26.7	1.93
80 μM aurovertin	14.6±0.7	72.3	28.5±1.2	73.3	13.9 ±0.7	74.3	1.95
5 μg mitochondrial inhibitor protein ($n=2$)	9.5	47.0	18.7	48.1	9.2	49.2	1.97
Brush border membrane fraction							
Control	62.3±2.4	100	79.5±2.6	100	17.2 ±0.6	100	1.28
40 μM oligomycin	54.8±2.0	88.0	70.5±1.0	88.7	15.7*±0.9	91.3	1.29
80 μM oligomycin	51.3±0.9	82.3	65.4±1.3	82.3	14.1*±0.7	82.0	1.27
80 μM aurovertin	55.8±4.0	89.6	71.0±1.7	89.3	15.2*±0.5	88.4	1.27
5 μg mitochondrial inhibitor protein ($n=2$)	61.8	99.2	76.3	96.0	14.5	84.3	1.23

The experiments were performed with freeze-thawed mitochondria or freeze-thawed brush border membranes, respectively. The mean values of five experiments are shown (with the exception of the mitochondrial inhibitor protein, $n=2$). The enzyme activities are given in μmol/hr/mg protein. The differences were statistically significant at the level of $P<0.001$, except for the cases marked with an asterisk, where $0.005<P<0.001$.

Table 5. The effect of carboxy-atractyloside on the Mg^{2+}-ATPase and the ($Mg^{2+}+HCO_3^-$)-ATPase activity in the mitochondrial fraction and in the brush border membrane fraction

	Added ions						$Mg^{2+}+HCO_3^-$
	Mg^{2+}	% of control	$Mg^{2+}+HCO_3^-$	% of control	ΔHCO_3^-	% of control	Mg^{2+}
Mitochondrial fraction							
Control	15.9±0.8	100	30.1±1.2	100	14.2±0.9	100	1.89
50 μM carboxy-atractyloside	7.6±0.5	47.8	14.6±0.6	48.5	7.0±0.6	49.3	1.92
100 μM carboxy-atractyloside	7.6±0.5	47.8	14.9±0.6	49.5	7.3±0.3	51.4	1.96
Brush border membrane fraction							
Control	57.3±1.9	100	71.5±2.1	100	14.2±0.8	100	1.25
50 μM carboxy-atractyloside	57.1±2.0	99.7	71.7±2.1	100.3	14.6±0.8	102.8	1.26
100 μM carboxy-atractyloside	68.2±1.6	101.6	72.7±1.9	101.7	14.5±0.7	102.1	1.25

The experiments were performed with intact mitochondria or freshly prepared brush border membranes, respectively. The mean values of 5 experiments are shown. The enzyme activities are given in μmol/hr/mg protein. All differences were statistically significant at the level of $P<0.001$.

Effect of Filipin on the Mg^{2+}-ATPase and the ($Mg^{2+} + HCO_3^-$)-ATPase Activity in the Mitochondrial Fraction and in the Brush Border Membrane Fraction

Filipin is a polyene antibiotic that complexes with cholesterol and cholesterol esters in the membrane [16]. Plasma membranes, including brush border membranes, are very rich in these components, whereas the mitochondria and especially the inner mitochondrial membrane contain only a small amount. We estimated the cholesterol content in pooled mitochondrial fractions as 11 µg cholesterol/mg protein and in pooled brush border membrane fractions as 128 µg cholesterol/mg protein. As shown in Table 6 filipin inhibits the Mg^{2+}-ATPase activity only in the cholesterol-rich brush border membrane fraction. The ($Mg^{2+} + HCO_3^-$)-ATPase activity is also reduced but to a smaller degree, so that the percent stimulation by bicarbonate is higher in the presence of filipin than in the absence. The same concentration of filipin as used in the ($Mg^{2+} + HCO_3^-$)-ATPase assay had no influence on the alkaline phosphatase activity (Table 7). The enzyme activity amounted to 131.4 µmol/hr/mg protein in the presence of filipin compared to 126.9 µmol/hr/mg protein in the absence of filipin (mean values of four experiments).

Effect of L- *and* D-*p-Bromotetramisole on the Mg^{2+}-ATPase and the ($Mg^{2+} + HCO_3^-$)-ATPase Activity of the Brush Border Membrane Fraction*

The effect of L-*p*-bromotetramisole, an inhibitor of alkaline phosphatase [5] on the Mg^{2+}-ATPase and the ($Mg^{2+} + HCO_3^-$)-ATPase activity was tested using a concentration which inhibits the alkaline phosphatase in our brush border membrane fraction completely. Under these conditions the inhibition of the Mg^{2+}-ATPase and the ($Mg^{2+} + HCO_3^-$)-ATPase amounted to maximal 10% (Table 7) thus making it very unlikely that the measured activity of a bicarbonate-stimulated Mg^{2+}-ATPase in the brush border membrane fraction is due to a hydrolytic action of the alkaline phosphatase on ATP. In contrast to the histochemical findings that D-*p*-bromotetramisole does not influence the alkaline phosphatase activity, we saw a 25% inhibition of the enzyme activity in the brush border membrane preparation when we used the dextro isomer of bromotetramisole in the same concentration which gave a 100% inhibition with the L-form. The Mg^{2+}-ATPase, however, remained unaffected.

Table 6. The effect of filipin on the Mg^{2+}-ATPase and on the ($Mg^{2+}+HCO_3^-$)-ATPase activity in the mitochondrial fraction and in the brush border membrane fraction

	Added ions						$Mg^{2+}+HCO_3^-$
	Mg^{2+}	% of control	$Mg^{2+}+HCO_3^-$	% of control	ΔHCO_3^-	% of control	Mg^{2+}
Mitochondrial fraction							
Control	21.3±1.7	100	39.8±2.5	100	18.5±1.2	100	1.87
35 μg filipin	21.8±2.5	102.3	39.9±2.3	100.3	18.1±1.2	97.8	1.83
70 μg filipin	20.8±1.4	97.6	40.0±1.4	100.5	19.2±1.8	103.8	1.92
Brush border membrane fraction							
Control	60.1±2.0	100	75.0±1.8	100	14.9±1.3	100	1.25
35 μg filipin	35.8±0.9	59.6	48.0±1.6	64.0	12.2±1.0*	81.9	1.34
70 μg filipin	23.3±0.5	38.8	32.9±1.4	43.9	9.6±1.0	64.4	1.41

The experiments were performed with freeze-thawed mitochondria or freeze-thawed brush border membranes, respectively. The mean values of 6 experiments are shown. The enzyme activities are given in μmol/hr/mg protein. The differences were statistically significant at the level of $P<0.001$, except for the case marked with an asterisk, where $0.005<P<0.001$.

Table 7. The effect of L-and D-*p*-bromotetramisole on the alkaline phosphatase activity, on the Mg^{2+}-ATPase, and on the $(Mg^{2+} + HCO_3^-)$-ATPase activity in the brush border membrane fraction

	Alkaline phosphatase	% of control	Mg^{2+}-ATPase	% of control	$(Mg^{2+} + HCO_3^-)$-ATPase	% of control	ΔHCO_3	% of control	$\frac{Mg^{2+} + HCO_3^-}{Mg^{2+}}$
Control	138.3±8.2	100	58.3±2.0	100	74.4±2.1	100	16.4±0.5	100	1.28
20 μM L-*p*-bromotetramisole	20.5±1.5	14.8	55.3±1.6*	94.8	71.2±2.1*	95.3	15.9±0.6	96.9	1.29
200 μM L-*p*-bromotetramisole	0		52.6±1.7*	90.2	68.3±1.5*	91.4	15.7±0.5	95.7	1.30
200 μM D-*p*-bromotetramisole	106.8±6.0	77.2	59.1±2.6	101.4	75.1±2.0	100.5	16.0±0.4	97.6	1.27

The experiments were performed with freeze-thawed brush border membranes. The mean values ±SD of 4 experiments are shown. The enzyme activities are given in μmol/hr/mg protein. The differences were statistically significant at the level of $P < 0.001$, except for the cases marked with an asterisk, where $P < 0.005$.

Discussion

The purpose of the isolation procedure used in this study (Table 1) was to obtain a mitochondrial fraction and a brush border membrane fraction under identical conditions. *Identical* means that the two fractions were derived from the same homogenate and were centrifuged in the same buffers within the same timespan in order to avoid influences of different kinds on the enzyme activities (activation or inactivation). The recovery of the enzyme activities and the protein as given in Table 3 proves that no serious effects happened during the isolation procedure.

Within our first attempts to isolate a mitochondrial fraction using the same buffer as for brush border membranes, it turned out that we could not simply transfer the directions given for the isolation of liver mitochondria to kidney tissue, because big brush border membrane fragments co-sedimented with mitochondria as indicated by the high alkaline phosphatase activity in the mitochondrial fraction prepared according to Mitchell et al. [18]. This problem was solved by an interposed, relatively low speed centrifugation step at $2000 \times g$ of the second supernatant, during which a sufficient sedimentation of the big brush border fragments occurs.

The brush border membrane fraction contains, besides microvilli, basal lateral membranes in a considerable amount. A separation of these two membrane structures would have been possible using the free-flow electrophoresis [12]. But since we already know that the bicarbonate stimulated Mg^{2+}-ATPase is only located in the brush border membrane and is lacking in the basal lateral membrane of the tubular cell in kidney cortex [14], we saw no reason to prolong the isolation procedure. Especially, because we would have risked our principle of identical treatment conditions for the two fractions during the isolation. The mitochondrial fraction as well as the brush border membrane fraction possesses a Mg^{2+}-ATPase activity which can be stimulated by bicarbonate. The question arose whether the ATPase activity observed in the brush border membrane fraction can be explained by the presence of mitochondria alone. The following arguments are against such an assumption: first, the specific activity of the Mg^{2+}-ATPase and the $(Mg^{2+} + HCO_3^-)$-ATPase in the brush border membrane fraction is about 3 to 4 times higher than that of the mitochondrial enzyme. Second, there is no parallelism between the enrichment of the ATPase and the reduction of the succinic dehydrogenase in the membrane fraction (Table 2). Regarding the higher specific activity, we considered the objection of a possible removal of the mito-

chondrial inhibitor protein [6] and measured the enzyme activities in the two fractions in the presence of the inhibitor protein (Table 4). Now the difference became even more clear. Only the mitochondrial enzyme was inhibited. There was no effect on the brush border membrane enzyme.

Another approach in the controversial discussion about the existence of a nonmitochondrial bicarbonate-stimulated Mg^{2+}-ATPase is the use of so-called mitochondrial inhibitors, for instance, aurovertin and oligomycin. Both substances inhibited the enzyme activity of the mitochondrial fraction as well as of the membrane fraction, the stronger inhibition of the ATPase in the mitochondrial fraction thereby supporting the view of a brush border membrane bound ATPase.

All arguments used so far in favor of the brush border membrane enzyme assumed that the accessibility to the enzyme molecules located in different membrane structures was the same for all inhibitors and that no changes of the enzymes occurred during the isolation procedure. The use of atractyloside and filipin represents a new approach to differentiate between the two enzyme activities. They are substances which do not interact with the enzyme molecule itself but with more or less unique components of the surrounding membrane in which the enzyme is embedded. In the inner mitochondrial membrane an adenine nucleotide translocator exists which is present in mitochondria but not in plasma membranes and which is responsible for the transfer of ATP across the membrane to the hydrolytic site of the ATPase. This system can be blocked specifically by atractyloside [15]. Using freeze-thawed mitochondria, that means with their structural organization destroyed, we could demonstrate that indeed atractyloside does not interact with the ATPase itself, but influences the uptake of the substrate into the mitochondria. Filipin, which forms complexes with cholesterol, should be expected to act only on the cholesterol-rich brush border membrane and not on the cholesterol-poor inner mitochondrial membrane. This was found to be the case and thereby at least part of the Mg^{2+}-ATPase and the $(Mg^{2+} + HCO_3^-)$-ATPase of the brush border membrane fraction could be identified positively as a plasma membrane bound enzyme not related to mitochondrial contamination.

A quantitative estimation of the contribution of mitochondria to the $(Mg^{2+} + HCO_3^-)$-ATPase activity in the brush border membrane fraction has been attempted based on the succinic dehydrogenase activity in the fraction and on the maximum effect of the various inhibitors. With respect to the succinic dehydrogenase activity a ratio Mg^{2+}-ATPase

vs. succinic dehydrogenase of 0.07 and a ratio ($Mg^{2+}+HCO_3^-$)-ATPase *vs.* succinic dehydrogenase of 0.14 is found in the mitochondrial fraction (Table 2). If the same ratio is assumed for the mitochondria present in the brush border membrane fraction, 2% of this ATPase activity (Mg^{2+}-ATPase as well as ($Mg^{2+}+HCO_3^-$)-ATPase) can be attributed to the action of a mitochondrial ATPase. Similar values ranging from about 2 to 9% are obtained from the inhibition of the membrane ATPase by the mitochondrial inhibitor protein (Table 4). The inhibitory effect of this substance on the Mg^{2+}-ATPase and on the ($Mg^{2+}+HCO_3^-$)-ATPase in the membrane fraction amounts to 0.8 and 4.0%, respectively. Considering the incomplete inhibition of the mitochondrial ATPase of about 50%, these values have to be corrected by multiplying them with a factor of 2.

In our view these values represent the minimum contribution of mitochondria because there are still some uncertainties about (i) a constant Mg^{2+}-ATPase/succinic dehydrogenase ratio due to the heterogeneity of renal mitochondria and (ii) because of the questionable equal accessibility to the mitochondria in the two fractions for the inhibitor protein.

The maximum contribution of mitochondria in the membrane fraction can be estimated from the experiments with filipin (Table 6) which show that at least about 60% of the Mg^{2+}-ATPase and the ($Mg^{2+}+HCO_3^-$)-ATPase activity derive from enzyme molecules located in brush border microvilli. However, this value is probably underestimated. It is quite possible that the enzyme activity in the membrane is not yet completely inhibited by the highest concentration of filipin used in this study.

An evaluation of mitochondrial contamination on the basis of the inhibition observed with oligomycin is, in our opinion, quite difficult because this compound has been shown to inhibit also plasma membrane ATPases and not only mitochondrial ATPases [11].

The results of this investigation confirm our previous results on the presence of a Mg^{2+}-ATPase in the brush border membrane which is stimulated by anions. They also demonstrate qualitative similarities of the enzyme activities in the two fractions with respect to the stimulatory action of anions and the inhibitory action of oligomycin. Therefore one might postulate that the mitochondrial Mg^{2+}-ATPase and the plasma membrane bound enzyme are similar or even the same. Quantitatively, however, the response of the brush border membrane bound enzyme to activators and inhibitors is smaller than that of the mitochondria. This might be due to the existence of more than one Mg^{2+}-ATPase in the brush border membrane: One enzyme molecule with a basic Mg^{2+}

activity which is anion-insensitive (probably an exo-enzyme) and another enzyme molecule with a basic Mg^{2+} activity which is anion- and oligomycin-sensitive and resembles the one located in the inner mitochondrial membrane.

The properties of the mitochondrial enzyme which is capable of performing ATP-driven proton transport have been recently studied in detail by Reckenwaldt and Hess [21]. They developed a model in which each subunit of the ATPase contains a catalytic as well as a regulatory site. The anions interfere with the regulatory site, thereby controlling allosterically the enzyme activity. If one assumes the same properties for the brush border membrane enzyme, one would postulate that the ATPase catalyzes an ATP-driven proton transport for which indirect physiological evidence has already been adduced in rat kidney proximal tubule [8]. In this view it is no longer justified to postulate from a stimulation of the ATPase by bicarbonate an ATP-driven HCO_3^- transport. It just seems to be an intrinsic property of the enzyme molecule itself due to an allosteric control of the enzyme activity by anions as ligands.

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References

1. Amelsvoort, J.M.M. van, Jansen, J.W.C.M., Pont, J.J.H.H.M. de, Bonting, S.L. 1978. Is there a plasma-membrane-located anion-sensitive ATPase? IV. Distribution of the enzyme in rat pancreas. *Biochim. Biophys. Acta* **512**:296
2. Amelsvoort, J.M.M. van, Pont, J.J.H.H.M. de, Bonting, S.L. 1977. Is there a plasma membrane-located anion-sensitive ATPase? *Biochim. Biophys. Acta* **466**:283
3. Amelsvoort, J.M.M. van, Pont, J.J.H.H.M. de, Stols, A.L.H., Bonting, S.L. 1977. Is there a plasma-membrane-located anion-sensitive ATPase? II. Further studies on rabbit kidney. *Biochim. Biophys. Acta* **471**:78
4. Bartlett, G.R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234**:466
5. Borgers, M., Thoné, F. 1975. The inhibition of alkaline phosphatase by L-*p*-bromotetramisole. *Histochemistry* **44**:271
6. Ferguson, S.J., Harris, D.A., Radda, G.K. 1977. The adenosine triphosphatase inhibitor content of bovine heart submitochondrial particles. *Biochem. J.* **162**:351
7. Fiske, C.H., Subbarow, Y. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**:375
8. Frömter, E. 1974. Ion transport across renal proximal tubule: Analysis of luminal, contraluminal and paracellular transport steps. *Fortschr. Zool.* **23**:248

9. Gibbs, G.E., Reimer, K. 1965: Quantitative microdetermination of enzymes in sweat glands. III. Succinic dehydrogenase in cystic fibrosis. *Proc. Soc. Exp. Biol.* **119**:470
10. Gluck, L., Kulovich, M., Brody, S.J. 1966. Rapid quantitative measurement of lung tissue phospholipids. *J. Lipid Res.* **7**:570
11. György, A.Z., Kinne, R. 1971. Energy source for transepithelial sodium transport in rat renal proximal tubules. *Pfluegers Arch.* **327**:234
12. Heidrich, H.-G., Kinne, R., Kinne-Saffran, E., Hannig, K. 1972. The polarity of the proximal tubule cell in rat kidney. Different surface charges for the brush border microvilli and plasma membranes from the basal infoldings. *J. Cell Biol.* **54**:232
13. Kinne, R., Schmitz, J.E., Kinne-Saffran, E. 1971. The localization of the $Na^+ + K^+$-ATPase in the cells of rat kidney cortex. A study on isolated plasma membranes. *Pfluegers Arch.* **329**:191
14. Kinne-Saffran, E., Kinne, R. 1974. Presence of a bicarbonate stimulated ATPase in the brush border microvillus membranes of the proximal tubule. *Proc. Soc. Exp. Biol. Med.* **146**:751
15. Klingenberg, M., Pfaff, E. 1966. Structural and functional compartmentation in mitochondria. *In:* Regulation of Metabolic Processes in Mitochondria. J.M. Tager, S. Papa, E. Quagliariello and E.C. Slater, editors. Vol. 7, p. 180. Elsevier, Amsterdam
16. Kruijff, B. de, Demel, R.A. 1974. Polyeme antibiotic-sterol interactions in membranes of *Acholeplasma Laidlawii* cells and lecitin liposomes. III. Molecular structure of the polyene antibiotic-cholesterol complexes. *Biochim. Biophys. Acta* **339**:57
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265
18. Mitchell, P., Moyle, J. 1967. Acid-base titration across the membrane system of rat liver mitochondria. Catalysis by uncouplers. *Biochem. J.* **104**:588
19. Møller, O.J. 1971. Activation by freezing of $(Na^+ + K^+)$-ATPase in a microsomal fraction from ox kidney cortex. *Exp. Cell Res.* **68**:347
20. Pfleger, R.C., Anderson, N.G., Snyder, F. 1968. Lipid class and fatty acid composition of rat liver plasma membranes isolated by zonal centrifugation. *Biochemistry* **7**:2826
21. Reckenwaldt, D., Hess, B. 1977. Allosteric influence of anions on mitochondrial ATPase of yeast. *FEBS Lett.* **76**:25