Ontogeny of Na/H Antiporter Activity in Rabbit Renal Brush Border Membrane Vesicles

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Abstract

The development of the Na/H antiporter was studied in renal brush border membrane vesicles (BBMV) from fetal and adult rabbits using isotopic and fluorescent techniques. The kinetics of the antiporter studied by $^{22}Na^+$ uptake revealed that the V_{max} was only 25% of that in the adult; however, the K_m's for Na^+ were not significantly different. These data were confirmed by a fluorescent assay using the pH-sensitive probe, acridine orange: the V_{max} was significantly lower in the fetal BBMV. Conductive Na^+ movement was estimated from amiloride-insensitive $^{22}Na^+$ uptake and the rate of alkalinization induced by K^+, an ion whose relative conductance was found to be similar to that of Na^+. Although relative Na^+ conductance was significantly greater in fetal BBMV, the lower V_{max} in fetal vesicles could not be ascribed to this factor. Maternal administration of betamethasone (50 μg/kg intramuscularly) for 2 d before delivery significantly increased the V_{max} of the antiporter to levels observed in the adult; K_m was unaffected. Na/K ATPase activity increased fourfold after betamethasone, but the specific activities of four brush border marker enzymes and the kinetics of Na^+-glucose cotransport were unchanged. These data indicate that there is a developmental increase in brush border Na/H exchange which is the result of an increase in the number and/or the turnover number of the carriers. Further, these data suggest that the postnatal increase in antiporter activity may be related to the surge in glucocorticoid concentration that occurs perinatally. (*J. Clin. Invest.* 1991. 87:2067–2076.) Key words: acridine orange • amiloride • betamethasone • development • ionic conductance

Introduction

Previous studies have shown that the threshold for bicarbonate reabsorption is reduced in newborn human (1), rat (2), and dog (3) kidneys compared with that in adults. In the juxtamedullary proximal convoluted tubules of the newborn rabbit, bicarbonate reabsorption is only 30–40% of that observed in the same segment of the adult kidney (4). This postnatal increase in tubular reabsorption of bicarbonate in proximal tubules could occur by a number of mechanisms including an increase in Na/H exchange. An increase in antiporter activity could derive from an increase in the driving force for lumen-to-cell flux of Na^+ via an increase in Na/K ATPase activity in the basolateral membrane and/or an increase in proton efflux into the tubular lumen via an increase in the number or turnover number of Na/H antiporters in the luminal membrane. Indeed, Na/K ATPase activity (5, 6) has been reported to increase postnatally. To determine whether there is a developmental increase in the Na/H antiporter, brush border membrane vesicles (BBMV)[1] were isolated from fetal kidneys and the activity of this transporter was assayed and compared to that from the adult.

Glucocorticoids are important modulators of postnatal differentiation (7, 8) and have been shown to increase Na/K ATPase in developing renal tissue (5, 9). Although it has been shown that glucocorticoids increase the activity of the renal proximal tubular Na/H antiporter in adult animals (10, 11), it is not known whether glucocorticoids play a role in the ontogeny of the antiporter. To this end, a synthetic glucocorticoid, betamethasone, was administered to pregnant rabbits late in gestation, renal BBMV were prepared from betamethasone-exposed fetuses, and the antiporter activity was estimated and compared to that in control fetal and adult BBMV.

These studies demonstrate that fetal BBMV possess an electroneutral Na/H antiporter that can be inhibited by amiloride. Further, there is a developmental change in the Na/H antiporter that is characterized by an increase in V_{max}, without a change in K_m. Maternal administration of betamethasone increases the V_{max} of the Na/H antiporter but does not affect the kinetics of the Na^+-dependent glucose transporter.

Methods

Isolation and preparation of membrane vesicles

Fetuses were obtained from gravid New Zealand white rabbits (Gingrich Animal Suppliers, Fredericksburg, PA) late in gestation (29–30 d). All does were anesthetized with an intramuscular injection of ketamine hydrochloride (Ketalar, 35 mg/kg body weight; Parke-Davis, Morris Plains, NJ) and xylazine (Rompun, 5 mg/kg body weight; Mobay Corp., Shawnee, KS) and the fetuses were delivered. In some studies, the synthetic glucocorticoid, betamethasone was administered intramuscularly to pregnant does for 2 d before delivery. The dose of betamethasone used, 50 μg/kg, is comparable to that used for replacement in adrenalectomized rabbits (12). Control adult kidneys were obtained from nonpregnant, untreated does. Renal microvillous membrane vesicles were isolated from adult and fetal kidneys using methods previously described from this laboratory (13). In brief, kidneys were rapidly removed and placed in ice-cold buffer (300 mM mannitol, 10 mM Tris, and 16 mM Hepes pH 7.5 [MTH]). The kidneys were decapsulated, renal cortical slices were obtained, weighed, and homogenized.

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1. *Abbreviations used in this paper:* BBMV, brush border membrane vesicles; EIPA, ethylisopropylamiloride; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone; MES, 2-(*N*-morpholino)ethane sulfonic acid; NMG; *N*-methyl glucamine.

In some studies, BBMV were simultaneously prepared from both inner cortical, outer medullary tissue and from outer cortical tissue of adult kidneys as described by Turner and Moran (14). In all studies, BBMV were isolated by the Mg^{++} aggregation method of Booth and Kenny (15) with the modifications of Aronson (16). Purified membranes were suspended in 10 ml of 300 mM mannitol, 100 mM 2-(*N*-morpholino)ethane sulfonic acid (MES) solution buffered to pH 5.5 with Tris (MMT), washed three times in this same medium by centrifugation at 27,000 *g* for 15 min, and finally suspended in the buffered mannitol medium (pH 5.5) at a protein concentration of 5–10 mg/ml. The protein concentrations of all suspensions were measured using the method of Lowry et al. (17) with bovine serum albumin as the standard. To assess the purity of the vesicle preparations, enzyme assays were carried out at 37°C on both whole homogenate and isolated brush border fractions prepared from fetal and adult kidneys. Alkaline phosphatase, measured with the Sigma Chemical Co. (St. Louis, MO) test kit, was used as a marker for the brush border membrane and K^+-dependent *p*-nitrophenyl phosphatase (K^+-PNPPase [18]), was employed as a basolateral membrane marker. β-Glucuronidase (determined with the Sigma Chemical Co. test kit), cytochrome *c* oxidase (19), and NADPH-cytochrome *c* reductase (20) were used as markers for lysosomes, mitochondria, and endoplasmic reticulum, respectively. In three studies the kidneys of one fetus were used to compare the morphology of decapsulated and nondecapsulated kidneys. The kidneys were fixed in 10% neutral buffered formalin and 6-μm sections were prepared and stained with hematoxylin and eosin for light microscopy.

Isotopic uptake studies

Time course of Na^+ uptake. Uptake of $^{22}Na^+$ (20 μCi/pmol; Dupont New England Nuclear, Wilmington, DE) was assayed at room temperature (22°C) by a previously reported rapid filtration technique using 0.65-μm filters (Millipore Corp., Bedford, MA [21]). 10 μl of membrane vesicles (pH_{in} = 5.5) were added to 40 μl of uptake medium: the final concentrations in the medium were 1 mM ^{22}Na gluconate, 100 mM mannitol, 100 mM *N*-methyl glucamine (NMG) gluconate, 64 mM Hepes, 20 mM MES, 48 mM Tris, pH 7.5, ±1 mM amiloride. At specified times, uptakes were terminated by addition of 4 ml of ice-cold 220 mM KCl, 10 mM Tris, 16 mM Hepes, pH 7.5, and 0.5 mM amiloride. Immediately thereafter the vesicle suspensions were filtered and the tubes and filters were washed with an additional 16 ml of ice-cold rinse solution. The filters were then removed from the filtration apparatus and dissolved in 10 ml of scintillation fluid (ACS; Amersham Corp., Arlington Heights, IL). Radioactivity was measured in a liquid scintillation counter (Tri-Carb 4430; Packard Instrument Co., Downers Grove, IL). Uptakes, expressed as nanomoles per milligram of protein, were calculated from the accumulated vesicular $^{22}Na^+$, the specific activity of Na^+ in the medium, and the protein concentration of the vesicles. A correction for $^{22}Na^+$ binding to the filters was made by subtracting activity retained on the filter at zero time.

Kinetics of the Na/H antiporter. 5 μl of BBMV (pH_{in} = 5.5) were added to 45 μl of medium containing $^{22}Na^+$ (1–25 mM as the gluconate salt), 150–200 mM mannitol (to maintain osmolarity constant as the [Na^+] was varied), 100 mM NMG gluconate, 16 mM Hepes, 10 mM MES, and 29 mM Tris, pH 8.0, ±1 mM amiloride. The reaction was initiated and terminated as described above. Since uptake was linear for 8 s in 25 mM Na^+, initial rates of Na^+ uptake were obtained from the slope between the 1- and 4-s time points. Initial rates were determined by subtracting the slope of the regression line for Na^+ uptake in the presence of amiloride from the slope of the regression line for total Na^+ uptake. The V_{max} and apparent K_m for Na^+ were determined from a Woolf-Augustinsson-Hofstee plot (22) of these initial rates at each [Na^+].

Assessment of potential dependence using $^{22}Na^+$ uptake. 5 μl of BBMV (pH_{in} = 5.5) were added to 45 μl of medium containing 1 mM ^{22}Na gluconate, 200 mM mannitol, 100 mM NMG gluconate, 16 mM Hepes, 10 mM MES, 29 mM Tris, pH 8.0, ±1 mM ethylisopropylamiloride (EIPA) ±100 μM carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone (FCCP). The reaction was terminated after 4 s as described above. Uptake in the presence of EIPA was subtracted from total uptake to yield the EIPA-sensitive component of Na^+ uptake. EIPA was dissolved in DMSO; FCCP, in ethanol. Control incubations contained both DMSO and ethanol, < 1% by volume.

Determination of intravesicular volume. 5 μl of BBMV (pH_{in} = 5.5) were suspended in 45 μl of medium containing 50 μM D-[^{3}H]glucose, 200 mM mannitol, 100 mM NMG gluconate, 16 mM Hepes, 10 mM MES, and 29 mM Tris at pH 8.0. Equilibrium uptake was measured after 120 min by the rapid filtration method described above. The intravesicular volume, calculated from the ratio of D-[^{3}H]glucose uptake at equilibrium and the external glucose concentration, was 1.10±0.06 μl/mg protein in the adult (n = 4) and 0.83±0.09 in the fetal BBMV (n = 4). These volumes are similar to those previously reported (13).

Assessment of the Na/H antiporter with acridine orange

Fluorescence measurements. The fluorescence of acridine orange was measured in a fluorescence spectrophotometer (model 650-10S; Perkin-Elmer Corp., Norwalk, CT) equipped with a magnetic stirring device and an injection port that permitted additions to the cuvette without interruption in the recording. Fluorescence was monitored at an excitation wavelength of 492 nm, an emission wavelength of 528 nm, and slit widths of 1 nm. Fluorescence was recorded in arbitrary units with a strip chart recorder (model 561; Perkin-Elmer Corp.) operated at a paper speed of 240 mm/min. Acrylic cuvettes (Sarstedt, Inc., Princeton, NJ) were used to minimize dye binding.

Calibration of acridine orange fluorescence in response to changes in the pH gradient. BBMV (33 μg of protein [pH_{in} = 5.5]) were added to 1 ml of Na^+-free media at varying pH's. The media contained 2 μM acridine orange, 200–225 mM mannitol (to maintain osmolarity constant as the [Tris] varied), 100 mM NMG gluconate, and 16 mM Hepes buffered with Tris to pH's that ranged from 6.5 to 8.0. The percent quench at each pH_{out} was determined by extrapolation to the initial fluorescence after the vesicles were added. The percent quench was plotted against the delta pH ($pH_{out} - pH_{in}$) to generate a calibration curve. The relationship between pH and fluorescence quench was 0.208±0.004 pH units/10% quench in adult BBMV and 0.296±0.008 pH units/10% quench in fetal BBMV.

The buffer capacity (nanomoles H^+ per milligram of protein per pH unit) of 50 μl of fetal and adult BBMV prepared in 300 mM mannitol, 1 mM MES/Tris, pH 5.5, was measured after dilution in 200 μl of a solution of identical composition. The buffer capacity of 250 μl of the same solution, without vesicles, was also determined. The pH was titrated from 5.5 to 6.5 with 5 mM NaOH. The endogenous buffer capacity of the vesicles, per se, was determined by subtracting the buffer capacity of the vesicle-free medium and the buffer capacity of fluid within the intravesicular space (estimated from the glucose space) from the total buffer capacity of the vesicle-containing suspension. The endogenous buffer capacity of the adult BBMV was 122±11 nmol H^+/mg protein per pH unit (n = 4); that of the fetal vesicles was 253±23 (n = 3).

Because the vesicles used in the studies were prepared in a highly buffered medium, the total buffering capacity of the vesicles was corrected for the additional buffer capacity provided by the 100 mM MES/Tris within the intravesicular space (calculated from the glucose space). The rate of H^+ efflux in nanomoles H^+ per second per milligram of protein was converted from fluorescence units per second (FU/s) using the formula:

$$H^+ \text{ efflux} = \frac{FU}{s} \times \frac{pH}{FU} \times \beta,$$

where pH/FU is the slope of the calibration curve of percent quench vs. pH, and β is the total buffer capacity in nanomoles H^+ per milligram of protein per pH unit. Total buffer capacity (β) is the sum of the endogenous buffer capacity (β_{end}) and exogenously added buffers (β_{ex}). β_{ex} is calculated from the intravesicular space and the buffer capacity of 100 mM MES/Tris, pH 5.5 (43.5 nmol/μl per pH unit).

Kinetics of the Na/H antiporter. The kinetics of the Na/H antiporter were measured with minor modifications of the technique of Warnock et al. (23). BBMV (33 μg of protein [pH_{in} = 5.5]) were injected into 1 ml of a solution containing 2 μM acridine orange, 100 mM NMG gluconate, 200 mM mannitol, 16 mM Hepes, and 20 mM Tris, pH 8.0. Subsequent to the initial quench of fluorescence, a 25-μl aliquot of a concentrated solution of Na gluconate, K gluconate, or NMG gluconate was injected into the cuvette; the final concentration of the added salt was 25 mM. Na and K gluconate concentrations ranged from 1 to 25 mM, while the concentration of NMG gluconate ranged from 25 to 0 mM. Initial rates of alkalinization were determined from the slope of the increase in fluorescence during the first 2.5 s after addition of the respective aliquots.

While it was possible to use amiloride to determine the component of $^{22}Na^+$ uptake that represented Na/H exchange, this technique could not be employed in the fluorescence studies. As previously reported (24), the concentration of amiloride that was required to induce maximal inhibition of the Na/H antiporter produced a significant artifact in fluorescence. Accordingly, in the fluorescence studies flux on the Na/H antiporter was estimated from the difference between the rate of alkalinization in the presence of identical concentrations of Na^+ and K^+. Conductive proton efflux in conjunction with depolarizing conductive cation influx was determined from the initial rate of alkalinization at each K^+ concentration. The V_{max} and apparent K_m for Na^+ were determined from a Woolf-Augustinsson-Hofstee plot (22) of the corrected (nonconductive) initial rates of alkalinization at each [Na^+].

Na^+ conductance relative to K^+ conductance (P_{Na}/P_K) was determined using the positively charged fluorescent, potential-sensitive probe 3,3'-dipropylthiadicarbocyanine iodide [diS-C_3-(5), Molecular Probes, Inc., Eugene OR], as previously described (25). In brief, 100 μg of BBMV incubated for 3 h at room temperature in 100 mM KCl and 300 mM MTH was added to 1 ml of media containing 100–0.5 mM K^+, 0–99.5 mM $choline^+$, 100 mM Cl^-, 3 μM diS-C_3-(5), and 300 mM MTH. After fluorescence stabilized, the potassium ionophore valinomycin was added (3 μM), and the resultant fluorescence recorded. $[KCl]_{in}$ was determined by the previously reported "null point" method; when intravesicular [K^+] and extravesicular [K^+] are equal, the addition of valinomycin causes no change in membrane potential or fluorescence (25). The null point was calculated by determining the $[K^+]_{out}$ at the intersection of the regression lines for the initial fluorescence and the fluorescence post-valinomycin fitted to the Goldman-Hodgkin-Katz constant field and Nernst equations, respectively. After $[KCl]_{in}$ was determined, fluorescence was converted to membrane potential (PD) in millivolts (25). Aliquots of KCl-loaded vesicles were added to a medium in which 100 mM NaCl replaced 100 mM KCl. Initial fluorescence in this medium was converted to PD, and relative ion permeability was calculated by substituting Na^+ for K^+ in the constant field equation.

Statistical analysis

All experiments were performed on at least three membrane vesicle preparations harvested on separate occasions. In each experiment, studies were performed at least in triplicate, and the mean of these multiple determinations served as one value in the calculation of the mean of all experiments. All data are expressed as the mean±SE. Statistical significance was determined with Student's *t* test for unpaired analyses. Regression lines were calculated by the method of least squares.

Results

Morphological studies. In newborn rabbit kidney the outermost layer (100–150 μm) of cortex containing primitive nephrons is removed when the kidney is decapsulated (26). To determine how much of the outer nephrogenic zone of undifferentiated nephrons is removed in fetal kidneys, nondecapsulated (Fig. 1 *A*) and decapsulated (Fig. 1 *B*) kidneys were examined by light microscopy. It is evident that the outer cortex

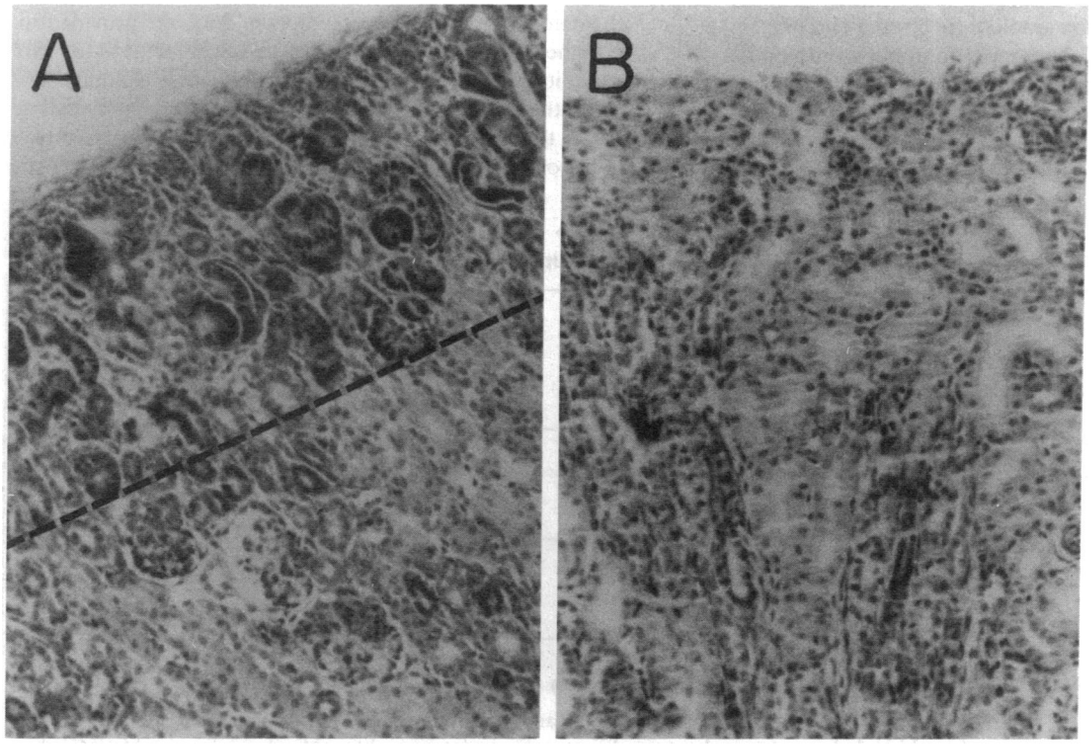

Figure 1. (*A*) Light micrograph of fetal kidney with capsule intact. The dotted line demarcates the line of densely staining immature nephrons which are removed when the kidney is decapsulated. (*B*) Light micrograph of fetal rabbit kidney after decapsulation. Approximately 35% of the cortex has been removed. Note the absence of any densely staining nephrons. ×275.

containing the nephrogenic zone was virtually completely removed by decapsulation: the residual cortical tissue contains a relatively homogeneous population of more mature midcortical and juxtamedullary nephrons (Fig. 1 *B*).

Purity of vesicle preparation. We have previously reported the specific activities, enrichments, and ontogeny of the brush border enzymes, alkaline phosphatase, leucine aminopeptidase, maltase, and γ-glutamyltransferase, and the basolateral enzyme Na^+/K^+ ATPase in fetal and adult BBMV prepared by techniques identical to those employed in the present study (13). As in our prior evaluation, the specific activities of alkaline phosphatase and Na^+/K^+ ATPase in the fetal membranes, 1,490±91 and 1.8±1.1 nmol/min per mg protein, respectively, were lower than those in the adult preparation, 1,830±105 and 3.5±1.1, respectively. However, the enrichments of alkaline phosphatase in the fetal (9.9±0.6) and adult (7.8±0.6) membranes were comparable and similar to values reported in an earlier study. Furthermore, there was no significant difference in the deenrichment of Na^+/K^+ ATPase in fetal (0.25±0.15) and adult vesicle preparations (0.13±0.01). In addition, the mitochondrial and endoplasmic reticulum markers cytochrome *c* oxidase and NADPH-cytochrome *c* reductase were not enriched in fetal (0.75±0.10 and 0.93±0.10, respectively) and adult (0.12±0.03 and 1.2±0.2, respectively) BBMV. The lysosomal marker, β-glucuronidase, is somewhat enriched in the fetal preparation (3.2±0.2) and deenriched in the adult (0.75±0.08). These findings are consistent with our prior conclusions that the fetal and adult vesicle preparations are comparably purified and suggest that neither preparation is significantly contaminated with other membranes or intracellular organelles.

Na^+ uptake into fetal and adult BBMV. The time course of Na^+ uptake into adult and fetal BBMV in the presence of an outwardly directed proton gradient (pH_i = 5.5 and pH_o = 7.5) is illustrated in Fig. 2 *A*. There was an uphill accumulation of Na^+ severalfold higher than the equilibrium value in both fetal and adult BBMV, suggesting the existence of a Na/H antiporter in both preparations. Although the total uptake of Na^+ in fetal BBMV was less at each time point, except equilibrium, this difference was not statistically significant. The equilibrium levels of Na^+ uptake were similar in fetal and adult BBMV. However, at early time points, amiloride (1 mM) produced a 75% inhibition of Na^+ uptake in the adult, but only a 45% inhibition in the fetal preparation. The amiloride-sensitive component of Na^+ uptake is illustrated in Fig. 2 *B*. At virtually all time points, the amiloride-sensitive Na^+ uptake was significantly less in the fetal BBMV compared to the adult, suggesting that the Na/H antiporter is less active in the fetal vesicles.

An alternative explanation for the lower amiloride-sensitive Na^+ uptake may be that the fetal antiporter is less sensitive to amiloride than the adult exchanger. To test this hypothesis, Na^+ uptake into adult and fetal BBMV was measured as a function of inhibitor concentration. Because of the limited solubility of amiloride, the more potent amiloride analogue, EIPA was used. The dose–response curves in the adult and fetal BBMV were strikingly similar at both 1 mM and 25 mM Na^+ (Fig. 3). The adult IC_{50} (3.1±0.7 μM) and the fetal IC_{50} (6.5±0.3 μM) were similar and approximate the value previously reported for rabbit BBMV (4.7 μM [27]). Of note, at the highest concentration of EIPA employed, the maximal inhibition obtained at 1 mM Na^+ was significantly less in fetal BBMV compared to adult [77.2±6.4% vs. 95.8±0.8%; $P < 0.05$]. Increasing the concentration of EIPA to 5 mM did not result in a greater inhibition of Na^+ uptake into either fetal or adult vesicles. These data are consistent with the suggestion that the inhibitor sensitivity of the fetal antiporter is similar to the adult and support the conclusion that there is less Na/H antiporter and more amiloride insensitive Na^+ uptake in fetal vesicles.

Kinetics of the Na/H antiporter from $^{22}Na^+$ uptake experiments. The kinetic properties of the Na/H exchanger were examined to probe for a possible mechanism for the apparent increase in antiporter activity with development. The K_m and V_{max}, determined from isotopic uptake data, are provided in Fig. 4: the K_m for Na^+ was not significantly different in the two preparations; however, the V_{max} was significantly greater (fourfold) in the adult than in the fetal BBMV. The latter finding suggests that the number of antiporters and/or the turnover number of the carriers is greater in the adult membranes. There

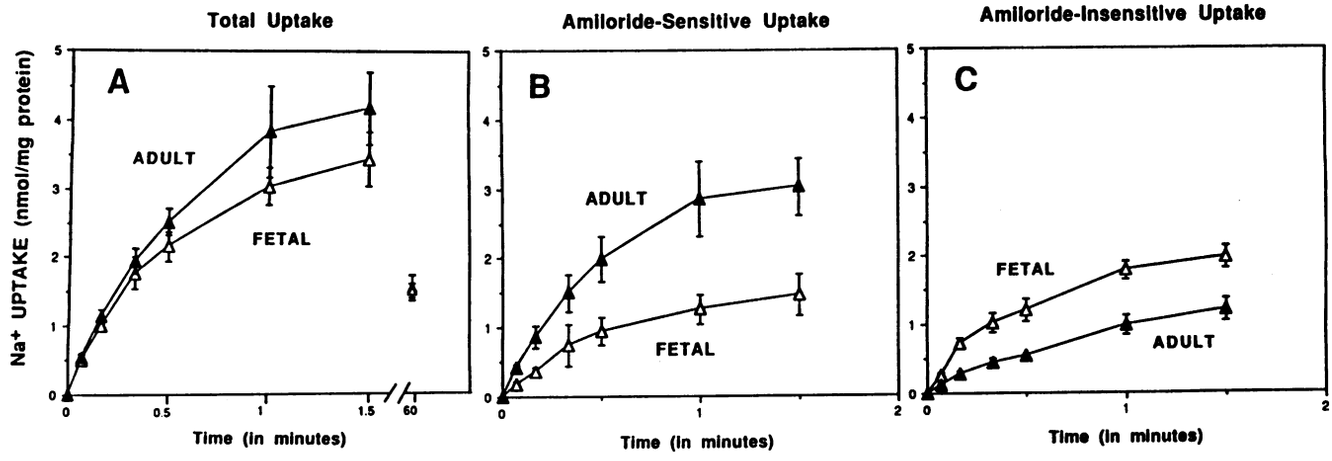

Figure 2. Time course of Na^+ uptake into BBMV. Vesicles were washed three times and resuspended in a medium containing 300 mM mannitol, 100 mM MES-Tris, pH 5.5. Uptake of 1 mM Na^+ gluconate was determined in a medium containing 200 mM mannitol, 100 mM NMG gluconate, 64 mM Hepes, 20 mM MES, 48 mM Tris, pH 7.5. (*A*) Total uptake of Na^+. (*B*) Na^+ uptake in the presence of 1 mM amiloride was subtracted from that determined in the absence of amiloride to yield the amiloride sensitive portion of uptake. (*C*) Na^+ uptake measured in the presence of 1 mM amiloride. Data represent the mean±SE of three experiments in the adult (▲) and four in the fetus (△).

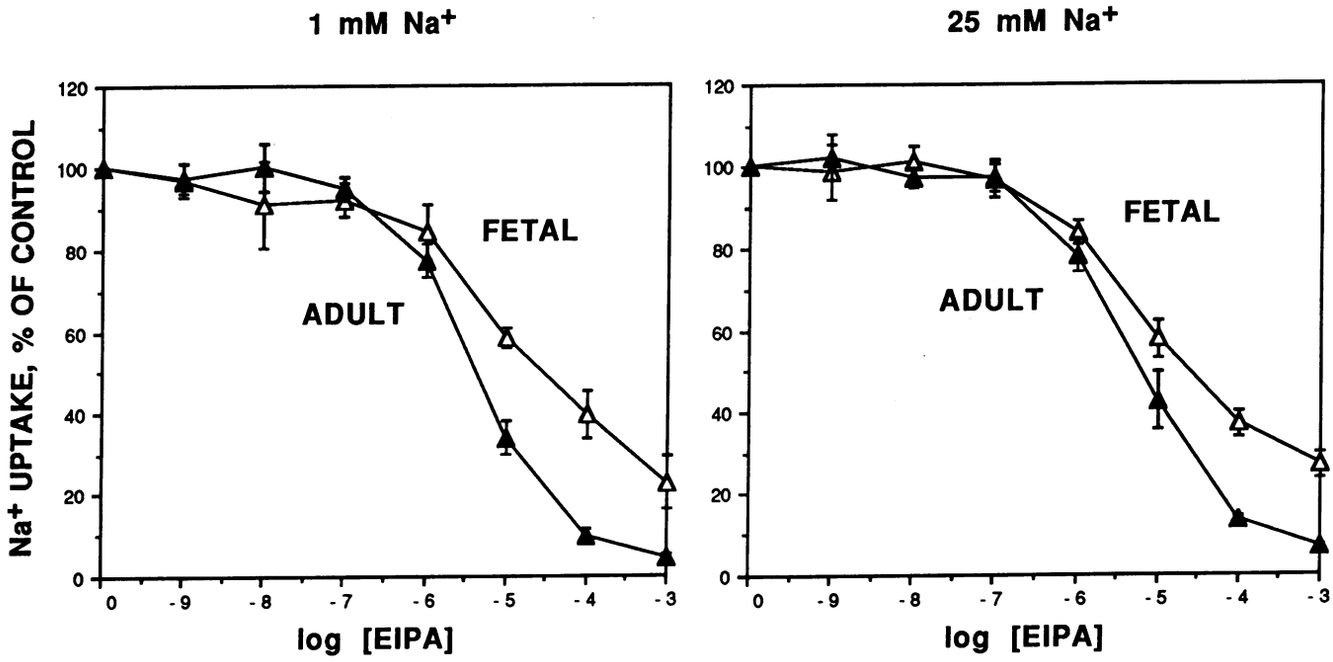

Figure 3. Inhibition of $^{22}Na^+$ uptake by EIPA in adult (▲) and fetal (△) BBMV. Uptake was measured after a 4-s incubation in the presence of 0–1 mM EIPA. Data represent the mean±SE of three experiments in the adult and three in the fetus.

are, however, several alternative explanations for the lower V_{max} in the fetal BBMV: the fetal antiporter, in contrast to that in the adult, could be electrogenic and therefore sensitive to the membrane potential; and the pH gradient could be dissipated more rapidly in fetal vesicles by conductive ion movements rather than by Na/H exchange.

To assess the possibility that the V_{max} of the fetal antiporter is dependent on membrane potential, the uptake of $^{22}Na^+$ was examined under voltage clamped conditions using the protonophore, FCCP (Table I). Total Na^+ uptake was not different in fetal and adult BBMV; however, EIPA-insensitive uptake was significantly greater in fetal vesicles. EIPA-sensitive Na^+ uptake was unaffected by FCCP in either the fetal or adult vesicles. The latter finding indicates that the activity of the fetal antiporter is independent of membrane potential and therefore electroneutral. Thus, the lower V_{max} in fetal vesicles cannot be ascribed to an effect of membrane potential.

In the absence of permeant anions the greater EIPA- and amiloride-insensitive Na^+ uptakes (Table I, Fig. 2 *C*) in fetal vesicles could be consequent to increased conductive Na^+ uptake coupled to conductive H^+ efflux, an effect that could dissipate the pH gradient more rapidly. To assess the relative rates of dissipation of the pH gradient in fetal and adult vesicles and evaluate the antiporter under conditions which are independent of nonspecific binding of Na^+, additional studies were performed using the pH sensitive fluorescent probe acridine orange.

Characterization of the Na/H antiporter using acridine orange fluorescence. For all studies, rates of change in fluorescence have been converted to rates of H^+ efflux in nanomoles H^+ per second per milligram of protein using the respective buffer capacity and calibration curves in fetal and adult vesicles. In each study, there was an immediate quench in fluorescence upon addition of vesicles (pH_i = 5.5) to the acridine orange medium (pH_o = 8.0). Thereafter, there was a slow, but constant increase in fluorescence which was not altered by the addition of 25 mM NMG gluconate. Although this basal change in fluorescence may not only reflect H^+ efflux from the intravesicular fluid, but also dissociation from membranes (28), these rates were converted to rates of H^+ efflux (6.75±1.43 and 0.965±0.083 nmol H^+/s per mg protein in fetal [n = 6] and adult vesicles [n = 8], respectively) and subtracted from the gross rates measured after addition of K^+ and Na^+ to determine the effects of these cations, per se.

Fig. 5 depicts the rates of H^+ efflux from fetal and adult vesicles as a function of the medium Na^+ or K^+ concentration. In the presence of K^+, the rate of H^+ efflux was significantly greater in fetal than in adult vesicles. In so far as H^+ efflux represents coupled conductive K^+ and H^+ fluxes, this finding indicates that the ionic permeability to K^+ (and H^+) is greater in fetal membranes. Since the ionic Na^+ permeability approximated that of K^+ (P_{Na}/P_K was 0.87±0.12 in fetal BBMV [n = 3] and 0.82±0.08 in adult BBMV [n = 8]), the K^+ induced rate of H^+ efflux has been assumed to equal the maximal rate of H^+ efflux due to conductive entry of Na^+. In this context, the greater rate of K^+ induced alkalinization in fetal vesicles also indicates that conductive Na^+ movement is significantly greater in fetal than adult membranes. This conclusion is consistent with that derived from a comparison of EIPA- and amiloride-insensitive $^{22}Na^+$ uptake in the two preparations (Table I, Fig. 2 *C*). However, the maximal change in internal pH due to conductive Na^+ influx ranged only from 0.025 to 0.037 pH U/s at [Na^+] of 1–25 mM in fetal and 0.007 to 0.014 pH U/s in adult BBMV. Therefore, at 4 s (the latest time point at which $^{22}Na^+$ uptake kinetic data were obtained), there would only be, at most, a decrease in the pH gradient from 3.00 pH U to 2.85 and 2.95 in fetal and adult vesicles, respectively. Thus, despite the greater conductive flux in fetal BBMV, it is not possible to explain the lower V_{max} on the basis of a more rapid dissipation of the pH gradient.

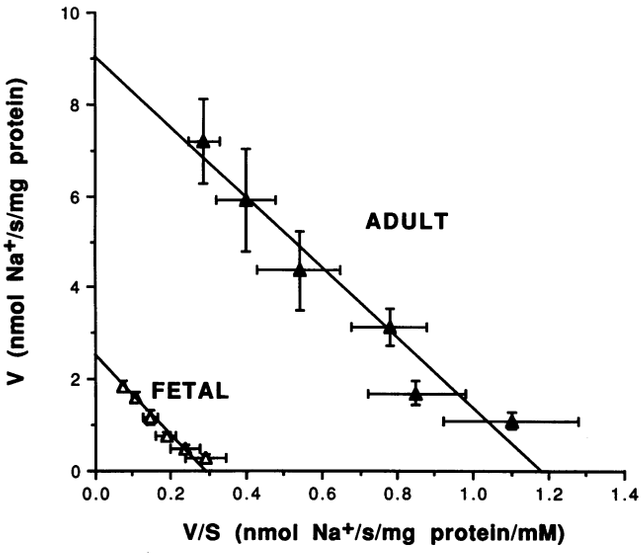

Figure 4. Woolf-Augustinsson-Hofstee plots of initial rates of amiloride sensitive $^{22}Na^+$ uptake into adult (▲) and fetal (△) BBMV. Vesicles were washed three times and resuspended in a medium containing 300 mM mannitol, 100 mM MES-Tris, pH 5.5. The incubation medium contained 150–200 mM mannitol, 100 mM NMG gluconate, 16 mM Hepes, 10 mM MES, 29 mM Tris, pH 8.0, $^{22}Na^+$ (1–25 mM as the gluconate salt), ±1 mM amiloride. Initial rates were determined by subtracting the slope of the regression line for 1- to 4-s Na^+ uptake in the presence of amiloride from the slope of the regression line for total Na^+ uptake. Kinetic parameters were calculated from a least squares analysis of the data: $r = 0.957$ and 0.982 for adult and fetal BBMV, respectively. Data represent the mean±SE of four experiments in the adult and four in the fetus.

Although the fluorescent assay precluded the use of amiloride, it was possible to determine the rate of H^+ efflux through the Na/H antiporter. Based on the similarities in ionic permeabilities to Na^+ and K^+, nonconductive H^+ efflux was calculated as the rate of H^+ efflux induced by Na^+ minus that due to K^+. Fig. 5 demonstrates that the Na/H antiporter is significantly less active in the fetal vesicles at all Na^+ concentrations. The kinetic parameters for the Na/H antiporter obtained in these studies are provided in Fig. 6 and compared to those obtained with the isotopic assay in Table II.

As stated above, using the calibration curve and buffer capacity, all fluorescence data have been converted to absolute rates of proton flux. The data in Table III present Na/H antiporter activity expressed as fluorescence units per second, i.e., before conversion, as well as pH units per second and nanomoles H^+ per second per milligram of protein. Of note, if simply expressed as fluorescence units per second or pH units per second, V_{max} increases approximately six- and fourfold, respectively, during development; when corrected for buffer capacity, there is only a 2.4-fold increase in the V_{max} as maturation occurs. Thus, had it been assumed that the buffering capacity of fetal and adult vesicles were identical, the disparity in V_{max} would have been significantly overestimated.

As demonstrated in Fig. 6 and Table II, the fluorescent assay confirms the isotopic data that the V_{max} is significantly lower in fetal than adult BBMV. It must be noted, however, that the calibration curves of acridine orange fluorescence were also different in fetal and adult BBMV (Fig. 7): the percent quench of acridine orange was linearly related to delta pH in both preparations (a delta pH between 1.5 and 3.0), but the percent quench was less at each pH gradient in fetal BBMV. The magnitude of acridine orange quenching by BBMV is the result of the pH gradient across the vesicles and membrane binding of dye (28). To evaluate the possibility that the lower V_{max} in fetal BBMV was due to a smaller initial pH gradient, the kinetics of the antiporter were reexamined in three adult BBMV preparations under conditions which yielded the same percent quench as in fetal vesicles. To accomplish this, pH_{in} was increased from 5.5 to 6.2 in adult vesicles and the pH_o was maintained at 8.0. Despite the fivefold decrease in the internal $[H^+]$, there was no significant change in either the K_m or V_{max} in the adult BBMV. Thus, the lower V_{max} in fetal BBMV cannot be ascribed to an effect of a smaller initial pH gradient. It seems likely, therefore, that the differences in calibration curves represent small differences in binding of acridine orange to fetal and adult membranes.

Of note, the V_{max} obtained with acridine orange was significantly higher than that obtained with $^{22}Na^+$ in both the fetal and adult vesicles (Table II). In an attempt to reconcile this disparity, both kinetic assays were performed in four additional adult preparations. In these paired studies, V_{max} was also significantly higher when estimated with the fluorescent technique than by the isotopic method (39.0±8.5 nmol H^+/s per mg protein vs. 9.2±1.8). Similarly disparate values for V_{max} have been previously reported in adult rabbit BBMV: based on $^{22}Na^+$ data, the V_{max} has ranged from 1 to 18 nmol Na^+/s per mg protein (29–32), while fluorescent assays have reported a V_{max} of 11–56 nmol H^+/s per mg protein (33–36). Thus, this disparity has been consistent, but its explanation remains unknown. Despite these methodologic differences in V_{max}, the K_m for Na^+ was not significantly different whether determined by the fluorescent or isotopic assay in either the fetal or adult BBMV.

Acridine orange studies with outer cortical and inner cortical adult BBMV. The developing rabbit kidney exhibits a centrifugal pattern of nephron growth which continues until about 3 wk postpartum. Whereas juxtamedullary proximal convoluted tubules are formed 10–15 d before birth, superficial tubules are not developed at birth. Therefore, the two- to threefold discrepancy in V_{max} between fetal and adult BBMV could reflect differences in the V_{max} of the antiporter in outer cortical and inner cortical/outer medullary regions of the kidney (37, 38). To examine this possibility, BBMV were prepared from both the outer cortical and inner cortical/outer medullary re-

Table I. $^{22}Na^+$ Uptake

	Total uptake	EIPA-insensitive	EIPA-sensitive
		nmol/mg protein	
Fetal (3)			
Control	1.03±0.14	0.22±0.05*	0.81±0.15
+FCCP	1.19±0.34	0.33±0.07*	0.86±0.27
Adult (3)			
Control	1.13±0.18	0.05±0.02	1.08±0.16
+FCCP	1.27±0.21	0.13±0.02	1.14±0.20

Data represent the mean±SE. The numbers in parentheses represent the number of experiments.
* Significantly different from adult ($P < 0.05$).

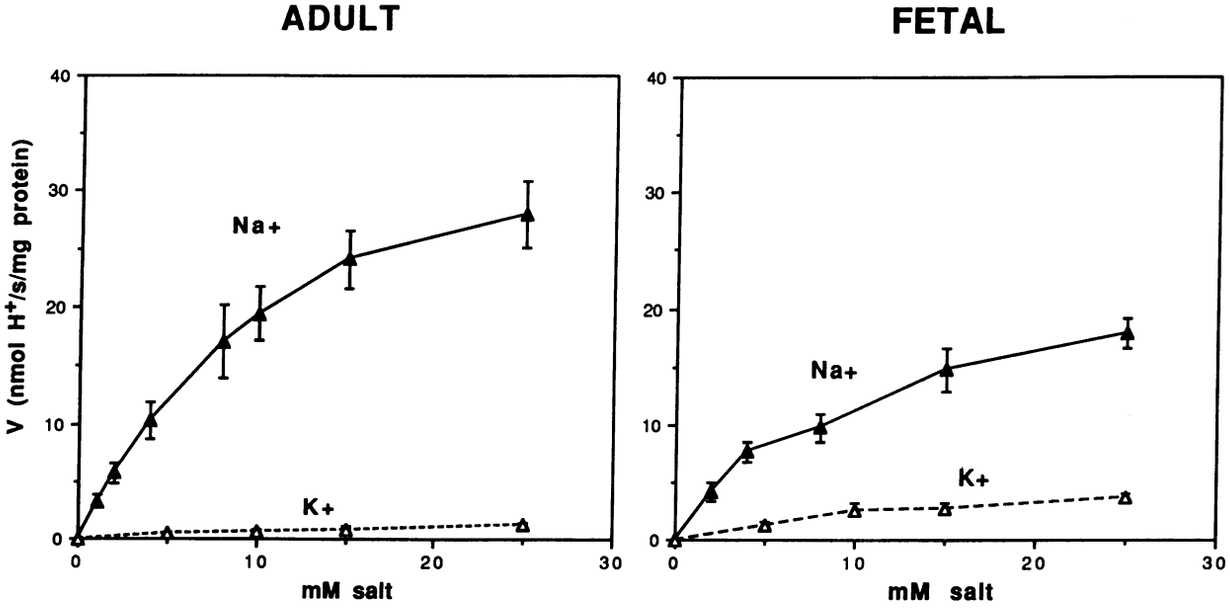

Figure 5. Effect of varying concentrations of Na^+ gluconate (▲) and K^+ gluconate (△) on the rate of collapse of the pH gradient in fetal and adult BBMV. Vesicles were washed three times and resuspended in a medium containing 300 mM mannitol, 100 mM MES-Tris, pH 5.5. BBMV (33 μg) were then added to 1 ml of medium containing 2 μM acridine orange, 100 mM NMG gluconate, 200 mM mannitol, 16 mM Hepes, and 20 mM Tris, pH 8.0. Subsequent to the initial quench of fluorescence, Na^+ gluconate or K^+ gluconate was added. Rates of alkalinization were calculated as described in Methods. Data represent the mean±SE for seven experiments in the adult and five in the fetus.

gions of the adult kidney and the kinetics of the Na/H antiporter were examined in each preparation using acridine orange fluorescence. In addition, since it has been shown that the concentrating ability of vesicles from the outer medullary region is greater than in outer cortical vesicles (14), glucose accumulation ratios were measured in each preparation (n = 4) to determine whether there was a clear separation of inner cortical/outer medullary and outer cortical segments. In inner cortical BBMV, the accumulation of glucose (at the overshoot) was 4.6±0.2-fold greater than the equilibrium value; in the outer cortex, the accumulation ratio was only 1.8±0.3-fold. In these same preparations, there was no significant difference in the V_{max} of the antiporter in the outer cortical and inner cortical BBMV, 30.8±3.2 and 25.6±2.2 nmol H^+/s per mg protein, respectively. Based on these findings it is concluded that the lower V_{max} in fetal BBMV reflects true developmental changes in the transporter rather than anatomical differences between fetal and adult kidneys.

Induction of the Na/H antiporter with glucocorticoids. The postnatal development of some transporters in several organ systems is dependent on adrenal corticoids, being retarded in adrenalectomized animals and accelerated by the administration of cortisone. To determine whether glucocorticoids affect the ontogeny of the renal antiporter, the effect of prenatal administration of betamethasone was assessed. Fig. 8, which depicts the kinetics of the Na/H antiporter in seven fetal and three betamethasone–exposed fetal preparations, demonstrates that the administration of betamethasone significantly increased the V_{max} of the antiporter, without altering the K_m for Na^+. In contrast, betamethasone had no effect on the kinetic parameters of the Na^+-dependent glucose transporter (untreated: K_m = 0.36±0.07 mM; V_{max} = 4.4±0.4 nmol/min per mg protein [n = 4] vs. treated: K_m = 0.49±0.16; V_{max} = 4.0±1.0 [n = 3]). The dose of betamethasone administered did not cause fetal growth retardation (untreated [52.0±4.6 g] vs. treated [49.1±0.9 g]) nor changes in fetal plasma glucose concentration (untreated [70.1 + 3.8 mg/dl] vs. treated [61.2±9.6 mg/dl]). Furthermore, the enrichments of alkaline phosphatase in the betamethasone–exposed fetal membranes was similar to those values for control fetal membranes (9.8±1.3 vs. 9.9±0.6, respectively). Betamethasone did significantly increase Na/K ATPase activity in the homogenate (19.3±2.3 nmoles/min per mg [treated]

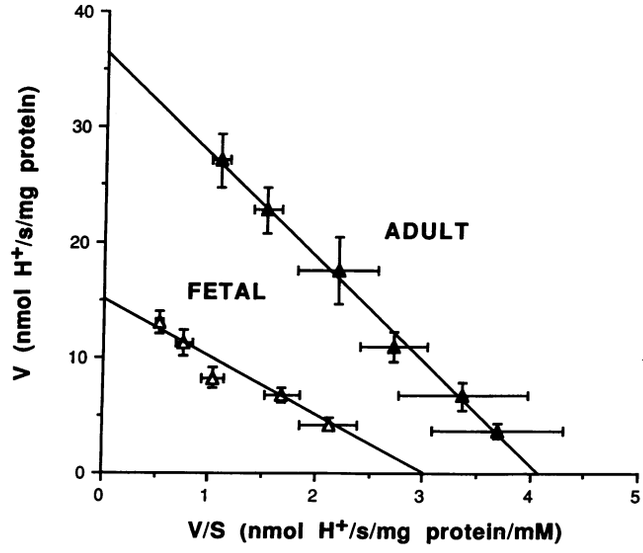

Figure 6. Woolf-Augustinsson-Hofstee plots of initial rates of alkalinization through the Na/H antiporter. Kinetic parameters were calculated from a least squares analysis of data: r = 0.997 and 0.972 for adult and fetal BBMV, respectively. Data represent the mean±SE for 11 experiments in the adult (▲) and 7 in the fetus (△).

Table II. Comparison of Kinetic Constants Estimated from Isotopic ($^{22}Na^+$) and Fluorescent (Acridine Orange) Assays

	K_m	V_{max}	n
	mM	*nmol/s per mg protein*	
Adult			
Isotopic	8.8±1.2	9.2±1.8	4
Fluorescent	10.8±1.2	38.4±3.3*	11
Fetal			
Isotopic	7.5±0.9	2.3±0.3‡	6
Fluorescent	6.5±1.2	16.1±1.2*§	7

Data represent the mean±SE. n indicates the number of experiments. In the columns labeled "isotopic," the V_{max} is expressed as nanomoles Na^+ per second per milligram of protein. In those columns labeled "fluorescent," the V_{max} is expressed as nanomoles H^+ per second per milligram of protein.
* Significantly higher than isotopically determined value ($P < 0.001$).
‡ Significantly lower in fetal than adult BBMV ($P < 0.005$).
§ Significantly lower in fetal than adult BBMV ($P < 0.001$).

vs. 5.7±0.8 [untreated]); however, the specific activities of four brush border marker enzymes in the homogenate remained unchanged. These cumulative findings suggest that betamethasone selectively enhances the activities of the Na/H antiporter and the Na/K pump in renal brush border and basolateral membranes, respectively.

Discussion

The present studies provide the first evidence that a Na/H antiporter is present in proximal tubule brush border membranes of the near-term fetus and that this transporter has several characteristics identical to those in the adult: in both, the apparent affinity for Na^+ approximates 7–11 mM, transport is electroneutral and therefore has a stoichiometry of 1:1 for Na^+ and H^+, and the carrier is inhibitable with amiloride. Of note, however, the V_{max} of the transporter is significantly lower in the fetal kidney. Insofar as there is currently no highly specific agent that would allow an accurate estimate of the number of Na/H carriers, it is not possible to determine whether this maturational increase in V_{max} represents a developmental increase in the number of antiporters inserted in the brush border membrane or a developmental increase in the turnover number of each antiporter. Despite the present inability to distinguish between these mechanisms, it is evident that there are significant postnatal changes in the Na/H antiporter which are kinetically similar to the postnatal changes which have been described for other proximal tubular transporters, i.e., Na^+-dependent glucose (13), taurine (39), and amino acids (40).

It is of note that the rate of bicarbonate reabsorption has been found to increase by ~ 60% in the rabbit juxtamedullary proximal convoluted tubule in the postnatal period despite a concurrent fall in plasma bicarbonate concentration (4). Although plasma bicarbonate concentrations have not been sequentially measured after birth, it seems likely that the fall in plasma bicarbonate occurs in association with weaning (rabbit milk has a very high calcium carbonate concentration [41]). Indeed, if the rate of proximal tubule bicarbonate reabsorption did not increase postnatally, the plasma bicarbonate concentration in adult rabbits might be significantly lower than that which is ultimately achieved and maintained. Since proximal tubule bicarbonate reabsorption is largely dependent on an apical Na/H exchanger and a basolateral Na-HCO_3 symporter, it appears likely that developmental changes in one or both of these transporters is responsible for increased bicarbonate reabsorption. Indeed, by monitoring changes in intracellular pH during in vitro microperfusion of juxtamedullary proximal convoluted tubules of neonatal rabbits, Baum has reported that the activities of the brush border Na/H antiporter and basolateral Na-HCO_3 symporter are both immature at birth: basolateral Na-HCO_3 activity was 50–60% of the adult level, while brush border Na/H activity was only one-third of the adult level (42). The present data are entirely consistent with both sets of observations in rabbit (4, 42) and provide the first evidence to indicate that the developmental increase in proximal tubule bicarbonate reabsorption is, at least in part, consequent to an increase in the number of antiporters inserted in the brush border membrane or an increase in their turnover number.

While the proximal tubule plays the major role in bicarbonate reabsorption in the adult, it is recognized that more distal sites also participate in the overall tubular reabsorption of bicarbonate. It is therefore of note that a maturational increase in the rate of bicarbonate reabsorption has also been described in

Table III. Na/H Antiporter Activity

	Fluorescence		pH		V_{max}	
Na^+	Adult	Fetal	Adult	Fetal	Adult	Fetal
mM	*U/s*		*U/s*		*nmol H^+/s per mg protein*	
1	1.05±0.17	—	0.022±0.004	—	3.69±0.60	—
2	1.91±0.34	0.49±0.06	0.040±0.007	0.015±0.002	6.73±1.19	4.23±0.53
4	3.10±0.37	0.78±0.08	0.064±0.008	0.023±0.002	10.9±1.3	6.71±0.67
8	4.98±0.82	0.96±0.10	0.104±0.017	0.029±0.003	17.5±2.9	8.24±0.89
15	6.49±0.57	1.32±0.13	0.135±0.012	0.039±0.004	22.8±2.0	11.3±1.1
25	7.71±0.65	1.52±0.12	0.160±0.014	0.045±0.003	27.1±2.3	13.0±1.0
V_{max}	11.0±0.9	1.88±0.14	0.229±0.020	0.056±0.004	38.4±3.3	16.1±1.2

Data represent the mean±SE for 11 experiments in the adult and 7 in the fetus.

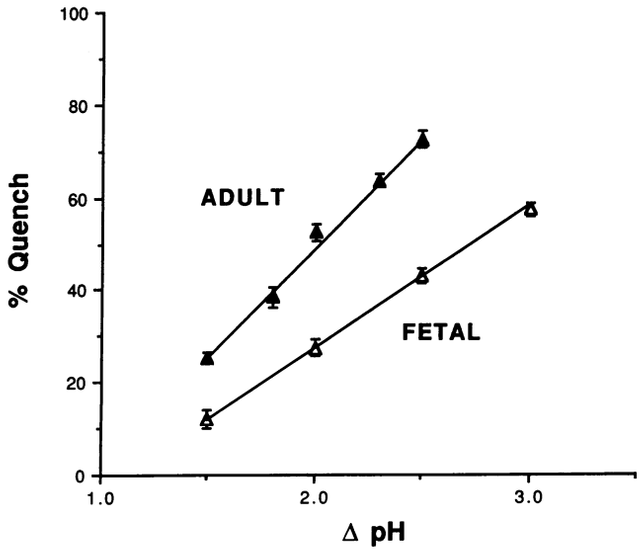

Figure 7. Calibration curves for acridine orange. Aliquots of BBMV (33 μg of protein) were incubated in 2 μM acridine orange in buffers at various pH's. The fractional decrease in fluorescence (%Quench) was determined as indicated in the Methods. Data represent the mean±SE for 11 experiments in the adult (▲) and 7 in the fetus (△).

the outer medullary collecting duct of rabbit (43). Thus, it is possible that maturational changes in both proximal and more distal nephron segments contribute to the overall increase in bicarbonate reabsorption seen postnatally. It remains to be determined, however, whether an increase in electrogenic H^+ secretion (44) or a decrease in bicarbonate secretion via Cl/HCO_3 exchange (45) is responsible for the maturational increase in collecting duct net bicarbonate reabsorption.

The ability of the tubule to reabsorb bicarbonate is also dependent on the presence of cytosolic and membrane bound carbonic anhydrase (46). Although the activity of this enzyme, at least in juxtamedullary nephrons of the human fetus (at 26 wk of gestation) is similar to that in the adult (47), carbonic anhydrase activity has not been quantitated in fetal or neonatal rabbit tubules. However, because of its exceedingly high turnover rate (48), it is unlikely that carbonic anhydrase activity would be rate limiting for proximal or distal bicarbonate reabsorption, even if it were lower in the immature rabbit.

Glucocorticoids play an important role in the control of numerous enzyme systems during mammalian development. It is quite possible that the normal pattern of antiporter development is the result of an increase in the availability of endogenous glucocorticoids. Several pieces of evidence suggest this hypothesis. Cortisol is the major active endogenous glucocorticoid in the rabbit (49). Late in gestation, the percentage of free cortisol, the physiologically important fraction of glucocorticoid, more than doubles (49); this rise continues postnatally, the time during which there is a significant increase in bicarbonate reabsorption (4). In addition, the number of glucocorticoid binding sites decreases during the differentiation of outer cortical tissue (50). In view of these cumulative observations and the fact that the synthetic glucocorticoid, betamethasone, binds with high affinity to the glucocorticoid receptor, we assessed the possibility that glucocorticoids might play a regulatory role in the maturation of the Na/H antiporter. These studies clearly demonstrate that the antiporter can be increased precociously on exposure to exogenous glucocorticoids. Although the mechanism for specific induction of the antiporter by glucocorticoids has not been addressed, it is conceivable that there is increased transcription of the gene for the Na/H antiporter. Glucocorticoids, when complexed with their specific receptor proteins, are known to bind to specific DNA sequences, thereby increasing the efficiency of transcription initiation from nearby promoters (51).

In addition to the maturational increase in antiporter activity, the present studies have demonstrated that there is a developmental decrease in conductive Na^+ and/or H^+ permeability. A similar finding has been observed in other developmental studies. Passive Na^+ conductance in both renal (52) and intestinal (53) BBMV is higher in young compared to adult animals. Because the Na/K ATPase is not fully developed in these immature animals (6), the increased conductance for Na^+ in young animals could result in intracellular accumulation of Na^+ and a reduction in the gradient for Na^+ across the luminal membrane. Such a decrease in driving force could limit the magnitude of all Na^+-linked transport systems in the immature kidney. In this context, the postnatal decrease in ionic Na^+ permeability and increase in pump activity may therefore also contribute to enhanced proximal tubule Na/H exchange (bicarbonate reabsorption) as well as contribute to the increased reabsorption of all substrates that are cotransported with Na^+.

In summary, these studies have demonstrated a developmental change in the Na/H antiporter that is kinetically similar to developmental changes of other brush border transporters, i.e., an increase in V_{max}. These studies also suggest that the development of the Na/H antiporter may be related to the normal postnatal increase in glucocorticoid production. In contrast, the development of another transporter, e.g., the Na^+-dependent glucose transporter, seems to be stimulated by alternate and as yet undefined mechanisms.

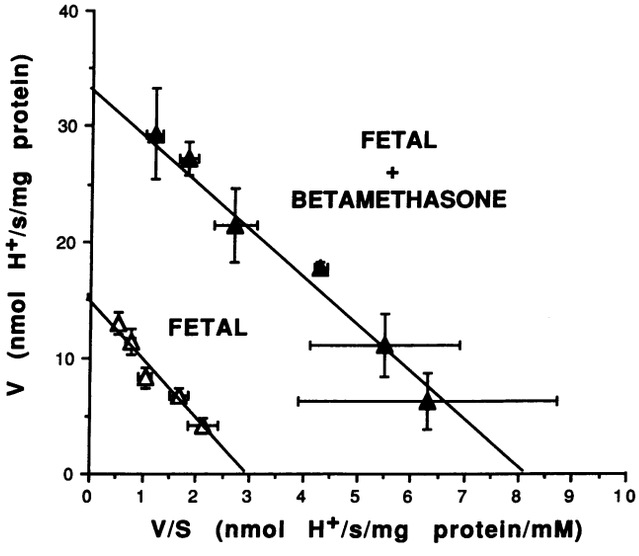

Figure 8. Woolf-Augustinsson-Hofstee plots of initial rates of alkalinization through the Na/H antiporter. The kinetic parameters were calculated from a least squares analysis of data: K_m = 3.4±1.0 mM; V_{max} = 32.6±3.5 nmol H^+/s per mg protein; r = 0.908 for fetal BBMV from betamethasone-exposed fetuses and K_m for Na^+ = 6.5±1.2 mM; V_{max} = 16.1±1.2; r = 0.972 for control fetal BBMV. Data represent the means±SE for three experiments in the betamethasone-exposed fetuses (▲) and seven in control fetuses (△).

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