L-Proline Transport by Newborn Rat Kidney Brush-Border Membrane Vesicles

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The transport of L-proline was studied in brush-border membrane vesicles isolated from the kidneys of newborn rats. In contrast with the rapid initial uptake with an 'overshoot' observed in adult vesicles, uptake by the newborn vesicle was slow, showed no 'overshoot', and proline continued to accumulate at a time when the adult vesicle had already equilibrated. L-Proline transport in the newborn rat occurs by Na^+ -dependent and independent mechanisms. There appeared to be essentially no uptake by anti-luminal vesicles isolated from newborn rat kidney. These observations may help to explain the prolinuria that occurs in the newborn animal.

The presence of marked prolinuria in the newborn rat (Baerlocher *et al.*, 1971*a,b*) and human (Brodehl & Gellissen, 1968) and the subsequent disappearance of proline from the urine during the course of development has prompted a good deal of work *in vitro* on rat kidney cortical slices in order to delineate the underlying mechanism. Baerlocher *et al.* (1971*a,b*) have shown that proline uptake by the newborn cortical slice is impaired compared with uptake by mature kidney slices. Also, very significant differences exist between the newborn and adult renal cortical cell with respect to efflux. Loss of accumulated substrate is characteristically much slower in the immature kidney, resulting in an increase in intracellular amino acid concentration (Segal *et al.*, 1971; Roth *et al.*, 1977). All of these studies, however, have in common the possible complication of simultaneous transport across both luminal and anti-luminal surfaces in the intact bipolar renal tubular cell, creating difficulties in interpretation. Furthermore, proline is extensively metabolized by the renal cortical cell (Baerlocher *et al.*, 1971*a,b*), thus raising the question of the relationship of intracellular metabolism to regulation of transport.

Using the technique described by Goldmann *et al.* (1976) for the preparation and characterization of a brush-border-enriched fraction from newborn rat renal cortex, we have investigated the transport of proline by brush-border membrane vesicles from the newborn rat. We have thus eliminated the complications alluded to above, while approaching more closely the membrane carrier site that is presumably the entity that undergoes changes during development.

Materials and Methods

Gravid female Sprague–Dawley rats were obtained from Charles River (Wilmington, MA, U.S.A.) at 14 days of gestation, and fed *ad libitum* on Purina Rat chow until parturition. Pups were killed by decapitation at less than 72 h of age. Adult male Sprague–Dawley rats weighing 150–200 g were obtained from the same source and used for the preparation of adult brush-border membrane vesicles. No distinction was made with regard to sex in newborn animals.

Membrane preparations were made by the method of Pockrandt-Hemstedt *et al.* (1972) as modified by McNamara *et al.* (1976), by using the principle of differential centrifugation and starting from whole kidneys in both groups of animals. The final pellet was resuspended in THM buffer {1 mM-Tris/Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/100 mM-mannitol (pH 7.4)} to a concentration of approx. 0.80 mg of protein/ml. Protein concentrations were determined by the method of Lowry *et al.* (1951). Compared with the homogenate, enrichment of alkaline phosphatase ranged from 6-fold in the newborn to a maximum of 8–10-fold in the adult, and (Na^+ + K^+)-dependent ATPase enrichment was 2-fold or less in all preparations.

Transport studies were carried out with membrane vesicles suspended in Na^+ -free THM buffer at pH 7.4. At the starting time, 96 μl of vesicle suspension at 22°C was added to 0.1 μCi of [^{14}C]proline, 0.1 μCi of 3-*O*-[methyl- ^3H]methyl-D-glucose, 5 μmol of NaCl and unlabelled amino acid to bring the incubation mixture to the desired amino acid concentration in a final volume of 113 μl . The mixture was stirred on a

Vortex genie for 6s. In order to study uptake under Na^+ -equilibrated conditions, membrane vesicles were preincubated in THM buffer containing 100mM-NaCl for 40min, and the above procedure was followed, omitting the 5 μmol of NaCl from the mixture and adding 106 μl of the equilibrated vesicle suspension. The use of 3-*O*-[methyl- ^3H]methyl-D-glucose as a diffusible spacer in studying proline transport has been described previously (McNamara *et al.*, 1976).

At various times, ranging from 15s to 20min, the incubation mixture was diluted with 1ml of ice-cold wash solution [154mM-NaCl/1mM-Tris/Hepes (pH7.4)], and immediately transferred by Pasteur pipette to a Millipore filter apparatus fitted with a 0.45 μm HAWP Millipore filter. The funnel and filter were then washed with 4ml of the wash solution. Filtration and washing required a total of 15s, and filtration of the incubation mixture was accomplished in less than 2s. The filters were air-dried overnight and counted for radioactivity in 5ml of phosphor dilute control (Yorktown Res., S. Hackensack, NJ, U.S.A.) in a Packard Tri-Carb liquid-scintillation spectrometer. Counting efficiency and cross-over values were determined by using an automatic external standard.

In each experiment, background values for retention of radioactivity by the Millipore filters were determined by filtration of incubation mixtures identical with those described above, with the use of membrane vesicles boiled for 90s before their addi-

tion. Medium samples were removed and counted in 0.5ml of Soluene 100 (Packard, Downer's Grove, IL, U.S.A.) and 10ml of phosphor. Background values were subtracted from the experimental data, and the diffusion component was calculated as previously described (McNamara *et al.*, 1976).

All chemicals were obtained from commercial sources and were of the highest purity available. Unlabelled proline was obtained from Mann Research Laboratories (New York, NY, U.S.A.). L-[U- ^{14}C]Proline (251mCi/ μmol) and 3-*O*-[methyl- ^3H]methyl-D-glucose (3.62Ci/ μmol) were obtained from New England Nuclear Corp. (Boston, MA, U.S.A.). Triethanolamine hydrochloride was purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.), and Tris/Hepes was purchased from Calbiochem (E. Rutherford, NJ, U.S.A.).

Results and Discussion

Assay of membrane-marker enzymes showed the preparation from newborn rats to be very similar to the brush-border fraction from newborn rats reported earlier by Goldman *et al.* (1976). Transmission-electron-microscopic inspection of a wet-mounted sample showed microvillar fragments indistinguishable from those shown in the earlier reports for newborn and adult brush-border membrane vesicles (Goldman *et al.*, 1976; Pockrandt-Hemstedt *et al.*, 1972).

Uptake of proline at a concentration of 0.06mM by the adult rat brush-border vesicle (Fig. 1b) illustrates

Fig. 1. L-Proline transport in newborn (a) and adult (b) rat membrane vesicles

The uptake of L-proline, at a concentration of 0.06mM, is shown in the presence of a Na^+ gradient (●) and after equilibration with Na^+ (○). The uptake of 3-*O*-[methyl- ^3H]methyl-D-glucose in the presence of a Na^+ gradient is also shown (▲). All points are means $\pm$ S.E.M. for at least nine determinations. For the sake of comparison, uptake of L-proline (0.06mM) by the anti-luminal membrane vesicle is shown under Na^+ -gradient conditions (□).

Fig. 2. (a) Na^+ -dependence of the uptake of L-proline (0.02 mM) in newborn brush-border membrane vesicles and (b) relationship of uptake of L-proline (0.06 mM) by newborn rat vesicles to medium osmolarity in the presence of a Na^+ gradient

(a) The medium Na^+ concentration was varied by equimolar replacement by choline chloride, as described in the text. Studies carried out at proline concentration of 2.0 mM showed no change in velocity of uptake with alteration of the medium Na^+ concentration. All points are means $\pm$ S.E.M. for at least nine determinations. v is the velocity of uptake expressed as nmol of substrate/0.5 min per mg of protein. (b) Osmolarity of the medium was varied by stepwise addition of increasing amounts of 2 M-sucrose. All points are means $\pm$ S.E.M. for at least nine determinations. v is the velocity of uptake expressed as nmol of substrate/2 min per mg of protein. The term 'relative osmolarity' is defined by arbitrarily setting 300 mOsmol equal to a value of 1.

the typical 'overshoot phenomenon' generally accepted as a major criterion for active Na^+ -dependent transport in the vesicle (McNamara *et al.*, 1976; Hammerman & Sacktor, 1977). Initial uptake is very rapid, reaching a peak by 0.5 min and falling to the level of the Na^+ -independent accumulation of proline by 20 min. Also shown (Fig. 1a) is the proline uptake, at the same concentration, by vesicles from newborn rats, which is notably different from that in the adult in all respects. The most remarkable feature of proline uptake in the newborn rat is the absence of an 'overshoot'. Instead, the initial uptake rate is low by comparison with that in the adult, although substrate accumulation persists beyond the time at which the adult vesicle has achieved equilibration.

The observations on proline uptake in newborn rat recorded in Fig. 1(a) also suggest that there are both Na^+ -dependent and Na^+ -independent systems present. Neither of the curves for proline uptake resembled the form of the curve obtained for the diffusion of 3-O-methyl-D-glucose, although the initial uptake of proline is more rapid under gradient conditions than under equilibrated conditions.

We investigated the possibility of the presence of Na^+ -dependent transport by using proline at a concentration of 0.02 mM and studying uptake for 0.5 min in the presence of various proportions of Na^+ and choline chloride. These data were then plotted by the double-reciprocal method and the lines fitted by computer (Fig. 2a). Under these conditions, proline

uptake was clearly Na^+ -dependent, an observation consistent with that already reported for the adult rat vesicle (McNamara *et al.*, 1976; Hammerman & Sacktor, 1977).

To obviate the possibility that we were studying substrate binding rather than transport into vesicles, experiments were performed in which the uptake of proline (0.06 mM) was examined after 2 min in media of various osmolarities (McNamara *et al.*, 1976). These data demonstrate diminishing degrees of uptake with increasing osmolarity (Fig. 2b), and are consistent with a decrease in intravesicular space.

Assessment of the contribution of anti-luminal membrane contaminants to total uptake in the newborn rat was made by studying uptake of proline (0.06 mM) in a plasma-membrane-enriched-vesicle suspension prepared by the method of Fitzpatrick *et al.* (1969). Measurement of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ and alkaline phosphatase indicated a 3-fold enrichment of anti-luminal membranes over the homogenate. The resulting uptake curve is shown in Fig. 1(a) for comparison with the data from the brush-border-membrane-vesicle preparation. Thus the curve shown for proline uptake by the brush-border vesicle from newborn rat includes no appreciable contribution from anti-luminal membrane contaminants.

Our results for uptake in brush-border vesicles are in agreement with the observations by Baerlocher *et al.* (1971a,b) that, in the cortical-slice model, proline uptake by the newborn Long-Evans rat kidney slice

is slower than in the adult tissue. In addition, the continued accumulation of substrate by vesicles from newborn rat suggests a lower rate of efflux compared with that in the adult, a possibility also consistent with previous observations in the slice (Baerlocher *et al.*, 1971a,b; Segal *et al.*, 1971). The intrinsic membrane differences responsible for the transport alterations in vesicles from newborn kidney may not, however, be the sole regulators of uptake by intact renal cells. Proline metabolism within the cell may be another important factor (Holtzapple *et al.*, 1973). It is, therefore, important that both of these aspects be kept in mind in evaluating developmental characteristics of transport systems. The study of transport in membrane vesicles in relation to development can, indeed, be a valuable tool in assessing physiological amino aciduria of the newborn animal.

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