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Molecular sizes of amino acid transporters in the luminal membrane from the kidney cortex, estimated by the radiation-inactivation method

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Renal brush-border membrane vesicles from rat kidney cortex were irradiated in frozen state with a γ-radiation source. Initial rates of influx into these vesicles were estimated for substrates such as L-glutamic acid, L-alanine, L-proline and L-leucine to establish the molecular sizes of their carriers. Transport was measured in initial-rate conditions to avoid artifacts arising from a decrease in the driving force caused by a modification of membrane permeability. Initial rates of Na^+-independent uptakes for those four substrates appeared unaffected in the dose range used (0–6 Mrad), indicating that the passive permeability of the membrane towards these substrates was unaffected. However, at higher doses of irradiation the Na^+ influx and the intravesicular volume evaluated by the uptake of glucose at equilibrium were altered by radiation. Thus Na^+-dependent influx values were corrected for volume changes, and the corrected values were used to compute radiation-inactivation sizes of the transport systems. Their respective values for L-glutamic acid, L-proline, L-leucine and L-alanine carriers were 250, 224, 293 and 274 kDa. The presence of the free-radicals scavenger benzoic acid in the frozen samples during irradiation did not affect the uptake of glucose, phosphate and alkaline phosphatase activity. These results indicate that freezing samples in a cryoprotective medium was enough to prevent secondary inactivation of transporters by free radicals. Uptakes of β-alanine and L-lysine were much less affected by radiation. The radiation-inactivation size of the Na^+-dependent β-alanine carrier was 127 kDa and that of the L-lysine carrier was 90 kDa.

INTRODUCTION

Radiation inactivation has been applied to study the size and structure of soluble enzymes and membrane-bound proteins [1,2]. The principal advantage of this method is that it allows the molecular size of integral membrane proteins to be determined without the need to purify or even to solubilize them from the membrane. We have previously used this method to determine the molecular size of the glucose and phosphate carriers [3] and the molecular size of the Na^+/H^+ exchanger [4] present in the luminal membrane of rat kidney. The values obtained for the radiation-inactivation size (RIS) represent the minimal structural organizations required to carry out the biological activities of the transporters.

Amino acid transport by brush-border membrane vesicles from kidney cortex has been studied extensively [5–8]. However, the nature of the proteins implicated in the molecular mechanism of amino acid re-absorption still remains unknown. Several transport systems have been proposed in intestinal and kidney tissues to explain the translocation of these essential cellular components. Kinetic evidence has shown heterogeneity among these systems [9,10]. The approach of Christensen [11] is used to delineate parallel transport pathways available for an amino acid. Care must be taken, however, to eliminate non-competitive inhibition caused by decreasing the electrochemical driving force.

In this study, we used radiation inactivation to see if we could find structural differences between the various transport systems present in brush-border membrane vesicles. We investigated the molecular size of some 'classical' Na^+ symporters such as L-alanine, L-glutamic acid, L-leucine and L-proline. A Na^+-independent system for cationic amino acids, designated system y^+ [12], was the second class of carriers to be studied, with L-lysine as the model substrate. β-Amino acid transport, which is known to require Cl^- in addition to Na^+ [13], was chosen as the third class of carrier, with β-alanine as the model substrate.

MATERIALS AND METHODS

Membrane preparation

Membrane vesicles were obtained from rat kidney cortex by the $MgCl_2$-precipitation method [14]. The final pellet was resuspended in a cryoprotective medium of high osmolarity [3] containing 150 mM-KCl, 14% (w/v) glycerol, 1.4% (w/v) sorbitol and 5 mM-Tris/Hepes, pH 7.5. Alkaline phosphatase enrichment was used as a quality control for the membrane preparation, and its specific activity routinely increased 12–14-fold with respect to the value in the cortex homogenate. Proteins were measured by the Bradford method, with the Bio-Rad protein assay.

Radiation inactivation

The vesicle samples were frozen and irradiated at −78.5 °C in a Gammacell model 220 instrument at a dose rate of about 2 Mrad/h [3]. Controls for non-irradiated preparations were made in the same conditions, but without irradiation. The molecular size (RIS) was computed from the equation:

$$\log \mathrm{RIS} = 5.89 - \log D_{37,t} - 0.0028t$$

where $D_{37,t}$ is the dose (Mrad) required to inactivate transport

Abbreviations used: RIS, radiation-inactivation size; RIFM, radiation-inactivation fragmentation method.

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activity to 37% of the initial value at an irradiation temperature t (°C).

$D_{37,t}$ values were obtained from a semi-logarithmic plot of uptake versus dose by using a least-squares fit. Unless otherwise noted, the errors quoted in the text and Tables represent S.E.M.

Transport measurements

A rapid-filtration technique was used to measure the transport. Uptake studies performed at least in triplicate at 25 °C were initiated by the addition of 80–120 μg of protein. The incubation media contained, in a volume of 30 μl, 25 μM-L-[^{3}H]amino acid (0.5 μCi), 14% glycerol, 1.4% sorbitol, 5 mM-Tris/Hepes, pH 7.5, and 150 mM-NaCl or -KCl. After incubation, the reaction was stopped by the addition of 1 ml of ice-cold stop solution, which consisted of 150 mM-KCl, 14% glycerol, 1.4% sorbitol and 5 mM-Tris/Hepes, pH 7.5. The suspension was applied to a 0.45 μm-pore-size Millipore filter under vacuum. Filters were washed with 8 ml of ice-cold stop solution and then processed for liquid-scintillation counting. Non-specific binding to the filter was determined by filtering [^{3}H]amino acid solutions that did not contain brush-border membrane vesicles. Non-specifically bound radioactivity (c.p.m.) was subtracted from observed radioactivity. Na^+-dependent uptake was calculated as the difference between uptake values in the presence of Na^+ and K^+.

For intravesicular-volume experiments, membrane vesicles were incubated for 60 min at 25 °C, in the presence of 50 μM-D-[^{14}C]glucose, 14% glycerol, 1.4% sorbitol, 5 mM-Tris/Hepes, pH 7.5, and 150 mM-NaCl or -KCl. They were then frozen in liquid N_2 and irradiated at −78.5 °C, under the conditions described above. After the irradiation, the vesicles were thawed by diluting them in 1 ml of the ice-cold stop solution described above and filtered immediately. The filters were washed as described for the uptake under initial-rate conditions. The correction for the loss of intravesicular volume was made as follows: for each irradiation dose, the decrease in volume was computed from the linear regression obtained for the log (loss of volume) as a function of the dose. Each value of amino acid transport was then corrected for this loss of volume by multiplying by the reciprocal of the percentage volume decrease, to obtain the true inactivation profile of the carrier protein, without the small interference generated by the destruction of vesicle integrity.

RESULTS

Proline uptake was measured in vesicles subjected to irradiation at doses up to 9 Mrad (Fig. 1*a*). There was a progressive loss of transport as a function of the dose. Na^+-independent proline influx, which is diffusional, was unaffected at these doses. The difference between these two fluxes was calculated as the Na^+-dependent influx. A semi-logarithmic plot was used to fit the data to a single exponential dependence on radiation dose (Fig. 1*b*).

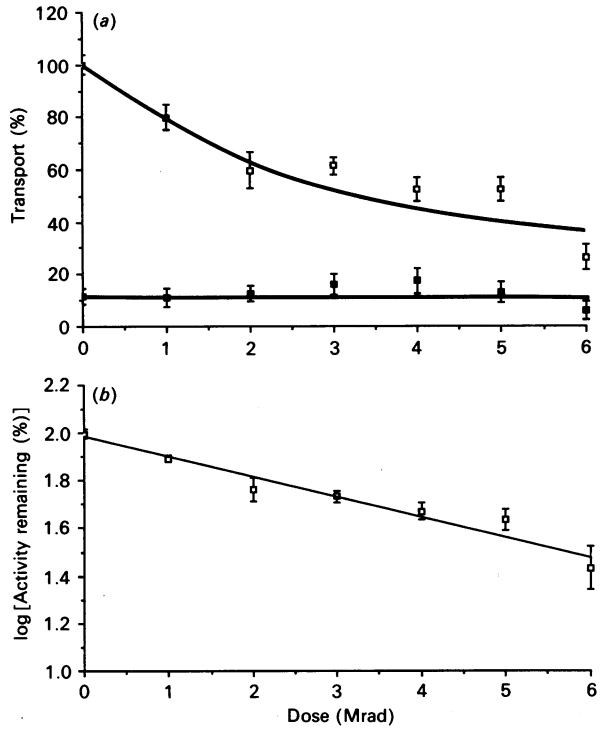

Fig. 1. Molecular-size determination of the L-proline carrier

(*a*) Vesicles were isolated and irradiated as described in the Materials and methods section. Proline uptake was measured at 3 s in a cryoprotective solution containing 25 μM-[^{3}H]proline (0.5 μCi), 14% glycerol, 1.4% sorbitol, 5 mM-Tris/Hepes, pH 7.5, and 150 mM-NaCl (□) or 150 mM-KCl (■). (*b*) Na^+-dependent L-proline transport was calculated as the difference between the uptakes measured in media containing Na^+ (□) and K^+ (■). The results are expressed as log of the percentage of activity remaining. Each value represents the average ± S.E.M. of four irradiation experiments each done in triplicate.

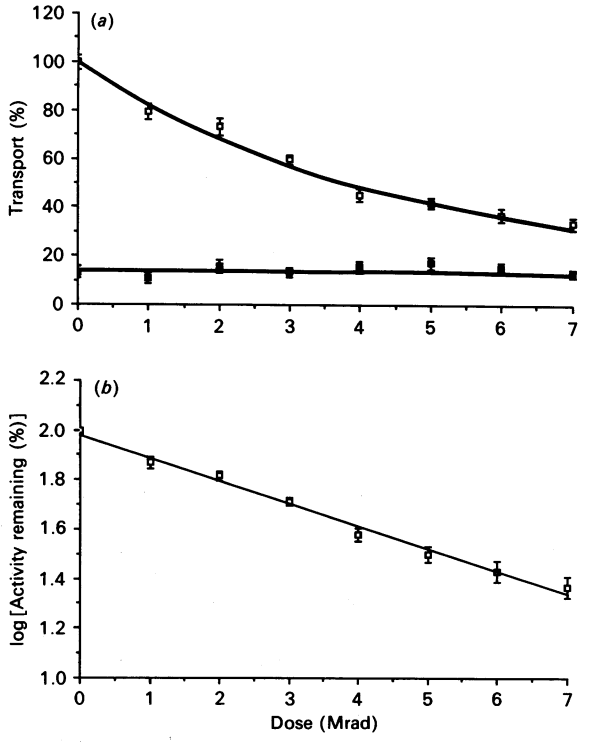

Fig. 2. Molecular-size determination of the L-glutamic acid carrier

(*a*) Glutamic acid uptake was measured at 3 s as described in the Materials and methods section. The incubation media contained 25 μM-[^{3}H]glutamic acid (0.5 μCi), 14% glycerol, 1.4% sorbitol, 5 mM-Tris/Hepes, pH 7.5, and 150 mM-NaCl (□) or 150 mM-KCl (■). (*b*) Na^+-dependent glutamic acid transport was calculated as the difference between the uptakes from incubation media containing Na^+ (□) and K^+ (■). The results are expressed as log of the percentage of activity remaining. Each value represents the average ± S.E.M. of data obtained from five experiments.

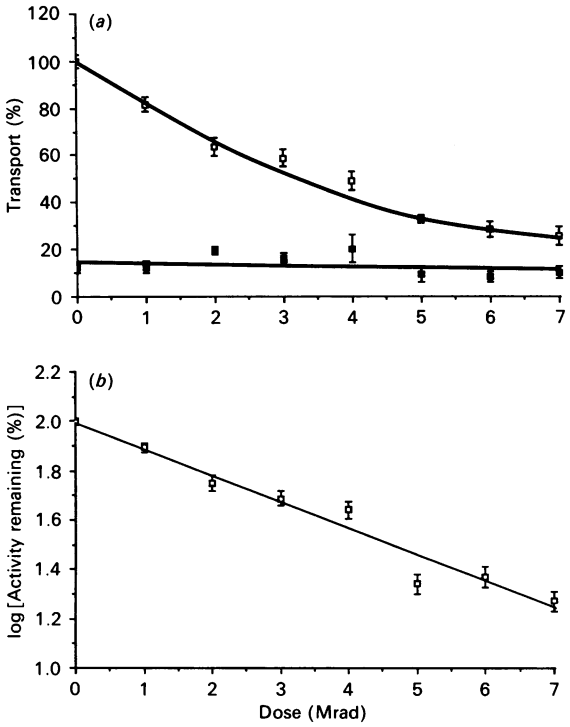

Fig. 3. Molecular-size determination of the L-leucine carrier in membrane vesicles

(*a*) Membranes were irradiated at the indicated doses. L-Leucine uptake at 3 s was measured from a solution containing 25 μM-L-[^{3}H]leucine, 14% glycerol, 1.4% sorbitol, 5 mM-Tris/Hepes, pH 7.5, and 150 mM-NaCl (□) or 150 mM-KCl (●). (*b*) The difference between uptakes from those two media was calculated as the Na^+-dependent uptake of L-leucine. The results were expressed as log of the percentage of activity remaining. Each value represents the average ± S.E.M. of four irradiation experiments each done in triplicate.

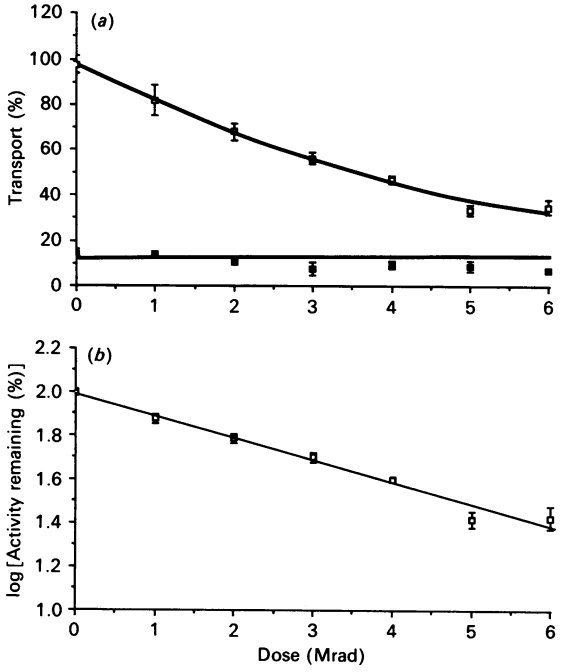

Fig. 4. Molecular-size determination of the L-alanine carrier

(*a*) The vesicles were prepared as described in the text, and exposed to different doses (0–6 Mrad). The transport of L-alanine was evaluated at 3 s with solutions containing 25 μM-L-[^{3}H]alanine, 14% glycerol, 1.4% sorbitol, 5 mM-Tris/Hepes, pH 7.5, and 150 mM-NaCl (□) or 150 mM-KCl (■). (*b*) The uptake measured with KCl was subtracted from the total transport measured with NaCl. The results were expressed as log of the percentage of activity remaining as a function of dose. Each value represents the average ± S.E.M. of five irradiation experiments each done in triplicate.

This assumption neglects the possibility of multiple carriers. The $D_{37,-78}$ value was 5.19, which corresponds to a molecular size of 248 ± 32 kDa for the proline transporter.

Glutamic acid (Fig. 2), leucine (Fig. 3), L-alanine (Fig. 4) and β-alanine (Fig. 5) uptakes were measured under the same experimental conditions as those described for proline. In every case, the Na^+-dependent uptake was inhibited progressively as a function of the dose, whereas the Na^+-independent uptake appeared unaffected by the irradiation procedure. The data were fitted to a single exponential. The $D_{37,-78}$ values were 4.67, 4.09, 4.30 and 8.47 Mrad for glutamic acid, leucine, L-alanine and β-alanine respectively, corresponding to molecular sizes of 276 ± 14, 315 ± 27, 300 ± 14 and 152 ± 14 kDa for these transporters.

One possible problem in the use of the radiation-inactivation fragmentation method (RIFM) is the generation of free radicals, which may cause secondary inactivation of transporters. To test for this possibility, we have irradiated membrane preparations in the presence of various concentrations of a free-radical scavenger (benzoic acid). Benzoic acid was shown to have no effect on the inactivation of alkaline phosphatase (Table 1) even at concentrations up to 50 mM. The inactivation was 28% in the absence of benzoic acid, compared with 23%, 36% and 30% in the presence of 10 mM-, 25 mM- and 50 mM-benzoic acid. Two additional controls were performed, with the glucose and the phosphate transporters. As shown in Table 1, the inactivation at 4.7 Mrad was similar, in the presence or absence of benzoic acid, for either the phosphate or the glucose transporter. For the

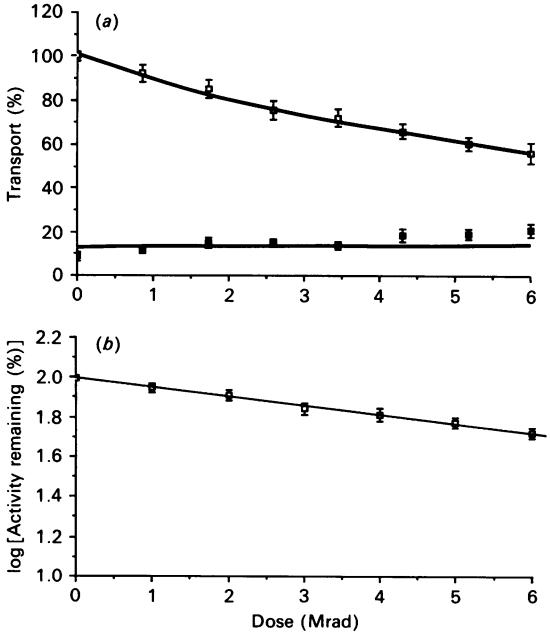

Fig. 5. Molecular-size determination of the β-alanine carrier

(*a*) Vesicles were incubated for 7 s in solutions containing 25 μM-β-[^{3}H]alanine, 14% glycerol, 1.4% sorbitol, 5 mM-Tris/Hepes, pH 7.5, and 150 mM-NaCl (□) or 150 mM-KCl (■). (*b*) Na^+-dependent β-alanine transport was calculated as the difference between the uptakes in the presence of NaCl or KCl in the incubation media and expressed as log of the percentage of activity remaining as a function of dose. Each value represents the average ± S.E.M. of five irradiation experiments each done in triplicate.

Table 1. Effect of free-radical scavenger on irradiated membrane vesicles

During the final centrifugation of the isolation, the membranes were resuspended in cryoprotective media consisting of 150 mM-KCl, 14% glycerol, 1.4% sorbitol and the indicated benzoic acid concentrations. The pH was adjusted to 7.5 with 50 mM-Hepes. The frozen samples (−78 °C) were irradiated at 0 Mrad and 4.7 Mrad as described in the Materials and methods section. Alkaline phosphatase activity was evaluated by the standard method. The rapid-filtration technique was used for measurement of glucose and phosphate uptakes. Incubation media for the glucose uptake contained 50 μM-D-[^{3}H]glucose (0.3 μCi), 50 mM-Tris/Hepes, pH 7.5, 14% glycerol, 1.4% sorbitol, 150 mM-NaCl or 150 mM-KCl. Phosphate transport was measured with the same solutions, but glucose was replaced with 200 μM-[^{32}P]P_i. Na^+-dependent uptake for both of these substrates as shown was calculated as the difference between the uptakes measured in the presence of Na^+ or K^+. Each value represents the mean ± S.D. of four experiments each done in triplicate.

[Benzoic acid] (mM)	Alkaline phosphatase (pmol/15 min per mg)		D-Glucose uptake (pmol/5 s per mg)		Phosphate uptake (pmol/5 s per μg)	
	0 Mrad	4.7 Mrad	0 Mrad	4.7 Mrad	0 Mrad	4.7 Mrad
0	92.9±2.9	66.9±3.7	131±20	52.2±13.4	1.16±0.1	0.34±0.07
10	85.7±7.3	65.1±2.7	110±19	51.0±12.3	0.81±0.2	0.41±0.15
25	100.4±7.3	64.8±9.7	109±20	41.8±18.1	0.84±0.2	0.29±0.08
50	89.7±12	63.6±4.9	123±34	48.9±12.7	0.95±0.2	0.29±0.11

glucose carrier, the inactivation was 60% in control vesicles, compared with 55%, 59% and 60% in the presence of 10 mM-, 25 mM- and 50 mM-benzoic acid respectively. The phosphate carrier was inhibited at 60%, 70% and 71% for the same benzoic acid concentrations, compared with a control value of 70% in the absence of benzoic acid. These results suggest that

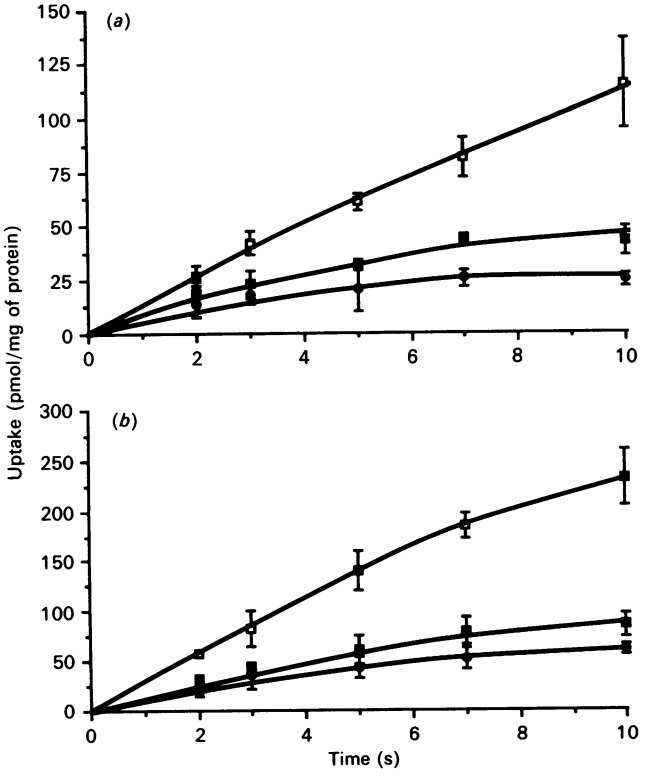

Fig. 6. Initial rates of L-glutamic acid and L-alanine uptake by brush-border membrane vesicles

Vesicles were isolated and irradiated as described previously. The membranes were exposed to different doses: □, 0 Mrad; ■, 2.1 Mrad; ●, 4.2 Mrad. (*a*) L-Glutamic acid uptake was measured as described in Fig. 2. (*b*) L-Alanine initial uptake was studied under the same conditions as in Fig. 4. Na^+-dependent L-glutamic acid and L-alanine uptake were calculated as the difference between the uptakes from incubation media containing Na^+ and K^+.

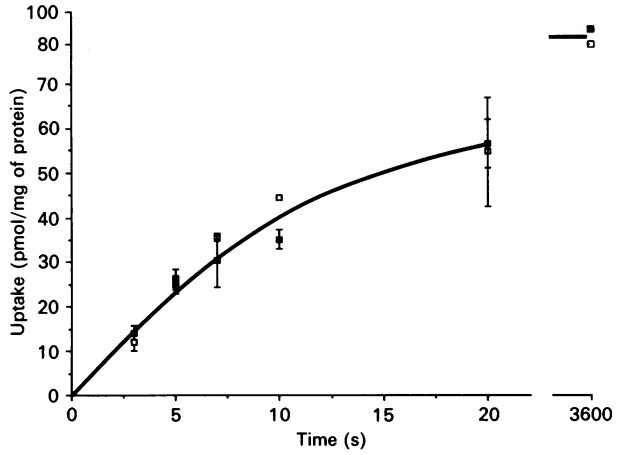

Fig. 7. Time course of L-lysine uptake by brush-border membrane vesicles

Brush-border membrane vesicles were prepared as described in the Materials and methods section. L-Lysine influx was measured at 25 °C in a cryoprotective medium that contained 150 mM-NaCl (□) or 150 mM-KCl (■). Both of these solutions contained also 14% glycerol, 1.4% sorbitol, 5 mM-Tris/Hepes, pH 7.5, and 20 μM-L-lysine (0.5 μCi). Each value represents the average ± S.D. of two experiments each done in triplicate.

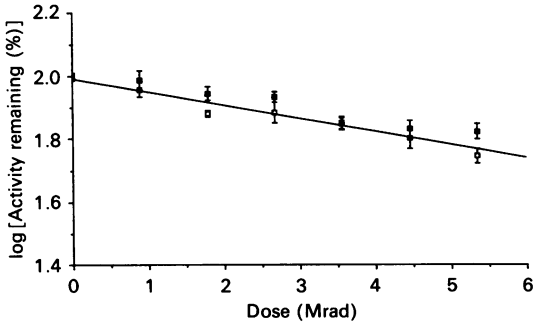

Fig. 8. Molecular-size determination of the L-lysine carrier

L-Lysine uptake was measured with vesicles irradiated at indicated doses. Transport of the substrate was evaluated for 3 s in solutions containing 25 μM-[^{3}H]lysine (0.5 μCi), 14% glycerol, 1.4% sorbitol, 5 mM-Tris/Hepes, pH 7.5, and 150 mM-NaCl (□) or 150 mM-KCl (■) The results are expressed as logarithm of the percentage of activity remaining. Each value represents the average ± S.E.M. of three irradiation experiments each done in triplicate.

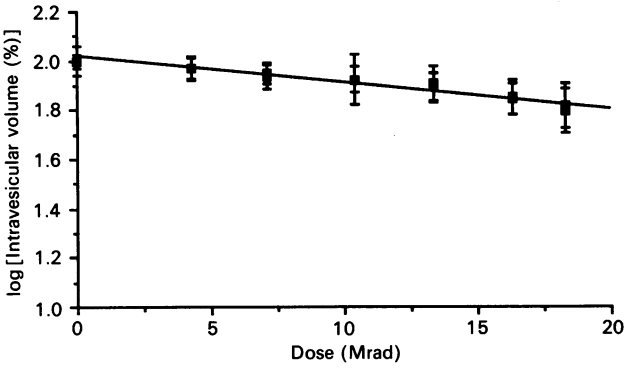

Fig. 9. Effect of irradiation on the intravesicular volume

Membrane vesicles were treated as described in the Materials and methods section. Uptake of glucose at equilibrium (60 min) was obtained by preincubating the vesicles in solutions containing 14% glycerol, 1.4% sorbitol, 5 mM-Tris/Hepes, pH 7.5, 50 μM-D-[^{3}H]glucose and 150 mM-NaCl (■) or 150 mM-KCl (□), before the irradiation was performed. The results are expressed as log of volume remaining. Each value represents the mean ± S.E.M. of data obtained during three experiments done in triplicate.

the destruction of the polypeptide chain caused by the primary inactivation is responsible for the loss of activity of these proteins. Any free radicals which are accessible to benzoate do not alter the rate of radiation inactivation.

To eliminate the possibility of a dose-dependent decrease in the driving force, initial rates of amino acid transport were compared between irradiated and non-irradiated vesicles (Fig. 6). The uptake was linear for at least the first 5 s of incubation independently of the irradiation dose, indicating that there was no alteration of the driving force for the short time of incubation used in our experiments. Any decrease in the driving force should have caused a pronounced effect on the linearity of the uptake.

To see if the RIFM method could also be used with the Na^+-independent carriers of the brush-border membrane, lysine uptake was studied. As shown in Fig. 7, lysine uptake was totally

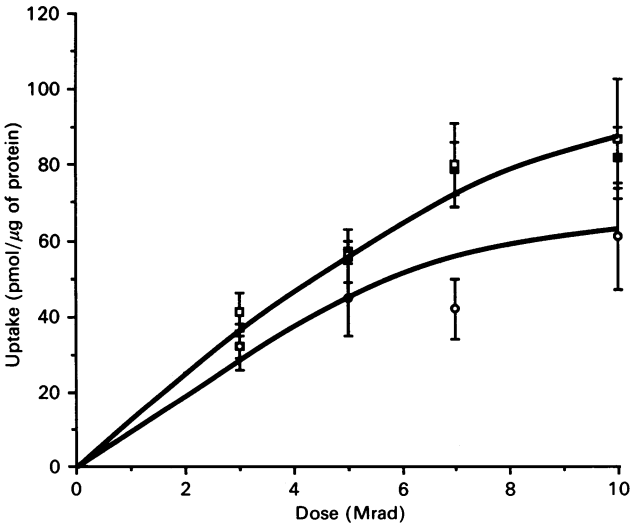

Fig. 10. Effect of irradiation on the time course of uptake of Na^+

Vesicles were irradiated at different doses: 0 Mrad (■), 3 Mrad (□) and 12 Mrad (○). Na^+ uptake was measured in a solution containing 14% glycerol, 1.4% sorbitol, 5 mM-Tris/Hepes, pH 7.5, and 150 mM-^{22}NaCl for the indicated times. Each value represents the average ± S.E.M. of three experiments.

Table 2. RIS determination of carriers in brush-border membrane vesicles

Substrate	RIS (kDa)	
	Measured value	Correction for volume change
L-Lysine	118 ± 13	90 ± 10
β-Alanine	152 ± 14	127 ± 12
Phosphate*	234 ± 14	205 ± 12
L-Proline	248 ± 32	224 ± 29
L-Glutamic acid	276 ± 14	250 ± 13
D-Glucose*	288 ± 19	256 ± 17
L-Alanine	300 ± 14	274 ± 13
L-Leucine	315 ± 27	293 ± 25
Na^+/H^+ exchanger†	321 ± 22	298 ± 20

* Data from Béliveau *et al.* [3].
† Data from Béliveau *et al.* [4].

insensitive to the presence of Na^+ in the incubation medium. The initial rate of Na^+-dependent uptake was 4.12 pmol/s per μg of protein, not different from K^+-dependent lysine uptake (4.18 pmol/s per μg of protein). Lysine uptake in the presence of Na^+ or K^+ was then measured as a function of the radiation dose (Fig. 8). The $D_{37,-78}$ values obtained were statistically identical: 10.9 Mrad, corresponding to a size of 118 kDa.

Intravesicular volumes as obtained by glucose uptake at equilibrium indicated a decrease in volume at higher doses (Fig. 9). Vesicle integrity thus appears to be significantly affected. This decrease in intravesicular volume is confirmed by the decrease in the uptake of Na^+ by diffusion into the vesicles (Fig. 10). The early time course of Na^+ uptake was unaffected with radiation doses up to 3 Mrad; initial rates were identical for 0 and 3 Mrad-treated vesicles, i.e. 11 pmol/s per μg of protein. A higher radiation dose (12 Mrad) caused a significant decrease in Na^+ uptake, especially after 5 s. At 12 Mrad, the initial rate of Na^+ uptake was 8.1 pmol/s per μg, i.e. a 33% decrease, in agreement with the 22% decrease in intravesicular volume at 12 Mrad (Fig. 9). This could be due to a decreased Na^+ permeability or to the destruction of a vesicle population. The RIS obtained for the loss of intravesicular volume was 28 kDa, suggesting that a protein of this size is responsible for the integrity of the vesicle structure; its destruction by radiation would cause an important leak that would be responsible for the decrease in intravesicular volume. This effect was not apparent in previous studies [3,4], because at low doses (6 Mrad) the effect is very small and is hidden in the standard deviation of experimental points. To correct for this loss of volume, we used the linear-regression equation in Fig. 9 to find the percentage decrease in intravesicular volume for each of the doses used in the estimation of the molecular sizes of the transport systems. Each value of Na^+-dependent transport was corrected for this decrease, and a new target size was calculated for every transport system. The results are given in Table 2.

DISCUSSION

The RIFM method was applied to study the transport of amino acids in renal brush-border membrane vesicles. The lack of effect of a free-radical scavenger confirms that the RIFM method can be used with these vesicles: in our experimental conditions, there appears to be little or no secondary inactivation caused by free radicals during the inactivation process, contrarily to what Eichler *et al.* [15] observed for other enzymes.

In addition, we have shown previously [3] that the size of alkaline phosphatase and 5'-nucleotidase obtained by the RIFM

in renal brush-border membrane vesicles is similar to the size of the purified enzymes. Any interference from free radicals generated should have caused a deviation (increase) in the size of these two enzymes. The lack of difference supports our suggestion that secondary inactivation should be quite small in our samples. Finally, the low temperature at which the irradiation was performed (−78.5 °C) and the presence of glycerol and sorbitol, as additional radical scavengers, decrease further the possibility of free-radical artefacts. It is thus the primary inactivation or the physical destruction of protein molecules that is responsible for the effects reported in this paper.

A protein (28 kDa) appears to be responsible for the structural integrity of the vesicles. Its destruction would cause an increase in the permeability of the vesicles, leading to a decrease in the mean intravesicular space of the whole population of vesicles. The vesicles hit by radiations would be destroyed (changed from closed vesicles to leaky membranes), and those that are not touched would remain active. This volume decrease, although small at low doses, significantly affects the RIS of carriers; it is probably the reason why previously reported RIS values for membrane carriers were higher than the sizes found by other methods.

The monomer size of the D-glucose carrier from the intestine has been reported to be 73080 Da, based on the amino acid sequence deduced from cloned cDNA [16]. The RIS value of this transporter (256 kDa) corresponds to a multimeric (3–4 subunits) arrangement. Previous studies have suggested a 97 kDa band as a candidate for the proline carrier, based on protection by proline of fluorescein isothiocyanate (FITC) labelling and fluorescence quenching of FITC [17]. The size reported here for the proline carrier (224 kDa) suggests that the active carrier in the membrane exists as a dimer.

Mutual inhibition of transport by L-alanine and L-leucine, together with glutamine and phenylalanine, have led Lynch & McGivan [18] to suggest a common transport system for all these four amino acids. The similarity of RIS values obtained for L-alanine (274 kDa) and L-leucine (293 kDa) transporters supports this conclusion.

The small molecular size (90 kDa) obtained for the L-lysine carrier indicates that this carrier belongs to a class of transporter different from that of the Na^+-symporter. It was previously shown that L-lysine transport was a carrier-mediated phenomenon in renal vesicles and that it differed from that of neutral and acidic amino acids, because protonmotive force instead of the Na^+ gradient could provide the driving force for reabsorption [19].

The RIS obtained for the β-alanine carrier (127 kDa) is also much lower than for other amino acid carriers. β-Amino acids are reabsorbed by a distinct transport system different from that for other amino acids, since the reabsorption is coupled to both Na^+ and Cl^- [13,20]. None of the α-amino acids, including L-α-alanine and other amino acids typical of each known transport system in the membrane, were inhibitory to β-alanine transport [20].

The similarity in the sizes found for the four Na^+-symporters studied here (proline, glutamate, leucine, alanine) suggests that a common molecular structure might be involved in the Na^+-coupled reabsorption of amino acids. These sizes are much larger than the RIS of two carriers (lysine and β-alanine) that are not Na^+-symporters. The Na^+-dependency could thus be a functional consequence of a structural similarity of the proteins involved in the Na^+-coupled reabsorption.

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