Biochimica et Biophysica Acta, 777 (1984) 201–208
Elsevier

BBA 72272

STRUCTURAL STATE OF THE Na^+/D-GLUCOSE COTRANSPORTER IN CALF KIDNEY BRUSH-BORDER MEMBRANES

TARGET SIZE ANALYSIS OF Na^+-DEPENDENT PHLORIZIN BINDING AND Na^+-DEPENDENT D-GLUCOSE TRANSPORT

JIANN-TRZUO LIN [a,*], KORNELIA SZWARC [a,**], ROLF KINNE [a,***] and CHAN Y. JUNG [b]

[a] *Department of Physiology and Biophysics, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York, NY 10461, and* [b] *Veterans Administration Medical Center, and Department of Biophysical Sciences, State University of New York at Buffalo, NY 14214 (U.S.A.)*

(Received December 9th, 1983)
(Revised manuscript received May 28th, 1984)

Key words: Phlorizin binding; Glucose transport; Na^+/glucose cotransporter; (Calf kidney)

Target sizes of the renal sodium-D-glucose cotransport system in brush-border membranes of calf kidney cortex were estimated by radiation inactivation. In brush-border vesicles irradiated at −50°C with 1.5 MeV electron beams, sodium-dependent phlorizin binding, and Na^+-dependent D-glucose tracer exchange decreased exponentially with increasing doses of radiation (0.4–4.4 Mrad). Inactivation of phlorizin binding was due to a reduction in the number of high-affinity phlorizin binding sites but not in their affinity. The molecular weight of the Na^+-dependent phlorizin binding unit was estimated to be 230000 ± 38000. From the tracer exchange experiments a molecular weight of 345000 ± 24500 was calculated for the D-glucose transport unit. The validity of these target size measurements was established by concomitant measurements of two brush-border enzymes, alkaline phosphatase and γ-glutamyltransferase, whose target sizes were found to be 68570 ± 2670 and 73500 ± 2270, respectively. These findings provide further evidence for the assumption that the sodium-D-glucose cotransport system is a multimeric structure, in which distinct complexes are responsible for phlorizin binding and D-glucose translocation.

Introduction

Glucose transport in the renal proximal tubule is known to be facilitated by a sodium-D-glucose cotransport system, which is competitively inhibited by phlorizin [1]. This transport system represents the best-studied sodium cotransport system to date but its complex function of sugar binding, sodium binding, phlorizin binding and sodium-glucose translocation is far from being understood. During recent years, several attempts have been made to determine the molecular weight of the transport system (or its subunits). Determinations based on affinity labeling or isolation of a protein exhibiting phlorizin binding or glucose transport function yielded molecular weights ranging from 25000 to 165000 [2–10]. All the above-mentioned studies involved, at some point or another, a dissociation of the membrane with

* To whom correspondence should be addressed at (present address): Department of Physiology, Cornell University Medical College, New York, NY 10021, U.S.A.
** Present address: Department of Hematology, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York, NY 10461, U.S.A.
*** Present address: Max-Planck-Institut für Systemphysiologie, 4600 Dortmund 1, F.R.G.

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

detergents, thus raising the possibility that the actual molecular weight of the transport system in the intact membrane might be higher than that of the isolated components. In 1982, Turner and Kempner [11] published a study which employed radiation inactivation to address this question. They observed that at low irradation doses about 50% of the phlorizin binding sites were inactivated, whereas at higher irradiation doses the residual binding sites behaved as a target site with an apparent molecular weight of 110000. In those experiments an attempt was also made to determine the molecular weight of the transport system by flux studies. This attempt failed, however, because of the increase in leak permeability of the rabbit kidney brush-border membranes after irradiation. Since inactivation of about 50% of the binding sites occurred even at very low irradiation doses, it was conceivable that larger complexes of phlorizin sites migth be present in the membrane, which should be amenable to investigations if only low irradiation energies are employed. At these low energies the unspecific membrane damage might also be minimal and, thus, flux studies should be possible. The following investigations concentrated, therefore, on the response of phlorizin binding and D-glucose transport by brush-border membranes exposed to irradiation doses of up to 4 Mrad, only. They show that the number of phlorizin binding sites, but not their affinity, decreases according to an apparent target size of 230 kDa. Sodium-dependent D-glucose tracer exchange is even more sensitive to irradiation. Its inactivation suggests a molecular weight of the tranporting unit of 345000. It is postulated that the sodium-D-glucose cotransport system consists of at least two different subunits, one involved in phlorizin binding ($\approx$ 115000) and another ($\approx$ 60000) required together with the former for the translocation of sodium and D-glucose. These latter complexes can apparently form dimers, which exhibit a molecular weight of approx. 345000.

Part of these results has been presented at the 15th meeting of the American Society for Nephrology, Chicago (Abstr. No. RW-53, 1982).

Materials and Methods

Materials

[^{3}H]Phlorizin (45 Ci/mmol) was synthesized in our laboratory [12]. D-[^{3}H]Glucose (15 Ci/mmol) was obtained from New England Nuclear. Phlorizin was purchased from Sigma Chemical Co.. Cellulose acetate filters (type HAWP; pore size 0.45 μM), obtained from Millipore Co., were used in the binding and transport studies. All other chemicals were of the highest purity commercially available. A DuPont-Sorvall ARC-1 centrifuge was used for high-speed and a Sorval RC-5B centrifuge for low-speed centrifugation.

Preparation of brush-border membrane vesicles from calf kidney

Slices of cortex were prepared from calf kidneys, provided by a private slaughterhouse, and stored at $-80\,^\circ$C. The $CaCl_2$-precipitation method, as described previously [13,14], was used for the preparation of membrane vesicles. The vesicles were repeatedly resuspended in a vesicle buffer (20 mM Hepes-Tris/200 mM mannitol, adjusted to pH 7.4 with Tris base) and sedimented twice at 100000 $\times$ g for 1 h to remove any residual sodium. The final membrane suspension had a protein concentration of approx. 10 mg/ml. In transport studies under gradient conditions, the membranes showed a 450% overshoot of sodium-dependent D-glucose uptake after 30 s of incubation. The enrichment of activities of marker enzymes in the vesicles was 12-fold for alkaline phosphatase, 14-fold for γ-glutamyltransferase and 11-fold for aminopeptidase M. No loss in D-glucose transport activity was found for at least 3 months of storage at $-80\,^\circ$C.

Irradiation of membrane vesicles

Approx. 700 μl membrane suspension (6–10 mg protein per ml) were spread onto an open aluminium tray and frozen in liquid nitrogen. Irradiation was carried out in a closed, anaerobic chamber at -40 to $-50\,^\circ$C, using Van de Graaff-generated 1.5 MeV electron beams [15]. The radiation dose was adjusted by varying the number of passages of the electron beam across the sample. The absolute radiation dose for each passage was measured at room temperature (25 °C), using Blue Cellophane (DuPont, dimethoxydiphenylbisazobis(8-amino-1-naphthol-5,7-disulfonic acid)). The response of the Blue Cellophane to radiation doses was calibrated

against a Fricke dosimeter with linear extrapolation. After irradation, the samples were stored at −80 °C. Membranes for control experiments were treated identically, but without irradiation. Enzyme assays, flux measurements, and phlorizin binding studies were conducted within 1 week after irradiation.

Transport studies

D-Glucose transport activities of control and irradiated membranes were measured by means of tracer exchange (equilibrium exchange) in the absence of ion gradients as follows. The control and irradiated membranes (protein content 3–5 mg/ml) were pre-equilibrated at 22 °C for 60 min with 0.1 mM D-glucose in a transport medium containing 100 mM NaSCN or KSCN, 200 mM mannitol and 20 mM Hepes-Tris (pH 7.4). Exchange experiments were started by adding a trace amount of D-[^{3}H]glucose to the transport medium at 22 °C, and aliquots of samples were taken after various incubation times. D-Glucose uptake was measured by the filtration technique according to Hopfer et al. [16,17]. In each experiment, samples were taken after 0.25, 1, 2 and 90 min of incubation time and each experimental series was performed in duplicate.

Phlorizin binding assay

Membrane suspensions (2–3 mg protein/ml) in Hepes-Tris buffer (20 mM, pH 7.4), containing 100 mM NaCl or KCl, were pre-incubated at 37 °C for 30 min with a trace amount of [^{3}H]phlorizin in the presence of various concentrations of phlorizin. After chilling to 4 °C, phlorizin bound to membranes was separated from free phlorizin in the medium by rapid filtration, followed by washing with 5 ml of ice-cold phlorizin-free buffer solution within 10 s. An appropriate correction was made for the amount of phlorizin bound to the filter. All assays were performed in duplicate.

Protein determination and enzyme assays

Routinely, the protein content of a sample was determined according to the method described by Lowry et al. [18], following precipitation with 10% trichloroacetic acid. The proteins remaining on filters during the transport experiments and phlorizin binding assay were determined by a modified dye-binding method (unpublished data) initially reported by Neuhoff et al. [19], using the dye Hoe-2485 (Hoechst Chemical Co., F.R.G.). Irradiation had no effect on the amount of membranes remaining on the filters. Activities of alkaline phosphatase (EC 3.1.3.1), aminopeptidase M (EC 3.4.11.2), and γ-glutamyltransferase (EC 3.2.2.2) were measured, as described previously [7].

Calculation of the target molecular weight

A simple form of the radiation target theory predicts that the inactivation of the biological activity of a protein by direct action of radiation would obey the relationship $E = E_o\, e^{KD}$, where E_0 and E express the biological activities before and after irradiation at a given dose, D. K is a constant for a single hit of target and corresponds to $1/D_{37}$, where D_{37} is the dose required for inactivation of the system to 37% of its initial activity. For calculation of the target molecular weight, M_r, the empirical equation of Kempner and Macey [20], $M_r = 6.4 \cdot 10^5/D_{37}$ (in Mrad), was employed. For calculating the target molecular weight of the sodium-dependent D-glucose transport protein, the apparent sodium-dependent exchange rate obtained in the tracer exchange experiments was used. It represents the difference between the uptake rate observed in the presence of sodium and the uptake rate observed in the presence of potassium. The exchange rates were obtained from the slopes of the regression lines calculated after plotting ($\ln C_\infty - C_t$) versus time. C_∞ represents the uptake after 90 min and C_t the uptake after 0.25, 1 or 2 min, respectively. The coefficients for linear regression, r^2, were at least 0.95. For calculating the target molecular weight of the sodium-dependent phlorizin binding protein, the number of the sodium-dependent phlorizin binding sites was estimated in Scatchard plots. In each case, results of seven independent radiation runs were used for the calculation and the inactivation data were analyzed by using a linear regression program. The target size of the protein was obtained by multiplying the estimated D_{37} value by a temperature correction factor of 1.56 [21,22]. This was necessary, because the radiation doses were measured at room temperature (25 − 30 °C), rather than at the sample irradiation temperature (−50 °C).

Results

Target size determination of the sodium-dependent phlorizin binding

The brush-border membrane is known to contain at least two binding sites for phlorizin, a low-affinity–high-capacity site, and a high-affinity but low-capacity site [1]. Fig. 1 illustrates the time-course of phlorizin binding to membranes in the presence of 0.2 μM phorizin and 100 mM sodium, before and after irradiation. Irrespective of the radiation dose, similar time-intervals of about 10 min are required for reaching binding equilibrium. In the same figure it is also evident that the equilibrium binding decreases progressively with an increasing radiation dose. As shown in Table I, irradiation induced a decrease only in the sodium-dependent phlorizin binding, but not in the sodium-independent binding.

When the high- and low-affinity phlorizin binding sites were distinguished in a Scatchard plot [1] (Fig. 2), it became obvious that the number of the phlorizin binding sites decreased with increased radiation doses, whereas the affinity of the binding sites for phlorizin remained unchanged (Table II).

When the number of sodium-sensitive phlorizin binding sites was plotted versus the irradiation dose in a semilog arithmetic plot (Fig. 3), a linear relationship was obtained, indicating the existence of a sole sodium-dependent phlorizing binding component in the membrane. The target size of this binding component was estimated to be 230 ± 38 kDa.

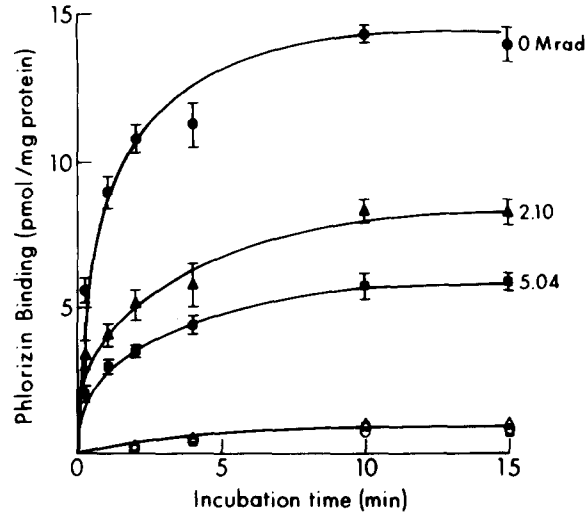


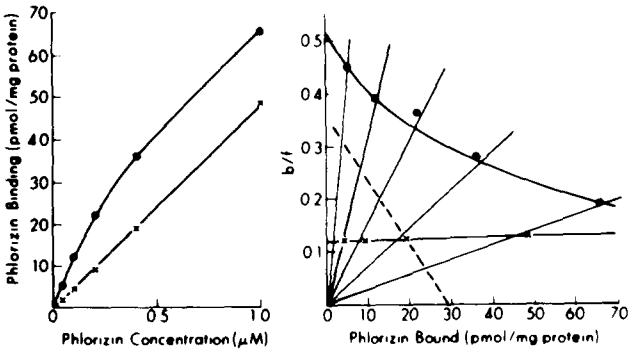
Fig. 1. Time course of phlorizin binding to irradiated and nonirradiated calf renal brush-border membranes. Aliquots of membrane vesicles in Hepes-Tris buffer (pH 7.4) were transferred into a medium containing 100 mM NaCl (closed symbols) or KCl (open symbols), plus 0.2 μM phlorizin with a trace amount of [^{3}H]phlorizin. The amount of phlorizin bound to the membranes was determined at 25 °C after various incubation times.

TABLE I

EFFECT OF IRRADIATION ON THE PHLORIZIN BINDING TO CALF RENAL BRUSH-BORDER MEMBRANES

Membranes irradiated with a given radiation dose were incubated with [^{3}H]phlorizin in Hepes-Tris buffer (20 mM, pH 7.4) containing 100 mM NaCl or KCl for 30 min at 25 °C, and phlorizin bound was measured as described in Methods and Materials. Data in column A represent the sodium-dependent phlorizin binding in pmol/mg protein, calculated as the difference in the amount phlorizin bound in the presence of sodium and that in the presence of potassium. Data in column B represent the sodium-independent phlorizin binding, determined from the amount of phlorizin bound in the presence of potassium. Data given are average values of triplicate determinations in a representative radiation-inactivation experiment.

[Phlorizin] (μM)	Radiation dose (Mrad)							
	0		1.6		4.0		20	
	A	B	A	B	A	B	A	B
0.04	3.2	2.0	2.1	2.4	1.4	2.3	0.6	1.9
0.1	7.3	4.4	3.9	4.9	3.4	4.7	1.0	4.5
0.2	13.1	9.2	8.3	10.5	6.7	9.0	1.3	8.6
0.4	17.6	18.7	10.5	20.4	10.3	18.1	2.7	17.5
1.0	17.7	48.1	12.6	49.0	10.0	48.5	2.6	44.4



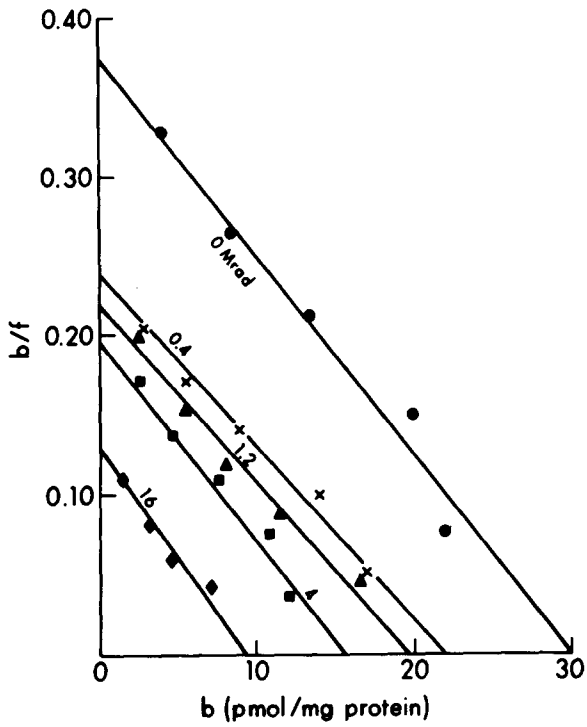


Fig. 2. Effect of irradiation on sodium-dependent and sodium-independent phlorizin binding in calf renal brush-border membranes. The calculation of sodium-dependent phlorizin binding is depicted in the upper two panels, using the following. symbols: ●, phlorizin binding in the presence of Na^+; ×, phlorizin binding in the presence of K^+; ------, sodium-dependent phlorizin binding.

Target size determination of the sodium-dependent D-glucose transport

The effect of low-dose radiation (0–4 Mrad) on the D-glucose exchange in the presence of sodium or potassium is summarized in Fig. 4. Sodium-dependent D-glucose exchange decreased with increasing radiation dose, whereas uptake in the presence of potassium was virtually unaltered. Semilog plots of the sodium-dependent exchange rates in irradiated membranes of seven independent radiation experiments revealed a straight line with a slope $m = -1.79$. From the D_{37} value a target size of 345 ± 24 kDa was estimated.

TABLE II

PROPERTIES OF THE SODIUM-DEPENDENT PHLORIZIN BINDING SITES IN IRRADIATED CALF RENAL BRUSH-BORDER MEMBRANES

The number of sodium-dependent phlorizin binding sites, n, and the dissociation constants, K_d, were calculated from the Scatchard plots, as shown in Fig. 2.

Dose (Mrad)	n(pmol/mg protein)	K_d (μM)
0	30	0.76
0.4	21.5	0.81
1.2	19.5	0.87
4	16.0	0.77
16	9.3	0.65

Target size determination of brush-border membrane enzymes

In order to establish the validity of the radiation-inactivation method for the determination of

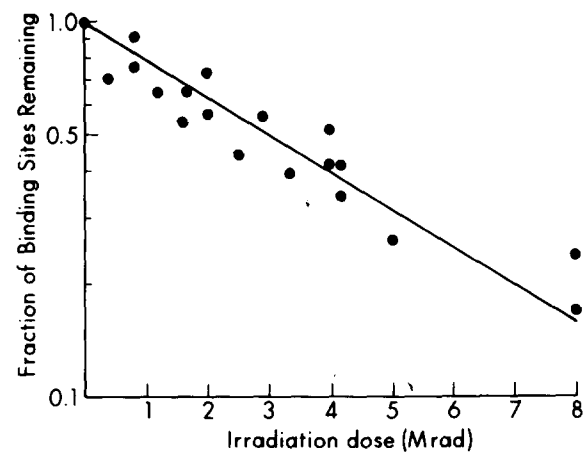


Fig. 3. Determination of the target size of the sodium-dependent phlorizin binding unit in calf renal brush-border membranes. The number of sodium-dependent phlorizin binding sites in the membranes is plotted against the radiation dose. Each point indicates an average of three determinations.

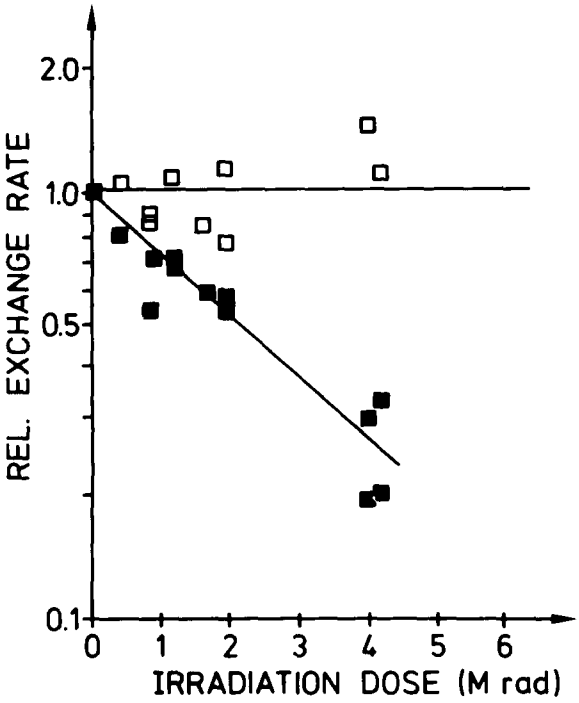


Fig. 4. Effect of irradiation on D-glucose transport in calf renal brush-border membranes. The normalized exchange rate is plotted against the irradiation dose. Closed squares indicate the sodium-dependent exchange rate, open squares the sodium-independent uptake. Each point represents an average calculated from duplicate determinations. The corresponding exchange rates in control membranes were: sodium-dependent, 0.35/min; sodium-independent, 0.1/min.

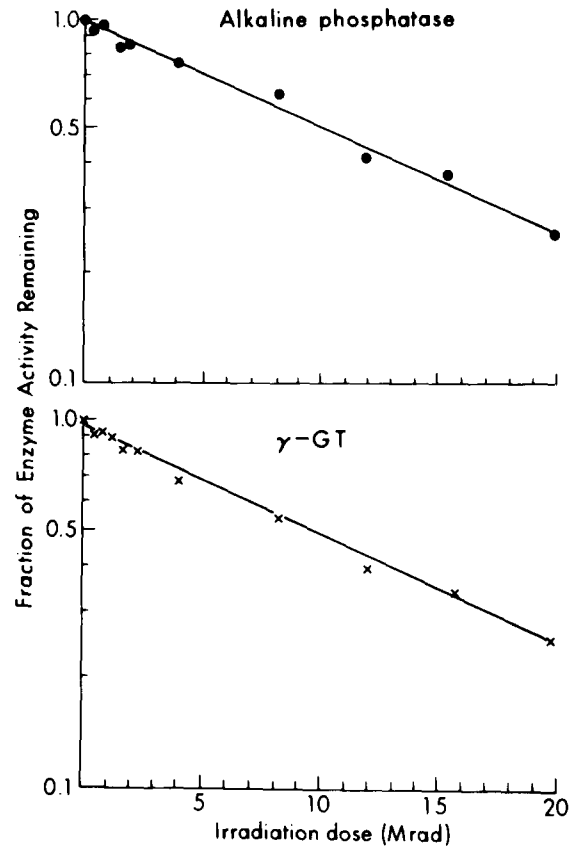


Fig. 5. Effect of irradiation on enzyme activities in calf renal brush-border membranes. The specific activities of alkaline phosphatase (upper part) and γ-glutamyltransferase (lower part) were determined in samples subjected to various doses of radiation. Data are from membranes in four independent radiation experiments. r^2 of the regression line is 0.998 for alkaline phosphatase and 0.996 for γ-glutamyltransferase.

molecular weights of brush-border membrane components, the inactivation of alkaline phosphatase, an intrinsic brush-border membrane protein, and of γ-glutamyltranspeptidase, an extrinsic membrane protein, was investigated. The results compiled in Fig. 5 show that the inactivation of both enzymes increases exponentially with the dose of irradiation, yielding a molecular weight of 68 570 ± 2670 ($n = 4$) for alkaline phosphatase and 73 500 ± 2270 ($n = 4$) for γ-glutamyltranspeptidase. For isolated alkaline phosphatase and γ-glutamyltranspeptidase similar values have been reported in the literature [23], demonstrating that radiation inactivation yields reliable values on the molecular weight of brush-border enzymes embedded in the membrane. A similar conclusion has also been reached by Turner and Kempner for alkaline phosphatase and aminopeptidase in rabbit brush border [11].

Discussion

In the present study, using the technique of radiation inactivation, we have observed two distinct target sizes for the D-glucose transport function and for the phlorizin binding activity in the brush-border membrane isolated from calf kidney. The target size of the sodium-dependent phlorizin binding unit was found to be 230 ± 38 kDa, which is significantly less than the target size of 345 ± 24 kDa observed for the sodium-dependent D-glucose transport function. This difference was also observed when the target size of the phlorizin binding component and the sodium-dependent trans-

port component were determined in parallel, using the same membranes ($0.001 < P < 0.01$). This indicates that the difference is real and not caused by inherent differences of the brush-border preparations or slight variations in the technical procedure of membrane irradiation.

With respect to the technical aspects of the experiments, the results on phlorizin binding can be considered as very reliable because the scatter is low and a decrease in the number of binding sites, but not in affinity, has clearly been demonstrated in the irradiated samples. The amount of protein remaining on the filters in the binding assay was not different before and after irradiation of membranes, indicating that the observed reduction in the number of binding sites is not due to any nonspecific loss of membrane protein, but due to inactivation of the binding sites. A similar conclusion can be drawn from the fact that sodium-independent phlorizin binding to the membranes remained constant.

With respect to the transport studies, several assumptions have been made in interpreting the data. First, since the transport studies were performed in the absence of salt gradients as tracer exchange experiments, the activity of the transport system would be independent of any change in ion permeability of the membrane that could affect the driving forces in transport experiments if performed under gradient conditions. Second, the uptake values were corrected for the decrease in the intravesicular space, which we assume to reflect a decrease in the number of vesicles rather than a decrease in the size of the vesicles. The latter would result in an increased surface/volume ratio and tend to increase the rate of uptake, quite opposite to the results obtained in this study. We also considered the possibility that irradiation increases the leakiness of the membrane, as shown for rabbit brush borders [11]. By simultaneously measuring D-glucose uptake in potassium- and sodium-containing media, we could show that, at least in the range of irradiation investigated, D-glucose uptake in the absence of sodium did not change in a consistent manner, whereas there was a clear-cut decrease both in total D-glucose uptake in the presence of sodium and in the sodium-dependent uptake (i.e., after correction for the uptake in the presence of potassium).

We also assume that the decrease in rate of sodium-dependent glucose transport is most probably due to a decrease in the number of transport sites, rather than due to a change in affinity. This conclusion is based on the finding that, in the range of radiation doses in which the decrease in exchange rate is observed, no change in the affinity for phlorizin binding to the protein is found. Since D-glucose inhibits phlorizin binding competitively, this would indicate that the affinity of the transport system for D-glucose is also unchanged. Furthermore, in isolated membranes, the phlorizin binding affinity is sodium-dependent [1]. Thus, the lack of radiation effect on phlorizin binding affinity would also suggest an unaltered sodium affinity of the transport system.

The experiments presented above extend those of Turner and Kempner [11] on rabbit brush borders in two aspects. One, with low doses of radiation similar to those we used for our flux studies, they found about 50% of the phlorizin binding sites were inactivated, suggesting a molecular weight of more than 1 000 000. In our studies, about 65% of the phlorizin binding sites were inactivated in the range between 0 and 4 Mrad, exhibiting a molecular weight of about 230 000. It is interesting to note that this value is about twice as high as that found by Turner and Kempner using higher radiation doses. Thus, it might be possible that the phlorizin binding sites exist in the membrane in a monomeric and in a dimeric form. If one assumes that even higher aggregates are possible, the occurrence of clusters could explain the high radiation sensitivity of half of the binding sites in the rabbit brush border.

With regard to the molecular weight obtained for the translocating unit, the value is again twice as high as the highest value of 165 000, reported by Malathi and Preiser for rabbit brush borders [9]. Again, it might be possible that we are dealing with a dimer for the functional transport unit. Therefore, we would tentatively arrive at the same picture for the translocator as Turner and Kempner, namely, that an additional protein is required to achieve glucose translocation as compared to phlorizin binding.

It is interesting to raise the question as to whether the hypothetical dimer exists indeed in the brush border membrane at the normal tempera-

ture of 37 °C or whether it is a result of analyzing the membrane at −50 °C. If the latter were the case, association and dissociation equilibria would be expected in the intact membrane. Such dissociation processes could occur not only between the various functional units but also between the different subunits, and thus a whole variety of different arrays is conceivable. Further experiments, especially involving crosslinking reactions or monoclonal antibodies, might be helpful in supporting the conclusions reached above.

References

1 Kinne, R. (1976) in Current Topics in Membrane Transport (Bronner, F. and Kleinzeller, A., eds.), Vol. 8, pp. 209–267, Academic Ress, New York
2 Thomas, L. (1973) Biochim. Biophys. Acta 291, 454–464
3 Smith, M.W., Ferguson, D.S. and Burton, K.A. (1975) Biochem. J. 147, 617–619
4 Lemaire, J. and Maestracci, D. (1978) Can. J. Physiol. Pharmacol. 56, 760–770
5 Koepsell, H., Menuhr, H., Ducis, I. and Wissmüller, T.F. (1983) J. Biol. Chem. 258, 1888–1894
6 Klip, A., Grinstein, S. and Semenza, G. (1979) J. Membrane Biol. 51, 47–73
7 Lin, J.T., DaCruz, M.E.M., Riedel, S. and Kinne, R. (1981) Biochim. Biophys. Acta 640, 43–54
8 Malathi, P., Preiser, H. and Crane, R.K. (1980) Ann. N.Y. Acad. Sci. 358, 253–266
9 Malathi, P. and Preiser, H. (1983) Biochim. Biophys. Acta 735, 314–324
10 Schmidt, U.M., Eddy, B., Fraser, C.M., Venter, J.C. and Semenza, G. (1983) FEBS Lett. 161, 279–283
11 Turner, R.J. and Kempner, E.S. (1982) J. Biol. Chem. 257, 10794–10797
12 Lin, J.T. and Hahn, K.-D. (1983) Anal. Biochem. 129, 337–344
13 Vannier, C., Louvard, D., Maroux, S. and Desnuelle, P. (1976) Biochim. Biophys. Acta 455, 185–199
14 Riedel, S. (1980) Dissertation, Rhein. Westf. Technische Hochschule, Aachen
15 Jung, C.Y., Hsu, T.L., Hah, J.S., Cha, C. and Haas, M.N. (1980) J. Biol. Chem. 255, 361–364
16 Hopfer, U., Nelson, K., Perrotto, J. and Isselbacher, K.J. (1973) J. Biol. Chem. 748, 25–32
17 Hopfer, U. (1977) J. Supramol. Struct. 7, 1–13
18 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
19 Neuhoff, V., Philipp, K., Zimmer, H.-G. and Mesecke, S. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 1657–1670
20 Kempner, G.R. and Macey, R.I. (1968) Biochim. Biophys. Acta 163, 188–203
21 Kempner, G.S. and Haigler, H.T. (1982) J. Biol. Chem. 257, 13297–13299
22 Jung, C.Y. (1983) in Membrane Receptor Purification and Characterization Techniques (Venter, J.C. and Harrison, L.C., eds.), Alan R. Liss Inc., New York, in the press
23 Kenny, J.A. and Maroux, S. (1982) Physiol. Rev. 62(1), 91–127