The high water permeability of kidney proximal tubules is of paramount importance for isotonic reabsorption of 70% of the glomerular filtrate, and water channel proteins have been postulated to account for the high water permeability. Target analysis following radiation inactivation was used to probe the molecular size of the water channel. Samples of brush border membranes from rat renal cortex were subjected to 3-MeV electron pulses from the Van de Graaff accelerator at a temperature of -130 °C. The inactivation of the renal brush border enzymes, alkaline phosphatase, and maltase was used for internal standardization of accumulated dose measurements in target analysis of the water channel. Osmotic water permeability was measured by following the change in scattered light intensity upon rapid mixing of vesicles with a hypertonic solution using stopped-flow spectrophotometry. The vesicle shrinkage response was biphasic and the rate of the fast phase decreased dose dependently by irradiation corresponding to a target size of 30 ± 3.5 kDa. The total change in scattered light intensity was unaltered, indicating that irradiation did not destroy the lipid barrier. Our results provide strong support for the hypothesis that the high osmotic water permeability of renal proximal tubules results from a water channel-specific protein with a functional unit of 30 kDa.

The water channel of renal proximal tubules, red blood cells, and vasopressin-stimulated epithelia of collecting ducts and amphibian urinary bladders constitutes the predominant route utilized by water in crossing the plasma membrane (1-3). In brush border and basolateral plasma membranes of proximal tubular epithelial cells, osmotic water permeability coefficients are among the highest reported for biomembranes (4-7). A specific water channel has been proposed to account for these high values and to discriminate against water movement across the lipid bilayer. Channel-mediated water transport is characterized by osmotic-to-diffusional water permeability ratios greater than unity and reduction in water permeability by mercurial sulphydryl reagents of one order of magnitude (4-7). In addition, dimethyl sulfoxide has been recently reported to affect channel-mediated osmotic water transport (7). Inhibition is accompanied by increased activation energies from 3-4 to 12-14 kcal/mol, leaving water transport through the lipid bilayer as the sole pathway.

Osmotic water permeability is measured in isolated renal brush border membrane vesicles using stopped-flow light scattering spectrophotometry. Rapid mixing of these membrane vesicles with hypertonic solutions causes osmotic shrinkage, which occurs at two distinct rates, a fast component comprising 70% of the signal amplitude, and a slow component (7). The fast component is ascribed to channels-mediated water transport and the slow one to lipid-mediated water transport, representing vesicles lacking water channels (6, 7). The separation is best observed at low temperatures (7), due to differences in activation energies of the two pathways.

Molecular identification of a water channel in biological membranes is a difficult task. No patch-clamp equivalents are available and ligand-protection methods are not feasible due to the ubiquitous nature of water. At present, it is still unknown whether water channels are proteins or specific lipid structures. Target analysis (8, 9) following radiation inactivation is an approach to measure the molecular size of a particular functional unit in intact membranes. The method is based on the probability of survival of biological activity in those cases where it decreases as a single exponential function of the radiation dose and inactivation of the functional unit. That the function of a single unit is either destroyed or fully intact, is a fundamental premise in target analysis, and when fulfilled, a plot of the ln(fractional activity) versus dose yields a straight line with a slope proportional to molecular weight. We report now that osmotic water flow via the water channel of proximal tubules was inhibited dose dependently by irradiation with a functional unit of 30 kDa, while osmotic flow via the lipid barrier remained unaltered.

**EXPERIMENTAL PROCEDURES**

*Radiation Inactivation*—Isolated brush border membrane vesicles, prepared as previously described (7), were concentrated to 25 mg of protein per ml and frozen in sealed ampules. The samples were subjected to 3-MeV electron pulses from the Van de Graaff accelerator in a cryostat at a temperature of -130 ± 10 °C of the Interfaculty Reactor Institute of the Technical University Delft. Pulses of 250-nsec duration with a peak current of ±2 A and a frequency of 10 Hz gave a dose rate of 850 Gv/s as determined from the ferrous-ferric sulphate Fricke dosimeter (10). Before thawing the irradiated samples, the ampules were opened and the produced ozone was blown off. The thawed samples were diluted 5-fold and aliquots were assayed for alkaline phosphatase (11) and maltase (12). Glucose/Na+ cotransport measurements were performed according to Stevens et al. (13), with minor alterations, such as 2-s uptakes of 3-n-[3H]glucose at 37 °C instead of 10 s at 20 °C and 100 mM NaSCN as initial gradient.

*Osmotic Water Permeability*—Osmotically induced changes in vesicular volume are followed by measuring changes in scattered light intensity with stopped-flow spectrophotometry as described previously (7). One-to-one mixing of vesicles (0.25 mg of protein per ml, 16633
RESULTS AND DISCUSSION

To verify the relationship between molecular weight and the slope from the ln(fractional activity) versus dose plot (Fig. 1) in this study, the target size of a known renal brush border enzyme, alkaline phosphatase (11), was determined. Measurement of survival after irradiation yielded linear ln(fractional activity) versus radiation dose plots. The absolute value of the slopes of these plots, in five different experiments, varied from $K = 0.0046$ to $0.0027$ kGy$^{-1}$, corresponding to a target size of $70 \pm 20$ kDa, in agreement with data from the literature (8, 14). Measurement of the target size of the membrane protein maltase (12) yielded 46 kDa with again a large variation of $\pm 20$ kDa ($n = 3$). The similarity, however, in the ratio of the slopes of the inactivation plot of maltase to alkaline phosphatase in each individual experiment (0.68 $\pm$ 0.08), prompted a normalization of the slope of alkaline phosphatase inactivation plots (Fig. 1). The normalization was used for internal standardization of accumulated dose measurements in target analysis of the water transport function.

Osmotic water permeability was monitored by following the change in vesicular volume via changes in scattered light intensity upon rapid mixing of vesicles with a 500 mM hypertonic sucrose solution using stopped-flow light scattering spectroscopy as described previously (7). Fig. 2 summarizes the radiation inactivation of the fast component, ascribed to channel-mediated osmotic water flow (7) (traces marked with a), and shows the sensitivity of the fast component to dimethyl sulfoxide (traces b) and mercuric chloride (traces c). The rate of the fast component decreased with increasing radiation dose. Radiation did not disturb the vesicles in their function as osmometers, since the amplitude of the signals remained virtually unaffected (Table I, Fig. 2). These results indicate that direct radiation damage on the water transport function.

1 The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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**Fig. 1.** Target size for renal brush border membrane alkaline phosphatase. Five different rat renal brush border membrane preparations (open and closed symbols of squares, circles, and triangles) and one pig kidney brush border membrane preparation (cross symbols) were subjected to radiation inactivation. Each preparation gave a different slope of the ln(fractional alkaline phosphatase activity) versus dose plot. The differences between slopes may be due to variation of temperature ($\pm 10^\circ C$) and scavenging properties in each preparation. According to the consideration in the text, the average slope $K = 0.0036$ kGy$^{-1}$ was used, which gave $r = 0.99$ and $M = 70$ kDa, so that the formula, $M = cKS$, $70$-kDa alkaline phosphatase, is obeyed, using $\ln(A/A_0)/KD$, where $c = 6400$ kGy kDa; $S = 2.98$; and $D$ represents the accumulated dose (8, 9).

in 50 mM mannitol, 2 mM MgCl$_2$, and 5 mM Hepes/Tris, pH 7.4) and test solute (500 mM sucrose, 2 mM MgCl$_2$, and 5 mM Hepes/Tris, pH 7.4) at 4°C produced a hypertonic gradient. Shrinkage of vesicles was recorded at different sampling rates and traces result from a summation of three runs under identical conditions (7). The traces were fitted with one or two exponential functions. Rate constants of the exponential functions are linearly related to water permeability constants as previously demonstrated (4).

**Fig. 2.** Effect of radiation dose on the time course of osmotic shrinkage of rat renal brush border membranes, using stopped-flow light scattering spectroscopy. Two different time scales were used, the sampling rate was decreased after 200 ms (the break in the plots) and the increase in scattered light intensity was followed until completion, as described in detail previously (7). The three traces in each panel show inhibition by dimethyl sulfoxide (traces b) and HgCl$_2$ (traces c) compared to the control (traces a). Applied accumulated dose: A, 0 kGy; B, 750 kGy; C, 1360 kGy.
Kinetic parameters of a typical stopped-flow experiment

In the absence of inhibitors, traces were fitted to two exponentials as published before (7). At high radiation dose (>1100 kGy), the trace could only be fitted to one exponential function, since \( k_b \) and \( k_i \) are not well separated. \( S \) represents amplitudes of change in scattered light intensity (arbitrary units), \( k_i \) values are rate constants (s\(^{-1}\)), which are proportional to osmotic water permeability (4). Indices: \( f \), fast; \( s \), slow; \( dmso \), in the presence of dimethyl sulfoxide; \( Hg \), in the presence of HgCl\(_2\).

<table>
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<th>( k_i )</th>
<th>( S_a )</th>
<th>( k_a )</th>
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Fig. 3. Target size analysis for renal brush border membrane vesicle osmotic water permeability and glucose/Na\(^+\) cotransporter. Symbols of closed squares and circles and open squares represent three different rat renal preparations. Open circles depict a pig kidney preparation. The lower curve (open triangles) represents the inactivation of the glucose/Na\(^+\) cotransporter. Absolute value of the slope of the upper curve = 0.0015 kGy\(^{-1}\); \( r = 0.96 \) (\( M = 30 \) kDa); absolute value of the slope of the lower curve = 0.0146 kGy\(^{-1}\); \( r = 0.92 \) (\( M = 290 \) kDa). Rate constants derived from stopped-flow experiments, which are proportional to osmotic water permeability (4), have been used to construct the upper curve. Glucose/Na\(^+\) cotransport measurements were performed as described earlier. Accumulated dose was determined as described in the text and in Fig. 1.

The analysis of the water transport function has been performed in the past (14, 17), but in these studies, the maximal dose was less than 100 kGy, which is insufficient to destroy rapid osmotic water flow. The recent finding that total mRNAs from water channel-containing tissues, injected into Xenopus oocytes, induces increased osmotic water permeability, is still no proof that a specific protein, serving as a functional target size for the water channel is now reported for the first time. The 30-kDa size provides strong support for the nature of a water channel as specialized protein and excludes the possibility that lipid structures constitute the water channel in renal proximal tubules.

**REFERENCES**