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THE EFFECT OF PAPAIN UPON PROLINE AND SODIUM TRANSPORT OF RAT RENAL BRUSH-BORDER MEMBRANE VESICLES

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Treatment of renal brush-border membrane vesicles with papain resulted in the removal of the activity of maltase, gamma-glutamyl transpeptidase and leucine aminopeptidase by 85, 50 and 75%, respectively. Stripping of these membrane enzyme activities constituted about 2% of the total membrane proteins and resulted in a widespread diminution in the ability of a variety of amino acids and sugars to be taken up by the membrane vesicles which remained osmotically responsive. Kinetic analysis of the uptake of proline, which was shown previously to be transported by both sodium-dependent and sodium-independent systems, revealed that the V_{max} for the sodium-dependent system and K_m for the sodium-independent system were halved, but other parameters were not affected indicating that the papain treatment altered sodium-gradient-stimulated entry and the affinity of the sodium-gradient-independent system for proline. Experiments on sodium entry and efflux demonstrate a marked enhancement of flux, so that equilibration of the sodium gradient occurred about 5-times more rapidly than in untreated vesicles. This occurred without any change in the osmotic properties of the vesicle with regard to sodium or amino acid uptake. Studies of fluorescence polarization suggest that incubation with papain does not alter the lipid domains of the membrane.

Introduction

The microvillus membrane of the renal proximal tubule cell is a complex structure, the architecture of which is currently being unravelled. Many studies have shown the existence of intrinsic proteins with enzyme activity such as L-leucine aminopeptidase (EC 3.4.11.2), γ-glutamyltranspeptidase (EC 2.3.2.2), maltase (EC 3.2.1.20) and alkaline phosphatase (EC 3.1.3.1) [1] which, though transmembranous, exist largely on the outer surface of the membrane. One of the important techniques used to assess the molecular organization within the membrane takes advantage of the presence of these diverse enzymes and the ability to remove them differentially by treatment with trypsin and papain. Also present in the brush-border membrane are transport systems for a variety of substrates such as amino acids and sugars [2–9]. Although no specific protein has been identified with amino acid transport processes in renal brush-border membranes, the kinetic experiments performed with microvillus vesicles are consistent with the presence of saturable binding sites on the membrane surface.

Our own interest has focused on whether the agents that remove enzymes and proteins from the

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Abbreviations: DPH, diphenylhexatriene; Hepes, 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

brush-border membrane alter the ability of membrane vesicles to transport amino acids and other substrates and, if so, whether this is the result of stripping out or altering proteins associated with the transport process. In examining the effects of graded trypsin digestion on rat renal brush-border membrane vesicles which releases L-leucine aminopeptidase activity and small amounts of protein, we observed a significant decrease in the sodium-gradient-stimulated uptake of a number of amino acids and sugars [7]. There was a linear correlation between the enzyme activity removed from the membrane and the decrease in the initial rate of sodium-stimulated proline uptake. A kinetic analysis indicated that only the V_{max} of proline uptake by its high-affinity sodium stimulated system was affected, without alteration of the characteristics of its high-K_m, sodium-independent mechanism. In the present paper we have extended our studies to the alterations in transport resulting from papain treatment of the membrane vesicle which removes not only leucine aminopeptidase but also most of the maltase and about half the γ-glutamyltransferase activity. Papain treatment produces a decrease in proline uptake under sodium-gradient conditions, but does not diminish proline uptake under conditions of sodium equilibration, suggesting that the uptake mechanism is not primarily affected. Papain treatment also significantly accelerated the flux of $^{22}Na^+$ by the vesicles. These results lead us to conclude that papain affects the transport of low-concentration amino acids and sugars secondarily beause of the rapid dissipation of sodium gradients by treated vesicles.

Materials and Methods

Preparation of brush-border membrane. Adult male Sprague-Dawley rats weighing 250–300 g and fed ad libitum on Purina rat chow were killed by decapitation. Kidneys were removed, decapsulated and placed in 0.9% NaCl solution at 4°C. Rat cortical tissue was removed by using a Stadie-Riggs microtome. Brush-border membranes were isolated by using the $MgCl_2$-precipitating method of Booth and Kenny [10]. The increase in alkaline phosphatase activity in the brush-border fraction over that in the cortical homogenate was between 10- and 13-fold. The average alkaline phosphatase specific activity of the membrane preparation was between 12 000 to 16 000 Sigma units per mg membrane protein [11]. Each final membrane fraction to be treated was suspended in buffer A (0.25 M sucrose/0.01 M triethanolamine hydrochloride (pH 7.6)). Membrane protein concentration was adjusted to between 2.5 and 3.0 mg per ml of buffer before papain treatment. The protein concentration of the suspension containing sucrose was determined by the method of Bradford [12] using the Bio-Rad protein assay kit from Bio-Rad, Rockville Center, NY. For membranes suspended in non-sucrose buffer, the protein concentration of the membrane preparation was determined by the method of Lowry et al. [13].

Papain-solubilization of enzyme activities from isolated brush-border preparation. Papain treatment of the membrane vesicles was carried out using a procedure similar to that previously reported [14]. Papain (EC 3.4.22.2, twice crystallized and suspended in 0.05 M sodium acetate (pH 4.5)) was purchased from Sigma. Papain was activated with 2 M NaCN according to the procedure of Emmelot et al. [15]. Isolated brush-border preparations in buffer A were incubated with 0.15 mg activated papain per ml of membrane preparation (or 1.01 Sigma unit papain per mg of membrane protein) at 37°C for 10 min. To a control sample was added an equal volume of buffer A. After incubation, the isolated brush-border fractions were centrifuged at 35 000 × *g* for 20 min at 4°C. The enzyme activities and proteins which did not sediment after centrifugation at 35 000 × *g* were designated the solubilized fraction of the brush-border fraction. The membrane pellets were resuspended in buffer B (100 mM mannitol/2 mM Tris-Hepes (pH 7.4)) with 6-times the incubation volume and spun at 35 000 × *g* for 20 min. The supernatant was discarded. The washed pellets were resuspended in buffer B. Protein concentration and enzyme activities both in the solubilized fractions and in the membrane pellets resuspended in buffer B after treatment were determined and expressed as percentage of total (supernatant fraction plus pellet fraction) recovered. Where papain digestion was involved, protein concentration in the fractions was corrected for added protein by the use of control samples.

Enzyme assays. The five marker enzymes measured were alkaline phosphatase, L-leucine aminopeptidase, γ-glutamyl transpeptidase, γ-glutamyl hydrolase (EC 3.4.22.12) and α-D-glucosidase (maltase). Alkaline phosphatase determinations were performed according to the procedures described in Sigma technical bulletin [11]. L-Leucine aminopeptidase was assayed according to the method of Goldman et al. [16]. The γ-glutamyl transpeptidase component activities, γ-glutamyl transpeptidase and hydrolase, were assayed according to the methods of Glossman and Neville [17]. Maltase activity was determined according to the procedure of Dahlqvist [18]. None of the enzymes assayed was inactivated by papain in the course of these experiments.

Uptake of amino acids and sugars. The membrane vesicles resulting from treatment with papain were separated from the solubilized fraction and washed as described in the papain-solubilization section. The final concentration of the washed membrane protein after these manipulations was adjusted to 0.20–0.25 mg protein per ml of buffer B. The resulting vesicle suspension was alllowed to equilibrate at 22°C for at least 30 min before the uptake studies. The uptake of amino acids at 22°C was determined in the presence of an 88.5 mM inward sodium gradient by using filtration techniques as described by McNamara et al. [9]. Where uptake in the absence of Na^+ gradient was to be studied, membrane vesicles in buffer B were preincubated with 88.5 mM NaCl for at least 60 min. Results of ^{14}C-labelled amino acid and sugar uptake studies were calculated as uptake in excess of the diffusion component as measured simultaneously by the entrance of L-[1-^{3}H]glucose into the vesicles. This sugar is thought to enter the brush-border vesicles by diffusion [6,9].

To determine the effect of papain solubilization of the brush-border membrane on the uptake of various substrates, 0.5 ml of the prepared membrane vesicles in buffer B at 22°C was added at the starting time to a disposable 10 mm × 75 mm test-tube containing 0.2 μCi of ^{14}C-labelled substrate, 0.6 μCi of L-[1-^{3}H]glucose, 50 μmol of NaCl and unlabelled substrate to bring the incubation mixture to the desired final concentration of substrates. The final volume of the mixture was 0.565 ml. The samples were vortex-mixed the first 3 s of incubation. After a total incubation time ranging from 6 s to 60 min, the incubation mixture was transferred by pasteur pipette to a filter apparatus. Uptake was stopped by rapid filtration of the mixture through a Sartorius filter (SM113, 0.45 μm) which was washed once with 5 ml of ice-cold washing buffer C (154 mM NaCl/100 mM mannitol/2 mM Tris-Hepes (pH 7.4)). The filter was air-dried and counted in a Packard Tricarb scintillation spectrometer in 4 ml of diluted concentrol (Yorktown Research, Miami, FL). Uptake of the substrates was expressed as nmol substrate taken up by the vesicles containing 1 mg membrane protein during the designated time of incubation at 22°C.

To study the effect of osmolarity of the medium, membrane vesicles suspended in buffer containing various concentrations of sucrose were incubated with 50 μmol of NaCl at 22°C for at least 60 min. Uptake was initiated by adding 0.2 μCi of labelled substrate. The vesicle preparations used for transport studies were also assayed for the five marker enzyme activities mentioned previously in order to measure and monitor the degree of solubilization of these enzymes by the papain treatiment.

Uptake of sodium. Transport of $^{22}Na^+$ at 22°C was determined in the presence of a 88.5 mM inward sodium gradient using filtration techniques as described in the above section except that the washing buffer C was replaced by sodium-free buffer D (154 mM choline chloride/100 mM mannitol/2 mM Tris-Hepes (pH 7.4)). 0.5 ml of membrane vesicles in buffer B was added at the starting time to a disposable 10 mm × 75 mm test-tube containing 0.2 μCi of carrier-free ^{22}NaCl, 44.3 μmol of unlabelled NaCl and unlabelled amino acid to bring the incubation mixture to the desired final concentration of substrate in a volume of 0.565 ml. Uptake of the sodium was calculated as μmol per mg membrane protein during the designated incubation time ranging from 6 s to 120 min. After filtering and washing, the filter was air-dried and counted in a Packard Tricarb gamma counter. To study the effect of osmolarity of the medium upon the uptake of 88.5 mM $^{22}Na^+$, vesicles were equilibrated with increasing amounts of sucrose in buffer B for at least 60 min at 22°C. Uptake was initiated by adding 0.020 ml of mix containing 0.2 μCi of $^{22}Na^+$, unlabelled NaCl and

L-proline to the 0.545 ml of vesicle suspension, which resulted in 88.5 mM ^{22}NaCl and 0.018 mM L-proline in the final incubation mixture. The mixture was incubated at 22°C for the designated time period before filtering and washing.

Efflux measurement of L-proline and sodium. For efflux studies, vesicles were preloaded with L-proline or sodium by incubating for 30 min in buffer B containing 88.5 mM NaCl and 0.018 mM L-[^{14}C]proline, or 88.5 mM ^{22}NaCl and 0.018 mM unlabelled L-proline. Efflux was initiated by addition of 0.050 ml of the preloaded vesicles (containing 0.13 to 0.23 mg membrane proteins together with 0.2 μCi of [^{14}C] proline or $^{22}Na^+$ labels) to 1 ml of buffer D. The amount of substrate retained within the vesicles was determined by using the rapid filtration procedure as described above using buffer D as washing solution. Substrates retained were calculated as nmol of substrate retained within the vesicles containing 1 mg membrane protein during the designated incubation period. Data were expressed as the percentage of total amount of the same substrate retained by the control vesicles at zero-time incubation.

Fluorescence polarization studies. Membrane suspensions were labelled with the lipid-soluble fluorophore, 1,6-diphenyl-1,3,5-hexatriene (DPH), according to the procedure described by Cooper et al. [19]. Measurement of fluorescence polarization and fluorescene intensity were performed with a Perkin-Elmer fluorescence spectrophotometer model 650-10S together with the automatic polarizer accessory. Temperature was controlled and determined by temperature programmer model C570-0701. For DPH-labelled membranes, excitation wavelength was set at 360 nm, emission wavelength was 430 nm. Readings were taken by recording emission intensities parallel and perpendicular to the plane of excitation ($I_{\parallel}$ and $I_{\perp}$) with automatic shutter at 5 s cycling time to reduce photoisomerization.

DPH was kept as a stock solution in tetrahydrofuran at a concentration of 2 mM. Immediately before use, it was diluted 1:2000 in buffer B with vigorous mixing for 2 h at room temperature. 1 vol. of the diluted DPH dispersion was added to 1 vol of the membrane preparation suspended in buffer B containing 0.4–0.5 mg/ml of membrane preparation. The mixture was incubated at 37°C for 30 min.

$I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities oriented parallel and perpendicular to the direction of polarization of the exciting light [19]. The polarization of fluorescence was expressed as the fluorescence anisotropy, r, which is derived from the Perrin equation

$$r = [(I_{\parallel} - I_{\perp}) \cdot C]/[(I_{\parallel} + 2I_{\perp}) \cdot C]$$

Corrections for the intrinsic polarization of the exciting beam were applied [20]. The correction factor, C, equal to $I'_{\parallel}/I'_{\perp}$, the primes indicating excitation polarized in a parallel direction, was used to correct for the contribution due to scattering. Scatter corrections were applied to all measurements. The C values measured ranged from 0.90 to 0.96. The polarization of fluorescence was also expressed as the parameter $[(r_0/r) - 1]^{-1}$. r_0 is the upper limit of r. For DPH, $r_0 = 0.362$ [21]. This anisotropy parameter so obtained represents the harmonic mean of the effective viscosity opposing the rotational diffusion of the probe in all possible directions. For examination of the effects of temperature, preparations were cooled initially to 5°C and the fluorescence polarization was measured every 0.5 °C as the suspension warmed at a rate of 1 deg C/min to 45°C. Excited state lifetimes (τ) were estimated from the relative fluorescence intensity, F, at each temperature and from the upper limit of τ_0 of 11.4 ns [21]. F is derived from $((I_{\parallel} + 2I_{\perp}) \cdot C)$. τ was obtained from the formula

$$\tau = \frac{\tau_0 \cdot F}{F_{max}}$$

F_{max} was obtained by plotting F versus each corresponding temperature (°C) and extrapolating to 0°C. According to Cooper et al. [19], the τ values obtained corresponded well to the independent life-time measurements made with an Ortec photon-counting fluorescence lifetime instrument [21]. One form of the Perrin equation, on which the fluorescence polarization determination were based, is:

$$\frac{r_0}{r} = 1 + 3\tau/\rho$$

where τ is the lifetime of the excited state of the fluorophore and ρ is its rotational relaxation time. Hence at constant τ, the parameter $[r_0/r) - 1]^{-1}$ is

directly proportional to the rotational relaxation time and provides a quantitive index of the resistance of the environment to the rotational motion [22].

Materials. Carrier-free sodium-22, L-[U-^{14}C]proline, [U-^{14}C]glycine, L-[U-^{14}C]leucine, L-[U-^{14}C]glutamine, L-[U-^{14}C]lysine, D-[U-^{14}C]glucose, L[U-^{14}C]methionine, L-[1-^{14}C]glucose, L-[1-^{3}H]glucose were purchased from New England Nuclear Corp. α-[U-^{14}C]methyl-D-glucoside was obtained from Rosechem Products. 1,6-Diphenyl-1,3,5-hexatriene was purchased from Aldrich and tetrahydrofuran 'photorex' reagent was obtained from J.T. Baker. All other chemicals were of the highest purity available.

Data analysis. In studies of concentration dependence of uptake of the substrates by renal brush-border membrane vesicles, the data were plotted by the Hofstee method. Then the data were fitted by the least-squares method. Apparent K_m and V_{max} values were determined from each experiment studied. The significance of the differences of uptake between control and papain-treated vesicles were analyzed by using the formula for Student's *t*-test given by Goldstein [23]. The kinetic parameters were also calculated from Eqn. 1 to give the best fit to the observed total uptake using a direct-grid search method described by Becsey et al. [24]:

$$V = \frac{V_1[S]}{K_1 + [S]} + \frac{V_2[S]}{K_2 + [S]} + K_3 S \quad (1)$$

This method will determine all five of the above parameters (V_1, V_2, K_1, K_2, K_3) simultaneously from the V vs. S data pairs. It operates by choosing values of the non-linear parameter (K_1 and K_2) and then solving the resultant equation for the best values of the linear term (V_1, V_2 and K_3) using standard least-squares equations. K_1 and K_2 are then iterated until the best fit is obtained. The best fit is defined as the minimum squared error after weighing the individual datum point as in Eqn. 2:

$$W = \frac{1}{V \cdot S} \quad (2)$$

This weighing factor, W, reflects the expected statistical uncertainty of each datum point, given the nature of the experiment.

Results

Effect of papain treatment on the marker enzymes of isolated renal brush-border membrane preparation

Almost all the maltase, 85% of L-leucine

TABLE I

EFFECT OF PAPAIN TREATMENT ON THE PERCENTAGE RECOVERY OF PROTEIN AND MARKER ENZYMES IN THE SOLUBILIZED FRACTION (35000 × *g* SUPERNATANT) OF RENAL BRUSH-BORDER PREPARATIONS

The brush-border fraction was treated for 10 min at 37°C with 0.015% papain. Supernatant and pellets were separated as described in the text. The solubilization factor (SF) is the ratio of the percentage of enzyme solubilized to that of the protein solubilized. A ratio above 1 indicates the preferential release of enzymes relative to protein and vice-versa. Relative enzyme activity (REA) is calculated as the ratio of enzyme activity in the papain-treated brush-border fraction (sum of 35000 × *g* supernatant and pellets) to that in the same amount of untreated preparation. Each value of the percentage recovery is the mean ± S.E. for six experiments of quadruple determinations from each experiment.

Protein and enzymes	Control		Papain-treated		REA
	% of total	SF	% of total	SF	
Protein	8.86 ± 1.54	–	10.88 ± 1.13 [a]	–	–
Alkaline phosphatase	9.23 ± 1.39	1.04	12.98 ± 3.64 [a]	1.19	1.01 ± 0.11
L-Leucine aminopeptidase	10.82 ± 1.38	1.22	85.07 ± 5.61	7.82	1.15 ± 0.06
γ-Glutamyl transpeptidase	11.40 ± 2.67	1.29	60.03 ± 8.88	5.52	1.09 ± 0.10
γ-Glutamyl hydrolase	9.89 ± 1.29	1.12	60.93 ± 9.82	5.60	0.95 ± 0.08
Maltase	10.25 ± 4.36	1.16	96.19 ± 1.56	8.84	1.05 ± 0.04

[a] $P > 0.3$ for difference from control.

aminopeptidase and about 60% of γ-glutamyl transpeptidase and hydrolase activities remained in 35 000 × *g* supernatant when brush-border membranes were digested with papain under the conditions given in Table I. Results in the table also show that under this selected condition, alkaline phosphatase was inaccessible to papain digestion, with 3% or less being solubilized after treatment. The values of the solubilization factor, SF, suggest that the four marker enzymes other than the alkaline phosphatase comprise the 2% membrane being solubilized by the papain treatment. The values for the solubilization factors of these enzymes after papain treatment are all larger than 1. This suggests the preferential release of these surface enzymes relative to other proteins contained in the membrane matrix such as alkaline phosphatase and other non-enzymic proteins. All the relative enzyme activity values are around 1, indicating the total recovery of all the enzyme activities after papain treatment. None of the enzymes assayed was inactivated by papain in the course of these experiments.

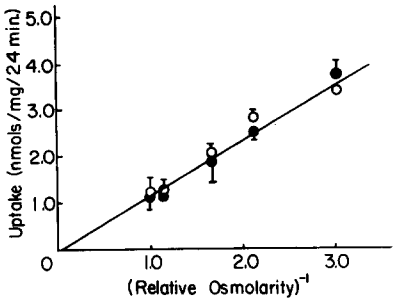

Fig. 1. Effect of medium osmolarity upon the uptake of L-[1-^{14}C]glucose by control and papain-treated vesicles. Brush-border vesicles were incubated at 37°C for 10 min in the absence or presence of 0.15 mg activated papain per ml of membrane preparation. Control (○) and papain-treated (●) vesicles were obtained as described in the text. The uptake was determined in vesicles equilibrated with 88.5 mM NaCl and increasing amounts of sucrose in the incubation medium. Values shown represent equilibrium values obtained after 24 min of incubation with 0.1 μCi L-[1-^{14}C]glucose at 22°C, and means ± S.E. for four determinations. Data points without brackets indicate that the S.E. is included within the point. The line was drawn by the least-squares method. The tonicity of the medium with respect to non-penetrating non-electrolytes (sucrose and mannitol) is expressed as relative osmolarity where 1 = isosmolar = 300 mosM and 0.33 = 100 mosM.

Effect of papain on the response of the membrane vesicles to osmotic perturbations and upon the friability of the membrane vesicles

Before examining the relationship between the surface membrane enzyme removal and the transport characteristics of the amino acids and sodium by the treated membrane vesicles, it is important to clarify whether the uptake of substrate by papain-treated vesicles represents transport into an intravesicular space. The relation of intravesicular volume to steady-state equilibration of substrates was examined. As shown in Fig. 1, steady-state accumulation of L-[1-^{14}C]glucose by control and papain-treated vesicles was found to be inversely proportional to medium osmolarity and thus directly related to intravesicular space. Extrapolation to infinite medium osmolarity, that is, zero intravesicular volume, resulted in no uptake, also indicating no binding to the membrane. No difference is apparent in the osmotic reactivity of the papain-treated vesicles and control vesicles which were also incubated at 37°C and washed in parallel with the papain-treated vesicles.

The amount of alkaline phosphatase retained on the 0.45 μm filter was used as a measure of the stability of the membrane matrix. Results have shown that a total of 1.94% of the alkaline phosphatase activity passed through the filter when papain-treated vesicles were filtered as compared to 0.25% of alkaline phosphatase activity of the control vesicles. Thus, papain treatment did not greatly affect or alter the friability of the membrane vesicles.

The uptake of amino acids and sugars by papain-treated vesicles

A survey of the uptake of various amino acids and sugars at low concentrations is shown in Table II. The data reveal that after papain treatment there is diminished uptake of a variety of amino acids and sugars. Because of the large amount of data on proline uptake [9,25,26], further study of the effect of papain on the vesicle transport systems and its relationship to the removal of marker enzymes was carried out.

Effect of papain upon the time-courses of L-proline uptake by the membrane vesicles

The removal of surface membrane enzymes as

TABLE II

EFFECT OF PAPAIN DIGESTION UPON THE UPTAKE OF VARIOUS 0.02 mM AMINO ACIDS AND SUGARS BY THE MEMBRANE VESICLES

Uptake of the amino acids and sugars was measured at the end of 0.5 min incubation at 22°C. The number of determinations are indicated in the parentheses after the mean value ± S.E.

% of rate of uptake of control vesicles		
^{14}C-labeled substrates	Control	Papain-treated
L-Proline	100 ± 2.08 (7)	37.36 ± 0.39 (8)
L-Leucine	100 ± 3.05 (10)	51.62 ± 2.49 (11)
L-Glycine	100 ± 2.52 (9)	64.46 ± 4.13 (9)
L-Methionine	100 ± 2.82 (12)	41.93 ± 2.20 (11)
L-Glutamine	100 ± 2.90 (12)	50.38 ± 1.62 (10)
L-Lysine	100 ± 1.68 (8)	52.63 ± 1.07 (7)
D-Glucose	100 ± 2.25 (16)	43.24 ± 2.47 (15)
α-Methyl-D-glucoside	100 ± 2.55 (14)	45.97 ± 2.01 (13)

the result of papain treatment exerts an inhibitor effect on the Na^+-gradient 'overshoot' phenomenon of the 0.018 mM L-proline uptake by brush-border vesicles. Fig. 2 shows the time-course of uptake of 0.018 mM L-[U-^{14}C]proline by control and papain-treated membrane vesicles in the presence of an Na^+ gradient. The uptake of 0.018 mM L-proline in the presence of 88.5 mM inward Na^+ gradient was decreased 40–54% by the papain-treated vesicles as compared to that of control vesicles over the first 3 min of incubation. Fig. 3 shows the time-course of uptake of 1.85 mM L-[U-^{14}C]proline by control and papain-treated vesicles under conditions similar to those described for Fig. 2. A slight 'overshoot' phenomenon is observed under the condition of Na^+ gradient. The small Na^+-gradient stimulation observed during the first minute of uptake is due to the 51.3% contribution from the low-K_m system (see below) which appears to be greatly stimulated by the 88.5 mM NaCl gradient. No significant difference in the uptake of 1.85 mM proline was observed between control and papain-treated

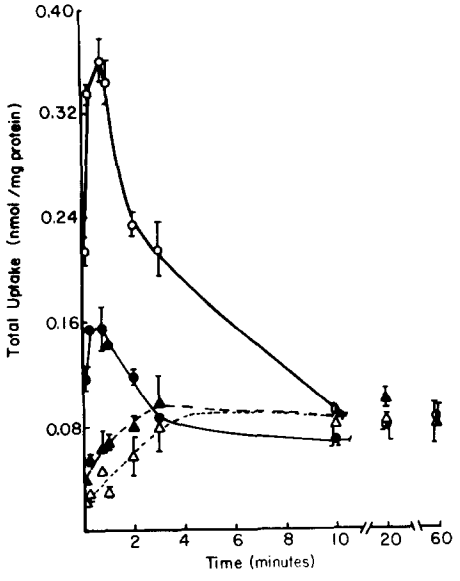

Fig. 2. Effect of papain upon the time-course of uptake of 0.018 mM L-proline under Na^+-gradient and Na^+-equilibrated conditions. Control (○, △) and papain-treated (●, ▲) vesicles were obtained as described in the text. The time-course of uptake was measured under the condition of 88.5 mM inward Na^+-gradient (○, ●) at 22°C as described in Materials and Methods. The time-course of uptake under the Na^+-equilibrated condition (△, ▲) was also measured. Values shown are the means ± S.E. for four determinations. Datum points without brackets indicate that the S.E. is included within the point.

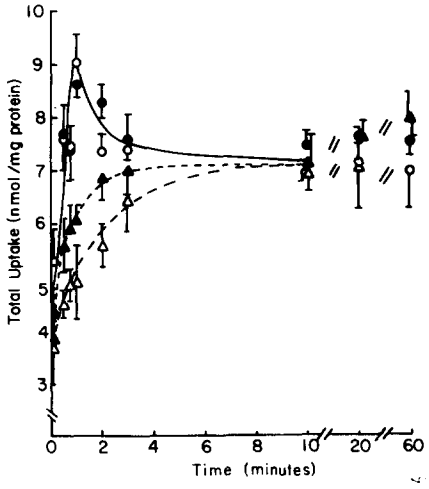

Fig. 3. Effect of papain upon the time-course of uptake of 1.85 mM L-proline under Na^+-gradient and Na^+-equilibrated conditions. The time-course of uptake by control (○, △) and papain-treated (●, ▲) vesicles was measured under the condition of 88.5 mM inward Na^+-gradient (○, ●) at 22°C in the presence of 1.85 mM L-proline medium concentration. The time course of uptake under the Na^+-equilibrated condition (△, ▲) was also measured. Values shown are the mean ± S.E. for eight determinations. Datum points without brackets indicate that the S.E. is included within the point.

vesicles under Na^+ gradient conditions. Also shown in Figs. 2 and 3 are the time-courses of uptake of L-[U-^{14}C]proline by control and papain-treated membrane vesicles under the condition of Na^+ equilibration across the vesicle membranes. There was slightly higher uptake of both 0.018 mM and 1.85 mM proline by papain-treated vesicles during the initial 2 min of incubation under Na^+-equilibrated conditions. However, at the end of 60 min of incubation, the same level of uptake had been reached by vesicles incubated under the conditions of Na^+ gradient and under the Na^+-equilibrated conditions.

Effect of papain treatment of vesicle membranes upon the kinetic parameters of proline transport systems

We have previously reported that the kinetics of proline transport by brush-border membrane indicate the presence of two saturable systems for uptake [9,25]. As shown in Fig. 4, the initial rate of uptake of proline by brush-border membrane vesicles at 22°C, after the membrane vesicles had been incubated at 37°C for 10 min in the presence of 0.015% papain, was still concentration-dependent and saturable. The Hofstee plot for the concentration dependence of proline uptake during the initial 15 s shows a two-limbed curve that indicates the presence of multiple transport systems for the vesicles without papain treatment. The transport parameters, determined from the Hofstee plot, where: K_{m1}, 0.10 ± 0.005 mM, V_{max1}, 3.35 ± 0.07 nmol/15 s per mg; K_{m2}, 1.34 mM ± 0.25 mM, V_{max2}, 9.36 ± 0.95 nmol/15 s per mg. The calculated values were: K_{m1}, 0.089 mM, V_{max1}, 2.56 nmol/15 s per mg; K_{m2}, 8.09 mM; V_{max2}, 12.91 nmol/15 s per mg and K_{m3}, 0 mM, which suggests that the diffusion component K_d was adequately measured by the entrance of L-[1-^{3}H]glucose into the vesicles as described in the uptake of amino acids and sugars of the Materials and Methods section. The overall standard deviation of the best-fit curve from the experimental data is S.D. = ±0.0187. The Hofstee plot values for proline uptake by papain-treated vesicles at 22°C were: K_{m1}, 0.11 ± 0.02 mM; V_{max1}, 2.36 ± 0.19 nmol/15 s per mg; K_{m2}, 1.56 ± 0.47 mM; V_{max2}, 8.26 ± 0.49 nmol/15 s per mg. The calculated values were K_{m1}, 0.074 mM, V_{max1} 1.16 nmol/15 s per mg; K_{m2}, 3.84 mM; V_{max2}, 10.23 nmol/15 s per mg; K_{m3}, 0 mM and the overall S.D. for this best fit is ±0.0083. The V_{max1} values for L-proline transport between the control and papain-treated vesicles are significantly different when compared by Student's t-test ($P < 0.001$). The K_{m1} values for L-proline transport by control and papain-treated vesicles are not statistically different ($P < 0.1$) Thus, the removal of 76% L-leucine aminopeptidase, 50% γ-glutamyl transpeptidase and 86% maltase, which comprised less than 2% of the total surface membrane protein, as the result of papain treatment of the membrane vesicles (Table I), appeared to lower the V_{max1} of proline entry on the low-K_m system by 55% when compared to that of control vesicles. The V_{max2} of proline entry on the high K_m system was lowered by 15% compared to that of control vesicles; however, the difference is not significant ($p < 0.8$). Papain treatment of the membrane vesicles does not seem to alter the substrate affinity in the low-K_m system, nor does it affect the diffusion component, K_{m3}. It appears that the K_{m2} value of proline is decreased as the result of papain treatment.

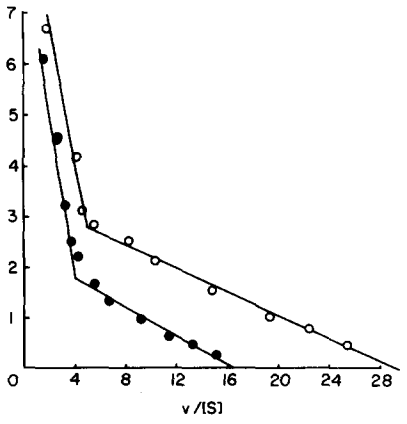

Fig. 4. Hofstee plots for the concentration-dependence of L-proline uptake by brush-border vesicles. Uptake of control (○) and papain-treated (●) vesicles after 15 s of incubation at 22°C with an 88.5 mM NaCl-inward gradient was measured over a range of 0.0184 mM to 3.913 mM L-proline. The velocity of uptake, V, was measured as nmol/mg protein per 15 s, and [S] is the proline concentration in the incubation medium. The lines represent the best fit for the data by using linear regression analysis. Each datum point represents the mean for 12 determinations.

Effect of papain upon the time-courses of efflux of L-proline from vesicles

The effect of papain treatment on the efflux of 0.018 mM L-proline from the vesicles is shown in Fig. 5. Both control and papain-treated vesicles demonstrated rapid efflux of L-proline from vesicles preloaded with this amino acid. After only 45 s, 77% and 74% of the vesicular L-proline was released fro control and papain-treated vesicles, respectively. There was essentially no difference in the efflux rate between control and papain-treated vesicles.

Effect of papain upon the relative contribution to total uptake by the low-K_m and high-K_m systems of proline

The relative contribution of each transport component of L-proline was obtained as nmol substrate per mg vesicle protein per 15 s from the calculated kinetic parameters for the two-component systems of L-proline transport and was expressed as percentage of the total uptake at the designated substrate concentrations for proline [8]. Fig. 6 shows that the papain treatment exerted an effect upon the relative contribution to total uptake by the two K_m systems of the membrane vesicles in the proline concentration range studied. Data show that the 50% contribution of low- or high-K_m system to total uptake of L-proline by papain-treated vesicles occurred at 0.42 mM L-proline medium concentration, while the 50% contribution of low- or high-K_m system to total L-proline uptake by control vesicles occurring at 1.77 mM L-proline medium concentration.

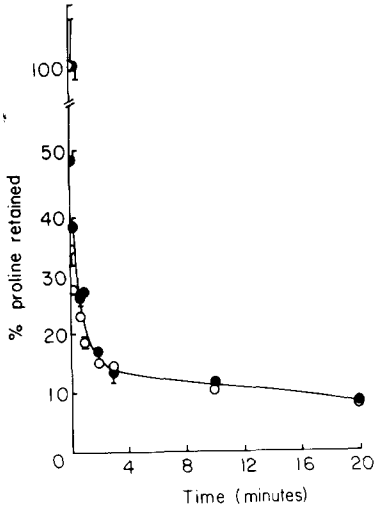

Fig. 5. Effect of papain upon the time-course of proline efflux from the brush-border membrane vesicles. Both the control (○) and papain-treated (●) vesicles were preloaded with 88.5 mM NaCl and 0.018 mM L-[^{14}C]proline at 22°C for 30 min. Efflux was initiated by addition of 0.050 ml of the preloaded vesicles (containing 0.13 to 0.23 mg membrane proteins together with 0.2 μCi of [^{14}C]proline) to 1 ml of buffer D. The results are expressed as the percentage of L-proline retained relative to the amount of L-proline found at zero incubation time without the 1:20 dilution with buffer D (100% retained). The average uptake of the L-proline by these vesicles were 1.02 ± 0.15 nmol/mg protein and 1.19 ± 0.04 nmol/mg protein for control and papain-treated vesicles, respectively. These values are designated as having a relative uptake of 100%. Each data point represents the mean ± S.E. for four determinations. Datum points without brackets indicate that the S.E. is included within the point.

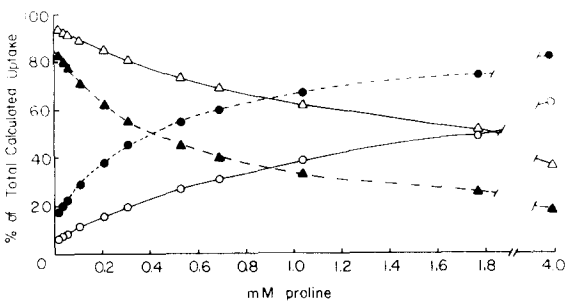

Fig. 6. The relative contribution to total uptake by the proline transport system of brush-border membrane vesicles. Control (△, ○) and papain-treated (▲, ●) membrane vesicles were obtained as described in the text. By using the calculated kinetic parameters for the low-K_m (△, ▲) and high-K_m (○, ●) systems for proline uptake, the relative contribution of each system was determined from Eqn. 1 in the text. Values shown are means for 12 determinations.

Effect of papain upon the time-course of $^{22}Na^+$ uptake and efflux by the membrane vesicles

Because papain treatment appeared to alter sodium-gradient-stimulated transport systems, the uptake of 88.5 mM $^{22}Na^+$ was determined, with the results shown in Fig. 7. The uptake of $^{22}Na^+$ appeared to be faster in vesicles exposed to papain, reaching equilibrium at 2 min compared to the control vesicles where equilibrium was reached at about 10 min (there was no statistical difference between the sodium content of papain-treated and control vesicles after 10 min). Thus papain ef-

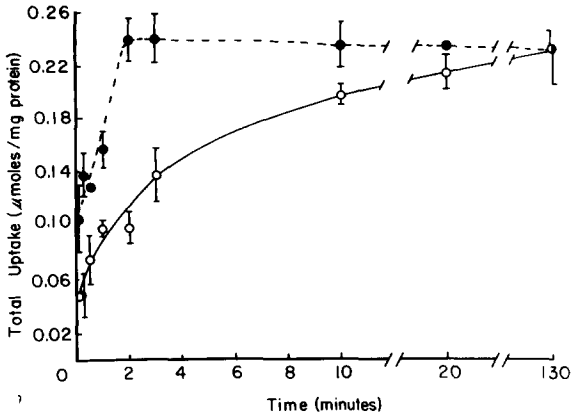

Fig. 7. Effect of papain upon the time-course of uptake of 88.5 mM ^{22}NaCl in the presence of 0.018 L-proline gradient. Time-course of uptake of ^{22}NaCl by control (○) and papain-treated (●) vesicles were measured under the condition of 0.018 mM inward L-proline gradient at 22°C as described in the text. Values shown are the mean ± S.E. for four determinations. Datum points without brackets indicate that the S.E. is included within the point.

fected a more rapid dissipation of the sodium gradient. The efflux of sodium was similarly changed as shown in Fig. 8, where the gradient for sodium was reversed, the vesicular sodium being

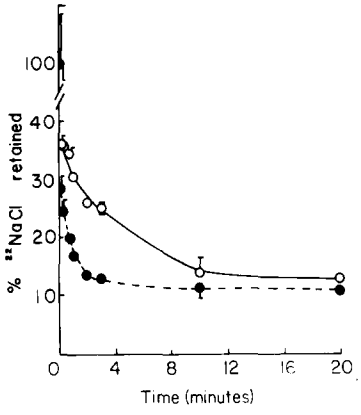

Fig. 8. Effect of papain upon the time-course of $^{22}Na^+$ efflux from brush-border membrane vesicles. Both the control (○) and papain-treated (●) vesicles were preloaded with 88.5 mM ^{22}NaCl and 0.018 mM L-proline. Efflux was initiated as described in the legend for Fig. 5. The average uptake of the $^{22}Na^+$ by these vesicles was 6.23 ± 1.09 μmol/mg protein and 7.78 ± 0.50 μmol/mg protein for control and papain-treated vesicles, respectively. The results were calculated and expressed as described in legend for Fig. 5 and in the text. Each datum point represents the mean ± S.E. for four determinations. Datum points without brackets indicate that the S.E. is included within the point.

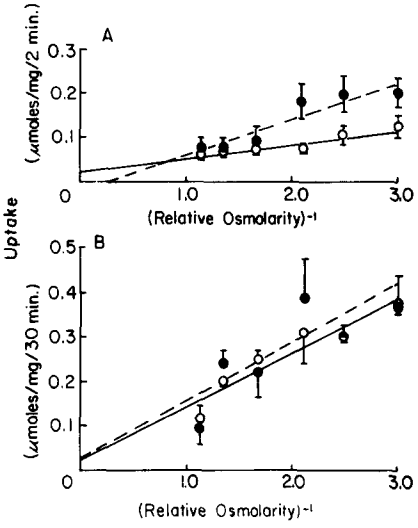

Fig. 9. Effect of osmolarity upon the uptake of 88.5 mM ^{22}NaCl by brush-border membrane vesicles. Vesicles were equilibrated with increasing amounts of sucrose in buffer B for at least 60 min at 22°C. Uptake measurement was initiated by adding to control (○) and papain-treated (●) vesicles a mixture of 0.2 μCi $^{22}Na^+$, unlabelled NaCl and L-proline as described in the text. The final incubation mixture contained 88.5 mM ^{22}NaCl and 0.018 mM L-proline. (A) represents uptake values after 2 min of incubation at 22°C. (B) represents uptake values after 30 min of incubation at 22°C. Each datum point represents the mean ± S.E. for eight determinations in (A) and four determinations in (B). Datum points without brackets indicate that the S.E. is included within the point. The line was fitted to datum points using linear regression analysis.

88.5 mM while the external sodium was 4.2 mM. Again, the sodium exited more rapidly from treated vesicles, reaching equilibrium at 2 min, while the control preparation reached an equilibrated state at 10 min paralleling the findings of the influx experiment. In studies of the effect of osmolarity, $^{22}Na^+$ uptake decreased linearly with increasing osmolarity, little uptake being indicated at infinite osmolarity by either papain-treated or control vesicles (Fig. 9). These data indicated that there was no binding component to the $^{22}Na^+$ uptake, either, at short incubation times (2 min) (Fig. 9A), or little binding (6%) at equilibrium (Fig. 9B).

Effect of papain upon the fluorescence polarization, excited-state lifetime and rotational relaxation-time studies of the vesicles

An effect by papain upon a variety of sub-

TABLE III

FLUORESCENCE POLARIZATION, EXCITED-STATE LIFETIME AND APPROXIMATE ROTATIONAL RELAXATION TIME STUDIES OF DIPHENYLHEXATRIENE IN CONTROL AND PAPAIN-TREATED BRUSH-BORDER MEMBRANES

Values are means ± S.E. from two preparations examined, except for those at 5°C, which were the results from one preparation.

Temperature (°C)	Membrane type	Anisotrophy parameter $[(r_0/r)-1]^{-1}$	Mean fluorescence anisotropy (r)	Excited-state life time τ (ns)	Approx. rotational relaxation time ρ (ns)
5	control	12.20	0.335	11.2	417
	papain-treated	9.97	0.329	11.0	330
10	control	8.24 ± 0.28	0.323	10.7	267
	papain-treated	7.51 ± 0.27	0.320	10.8	243
25	control	3.83 ± 0.03	0.288	10.2	118
	papain-treated	3.58 ± 0.12	0.283	10.0	107
37	control	2.25 ± 0.08	0.251	9.6	65
	papain-treated	2.12 ± 0.03	0.246	9.0	57
45	control	1.70 ± 0.06	0.228	9.5	48
	papain-treated	1.52 ± 0.08	0.218	8.6	39

strates transported by the vesicle was observed (Table II). This could indiate a general modification of membrane bilayer as the result of papain treatment.

The particular usefulness of the fluorescence polarization methods to study the vesicular membranes stems from the fact that the polarization of the fluorescence of a molecule depends upon its rate of rotation [27–29]. Since the rotation rate depends on the resistance offered by the microenvironment to the motion of the probe [30,31], the fluorescence polarization might be expected to provide an insight into and an estimate of the environmental resistance as the result of papain treatment of the vesicular membranes. Experiments were performed to determine the fluorescence polarization of DPH in control and papain-treated vesicle membranes. The results shown in Table III demonstrate that the removal of surface marker enzymes from the membrane as the result of papain treatment did not affect the anisotropy parameter, $[(r_0/r)-1]^{-1}$, indicating that the removal of the marker enzymes did not interfere with the DPH-lipid interaction of the membranes and that these enzymic proteins occupied a different domain which was distant from the lipid bilayer. The excited-state lifetime, τ, of DPH in papain-treated membranes when measured at 37 and 45°C, and the approximate rotational relaxation time, ρ, of DPH in papain-treated membranes at all temperatures measured (Table III) are slightly lower than that of control membranes. However, the differences are statistically

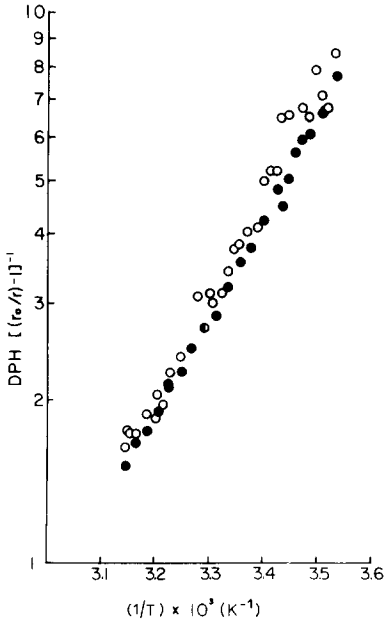

Fig. 10. Arrhenius plots of the anisotropy parameter of DPH in samples of brush-border membranes. Control (○) and papain-treated (●) vesicles were prepared as described in the text and suspended in buffer B, pH 7.4. $\lambda_{exc} = 360$ nm; $\lambda_{em} = 430$ nm. Each datum point represents the mean for two preparations.

insignificant. Arrhenius plots of the anisotropy parameter of DPH in control and papain-treated membranes (Fig. 10) indicates that the removal of enzymic proteins from the membrane surface did not change the 'fluidity' of the lipid layer in the membranes.

Discussion

Under the conditions of our experiments, the treatment of brush-border membrane vesicles with papain resulted in an insignificantly small loss of protein above that of control vesicles, but at the same time removed the activity of maltase, γ-glutamyl transpeptidase and leucine aminopeptidase by a further 85, 50 and 75%, respectively. Stripping of these external membrane enzymes resulted in a widespread diminution in the ability of a variety of amino acids and sugars to be taken up by the membrane vesicles which remained osmotically responsive. A careful analysis of the kinetics of the uptake of proline, which was shown previously to be transported by both sodium-dependent and sodium-independent systems [25], revealed a marked diminution of the sodium-stimulated 'overshoot' phenomenon. By non-linear regression analysis, the V_{max} for the sodium-dependent system and the K_m for the sodium-independent system were halved, but other parameters were not affected, indicating that the papain treatment altered not only the sodium-gradient-stimulated entry but also the affinity of the sodium-gradient-independent system for proline. No decrease in proline entry was seen under sodium-equilibrated conditions, which is consistent with the conclusion that the proline transporter itself was not removed in parallel with the enzyme stripping. In fact, the entry rate under sodium equilibration was slightly but significantly enhanced, suggesting that membrane translocation of the proline-loaded transporter may be enhanced. Thus, the proline kinetic data signaled that any major effect of papain treatment was secondary to an alteration in the sodium-related aspects of the uptake process.

The latter supposition was shown to be the case by examination of the kinetics of $^{22}Na^+$ uptake. Experiments on sodium entry and efflux demonstrate a marked enhancement of flux such that equilibration of the sodium gradient occurred about 5-times more rapidly than in untreated vesicles. This occurred without any change in the osmotic properties of the vesicle with regard to sodium or amino acid uptake. An apparent explanation for marked decrease in the sodium-stimulated 'overshoot' in papain exposed vesicles is the extremely fast disspation of the sodium gradient. We can only speculate on the mechanism involved in enhanced flux of sodium. Sodium ion movement, as discussed by Babcock et al. [32] and applied to a model of proline transport by rat renal brush-border membrane vesicles by Weiss et al. [26] is a first-order differential in time:

$$\frac{d[Na_i^+]}{dt} = L^{Na^+}([Na_o^+] - [Na_i^+])$$

where $[Na_i^+]$ is the concentration inside, $[Na_0^+]$ that outside, the vesicle and L^{Na^+} is the sodium-ion flux constant. The latter is a composite of several factors, including diffusion and membrane potential. It may be that papain, by removal of surface enzyme proteins, also removes a diffusional barrier to sodium so that the sodium ion flux constant is increased. Whether this involves the opening of new sodium 'channels' remains to be determined. According to the model for proline transport proposed by this laboratory, an increase in L^{Na^+} should result in a faster proline entry and a much earlier 'overshoot', which were not observed in these experiments. It is possible that the membrane translocation time of the proline transporter is so slow in relation to the accelerated sodium entry that the sodium gradient is dissipated before proline entry can effectively take place.

The non-linear regression analysis indicated that there was an increase in affinity of the high-K_m transport system, although none of the observed data (Fig. 4) indicated this to be the case. Since McNamara et al. [25] have shown there is no sodium requirement for the high-K_m proline transport system, it thus appears that papain treatment has an effect which is independent of sodium ion. The smaller K_{m2} after papain treatment contributes to the larger component of the uptake by the high-K_m system, as shown in Fig. 6, and perhaps to the higher uptake observed in the sodium-equilibrated state (Figs. 2 and 3).

Our studies of fluorescence polarization suggest

that incubation with papain did not alter the lipid domains of the membrane. This result would not be unexpected, since the enzyme acts to strip out membrane surface enzyme activity and does not solubilize alkaline phosphatase which is more intimately associated with the lipid skeleton.

In a prior communication [7] we reported that trypsin treatment of the brush-border membrane, which strips out only leucine aminopeptidase, diminishes sodium-stimulated uptake of various amino acids and sugars. The decrease in the sodium-gradient-stimulated proline 'overshoot' was proportional to the amount of leucine aminopeptidase removed from the membrane. Trypsin also decreased the velocity of proline entry (V_{max}) of the sodium-dependent transport system. These results parallel those observed in the present work. The papain treatment removed about the same percentage of leucine aminopeptidase as the maximum exposure to trypsin. It appears from our present results that the explanation for the results of trypsin treatment may be the same as for papain, an alteration in the kinetics of sodium flux.

The nature of the amino acid transport systems is unknown. One might expect that the binding sites for transport would be on the surface of the membrane and subject to enzymatic cleavage just as the surface enzyme activities. This appears not to be the case. The resistance to cleavage from the membrane of the transport systems while other proteins are removed may indicate a starting point for purification and subsequent isolation of the proteins involved in the transport process.

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