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Assessment of binding of L-cystine and L-lysine by rat renal brush-border membranes

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Cystine and lysine bind to isolated rat renal brush-border vesicles. Three methods to determine the extent of amino acid binding to the membranes have been compared, one relying on the osmotic reactivity of the vesicle, a second by trichloroacetic acid precipitation of membrane-bound material and a third by initial rate analysis. For cystine, all methods yield comparable results at early time points, indicating the trichloroacetic acid method is a simple and valuable tool for binding estimation under initial-rate or near initial-rate conditions. For lysine, initial rate analysis and osmotic perturbation are the methods of choice since lysine co-precipitates with trichloroacetic acid.

The use of isolated brush-border membrane vesicles to measure the uptake of various substrates has become an important technique for the evaluation of the process whereby intestinal and renal cells perform their transport functions [1]. In such experiments, a basic issue has been whether the solute enters the intravesicular space and/or is adsorbed to the membrane itself. A standard procedure has been devised in which vesicular uptake under conditions of increasing external osmolality is measured and uptake at infinite osmolality is estimated by extrapolation [2,3]. With a substance such as L-glucose for which no membrane transport system has been postulated, the extrapolated plot passes through the origin, a finding interpreted as an absence of adsorption or binding of the substance to the membrane itself [2,4]. Such studies require enough time to entirely fill the vesicle in order to see it react as an osmometer. For a number of amino acids [3,5] little or no binding has been observed. However, studies with cystine and the basic amino acids, lysine and arginine, which are known to share transport systems in brush-border membranes [6] demonstrate significant binding. Therefore, in investigating the transport properties of L-cystine and the basic amino acids by renal brush-border membrane vesicles, the discrimination between binding to the membrane and uptake into vesicle space is important. In addition, at initial rate measurements when the vesicle is not filled, an alternative method for determining binding during the transport process is necessary.

As an alternative to osmotic perturbation, McNamara et al. [6], in their experiments on cystine uptake by rat renal brush-borders, utilized the technique of precipitating membrane-bound L-[^{14}C]cystine with trichloroacetic acid. This permitted the determination of cystine binding in

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every incubation as well as the assessment of the difference between total uptake and membrane bound uptake. The percentage of membrane-bound cystine was small at very early incubation times, but increased with time to more than 50% of uptake after thirty minutes. Whether trichloroacetic acid-precipitable membrane-bound cystine corresponded to the extrapolated value obtained at 'infinite' osmolality under similar conditions had never been determined before. In the present study, we have compared the extent of binding of L-[^{35}S]cystine and L-[^{14}C]lysine to brush-border membranes under sodium gradient conditions by several techniques. Uptake of both substrates was assayed by trichloroacetic acid precipitation and osmotic perturbation to determine the percentage of total uptake represented by membrane-bound substrate. For cystine binding at early time points both methods give similar results. However the trichloroacetic acid method proved to be unusable for assessment of lysine binding and a method involving extrapolation of initial rate was used.

Brush-border membrane vesicles were prepared by the $MgCl_2$ precipitation method of Booth and Kenny [7]. Vesicles were suspended in THM buffer (100 mM mannitol in 2 mM Hepes adjusted to pH 7.4 with Tris). Details of the experimental conditions are listed in the figure legends. The final concentration of L-[^{35}S]cystine in the incubation was 0.017 mM and the final concentration of L-[^{14}C]lysine was 0.018 mM.

Transport measurements of uptake at 22°C were performed by rapid filtration techniques described previously [3] using Sartorius filters (SM 113, 0.45 μm). For binding determinations by trichloroacetic acid precipitation, the trichloroacetic acid-treated incubations were filtered through glass fiber filters using the technique described by McNamara et al. [6]. The filters were air dried and counted in a Packard Tricarb Scintillation Spectrometer. Total uptake was measured as membrane-associated radioactivity present on the filter with appropriate background subtracted. Protein was determined by the Bio-Rad procedure [8] and uptake of the amino acids expressed as nanomoles substrate taken up per milligram of vesicle protein.

Simple linear regression using the least-squares trend line was used to draw the lines for the osmotic reactivity plots and for initial rate analysis. The Y intercept $\pm$ the 95% confidence interval for that intercept for all data on osmotic reactivity and on initial rate of lysine uptake were determined using standard inference tests according to methods described by Dowdy and Wearden [9].

Fig. 1 shows a series of curves for [^{35}S]cystine uptake under sodium gradient conditions where membranes equilibrated in buffer to which were added increasing amounts of sucrose [2,3] were incubated for various times from 0.25 to 60 min. The linear decrease in uptake with increasing

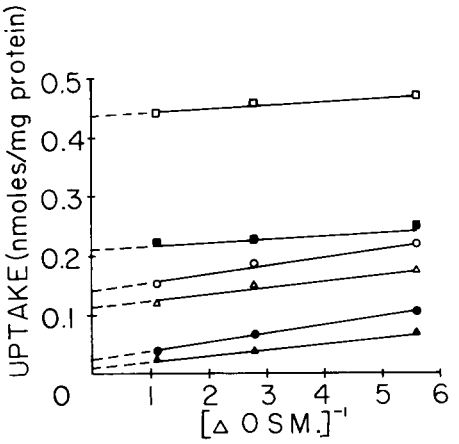

Fig. 1. Effect of medium osmolality on the uptake of L-[^{35}S]-cystine by isolated rat renal brush-border vesicles. Osmotic perturbations were used to assess the degree of cystine binding to brush-border membranes under NaCl gradient conditions at 22°C. The basal incubation consisted of 0.05 ml brush-border vesicles in THM buffer (pH 7.4), which were equilibrated with 0.45 ml buffer A (60 mM mannitol/20 mM Tris/20 mM Hepes) for 30 min. The uptake period was started by the simultaneous addition of 0.01 ml of L-[^{35}S]cystine in 0.5 M HCl and 0.05 ml 100 mM NaCl such that the final incubation mixture contained 0.017 mM L-[^{35}S]cystine, and 90 mM NaCl, and was at a final pH of 7.4. Osmotic perturbations were accomplished by sequential replacement of buffer A with increasing aliquots of 2 M sucrose dissolved in buffer A. The final sucrose concentrations used were 177 mM, 354 mM, and 885 mM. The change in osmolality of the incubations was determined by the concentration of the sucrose added. Uptake was plotted against the inverse of this change in osmolality and the line extrapolated to the Y intercept to indicate the amount bound. Time periods for cystine uptake after 30 min of equilibration with the buffer and sucrose were: ▲, 0.25 min; ●, 1 min; △, 5 min; ○, 10 min; ■, 20 min and □, 60 min. The points plotted are the means of 8–16 determinations per data point. Standard errors of the means fit within the size of the symbol used to designate the mean.

TABLE I

COMPARISON OF CYSTINE BINDING AS DETERMINED BY TWO METHODS

Standard incubation conditions as described in Fig. 1 for cystine were used to assess total uptake. For total uptake at each time point, the values given are the mean ± S.E. for 8–16 determinations. To determine binding by the osmotic method, the data in Fig. 1 were used and the *Y* intercept ± 95% confidence intervals determined by standard linear regression analysis according to Dowdy and Wearden [9]. The number of determinations for each time period varied from 23 to 48. The percent binding was obtained by dividing the *Y* intercept by the total uptake where no sucrose had been added. The trichloroacetic acid (TCA) method for evaluation of cystine binding has been described [6]. The values given are the means ± S.E. for 8–16 determinations for each time point. The percent binding was calculated by dividing the trichloroacetic acid-precipitable uptake by the average total uptake in each individual experiment. The results are given as the means ± S.E. for 8–16 determinations.

Time (min)	Basal incubation Total uptake	Osmotic method		TCA method	
		Y intercept ± 95% confidence interval	% binding	TCA-precipitable uptake	% binding
0.25	0.114 ± 0.0088	0.010 ± 0.0105	9.1	0.015 ± 0.0021	13.1 ± 1.46
1	0.162 ± 0.0036	0.024 ± 0.0095	14.7	0.027 ± 0.0022	16.6 ± 1.30
5	0.233 ± 0.0058	0.112 ± 0.0166	48.0	0.066 ± 0.0059	28.1 ± 2.41
10	0.277 ± 0.0058	0.140 ± 0.0144	50.6	0.094 ± 0.0056	33.9 ± 2.27
20	0.294 ± 0.0148	0.212 ± 0.0329	72.0	0.130 ± 0.0113	44.1 ± 2.69
60	0.506 ± 0.0087	0.438 ± 0.0174	86.6	0.206 ± 0.0051	40.7 ± 1.07

osmolality when extrapolated to the ordinate gives a definable intercept at the lowest time studied (0.25 min) which increases with time. In Table I, the percent of cystine binding by the osmotic method was about 9% of uptake at 0.25 minutes but increased to about 70% after 30 min of incubation. Table I also reveals the percent of total cystine uptake as determined by the trichloroacetic acid precipitable method in incubations identical to the basal incubations in the osmolality experiments. As reported earlier [6] cystine did not adhere to filters and there was no co-precipitation of cystine with membranes after trichloroacetic acid treatment. A comparison of the percent of binding by the two methods reveals comparable values at early incubation times up to 1 min.

While the extent of cystine binding to the brush-border membrane vesicle appears to depend on the incubation conditions employed, the correlation of percent binding at early time points as determined by the osmotic method and by trichloroacetic acid precipitation remains. Under conditions similar to those of Biber et al. [10] where the vesicles were prepared in 300 mosmolar buffer (260 mM mannitol/20 mM Tris/20 mM Hepes (pH 7.4)) and incubated in medium containing 60 mM mannitol, 20 mM Tris, 20 mM Hepes (pH 7.4), and 100 mM NaCl, the binding component measured by osmotic perturbation experiments was 28.5% at 0.25 min and 35.3% at 0.5 min incubation time. This compared to 27.1 ± 3.3% (six determinations) and 38.3 ± 3.0% (10 determinations) when binding was assessed by trichloroacetic acid precipitation at these times, respectively. Biber et al. [10] demonstrated binding of cystine to renal brush-border membranes by extrapolating the linear uptake at one second intervals over a period of 6 s of incubation back to zero-time. The intercept above the origin can be estimated to be about 25–30% of the uptake at that time, a value comparable to that reported here by osmotic perturbation and trichloroacetic acid precipitation.

Fig. 2 shows the effects of osmotic perturbations on L-lysine uptake by vesicles under sodium gradient conditions. The protocol for these experiments was the same as that employed for cystine except for the use of different buffers. Extrapolation of the linear decrease in uptake with increasing osmolality intercepted the ordinate above the origin for all times of incubations examined. The percent of lysine thus shown presumably bound to the membranes in totally collapsed vesicles is listed in Table II. About 24% of the uptake represented binding at 1 and 5 min which increased to a higher percentage by 10 min. However, the ab-

solute amount of lysine bound did not increase with time.

The use of trichloroacetic acid to determine lysine binding in a manner similar to that described above for cystine was not feasible. Significant amounts of radioactive lysine were found to be associated with the trichloroacetic acid-precipitated membranes on glass fiber filters. However, after the addition of trichloroacetic acid to the incubation media without membranes or addition of radioactive lysine after trichloroacetic acid had been added to a membrane incubation, a similarly large amount of radioactivity adhered to the filters. This high 'blank' indicates that lysine coprecipitates with trichloroacetic acid necessitating the use of an alternative method for assessing the transport related binding component for lysine. The method chosen was initially demonstrated to be valid by Turner and Silverman [11] who measured phlorizin binding at zero-time and showed it to be linear with early time points. Extrapolation of the initial phase of transport to zero-time has been used to determine the binding of amino acids to isolated membranes [10,12] and whole cells [13]. Data on the uptake of 0.02 mM lysine measured at incubation times ranging from 3 s to 1 min are shown in Table II. Uptake was linear from 3 to 18 s and the line determined from these time points was extrapolated to the ordinate. This intercept was 0.080 nmol/mg protein, the theoretical amount of lysine taken up at zero-time which is presumed to be the amount bound and represented 19.3% binding in a 1 min incubation. The average of the amount of lysine bound (Y intercept) as determined by the osmotic method was 0.084 nmol/mg protein and represented 23.1% of the uptake at 1 min (Table II). Thus transport related binding of lysine can be determined by both initial rate analysis and osmotic perturbation studies.

That cystine and lysine bind to renal brush-border membrane vesicles is quite clear. Our studies with cystine under sodium gradient conditions show that the theoretical calculation of binding made from osmotic perturbation corresponds to the actual estimation of binding by precipitation

TABLE II

COMPARISON OF LYSINE BINDING AS DETERMINED BY TWO METHODS

Standard incubation conditions as described for Fig. 2 for lysine were used to assess total uptake. Binding by the osmotic method was determined using the data from Fig. 2. Total uptake is shown as the mean ± S.E. for 3–8 determinations. The Y intercept ± 95% confidence intervals osmotic for data and when using the linear range (3–18 s time points) of the initial rate data was determined by standard linear regression analysis according to Dowdy and Wearden [9]. Number of determinations used to draw and extrapolate each line to the ordinate intercept varied from 18 to 24. Percent binding was calculated by dividing the Y intercept by total uptake at each time point.

Method	Time	Total uptake	Y intercept ± 95% confidence interval	% binding
	(min)	(basal incubation)		
Osmotic perturbation	1	0.340 ± 0.0136	0.079 ± 0.0130	23.1
	5	0.380 ± 0.0069	0.092 ± 0.0098	24.2
	10	0.269 ± 0.0022	0.085 ± 0.0067	31.5
	20	0.247 ± 0.0055	0.089 ± 0.0072	35.9
	60	0.203 ± 0.0055	0.075 ± 0.0070	37.0
	(s)			
Initial rate	3	0.106 ± 0.0039		75.8
	6	0.143 ± 0.0025		55.9
	9	0.172 ± 0.0091		46.4
	12	0.183 ± 0.0058	0.080 ± 0.0168	43.7
	15	0.224 ± 0.0047		35.8
	18	0.256 ± 0.0121		31.3
	60	0.414 ± 0.0104		19.0

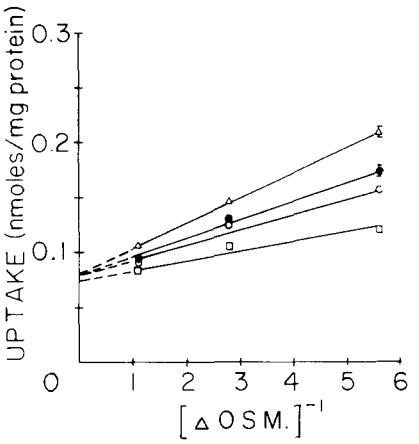

Fig. 2. Effect of medium osmolality on uptake of L-[^{14}C] lysine by isolated rat renal brush-border vesicles. Experiments were performed as in the legend to Fig. 1 except that the basal incubation conditions differed. The basal incubation for lysine consisted of 0.45 ml of THM buffer (pH 7.4) to which 0.05 ml brush-border vesicles in THM were added and allowed to equilibrate at 22°C for 30 min. The uptake of lysine was started by the addition of 0.06 ml of L-[^{14}C] lysine and NaCl and resulted in a final lysine concentration of 0.018 mM and a final NaCl concentration of 90 mM. Increasing amounts of 2 M sucrose in THM buffer were used to replace THM buffer alone in the incubations. The data presented represent the means ± S.E. of 6–8 determinations per data point. If no S.E. bars are indicated, the standard errors of the means are included within the size of the symbol used to designate the mean. Time periods for lysine uptake shown are: ●, 1 min; △, 5 min; ○, 10 min; □, 60 min. The data for a 20-min incubation are not shown because they were virtually superimposable with the 10-min points.

of membranes with trichloroacetic acid if early incubation times are considered. Since the experiments with trichloroacetic acid are relatively easy and can determine cystine binding directly, at very early times of incubation the difference between total uptake and trichloroacetic acid-precipitable uptake can be determined and used to represent free cystine uptake into the intravesicular space. This procedure, then, appears to be a valuable method to examine the characteristics of cystine transport as was employed previously by us [6] in examining concentration dependence of cystine uptake at 15 s. No difference was observed in the K_m values of either the high- or low-affinity systems when calculations were based on free cystine uptake instead of total uptake, but the V_{max} of the high K_m system was 25% lower under that mode of calculation. The nature of the cystine binding is not known. The data here indicates it increases with time but appears to be unsaturable. It is possible that after 1 min of incubation there occurs a second form of cystine binding unrelated to the transport system itself. This non-transport related binding could be reversible with trichloroacetic acid.

Our results indicate that trichloroacetic acid precipitation cannot be used to assess the binding of lysine. Initial rate analysis or osmotic perturbation appear to be the methods of choice for this amino acid. Under the incubation conditions we employed, there was 19–24% binding at 1 min and the amount of lysine bound per mg protein did not change with time.

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