Albumin Absorption and Catabolism by Isolated Perfused Proximal Convoluted Tubules of the Rabbit

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Abstract. Overall characteristics and kinetics of tubular absorption of albumin (Alb) were studied in isolated perfused proximal convoluted tubules of the rabbit. The fate of absorbed Alb was determined in tubules perfused with low [Alb]. Alb was labeled with tritium by reductive methylation ([^3H_3C]Alb). At [Alb] = 0.03 mg/ml, ~80% of the absorbed [^3H_3C]Alb was released to the peritubular bathing solution as catabolic products. Transcellular transport of intact [^3H_3C]Alb was negligible. Iodoacetate (IAA, 4 mM) inhibited albumin absorption (J_{Alb}) by >95% and fluid reabsorption (J_v) by 55%. At [Alb] = 0.1 mg/ml the absorption rate of a derivatized cationic Alb (pI = 8.4) was fivefold greater ($P < 0.01$) than that of anionic Alb. Higher cationic [Alb] had deleterious effects on tubular functions. Overall Alb absorption was of high capacity and low affinity (J^{max}_{Alb} = 3.7 ng/min per mm tubule length, apparent Michaelis constant (K_m) = 1.2 mg/ml). A low capacity system that saturates at near physiological loads was also detected (J^{max}_{Alb} = 0.064 ng/min per mm, apparent K_m = 0.031 mg/ml). High [Alb] did not alter the rate of endocytic vesicle formation as determined by the tubular uptake of [^{14}C]inulin. Results show that Alb absorption is a saturable process that is inhibited by high IAA concentrations and is affected by the charge of the protein. Absorbed Alb is hydrolyzed by tubular cells and catabolic products are readily released to the peritubular side. The dual kinetics of Alb absorption may be due to a combination of adsorptive endocytosis (low capacity system) and fluid endocytosis of albumin aggregates (high capacity system). Results indicate that albuminuria occurs much before albumin absorption is saturated. The kinetic characteristics of the process of tubular absorption of albumin helps to explain the concomitance of albuminuria, increased renal catabolic rates of albumin, and renal cell deposition of protein absorption droplets in severe glomerular proteinurias.

Introduction

Renal metabolism of circulating proteins involves glomerular filtration tubular absorption by endocytosis and intracellular hydrolysis of absorbed proteins (for review see reference 1). Studies with low molecular weight proteins have shown that tubular absorption is a selective, energy dependent, and saturable process, characterized by a high capacity and a relatively low affinity as compared with the normal filtered loads of these proteins (1–7). Net charge of the molecule has been shown to influence renal cell uptake of proteins (7–10). In addition, other physico-chemical characteristics of protein molecules as well as geometric and electrical constraints on the access of filtered proteins to the endocytic sites may play an important role on the selectivity and kinetics of tubular absorption of proteins (7). Absorbed low molecular weight proteins are hydrolyzed to completion within renal cells and the resulting amino acids are returned to the circulation (1). Much less is known about the quantitative aspects of tubular absorption and catabolism of albumin. Since this protein is the most prevalent of the plasma proteins and albuminuria and hypoalbuminemia are such prominent features in many renal diseases, elucidation of the tubular absorption, and catabolism of albumin assumes particular importance.

Morphological studies have shown that albumin is also absorbed by endocytosis and eventually hydrolyzed within lysosomes of proximal tubular cells (11, 12). Microperfusion experiments in the intact rat indicate that the bulk of albumin absorption takes place in the proximal convoluted tubule (13). There is still uncertainty as to the normal range of albumin concentrations in the glomerular filtrate, but most authors seem

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to concur that it is <3 mg/dl (14–22). Contrary to what has been found for small proteins, several investigators claim that albumin absorption saturates at near physiological loads of this protein (16, 17, 19–22). This conclusion is based on micropuncture studies in normal rats and in rats made proteinuric by the administration of aminonucleoside (16, 17), anti-glomerular basement membrane antibodies (20–22), or angiotensin (19). Results of these micropuncture experiments are, however, difficult to interpret in view of the technical problems of measuring very low concentrations of albumin and the large variability of the reported values of albumin concentrations in the glomerular filtrate and tubular fluid (14–22).

In the present study quantitative aspects of tubular absorption and catabolism of albumin were examined in the isolated perfused proximal convoluted tubule of the rabbit. Albumin was labeled with tritium by reductive methylation, a procedure which results in very high radioactive specific activities without altering the physico-chemical characteristics of protein molecules (23). The approach used in the present experiments allowed for the precise determination of albumin absorption rates at loads ranging from physiological to very high levels. To quantitate the influence of net charge on the renal absorption of albumin, we also compared absorption rates of native anionic albumin and a derivatized cationic albumin.

The results show that: (*a*) albumin absorption is a saturable process that is inhibited by high concentrations of iodoacetate; (*b*) at relatively low loads, the rate of intracellular hydrolysis of absorbed albumin is nearly equivalent to its rate of absorption and the hydrolytic products are readily released to the peritubular bathing solution; (*c*) at low loads, cationic albumin is absorbed at much higher rates than anionic albumin while high concentrations of cationic albumin have deleterious effects on tubular functions, decreasing fluid reabsorption, and albumin uptake; (*d*) the overall kinetics of tubular uptake of albumin are characterized by a very high capacity and relatively low affinity compared with normal filtered loads of this protein; (*e*) in addition to this overall high capacity system there is another transport system for albumin that saturates at near physiological loads. The hypothesis is raised that the dual transport system for albumin uptake is due to a combination of adsorptive endocytosis (low capacity system) and bulk uptake of albumin aggregates by fluid endocytosis (high capacity system). The results permit new insights into the phenomena of albuminuria, deposition of protein absorption droplets within renal cells, and increased renal catabolic rates of albumin in proteinuric conditions.

Methods

Perfusion procedure. General protocol

Renal proximal convoluted tubules of New Zealand white rabbits (1.0–1.5 kg body wt) were perfused according to the technique of Burg and Orloff (24) as slightly modified by Friedman et al. (25). The S_1 segments of proximal tubules were dissected from superficial cortical slices of the right kidney in a solution identical to the peritubular bathing solution and maintained at 4°C. Tubules with no observable structural damage and measuring 0.6–1.9 mm in length were perfused at 37°C at perfusion rates varying between 10 and 15 nl/min (11.2±0.2 nl/min, n = 105 tubules). The bathing solution was constantly gassed with 95% O_2/5% CO_2. After a 30–40-min equilibration period three to seven 10-min perfusion samples were collected by a constant-bore collection pipette (inner diameter = 200 μm). At the end of each sampling period the bathing solution was collected by aspiration followed by several washes with fresh solution. At the end of all sampling periods, the perfusion solution was rapidly perfused through the tubules at ~500 nl/min and a triplicate sample of this rapidly perfused fluid was collected as the standard for the perfusate. In the experiments in which the fate of absorbed albumin (Alb)[1] was determined, six tubules were perfused with an [Alb] = 0.03 mg/ml for 121±4 min. The perfusion and bathing solutions were collected over the entire span of the perfusion. At the end of the perfusion time the tubules were extracted overnight in 1.0 ml of 0.1 N HNO_3 in order to determine the tubule cellular content of ^{3}H- or ^{125}I-labeled albumin and [^{14}C]inulin. Before the acid extraction, tubules were thoroughly perfused and washed with radioisotope-free perfusate and frequent changes of the bathing solution. In the experiments in which iodoacetate was used, this substance was added to the bathing solution at a final concentration of 4 mM. Two to three control samples were collected in the absence of the inhibitor followed by a 15–20-min equilibration period and one or two experimental periods.

Composition of perfusion and bathing solutions

The composition of the solutions used for bathing and perfusing the proximal convoluted tubules was as follows (in millimolar): NaCl, 105; $NaHCO_3$, 25; Na acetate, 10; NaH_2PO_4, 0.92; KCl, 4.84; KH_2PO_4, 0.6; $CaCl_2$, 1.97; $MgSO_4$, 0.81; D-glucose, 8.0; L-alanine, 5.0. The pH of the solutions, equilibrated with 95% O_2/5% CO_2, was 7.4. In addition the bathing solution contained 6.5 g/dl fraction V bovine albumin (Reheis Chemical Co., Kankakke, IL or Pentax, Miles Laboratories, Inc., Elkhart, IN). The perfusate also contained 5 μCi/ml of [*carboxyl*-^{14}C]inulin (New England Nuclear, Boston, MA), 10–70 μCi/ml of [^3H_3C]albumin (see below) and 0.0012–10 mg/ml crystalline bovine albumin (C. F. Boehringer and Sons, Mannheim, Federal Republic of Germany). In some experiments ^{125}I-albumin (5–10 μCi/ml, see below) was used instead of the tritiated albumin. Before the addition of [^{14}C]inulin, the perfusate was dialyzed against a great excess of bathing solution in order to reduce the concentrations of phosphate buffer used in the labeling procedure of albumin and to equilibrate the diffusable components of the perfusate and bathing solutions. Osmolalities of the perfusate and bathing solutions were within 3 mosmol/kg of each other.

Labeling of albumin

Albumin was labeled with tritium by the reductive methylation procedure of Tack et al. (23). For this purpose crystalline bovine albumin and [^{3}H]$NaBH_4$ (60 Ci/mMol, New England Nuclear) were used. The volume of the reaction was 0.32 ml, the molar ratio HCHO/lysine residues of albumin was 2.6 and the molar ratio [^{3}H]$NaBH_4$/HCHO was 0.2. Labeled albumin was separated from the bulk of the unreacted labeled material by gel chromatography using disposable Sephadex G-25 columns (PD-10 Sephadex G-25, Pharmacia Fine Chemicals, Upsalla, Sweden), pre-equilibrated and eluted with phosphate buffer. The labeled albumin was then extensively dialyzed for 2 d against a 100-fold excess phosphate

1. *Abbreviations used in this paper:* Alb, albumin; J_{Alb}, Alb absorption rate; J_v, fluid reabsorption rate.

buffer with repeated changes of the buffer solution to remove the remaining free label. The final specific activity of [^3H_3C]albumin was ~200 Ci/mmol. Fig. 1 *A* shows that the labeled albumin coeluted with unlabeled crystalline albumin. [^3H_3C]albumin was kept at −70°C for several months without any apparent dissociation of the label or denaturation of the protein. Albumin was labeled with carrier-free Na ^{125}I (New England Nuclear) using an immobilized preparation of lactoperoxidase and glucose oxidase (Enzymobeads, Technical Bulletin 1071, Bio-Rad Laboratories, Rockville Centre, NY). The specific activity of the prepared ^{125}I-albumin was 42 Ci/mmole and free ^{125}I was ~1%. The labeled and unlabeled albumin coeluted in Sephadex G-100.

Cationization of albumin

Native anionic crystalline albumin was cationized by the method of Hoare and Koshland (26). The method consists of the activation of carboxyl groups of albumin by carbodiimide and subsequent amination by ethylenediamine. Anhydrous ethylenediamine solution (Fisher Scientific, Fairlawn, NJ) was diluted 1:7.5 in 500 ml distilled water and the pH was adjusted to 4.75 with ~350 ml of 6 N HCl. To this solution we added 2 g of crystalline bovine albumin and 0.725 g of 1-ethyl-3 (3-dimethyl-amino-propyl)-carbodiimide (Pierce Chemical Co., Rockford, IL). The reaction continued for 120 min with stirring and was stopped by the addition of 30 ml of 4 M acetate buffer (pH = 4.75). The derivatized albumin solution was dialyzed against distilled water with several changes of the bath water and lyophilized. This procedure was repeated twice and the final lyophilizate was stored at −20°C.

Isoelectric focussing was performed as described by Tate and Meister (27) using 6% polyacrylamide gels containing 2% ampholines (pH range 3–10). Fig. 2 shows the gels of the native and derivatized cationic albumin. Both migrated in single sharp bands. The pIs were 4.6 and 8.4 for anionic and cationic albumin, respectively.

Derivatized cationic albumin was labeled with ^3H by the reductive methylation procedure of Tack et al. (23) as described above. The specific activity was also ~200 Ci/mmole. Fig. 1 *B* shows that the cationic labeled protein coeluted with unlabeled anionic albumin.

Siliconization and protein-coating of glass micropipettes

Pilot studies showed that siliconization of the pipettes of the collection side was not sufficient to prevent binding of albumin. To avoid this artifact, siliconized glass micropipettes on the collection side were precoated with albumin. All glass pipettes used in the microperfusion system were siliconized with a 5% solution of Siliclad (Clay-Adams, Div. of Becton, Dickinson & Co., Parsippany, NJ). This solution was aspirated into the pipettes several times for ~30 min. Then the siliconized pipettes were washed with distilled water and dried at 100°C for 30 min. The siliconized perfusion pipettes were equilibrated with the perfusate of each experiment for at least 30 min before the start of the perfusion. From this time on, the [Alb] in the fluid delivered from the perfusion pipettes remained constant. At very low [Alb] (0.0046 mg/ml and less) the [Alb] in the fluid delivered from the perfusion pipettes was slightly lower than the [Alb] in the solution used to load the pipettes. Since the standard used to determine albumin absorption was measured in the delivered fluid and not in the loading solution (see above) this small

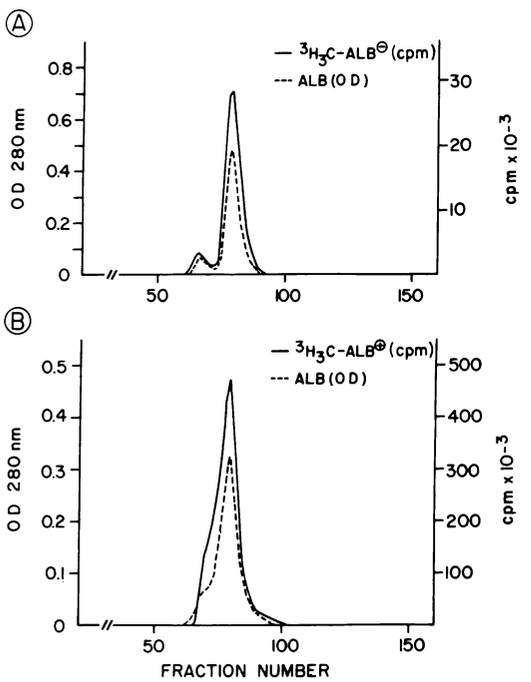

Figure 1. Representative chromatogram of the preparations of native anionic albumin (ALB), labeled anionic albumin (^3H_3C-ALB$^{\ominus}$) and labeled derivatized cationic albumin (^3H_3C-ALB$^{\oplus}$) used in the present study. Samples (1 ml) were layered on a Sephadex G-100 column (void volume = 50 ml), preequilibrated with saline-phosphate buffer (pH = 7.4). The samples were eluted with the same buffer at a flow rate of 8 ml/h and 0.8-ml fractions were collected. The optical density (OD) was measured at 280 nm and the ^3H-radioactivity was determined by liquid scintillation counting. (*A*) Elution of sample containing ALB and ^3H_3C-ALB$^{\ominus}$. Recovery of radioactivity added to the column = 101%. (*B*) Elution of sample containing ALB and ^3H_3C-ALB$^{\oplus}$. Recovery of radioactivity added to the column = 98%. The three forms of albumin coeluted in Sephadex G-100.

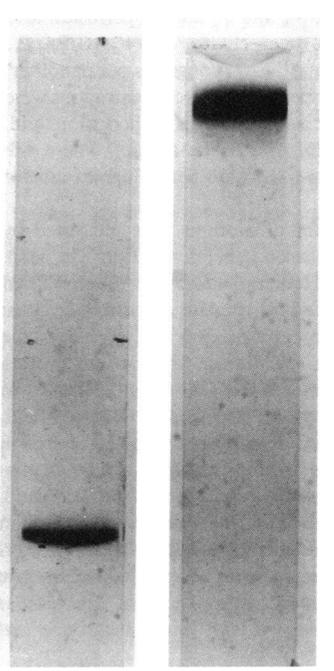

Figure 2. Isoelectric focussing gels of the native anionic albumin (*left*) and the derivatized cationic albumin (*right*). The pH gradient of the ampholite solution was 3–10 (bottom to top). The albumins focussed as single bands at the pH of 4.6 and 8.4 for the anionic and cationic albumin, respectively.

decrease did not influence the calculations. Therefore, there was no need to precoat the perfusion pipettes with protein. On the other hand, because of the much lower fluid volume/glass surface area ratio in the collection pipettes, protein binding markedly decreased the [Alb] in collected fluid. To circumvent this problem the siliconized pipettes on the collection side (holding and constant-bore pipettes) were coated with a 10 mg/ml albumin solution using the same procedure as that used for the siliconization. Recovery of labeled albumin from the glass micropipettes was tested by comparing the concentration of radioactivity in samples collected from tubules perfused at a very rapid rate (see above) with the concentration of a 5-μl sample of the original perfusate delivered from an automatic pipette with a plastic tip. At a perfusate [Alb] of 0.0046 mg/ml, siliconization alone resulted in <5% (3.5±1.1%, $n = 4$) recovery, while siliconization and precoating with protein resulted in a >95% (95.5±3.0%, $n = 8$) recovery. It should be pointed out that the degree of binding of Alb to siliconized glass micropipettes is concentration dependent. At the higher [Alb] (> 1 mg/ml) the binding of Alb to siliconized collection micropipettes becomes negligible. Although the collection pipettes used in the present experiments were coated with albumin solutions, the same results are obtained if the glass pipettes are coated with a 2 mg/ml solution of insulin. The binding of protein to the glass micropipettes was so strong that protein-coated collection pipettes could be reused for several experiments after extensive washing with distilled water.

Measurements, calculations, and statistics

Fluid reabsorption (J_v, nanoliters per minute per millimeter of tubule length) was determined using [^{14}C]inulin according to the equation: $J_v = V_c[(C/P)_{inulin} - 1]/L$, where V_c is the collection rate in nanoliters per minute, $(C/P)_{inulin}$ is the ratio of the ^{14}C-radioactivity per nanoliter of collected fluid (C) and perfusate standard (P), and L is the perfused length of the tubule in millimeters, measured at the end of the experiment with an eyepiece micrometer.

Alb absorption rates (J_{Alb}, nanograms per minute per millimeter of tubule length) were determined according to the following equation: $J_{Alb} = (V_p \cdot P_{Alb} - V_c \cdot C_{Alb})/L$, where V_p and V_c are the perfusion and collection rates (nanoliters per minute), respectively, $V_p = V_c \cdot (C/P)_{inulin}$, P_{Alb} and C_{Alb} are the albumin concentrations (nanograms per nanoliter) in the perfused and collected fluid, respectively. Alb concentrations were calculated from their radioactive specific activities in the perfusion fluid, i.e., counts per minute of [^3H_3C]Alb/Alb specific activity, where Alb specific activity is counts per minute [^3H_3C]Alb per milligram labeled Alb and milligram unlabeled Alb.

Na and K in perfusate and bathing solutions were measured by flame photometry (IL 143, Instrumentation Laboratory, Lexington, MA). The osmolalities of the perfusion and bathing solutions were determined by freezing-point depression (Advanced Instruments, Needham Heights, MA). Albumin concentration of the perfusate was determined by the Bradford microassay method with a commercial kit (Bio-Rad Protein Assay Kit II, Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as a standard. ^{3}H- and ^{14}C-counts in perfusate, collected fluid, bathing solution, and tubular tissue were determined by liquid scintillation using gellified Hydrofluor (National Diagnostics, Inc., Advanced Applications Institute, Inc., Somerville, NJ) as the liquid scintillation fluid. Appropriate settings for double isotope counting with quench correction were used. ^{125}I-counts were determined in a gamma scintillation counter. The perfusate and collected fluid counts were at least 30 times above background and the samples were counted to <1% error. The tubular tissue and bathing solution counts were at least twice background and were counted to a <10% error.

The t test was used for the statistical analysis of the results (28). Values are expressed as mean±SE except where noted.

Results

Validation of the methodology used to determine the tubular absorption of albumin. Tubular absorption and fate of absorbed albumin at low perfusate albumin concentration

Proximal tubule uptake of [^3H_3C]Alb was determined by the rate of removal of total ^{3}H-radioactivity from the tubular lumen. To test whether this parameter effectively measures J_{Alb}, we compared in the same tubules the disappearance rate of total ^{3}H-radioactivity from the tubular fluid to the accumulation rate of ^{3}H-radioactivity in tubular tissue and its rate of appearance in the peritubular bathing solution. In addition we determined whether the ^{3}H-radioactivity remains protein-bound in the tubular fluid and bathing solutions. Fig. 3 presents the results of these experiments performed in six tubules perfused with an [Alb] of 0.03 mg/ml. Fig. 3 *A* shows that only ~20% of the radioactivity removed from the tubular fluid accumulated in tubular tissue. The bulk of the radioactivity removed from the tubular fluid (~80%) appeared in the bathing solution. Fig. 3 *B* shows that the ^{3}H-radioactivity in tubular fluid remains protein-bound since 94.8±1.4% of the total radioactivity is precipitated by trichloroacetic acid (TCA). On the other hand the ^{3}H-radioactivity appearing in the bathing solution consisted almost totally of small catabolites, since 92.1±2.4% of the radioactivity was TCA soluble.

Efflux of TCA-soluble ^{3}H-radioactivity from the lumen and nonspecific leaks of [^3H_3C]Alb may contribute to the determination of J_{Alb} when this parameter is calculated from the efflux of total ^{3}H-radioactivity from the lumen. The contribution of these two possible artifacts was quantitated in the same six experiments described above. The efflux of TCA-soluble ^{3}H-radioactivity (perfused-collected TCA-soluble radioactivity) was only 11.7±2.5% of the efflux of total ^{3}H-radioactivity from the lumen. The rate of appearance of TCA-precipitable ^{3}H-radioactivity in the bathing solution was taken as a maximal estimate of the nonspecific leak of [^3H_3C]Alb. It corresponded to only 0.9±0.3% of the perfused load of [^3H_3C]Alb. This value is actually smaller than the concomitantly measured appearance of [^{14}C]inulin in the bathing solution (1.6±0.4% of the perfused load of [^{14}C]inulin). In view of the small values of the efflux of non–protein-bound radioactivity and of the maximal nonspecific leak of labeled albumin as compared with the efflux of protein-bound radioactivity from the lumen, no corrections were performed for the values of these parameters in all subsequent determinations of J_{Alb}.

The tubular absorption of ^{125}I-Alb was determined in six separate tubules. Table I presents the comparative results of the tubular handling of [^3H_3C]Alb and ^{125}I-Alb at perfusate [Alb] of 0.03 mg/ml. As shown, there was no significant difference between $J_{^3H_3C\text{-}Alb}$ and $J_{^{125}I\text{-}Alb}$ or between the proportion of ^{3}H or ^{125}I radioactivity accumulated in kidney tissue and appearing

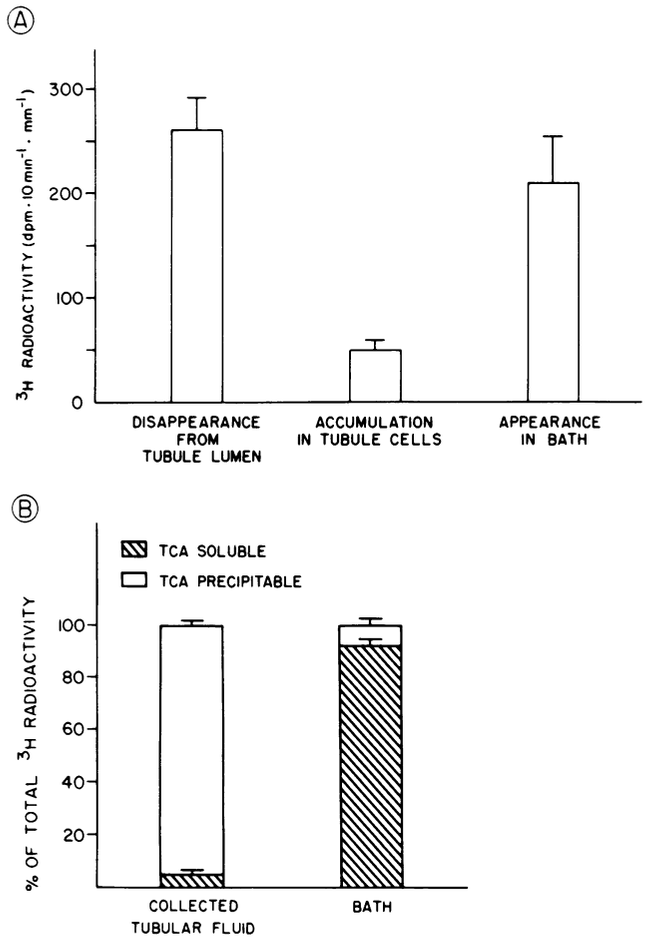

Figure 3. Fate of absorbed [^3H_3C]albumin in proximal convoluted tubules. Six tubules were perfused with an albumin concentration of 0.03 mg/ml for 121±4 min. Efflux of ^{3}H-radioactivity and appearance of this radioactivity in the bathing solution were determined over the entire span of perfusion. At the end of perfusion the radioactivity remaining in the tubules was also determined (see Methods). (*A*) The rate of efflux of ^{3}H-radioactivity from the lumen and the rates of accumulation in tubular cells and in the bathing solution were determined in each tubule. Results (mean±SE) are expressed as disintegrations per minute per 10 minutes per millimeter of tubule length to normalize for differences in perfusion time and tubule length. (*B*) Corresponding values for the TCA-soluble and TCA-precipitable radioactivity in the collected perfusion fluid and in the bathing solution.

in the peritubular bathing solution relative to the radioactivity removed from the tubular lumen.

Taken as a whole, the results of the experiments described to this point validate the use of the efflux of total ^{3}H-radioactivity from the lumen as a measure of the tubular absorption of [^3H_3C]Alb and the use of the latter to study the tubular handling of albumin. The data show that at low perfusate [Alb] the bulk of the absorbed albumin is catabolized by tubular cells and the hydrolytic products are delivered to the bathing solution.

Overall characteristics of tubular absorption of albumin

Table II presents individual data on perfusion rates, concentrations of inulin and albumin in perfusate and collected tubular fluid, J_{Alb} and J_v in the six proximal convoluted tubules perfused with [Alb] of 0.03 mg/ml. Of note is the finding that the [Alb] in tubular fluid does not change significantly since the ratio of albumin per nanoliter of collected fluid and perfusate standard of 1.02±0.01 is not statistically different from 1.00 ($P > 0.05$). This indicates that at the particular perfusate [Alb] used in these experiments (0.03 mg/ml) the proximal tubular cells remove albumin from the tubular fluid in the same proportion as they reabsorb fluid.

To test whether the absorption of albumin is influenced by metabolic inhibitors, proximal convoluted tubules were perfused under control conditions and after the addition of 4 mM iodoacetate to the bathing solution. Fig. 4 presents the results of individual experiments. The 14 proximal tubules used in these experiments were perfused with perfusate [Alb] ranging from 0.0012 to 2.0 mg/ml. In every instance iodoacetate markedly inhibited the tubular absorption of albumin. J_{Alb} after iodoacetate was only 3.9±5.8% of its value in the control periods. As expected, the high concentrations of iodoacetate used in these experiments also inhibited fluid reabsorption. However, the inhibition of J_v (45.4±8.1% of control value) was significantly smaller ($P < 0.01$) than the inhibition of J_{Alb} (see Fig. 4).

Kinetics of albumin absorption

Figs. 5 and 6 depict the absorption rates of albumin as a function of perfusate [Alb] and Alb perfused loads, respectively. To obtain the absorption curves for Alb, 57 tubules were perfused, each with a fixed perfusate [Alb]. As can be seen, tubular absorption of albumin is a saturable process, with maximal absorption rates significantly above the Alb concentrations and loads expected to be present in tubular fluid under physiological conditions. Kinetic parameters were calculated from a Lineweaver-Burk transformation of the data presented in Fig. 5. The overall J^{max}_{Alb} was 3.7 ng/min per mm and the apparent Michaelis constant (K_m) was 1.2 mg/ml. It is clear from Figs. 5 and 6 (insets) that, in addition to the overall high capacity-low affinity absorption system, there is another system that tends to saturate at much lower luminal fluid [Alb] with a J^{max}_{Alb} of 0.064 ng/min per mm and an apparent K_m of 0.031 mg/ml. The overall J_v in all tubules used in the determination of the absorption curve of Alb was 0.94±0.05 nl/min per mm. The J_v in the tubules perfused with [Alb] in the range of the high capacity-low affinity transport system ([Alb] > 0.3 mg/ml, J_v = 0.84±0.08 nl/min per mm, n = 16 tubules) was not significantly different ($P > 0.2$) from the J_v in tubules perfused with [Alb] in the range of the low capacity transport system ([Alb] < 0.3 mg/ml, J_v = 0.97±0.07, n = 41 tubules).

To test whether the rate of formation of endocytic vesicles per se was altered by the presence of albumin in the tubular

Table I. Comparison of Proximal Convoluted Tubule Handling of [^3H_3C]Albumin and ^{125}I-Albumin

	Alb perfused load*	Removal of Alb from tubular fluid (J_{Alb})	Accumulation in tubular tissue	Accumulation in bathing solution
	ng/min	*ng/min per mm*	*% cpm removed from tubular fluid*	*% cpm removed from tubular fluid*
[^3H_3C]Alb (n = 6)	0.325±0.020‡	0.0231±0.0030	20.8±4.5	78.2±9.3
^{125}I-Alb (n = 6)	0.333±0.024	0.0331±0.0056	16.0±4.7	83.9±8.9
	$P > 0.5$§	$P > 0.1$	$P > 0.4$	$P > 0.5$

* Albumin (Alb) concentration in perfusate = 0.03 mg/ml. ‡ Results are expressed as mean±SE. n = number of tubules. § Differences between values of [^3H_3C]Alb and ^{125}I-Alb groups were not statistically significant (independent t test).

fluid, the uptake of [^{14}C]inulin by tubular cells was measured in tubules perfused in the absence or presence of high perfusate [Alb] (2.0 mg/ml). Since inulin does not bind significantly to the luminal membrane and does not cross cellular membranes directly, its incorporation into tubular cells must occur by fluid endocytosis (nonadsorptive endocytosis). Consequently, tubular cell uptake of inulin must be directly proportional to the rate of formation of endocytic vesicles. Table III summarizes the results of the [^{14}C]inulin accumulation experiments. As expected, uptake of inulin was extremely small, ~0.05% of the perfused load of [^{14}C]inulin. More importantly, the rate of tubular uptake of inulin was not significantly different when tubules were perfused with 2.0 mg/ml Alb or without Alb. These data indicate that the rate of endocytic vesicle formation is not altered by the presence of Alb in tubular fluid and suggests that the high capacity transport system for Alb is not simply due to a stimulation of endocytosis by increasing concentrations of this protein in the tubular fluid.

Influence of charge

The effects of the net charge of albumin on its tubular absorption were tested by perfusing a separate group of tubules with derivatized cationic albumin (Alb$^{\oplus}$) at perfusate concentrations of 0.1–1.0 mg/ml. Fig. 7 shows that at [Alb$^{\oplus}$] of 0.1 mg/ml, $J_{Alb^{\oplus}}$ was 0.25±0.04 ng/min per mm (n = 6), a value fivefold higher ($P < 0.001$) than the corresponding rate of absorption of the native anionic Alb (J_{Alb} = 0.05±0.005 ng/min per mm, n = 6). On the other hand at [Alb$^{\oplus}$] of 1.0 mg/ml the $J_{Alb^{\oplus}}$ of 0.64±0.12 ng/min per mm (n = 6) was significantly smaller ($P < 0.01$) than the absorption rate of native Alb at the same perfusate concentration (J_{Alb} = 1.63±0.32 ng/min per mm, n = 3). This may be due to the toxic effects of high concentrations of Alb$^{\oplus}$ which could affect the integrity of tubular functions and, consequently, its own cellular uptake. Indeed, as shown in Fig. 8, higher concentrations of Alb$^{\oplus}$ but not of native Alb significantly decrease fluid reabsorption in isolated perfused proximal convoluted tubules of the rabbit.

Discussion

In the present study [^3H_3C]albumin was used to measure the tubular absorption of albumin in isolated perfused proximal convoluted tubules of the rabbit. The labeling of proteins with ^{3}H by the reductive methylation procedure of Tack et al. (23) leads to radioisotopic specific activities as high or higher than

Table II. [^3H_3C]Albumin Absorption and Fluid Reabsorption in Proximal Convoluted Tubules

Experiment	$\dot{V}_p$	P_{in}	C_{in}	$(C/P)_{in}$	P_{Alb}	C_{Alb}	$(C/P)_{Alb}$	J_{Alb}	J_v
	nl/min	*dpm/nl*	*dpm/nl*		*mg/ml*	*mg/ml*		*ng/min per mm*	*nl/min per mm*
1	11.3	9.0	10.4	1.15	0.030	0.031	1.04	0.0261	1.29
2	11.1	9.1	10.2	1.12	0.030	0.030	1.00	0.0323	1.11
3	13.3	9.0	9.4	1.04	0.030	0.029	0.97	0.0270	0.56
4	11.9	11.8	14.3	1.22	0.030	0.031	1.03	0.0250	1.04
5	11.1	11.8	12.6	1.07	0.030	0.031	1.03	0.0147	0.72
6	10.1	11.9	12.5	1.05	0.030	0.031	1.02	0.0134	0.74
mean±SE	11.5±0.4	10.4±0.6	11.6±0.8	1.11±0.03	0.030	0.0305±0.0003	1.02±0.01	0.0231±0.0030	0.91±0.11

$\dot{V}_p$ is the perfusion rate; P_{in} and C_{in} are [^{14}C]inulin concentration of the perfusate standard and collected fluid respectively; $(C/P)_{in}$ is the ratio of [^{14}C]inulin concentration in the collected fluid and perfusate standard; P_{Alb} and C_{Alb} are albumin (Alb) concentrations of the perfusate standard and collected fluid, respectively. $(C/P)_{Alb}$ is the ratio of albumin concentration in the collected fluid and perfusate standard.

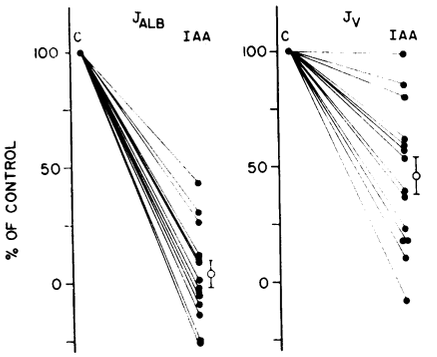

Figure 4. Effect of 4 mM iodoacetate on albumin absorption and fluid reabsorption by proximal convoluted tubules. 14 tubules were perfused with albumin concentrations ranging from 0.0012 to 2 mg/ml. After control periods (C) 4 mM iodoacetate (IAA) was added to the bathing solution. Values for J_{ALB} and J_v after IAA are expressed as percent of control values (C = 100%). Each individual experiment is represented and the mean±SE values for all 14 tubules are depicted by the open circles and bars. IAA almost completely abolished J_{ALB} and significantly decreased J_v ($P < 0.0001$, paired t test). The IAA-induced inhibition of J_{ALB} was significantly more pronounced than that of J_v ($P < 0.05$, independent t test).

those obtained by iodination of albumin with ^{125}I. The procedure has the advantage of not altering the physico-chemical or biological properties of protein molecules (23). The coelution of $[^3H_3C]$albumin and unlabeled albumin shows that the molecular

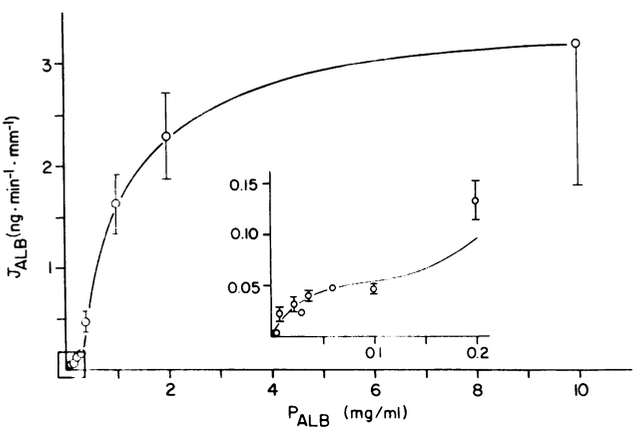

Figure 5. Kinetics of albumin absorption in proximal convoluted tubules. J_{Alb} are plotted against perfusate concentrations of albumin (P_{ALB}). Each of 57 tubules was perfused with a fixed P_{ALB} ranging from 0.0012 to 10 mg/ml. Values are mean±SE of at least three tubules. The absorption curve has at least two components: an overall high capacity-low affinity system (J^{max}_{Alb} = 3.7 ng/min per mm tubule length, K_m = 1.2 mg/ml) and a low capacity system (inset), which saturates near the physiological ranges of tubular fluid albumin concentrations in mammals (J^{max}_{Alb} = 0.0064 ng/min per mm tubule length, K_m = 0.031 mg/ml).

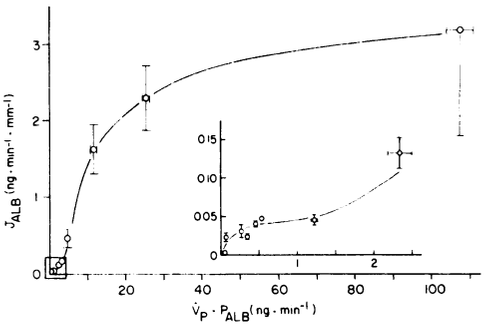

Figure 6. J_{Alb} as a function of albumin perfused loads ($\dot{V}_p \cdot P_{ALB}$) in proximal convoluted tubules. From the same experiments depicted in Fig. 5. Perfusion flow rates ($\dot{V}_p$) varied between 10 and 15 nl/min.

size of albumin is not altered by the labeling procedure (Methods, Fig. 1). The tubular handling of $[^3H_3C]$albumin did not differ substantially from that of ^{125}I-albumin (Results, Table I), indicating that reductive methylation per se does not alter the tubular absorption of albumin. Other possible artifacts such as absorption of spontaneously dissociated label and nonspecific leaks of $[^3H_3C]$albumin were shown to play a minimal role under the conditions of the present experiments (Results, Fig. 3). The methodology used in the present study cannot unmask possible subtle molecular differences between labeled and unlabeled albumins. It is unlikely, however, that such differences, if present, will markedly influence the tubular processing of the protein. Consequently, the tubular handling of $[^3H_3C]$albumin was considered to accurately reflect that of unlabeled albumin. The main advantages of using labeled albumin are: (*a*) radioisotopic determinations are inherently more precise than any of the chemical and immunological methods available for the measurements of albumin, particularly in the nanogram range used in the microperfusion experiments; (*b*) the nonspecific leak of albumin can be precisely quantitated by measuring the rate

Table III. Proximal Convoluted Tubule Uptake of $[^{14}C]$Inulin

P_{Alb}	$\dot{V}_p$	J_v	Accumulation of ^{14}C-inulin in tubular tissue*	
mg/ml	*nl/min*	*nl/min per mm*	*cpm/h per mm*	*% of perfused load*
2.0 (n = 4)	10.31±0.31	0.49±0.15	6.4±1.6	0.05±0.02
0 (n = 4)	11.45±0.51	0.59±0.15	9.7±1.7	0.06±0.02
	$P > 0.1$‡	$P > 0.5$	$P > 0.2$	$P > 0.5$

P_{Alb}, perfusate concentration of albumin; $\dot{V}_p$, perfusion rate; J_v, fluid reabsorption rate; n, number of tubules.
* Perfusate concentration of $[^{14}C]$inulin was 17 μCi/ml (8.0 mg/ml).
‡ Differences between the two groups were not statistically significant (independent t test).

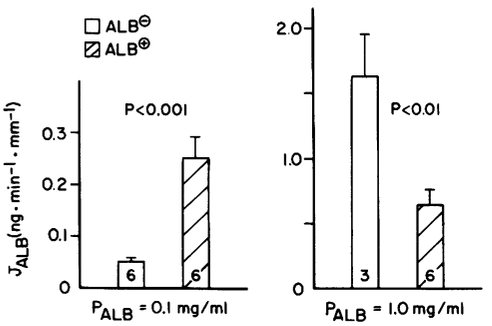

Figure 7. Effects of net charge on tubular absorption of albumin in proximal convoluted tubules. Tubules were perfused with native anionic albumin ($ALB^{\ominus}$) or derivatized cationic albumin ($ALB^{\oplus}$) at perfusate albumin concentrations (P_{ALB}) of 0.1 or 1.0 mg/ml. J_{ALB} are expressed as mean±SE and the numbers within the bars indicate the number of tubules; P = probability that the values are identical (independent t test). The low value of $J_{ALB^{\oplus}}$ at the higher concentration is probably due to the deleterious effect of high concentrations of cationic albumin on tubular function (see text and Fig. 8).

of appearance of intact [^3H_3C]albumin in the bathing solution; and (*c*) the fate of absorbed albumin (accumulation in tubular tissue, intracellular hydrolysis, delivery of hydrolytic products to the peritubular bathing solution) can be determined by the measurement of the radioactivity accumulated in tubular tissue and analyzing the TCA solubility of the radioactivity present in the peritubular bathing solution. The use of the isolated perfused proximal convoluted tubule of the rabbit allows for the establishment of known perfused loads of albumin and, therefore, tubular absorption rates and the fate of the absorbed albumin can be precisely determined.

The accumulation rate of [^3H_3C]albumin and ^{125}I-albumin in proximal tubular tissue is equivalent to that determined by Bourdeau et al. (12) using ^{125}I-albumin in the same preparation. However, as shown by the present study this rate grossly underestimates the tubular absorption of albumin, since at perfusate [Alb] = 0.03 mg/ml it accounts for only ~20% of the rate of

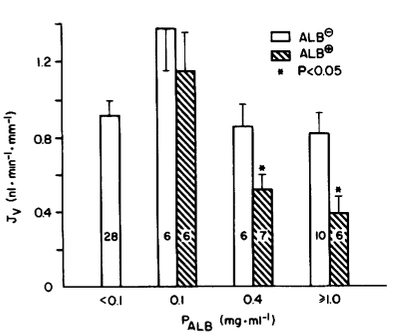

Figure 8. Effect of native anionic albumin ($ALB^{\ominus}$) and derivatized cationic albumin ($ALB^{\oplus}$) on fluid reabsorption in proximal convoluted tubules. Tubules were perfused with the indicated concentrations of albumin. Higher concentrations of $ALB^{\oplus}$ (>0.4 mg/ml) but not $ALB^{\ominus}$ significantly decreased J_v.

removal of [^3H_3C]albumin or ^{125}I-albumin from the tubular fluid. The bulk (80%) of the absorbed labeled albumins is delivered to the bathing solution as TCA-soluble labeled catabolites. It is known that absorbed albumin is localized within phagolysosomes of proximal tubular cells and eventually hydrolyzed within these cell organelles (11, 12). The present results demonstrate that at low tubular fluid [Alb], the rate of intracellular hydrolysis accompanies paripassu the rate of absorption of albumin and that the resulting catabolites are rapidly returned to the peritubular bathing solution. In steady state conditions the actual rate of hydrolysis of albumin must equal its rate of absorption, otherwise there would be an infinite accumulation of this protein in the tubular cells. In the present conditions, this steady state was at least nearly accomplished since 80% of the absorbed albumin was hydrolyzed in the course of the perfusion. It is possible that the 20% accumulation in tubular tissue represents the loading of lysosomes in the intitial period of perfusion. Thereafter the rate of hydrolysis may have been equal to the rate of absorption. It cannot be ruled out, however, that in the conditions prevailing in the present experiments there is a small continued cell accumulation over the course of the perfusion. The relative proportion of absorbed albumin that remains in tubular tissue by the end of perfusion was determined in the present experiments at one particular low perfusate [Alb]. Since the kinetics of hydrolysis of absorbed Alb is unknown, it is not possible to predict from the present data the relative accumulation of albumin at higher perfused loads of the protein. In any event, the results demonstrate that tubular absorption of albumin must be determined by the rate of removal of protein from the tubular fluid rather than by its rate of accumulation in renal cells.

The general characteristics of the process of tubular absorption of albumin, as determined in the present study, do not differ qualitatively from those of low molecular weight proteins, as previously determined in our laboratory in the isolated perfused rat kidney (1–7). Thus, tubular absorption of albumin is a saturable process which is inhibited by iodoacetate. Since it was previously shown that absorption of low molecular weight proteins is inhibited by other metabolic inhibitors as well as iodoacetate it is likely that albumin absorption is also directly or indirectly dependent on cell energy (1). The present data demonstrate that at low loads the albumin concentration in collected tubular fluid does not differ substantially from the albumin concentration in the perfused fluid, indicating that under physiological conditions albumin absorption is proportional to fluid reabsorption. Although the large scatter of the data in micropuncture studies preclude definitive comparisons, apparently there is also a parallel albumin and fluid reabsorption in proximal convoluted tubules in the intact dog and rat (14–21).[2] However, protein uptake and fluid reabsorption occur by

2. The reported individual values for the normal albumin concentration in glomerular filtrate and proximal tubular fluid scatter over a range of 10-fold or more even in micropuncture samples obtained by the same group of investigators in a single animal (14–22). The scatter is still

different mechanisms and can be dissociated. Thus, in previous studies we have demonstrated that cytochalasin B markedly inhibits the tubular absorption of several low molecular weight proteins at concentrations that do not affect fluid reabsorption (1, 7). In addition, the present data show that 4 mM iodoacetate inhibits albumin absorption to a much greater extent than fluid reabsorption. Finally, we have recently reported that 0.1 mM chloroquine almost completely inhibits albumin uptake in isolated perfused proximal convoluted tubules of the rabbit while fluid reabsorption remains normal (29). These data taken as a whole indicate that there is no tight coupling between albumin uptake and fluid reabsorption. Whether the proportionality of these two processes under normal conditions is simply coincidental or due to some physiological interaction cannot be decided by the present experiments.

The kinetics of albumin absorption is characterized by an overall high capacity (J_{Alb}^{max} = 3.7 ng/min per mm tubule length) and a relatively low affinity (apparent K_m = 1.2 mg/ml) compared with the normal filtered loads of this protein. This kinetic characteristic is similar to that previously described by our laboratory for low molecular weight proteins (1–7). If the length of the proximal convoluted tubule is taken as 6 mm, maximal absorption of albumin by this tubule segment would be ~20 ng/min, or about two orders of magnitude greater than the estimated normal nephron loads of albumin in the mammalian kidney (14–22). In the linear range of the albumin absorption curve (Figs. 5 and 6) fractional absorption of albumin is relatively constant, amounting to ~10% of the load per millimeter tubule length, which corresponds to an absorption of 50–60% of the load by the end of the proximal convoluted tubule. This value closely agrees with that derived from micropuncture studies in the intact dog and rat (14–21).

In addition to the high capacity transport system the present study unveiled the presence of a lower capacity system with a J_{Alb}^{max} = 0.064 ng/min per mm tubule length and an apparent K_m = 0.031 mg/ml. Based on clearance studies in the rabbit, Nomiyama and Foulkes (30) suggested that absorption of cadmium-metallothionein is also characterized by the presence of a dual kinetics of absorption. The kinetic parameters of the low absorptive capacity system for albumin are about two orders of magnitude lower than those of the high absorptive capacity system and, therefore, this system is operational in the lower range of tubular fluid [Alb] (up to 0.1–0.2 mg/ml). Based on micropuncture data in normal rats and rats made proteinuric by the administration of aminonucleoside (16, 17), angiotensin (19), or relatively low doses of anti-glomerular basement membrane antibody (20–22), several investigators concluded that albumin absorption saturates at near physiological albumin filtered loads. Tubular fluid [Alb] in these studies were reported to range from about 0.01 mg/ml in normal rats to about 0.1 mg/ml in proteinuric rats with a very large scatter of the data in both conditions (16, 17, 19–22, see footnote 2). The reported values for the glomerular filtrate concentrations of albumin in the micropuncture studies encompass the operational range of the lower absorptive capacity system described in the present study. Consequently, the failure to detect a high absorptive capacity system may have been due to the relatively low tubular fluid [Alb] attained in the proteinuric models in the micropuncture experiments. In this regard it is of interest that much higher albumin rates were reported in nephritic rats with a tubular fluid [Alb] > 1 mg/ml (18, 22). Under these experimental conditions, however, the decreased tubular flow rate and tubular lumen dilation made it difficult to interpret the results (18, 22). The present study demonstrates that the high absorptive capacity system for albumin may operate in conditions of normal tubular function and flow rates. Indeed, there was no significant difference between perfusion and fluid reabsorption rates in tubules perfused with low or high concentrations of native anionic albumin.

The exact mechanism(s) of the dual kinetics of albumin absorption in the isolated perfused proximal tubule cannot be fully clarified by the present experiments. It cannot be ruled out that the luminal membrane has two or more binding sites for albumin. Another possible mechanism to account for the present observations would be the stimulation of the rate of endocytic vesicle formation by high luminal fluid [Alb]. This is apparently not the case, however, since inulin uptake (a measure of fluid volume endocytosis and, consequently, of the rate of formation of endocytic vesicles) was not increased by tubular fluid [Alb] as high as 2.0 mg/ml (Table III, see Results). The possibility that we favor is that the dual kinetics of albumin uptake is due to a combination of adsorptive endocytosis and bulk incorporation of albumin aggregates by fluid endocytosis. Fig. 9 depicts diagrammatically and the text below describes how the combination of these two modes of endocytosis could account for the present observations.

At low tubular fluid [Alb] (<0.2 mg/ml) adsorptive endocytosis (Fig. 9 *A*) would account for the bulk of albumin absorption. Saturation of adsorptive endocytosis would occur when endocytic site binding capacity is exceeded. Since albumin is a relatively large anionic protein it is not surprising that the capacity of the adsorptive endocytic mechanism for this protein is relatively low. On the other hand, at the higher tubular fluid [Alb], bulk incorporation of albumin aggregates by fluid endocytosis may be responsible for the high absorptive capacity

larger in proteinuric models (16, 17, 19–22). As pointed out by Oken and Flamenbaum (16) and Oken et al. (17) contamination of tubular fluid samples with extratabular fluid rich in albumin may contribute to the scatter and to an overestimate of the normal concentrations of albumin in tubular fluid. On the other hand it is also possible that binding of albumin to the siliconized glass micropipettes, particularly at the low albumin concentrations expected to be present in the tubular fluid, contributed significantly to the scatter found in micropuncture experiments (see Methods). This artifact, if present, would tend to underestimate the concentrations of albumin in tubular fluid. Finally, it cannot be ruled out, particularly in proteinuric models, that the scatter is due to a true heterogeneity of glomerular permselectivity among different nephrons of the same kidney. In any case, the wide scatter of the data in micropuncture experiments precludes a precise quantitation of the kinetics of albumin absorption and its relationship with fluid reabsorption in proximal tubules of the intact rat or dog.

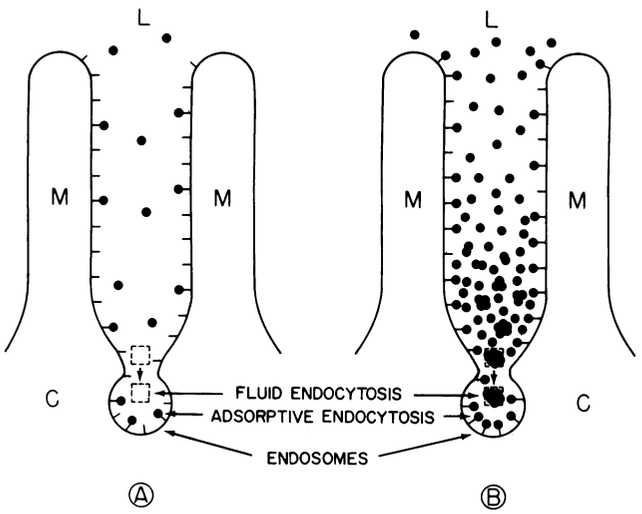

Figure 9. Diagrammatic representation of a hypothetical qualitative model to explain the dual kinetics of albumin absorption described in the present study. The luminal membrane of proximal convoluted tubules is represented with the tubular lumen (*L*), microvilli (*M*), endosomes, and the cell interior (*C*). Albumin molecules are represented by filled circles, binding sites by bars, and vectorial incorporation into endosomes (fluid endocytosis) by the squares with the arrows. Albumin may be absorbed by two distinct kinetic processes. (*A*) binding of albumin to a finite number of low affinity binding sites with subsequent incorporation into the endosome (adsorptive endocytosis). This mechanism may be responsible for the low capacity system that operates at near physiological ranges of tubular fluid albumin concentrations. (*B*) Aggregation of albumin molecules near the base of the microvilli with subsequent bulk incorporation into endosomes (fluid endocytosis). This mechanism may be responsible for the high capacity system of albumin adsorption and operates at abnormally high tubular fluid concentrations of albumin. For details, see text.

system (panel B, Fig. 9). The ultrastructural and functional characteristics of the proximal tubule favor such a possibility. Thus, the rapid removal of fluid via active Na transport in all likelihood increases the concentration of free albumin near the endocytic sites at the base of the microvilli. The aggregation of protein molecules is more likely to occur at the endocytic sites than in the tubular lumen proper, in view of the narrower geometric spaces at the base of the microvilli. Aggregation will occur when there is a sufficient number of free albumin molecules in the fluid phase near the endocytic sites. Consequently, bulk uptake of albumin aggregates is only likely to play an important role at tubular fluid [Alb] above the saturation of the adsorptive endocytic mechanism. Access of albumin to the narrow spaces between the microvilli becomes the rate-limiting step in this mode of uptake. As proposed previously by our laboratory (see selective constraint model for protein absorption, reference 7), access of proteins to the endocytic sites at the base of the microvilli is limited by electrical and geometrical constraints. The relatively large size and negative net charge of albumin makes it likely that the luminal negative surface charges will eventually impede further access of this protein to the endocytic sites. At this point the overall capacity of albumin absorption would reach its limit. Although the above proposed hypothesis conforms to the present data and to our present knowledge of the endocytic process in renal tubular cells, further work is needed to test its validity.

Cationization of albumin led to an enhanced tubular uptake of this protein when tubules were perfused with low cationic [Alb](<0.1 mg/ml). This finding is in agreement with the previous morphological data of Christensen et al. (9) which show that derivatized cationic ferritin binds to the luminal membrane and is incorporated into endocytic vesicles to a greater extent than the native anionic ferritin. These observations were performed after perfusion of rat proximal tubules with relatively high concentrations of ferritin (7 mg/ml). In our case, perfusion of rabbit proximal tubules with higher tubular fluid cationic [Alb] (1.0 mg/ml) showed a decrease rather than an increase in tubular absorption of this protein as compared with that of native anionic albumin at the same concentration (Fig. 7). This decrease was accompanied by a marked inhibition of fluid reabsorption (Fig. 8). Therefore, at higher concentrations this cationic compound had deleterious effects on proximal tubular function. This is not surprising since previous authors have shown that cationic macromolecules, including cationized albumin, alter the morphology and function of epithelial structures (31–34). Furthermore, perfusion of isolated rat kidneys with 5 mM of the cationic amino acid lysine leads to a disruption of microvilli and inhibition of tubular absorption of β_2-microglobulin (7). All of these findings indicate that care must be exercised in the interpretation of the results of experiments testing epithelial cell uptake of cationic macromolecules. In any event, the higher absorption rate of cationic albumin at low tubular fluid concentrations of this protein is consistent with the view that net charge is an important determinant of the tubular uptake of proteins (7–10). As previously pointed out, the enhanced tubular absorption of cationic proteins may be due to a combination of its more extensive binding to anionic sites at the luminal membrane and its easier access to the endocytic sites at the base of the microvilli (7).

In view of the kinetic characteristics of the tubular absorption process of albumin, an increase in filtered loads of albumin may lead to albuminuria much before the saturation of tubular uptake of albumin has been reached. In overload conditions, due to the low affinity of the absorption system, there will be a proportionality between filtered loads and urinary excretion rates of albumin, a phenomenon that can be mistakenly interpreted as saturation of absorption. However, tubular absorption rates will continue to increase as filtered loads rise. Consequently, in severe proteinurias, albuminuria may be accompanied by an increase in renal catabolism of albumin (35) and possibly by the deposition of albumin absorption droplets within renal tubular cells (36). Both phenomena may be of pathophysiological significance in humans. Increased renal catabolism of albumin may contribute to the accelerated plasma turnover of this protein, e.g., in the nephrotic syndrome. Deposition of albumin absorption droplets within renal cells may cause tubular damage

and contribute to the deterioration of renal function in glomerular diseases.

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