Disposition Characteristics of Protein Drugs in the Perfused Rat Kidney

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The renal disposition characteristics of ^{111}In-labeled neocarzinostatin (NCS), soybean trypsin inhibitor (STI), and superoxide dismutase (SOD) were studied in the perfused rat kidney. In a single-pass indicator dilution experiment, venous and urinary recovery profiles and tissue accumulation of proteins were determined under filtering or nonfiltering conditions. In the nonfiltering kidney perfusion experiment, no significant tissue accumulation was observed, suggesting minimal uptake from the glomerular and peritubular capillary sides. Therefore, tissue recovery corresponded to that with tubular reabsorption after glomerular filtration. The total amount of NCS or STI being filtrated through glomeruli, the sum of tissue and urinary recoveries, was similar to that of inulin, but that of SOD was about half. Similarly, the steady-state distribution volumes (V_d) of NCS and STI obtained by moment analysis of their venous outflow curves were similar to that of inulin, while the V_d value of SOD was significantly lower. These results suggest the restricted passage of SOD through the glomerular and postglomerular capillary wall. The tubular reabsorption ratio of proteins against the total filtrated amount decreased with an increase in the administered dose, suggesting nonlinearity of reabsorption. SOD had the largest reabsorption ratio. Thus, this experimental system is useful for quantitative analysis of renal disposition of proteins.

KEY WORDS: protein drugs; rat kidney perfusion; moment analysis; glomerular and postglomerular permselectivity; tubular reabsorption.

INTRODUCTION

With the progress of biotechnology, various peptides and proteins have attracted great interest as drug candidates. However, their clinical application is often limited by their rapid *in vivo* clearance. The kidney plays an important role in the disposition of protein drugs since proteins with molecular weights of less than 30 kDa are filtrated through glomeruli (1) and proteins are also metabolically degraded in this organ (2,3).

We have examined the *in vivo* behavior of some protein drugs and their conjugates with macromolecules (4–6). The results showed that the small proteins accumulate in the kidney and/or are excreted in the urine. Since the renal disposition of proteins involves several processes in the kidney such as glomerular filtration, tubular reabsorption, and interaction at the capillary side, it is necessary to quantitatively clarify these processes in order to construct a strategy for their administration and design protein–macromolecule conjugates.

In a previous study, we developed an experimental system to quantitatively investigate the renal disposition characteristics of macromolecules using a single-pass indicator dilution technique and statistical moment analysis in the perfused rat kidney (7). In this study, the system was applied to assess the renal disposition characteristics of three types of proteins with different molecular weights, i.e., an antitumor antibiotic neocarzinostatin (NCS; MW 12,000), soybean trypsin inhibitor (STI; MW 20,100), and human recombinant superoxide dismutase (SOD; MW 32,000).

MATERIALS AND METHODS

Chemicals

NCS (NW = 12,000, p*I* = 3.2), STI (MW = 20,100, p*I* = 4.5–4.6), and SOD (MW = 32,000, p*I* = 5.0–5.2) were supplied by POLA Cosmetics, Yokohama, Fuji Seiyu, Osaka, and Asahi Kasei, Tokyo, respectively. [methoxy-^{14}C]Inulin (185 MBq/g) was purchased from New England Nuclear (Boston, MA). [^{111}In]Cl_3 (74 MBq/mL) was a gift from Nihon Mediphysics, Co., Takarazuka, Japan. All other chemicals were reagent-grade products obtained commercially.

Radiolabeling of Proteins

Proteins were radiolabeled with ^{111}In using a bifunctional chelating agent, diethylenetriaminepentaacetic acid (DTPA) anhydride (Dojindo Labs, Kumamoto, Japan), according to the method of Hnatowich *et al.* (8).

Isolated Rat Kidney Perfusion

The kidney was isolated according to the method reported previously (7). The perfusate consisted of Krebs–Henseleit bicarbonate buffer (pH 7.45) containing glucose (5 m*M*) and BSA (5%), gassed with 95% O_2/5% CO_2. The filtering kidney was prepared by maintaining renal arterial pressure at 70–80 mm Hg by adjusting the perfusate flow rate. The nonfiltering kidney was prepared by tying off the ureters, raising the perfusate albumin concentration to 10%, and lowering the renal arterial pressure to 55 mm Hg.

In this perfusion system, the perfusate flow rate (PFR; 17.9 ± 1.9 mL/min, mean ± SD; n = 44), glomerular filtration rate (GFR; 0.41 ± 0.10 mL/min; n = 4), perfusion pressure (73 ± 9 mm Hg; n = 44), urine flow rate (50–100 μL/min), and glucose reabsorption ratio (>90%) were maintained almost constant, comparable with those reported in other studies (9,10). Although erythrocytes were not added to the perfusate and the PFR was three to four times greater than normal, kidney function was maintained (11).

Indicator Dilution Experiment

Saline (0.14 mL), dissolved BSA (5%), and test protein

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drugs were introduced into the arterial catheter using a six-position rotary valve injector (Type 50 Teflon rotary valves, Rheodyne, Cotati, CA) (7). Venous outflow samples were collected into the tube at 0.3- to 3-sec intervals for 20 sec and urine samples were collected at 1- or 2-min intervals for 14 min after injection. After the perfusion, the kidney wet weight was determined and the renal cortex and medulla were separated for assay.

Gel Filtration

To detect degradation products of protein drugs, gel filtration was performed using Sephadex G-25 (0.8 × 20 cm). Urine and perfusate samples (0.1 mL) were applied to the columns and eluted with 0.25 *M* phosphate buffer.

Assay

^{14}C radioactivity was determined with a liquid scintillation counter (LSC-5000, Beckman, Tokyo) and the ^{111}In radioactivity was measured with a well NaI scintillation counter (ARC-500, Aloka, Tokyo) (7).

Data Analysis

The statistical moment parameters for the outflow pattern were calculated as follows (12,13):

$$AUC = \int_0^\infty C dt$$

$$MTT_{kid} = \int_0^\infty t\, C dt / AUC$$

where t is time and C represents the concentration of compounds normalized by the injection dose. AUC and MTT_{kid} denote the area under the concentration–time curve and mean transit time of the drug in the kidney, respectively. These parameters were calculated by numerical integration using a linear trapezoidal formula and extrapolation to infinite time, based on a single-exponential equation. The steady-state distribution volume was calculated from the moment parameters as follows:

$$F_o = AUC\ Q$$
$$V_d = Q\ MTT_{kid}/F_o$$

where F_0 corresponds to the venous outflow recovery ratio, Q represents the perfusion flow rate, and V_d is the steady-state distribution volume. MTT_{kid} and V_d were corrected by the subtraction of catheter transit time and catheter volume, respectively.

The urinary excretion rate–time curve is also analyzed based on statistical moment theory as follows:

$$F_u = \int_0^\infty (dX_u/dt)dt$$

$$MTT_u = \int_0^\infty t(dX_u/dt)dt/F_u$$

where F_u corresponds to the urinary recovery ratio, and dX_u/dt is the urinary excretion rate normalized with the injection dose and MTT_u is the urinary mean excretion time.

RESULTS

Venous Recovery of Proteins

Figure 1 illustrates typical outflow concentration–time curves of ^{111}In-labeled proteins together with those of ^{111}In-BSA and ^{14}C-inulin after bolus injection. In all cases, more than 95% of the compounds was detected in venous outflow samples during the first 10 sec after injection. The outflow pattern of SOD was similar to that of BSA. In contrast, NCS and STI showed a decreased peak concentration identical to that of inulin. The moment parameters and steady-state distribution volumes (V_d) for these proteins and inulin at different doses were calculated from these outflow patterns and are summarized in Table I. The V_d values of inulin and BSA, which correspond to extracellular volume and intravascular volume, respectively, were 0.45 and 0.25 mL/g (14). At all doses, SOD and BSA showed almost identical V_d values, while those of NCS and STI were slightly higher than that of BSA.

Urinary Excretion Patterns of Proteins

Figure 2 shows typical urinary excretion rate–time curves of three ^{111}In-proteins, ^{111}In-BSA, and ^{14}C-inulin. All test substances except for BSA were excreted in the urine within 14 min after bolus injection. Moment parameters were calculated from these patterns as shown in Table I. Similar MTT_u values were obtained for all substances at all injection doses. The urine samples subjected to gel filtration contained no degradation products with low molecular weights.

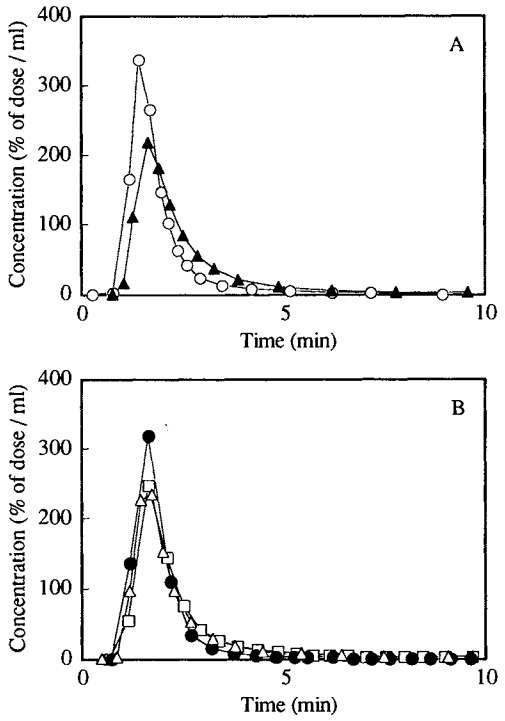

Fig. 1. Typical outflow curves for proteins and inulin in the perfused rat kidney after bolus injection. (A) ○, ^{111}In-BSA; ▲, ^{14}C-inulin. (B) ●, ^{111}In-SOD; △, ^{111}In-STI; □, ^{111}In-NCS.

Table I. Moments and Distribution Volumes of Protein Drugs, BSA, and Inulin in the Isolated Rat Kidney Perfusion Experiment[a]

	Dose (mg/kidney)	AUC (% of dose sec/mL)	MTT_{kid} (sec)	V_d (mL/g kidney)	MTT_u (min)
^{111}In-NCS	0.014	315.8 ± 33.1	1.519 ± 0.240	0.414 ± 0.027	1.779 ± 0.872
	0.14	336.2 ± 35.9	1.298 ± 0.164	0.384 ± 0.013	2.049 ± 0.392
	1.4	303.6 ± 14.8	1.455 ± 0.106	0.414 ± 0.028	2.195 ± 0.733
^{111}In-STI	0.014	341.5 ± 29.5	1.540 ± 0.189	0.429 ± 0.009	1.843 ± 0.509
	0.14	309.8 ± 13.4	1.401 ± 0.138	0.411 ± 0.034	1.810 ± 0.320
	1.4	325.5 ± 22.4	1.440 ± 0.177	0.412 ± 0.008	1.417 ± 0.199
^{111}In-SOD	0.014	310.3 ± 30.6	0.913 ± 0.125	0.275 ± 0.025	2.452 ± 0.625
	0.14	353.4 ± 14.1	0.905 ± 0.071	0.275 ± 0.018	1.844 ± 0.550
	1.4	320.4 ± 40.1	0.925 ± 0.120	0.255 ± 0.022	1.244 ± 0.259
^{111}In-BSA		325.6 ± 32.3	0.827 ± 0.077	0.254 ± 0.026	N.D.
^{14}C-inulin	0.01	337.8 ± 48.8	1.418 ± 0.095	0.450 ± 0.022	1.917 ± 0.299

[a] Results are expressed as the mean ± SD of at least three experiments.

Tissue Accumulation and Urinary Excretion Amounts of Proteins

Figure 3 shows the tissue accumulation and urinary excretion of radioactivities after bolus injection of ^{111}In-proteins and ^{14}C-inulin at different doses in the filtering kidney perfusion experiment. At all doses, the sum of the amount recovery in the kidney and urine were unchanged for all protein drugs. Absolute amount for NCS and STI were large and comparable to that of inulin, while that of SOD was small (about 40% against inulin) and that of BSA was nearly zero.

As for tissue accumulation, STI showed the largest value and NCS gave the smallest at any dose. However, the ratio of kidney recovery to urinary recovery was the highest for SOD, at 0.14 and 0.014 mg/kidney, while NCS showed the lowest ratios. Recoveries of STI and SOD in the kidney decreased and those in the urine increased with an increase in the injection dose.

Nonfiltering Kidney Perfusion Experiments

Radioactivity recovered in the kidney tissue after injection of ^{111}In-SOD, STI, and NCS at a dose of 0.14 mg/kidney were 0.018, 0.022, and 0.010%, respectively, in the nonfiltering kidney perfusion systems. Thus little accumulation of radioactivity in the kidney was observed for all types of proteins.

Suborgan Distribution of Proteins

Figure 4 shows the radioactivity concentrations of proteins in the cortex and medulla. Concentrations of STI and SOD in the cortex were much higher than those in the medulla at low doses. The percentage doses of STI and SOD in the cortex were inversely related to the injection dose while those in the medulla remained almost constant. Intrarenal distribution of NCS was relatively uniform.

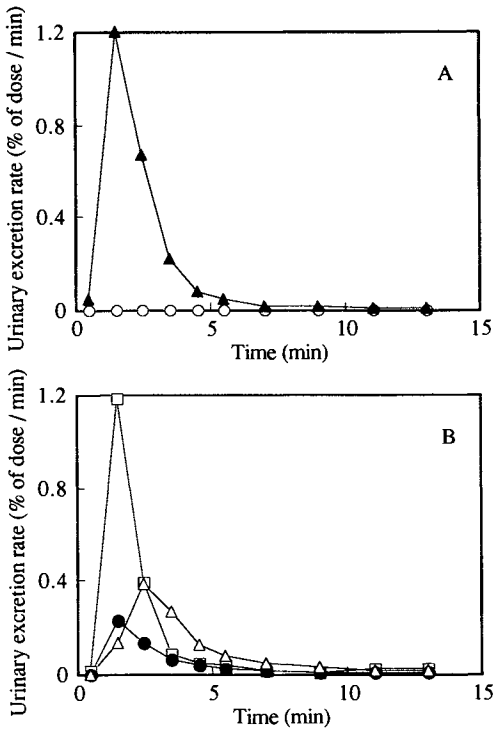

Fig. 2. Typical urinary excretion rate versus time curves for proteins and inulin in the isolated perfused rat kidney after bolus injection. (A) ○, ^{111}In-BSA; ▲, ^{14}C-inulin. (B) ●, ^{111}In-SOD; △, ^{111}In-STI; □, ^{111}In-NCS.

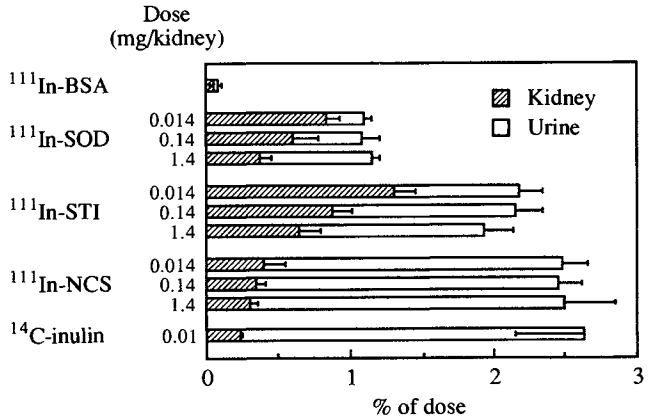

Fig. 3. Amount recovery of proteins and inulin in the kidney and urine at various doses in the filtering kidney perfusion experiment. Results are expressed as the mean ± SD.

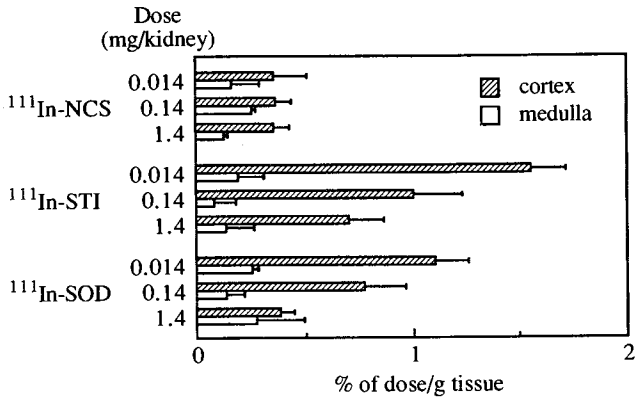

Fig. 4. Concentration of NCS, STI, and SOD in the kidney cortex and medulla. Results are expressed as the mean ± SD.

DISCUSSION

In this study, we selected three model protein drugs: NCS (MW = 12,000, p*I* = 3.2), STI (MW = 20,100, p*I* = 4.5–4.6), and SOD (MW = 32,000, p*I* = 5.0–5.2), because most protein drugs developed by genetic recombination such as interferons (15–25 kDa), interleukin 2 (15 kDa), granulocyte colony-stimulating factor (20 kDa), and erythropoietin (30 kDa) have molecular weights within this range, and the kidney plays a major role in their clearance from the body (15–17). To evaluate the disposition characteristics of proteins separate from metabolic degradation, the proteins were labeled with ^{111}In, which is accumulated in the tissue by being converted to an iron-binding protein after intracellular degradation (18).

In the nonfiltering kidney perfusion experiments, all tested proteins showed little accumulation in the kidney. In addition, we observed the accumulation of eel calcitonin, which is taken up by the kidney via receptor-mediated endocytosis (24). Thus, the validity of the present experimental conditions was confirmed. All proteins used in this study were not taken up from the glomerular and peritubular capillary sides. This suggests that the total amounts recovered in the kidney are equivalent to those taken up by tubular reabsorption after glomerular filtration. Therefore, we can also quantify the extent of glomerular filtration as the sum of the amount recoveries in the kidney and urine in the filtering kidney perfusion experiment.

Figure 5 schematically summarizes the renal disposition profiles of proteins and inulin obtained. We quantitatively determined their renal disposition by separating the processes according to glomerular filtration, tubular reabsorption, and capillary side uptake.

Since the total filtrated amounts of NCS and STI were similar to that of inulin, a marker of the glomerular filtration rate (Fig. 3), the passage of NCS and STI through the glomerular capillary wall seemed to be negligibly restricted. On the other hand, filtration of SOD was considerably reduced suggesting a marked decrease in the permeability of the glomerular capillary wall to macromolecules in this molecular weight range (20,000–32,000). We obtained similar data on urinary excretion of these proteins in *in vivo* studies. These results are in good agreement with the findings on the permselectivity of the glomerular capillary to proteins based upon their molecular sizes (1). In addition, the total filtrated amounts of proteins were unchanged at all doses (Fig. 4), suggesting the linearity of the glomerular filtration process.

Renal tubular epithelial cells, particularly those in the proximal tubule, have the ability to reabsorb proteins from the tubular lumen. In this process, proteins are bound to the luminal cell surface, endocytosed, and then digested in ly-

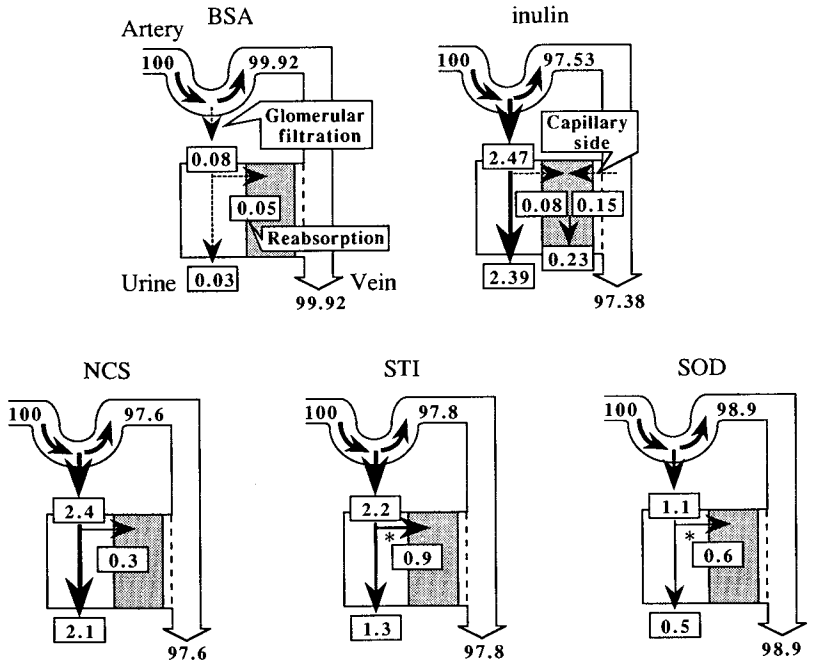

Fig. 5. Renal disposition profiles of proteins and inulin in the kidney perfusion experiment. Values in each process are expressed as percentage of dose. The dose for NCS, STI, and SOD was 0.14 mg/kidney. (*) Nonlinear process.

sosomes of the cells. Although many studies have suggested the participation of physiocochemical properties of proteins in their reabsorption, a clear understanding has not been obtained. One reason might be that most studies have used radioiodinated proteins, which complicated the results because the trace radioactivities were derived largely from metabolic degradation and successive redistribution.

In the present study, the observed luminal sequestration process would be a reflection of reabsorption of intact proteins. The test proteins might be degraded in the tubular lumen by membrane-bound hydrolytic enzymes, followed by rapid reabsorption of ^{111}In-labeled peptides or amino acids. However, this is unlikely since degradation products with small or large molecular weights were not detected in the urine.

Renal reabsorption of proteins can be estimated from the ratio of the amount recovered in the tissue to that being filtrated through glomeruli (sum of recovery amounts in the kidney and urine). The reabsorption process was shown to be a saturable process since the ratio decreased with an increase in the dose. The nonlinear reabsorption could be explained by the saturation of binding of intact proteins to the luminal surface, an initial step of endocytosis. SOD had the highest reabsorption ratio among the tested proteins. Net charge may be an important determinant of renal cell uptake of proteins since cationic proteins were reported to be more susceptible to reabsorption than anionic proteins (19,20). On this point, several studies have suggested that cationic groups such as an amino group of lysine bind to anionic sites of the tubular cell membrane. In the present study, however, all the tested proteins had net negative charges. The difference in numbers of free amino groups (20, 12, and 2 for SOD, STI, and NCS, respectively) or p*I* may account for the differences in uptake. The concentrations of STI and SOD accumulated in the cortex were much higher than those in the medulla at doses of 0.014 and 0.14 mg per kidney. These findings suggest that the renal uptake of proteins mainly occurred at the proximal tubule (21).

Concerning the distribution of proteins in the vascular space, the steady-state distribution volumes (V_d) of STI and NCS were similar to that of inulin, suggesting that STI and NCS readily passed through the postglomerular peritubular capillary wall. On the other hand the V_d value of SOD was comparable to that of BSA, which showed that SOD could not pass through the capillary wall. The postglomerular capillaries have many fenestrae, and small macromolecules such as insulin, calcitonin, and low molecular weight dextran may pass through them and reach the basolateral membranes of tubular epithelium (22–24).

Our findings quantitatively confirmed these phenomena and showed that the critical point existed at a molecular size of 20,000–30,000. The present experimental system using the perfused rat kidney is useful for quantitative analysis of renal disposition of proteins and their various derivatives.

REFERENCES

1. T. Maack, V. Johnson, S. T. Kau, J. Figueiredo, and D. Sigulem. Renal filtration, transport, and metabolism of low-molecular-weight proteins: A review. *Kidney Int.* 16:251–270 (1979).
2. R. Rabkin, M. P. Ryan, and W. C. Duckworth. The renal metabolism of insulin. *Diabetologica* 27:351–357 (1984).
3. H. Sato, K. Yoshioka, T. Terasaki, and A. Tsuji. Receptor-mediated endocytosis of A_{14}-^{125}I-insulin by the nonfiltering perfused rat kidney. *Biochim. Biophys. Acta* 1073:442–450 (1991).
4. T. Fujita, Y. Yasuda, Y. Takakura, M. Hashida, and H. Sezaki. Alteration of biopharmaceutical properties of drugs by their conjugation with water-soluble macromolecules: Uricase-dextran conjugate. *J. Control. Release* 11:149–156 (1990).
5. T. Fujita, M. Nishikawa, C. Tamaki, Y. Takakura, M. Hashida, and H. Sezaki. Targeted delivery of human recombinant superoxide dismutase by chemical modification with mono- and polysaccharide derivatives. *J. Pharmacol. Exp. Ther.* 263:971–978 (1992).
6. Y. Takakura, T. Fujita, M. Hashida, and H. Sezaki. Control of pharmaceutical properties of soybean trypsin inhibitor by conjugation with dextran. II. Biopharmaceutical and pharmacological properties. *J. Pharm. Sci.* 78:219–222 (1989).
7. K. Mihara, M. Mori, T. Hojo, Y. Takakura, H. Sezaki, and M. Hashida. Disposition characteristics of model macromolecules in the perfused rat kidney. *Biol. Pharm. Bull.* 16:158–162 (1993).
8. D. J. Hnatowich, W. W. Layne, and R. L. Childs. The preparation and labeling of DTPA-coupled albumin. *Int. J. Appl. Isot. Radiat.* 33:327–332 (1982).
9. F. H. Epstein, J. T. Brosnan, J. D. Tange, and B. D. Ross. Improved function with amino acids in the isolated perfused kidney. *Am. J. Physiol.* 243:F284–F292 (1982).
10. S. Hall and M. Rowland. Influence of fraction unbound upon the renal clearance of furosemide in the isolated perfused rat kidney. *J. Pharmacol. Exp. Ther.* 232:263–268 (1985).
11. D. Alcorn, K. R. Emslie, B. D. Ross, G. B. Ryan, and J. D. Tange. Selective distal nephron damage during isolated kidney perfusion. *Kidney Int.* 19:638–647 (1981).
12. K. Yamaoka, T. Nakagawa, and T. Uno. Statistical moments in pharmacokinetics. *J. Pharmacokinet. Biopharm.* 6:547–558 (1978).
13. T. Kakutani, K. Yamaoka, M. Hashida, and H. Sezaki. A new method for assessment of drug disposition in muscle: Application of statistical moment theory to local perfusion systems. *J. Pharmacokinet. Biopharm.* 13:609–631 (1985).
14. S. W. O'Connor and W. F. Bale. Accessibility of circulating immunoglobulin G to the extravascular compartment of solid rat tumors. *Cancer Res.* 44:3719–3723 (1984).
15. V. Bocci. Metabolism of protein anticancer agents. *Pharmacol. Ther.* 34:1–49 (1987).
16. V. Bocci. Interleukins–Clinical pharmacokinetics and practical implications. *Clin. Pharmacokinet.* 21:274–284 (1991).
17. V. Bocci. Physicochemical and biologic properties of interferons and their potential uses in drug delivery systems. *CRC Crit. Rev. Ther. Drug Carrier Syst.* 9:91–133 (1992).
18. B. A. Brown, R. D. Comeau, P. L. Jones, F. A. Liberatore, W. P. Neacy, H. Sands, and B. M. Gallagher. Pharmacokinetics of the monoclonal antibody B72.3 and its fragments labeled with either ^{125}I or ^{111}In. *Cancer Res.* 47:1149–1154 (1987).
19. B. E. Sumpio and T. Maack. Kinetics, competition, and selectivity of tubular absorption of proteins. *Am. J. Physiol.* 243:F379–F392 (1982).
20. E. I. Christensen, H. G. Rennke, and F. A. Carone. Renal tubular uptake of protein: Effect of molecular charge. *Am. J. Physiol.* 244:F436–F441 (1983).
21. M. A. Cortney, L. L. Sawin, and D. D. Weiss. Renal tubular protein absorption in the rat. *J. Clin. Invest.* 49:1–4 (1970).
22. C. Whiteside and M. Silverman. Determination of postglomerular permselectivity to neutral dextrans in the dog. *Am. J. Physiol.* 245:F496–F505 (1983).
23. C. I. Whiteside, C. J. Lumsden, and M. Silverman. In vivo characterization of insulin uptake by dog renal cortical epithelium. *Am. J. Physiol.* 255:E357–E365 (1988).
24. R. E. Simmons, J. T. Hjelle, C. Mahoney, L. J. Deftos, W. Lisker, P. Kato, and R. Rabkin. Renal metabolism of calcitonin. *Am. J. Physiol.* 254:F593–F600 (1988).