Macromolecule–Macromolecule Interaction in Drug Distribution. III. Kinetic Characterization of the Uptake of Fractionated [^3H]Heparin and the Effect of Plasma Proteins in the Perfused Rat Liver

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The concentration-dependent hepatic uptake of fractionated [^3H]heparin, a macromolecular model drug, was kinetically characterized and the effect of plasma proteins, albumin and α -globulin, was evaluated in the perfused rat liver as part of an ongoing effort to elucidate the mechanism of interaction of macromolecular drugs with biological macromolecules and the role of this interaction in the drugs' distribution. In the absence of proteins, the uptake of fractionated [^3H]heparin was saturable with the maximum uptake velocity ($V_{\max}$) of 7.6 pmol/min/g liver and the Michaelis constant (K_m) of 32.2 nM, suggesting the involvement of a specialized transport. α -Globulin (8.0 mg/ml) reduced the uptake of fractionated [^3H]heparin at lower heparin concentrations. However, albumin (40 mg/ml) did not affect the uptake of fractionated [^3H]heparin, suggesting an insignificant interaction. Assuming that fractionated [^3H]heparin bound to α -globulin cannot be uptaken and that the reduction in uptake was solely attributable to the saturable Scatchard-type binding of fractionated [^3H]heparin to α -globulin, the dissociation constant (K_d) and the binding capacity (n) were estimated to be 2.1 nM and 0.002, respectively.

In *in vitro* binding experiments by ultrafiltration, K_d and n were estimated as 168 nM and 0.5, respectively, for α -globulin and 1021 nM and 0.02, respectively, for albumin, suggesting lower affinity and higher capacity *in vitro* for each protein. The results of *in vitro* binding experiments did not agree with those of perfusion experiments in that the binding of fractionated [^3H]heparin to albumin was significant and the unbound fraction in the presence of α -globulin was smaller than that estimated in the analysis of hepatic uptake. The apparent enhancement of dissociation of fractionated [^3H]heparin in perfusion suggests the participation of "protein-mediated transport," as reported for low molecular weight compounds in the transport of macromolecular compounds.

Thus, the present study successfully characterized the hepatic uptake of fractionated [^3H]heparin as a Michaelis–Menten type process in the perfused rat liver and confirmed the involvement of protein-mediated transport.

Keywords hepatic uptake; fractionated [^3H]heparin; α -globulin; albumin; protein binding; protein-mediated transport

Heparin is a water soluble mucopolysaccharide and has been widely used for more than fifty years as an anti-coagulant.^{1,2)} To accomplish greater control in heparin therapy and to take advantage of newly found biological activities such as anti-human immunodeficiency virus (anti-HIV) activity,³⁾ inhibitory effect on the multiplication of vascular smooth muscle cells⁴⁾ and inhibitory action on the metastasis of cancer,⁵⁾ it is important to clarify the mechanism of disposition in the body. However, the reported disposition characteristics of heparin in the body are quite variable,^{6–8)} partly because commercially available heparin is polydisperse with respect to molecular weight and heterogeneous with respect to its biological and chemical properties. One of our earlier studies using fractionated [^3H]heparin, which has an affinity to protamine and of which molecular weight is approximately 20000 Da, successfully demonstrated this point.^{9,10)} The disposition of fractionated [^3H]heparin after i.v. bolus administration to rats was characterized by the first-order elimination from plasma with extensive accumulation in the liver followed by gradual release to plasma to maintain the pseudo-steady state,¹⁰⁾ whereas the elimination of unfractionated [^3H]heparin from plasma followed the ordinary 2-compartment model.⁹⁾ Thus, the fractionation of heparin is prerequisite for pharmacokinetic studies.

Meanwhile, the plasma protein binding is one of the important pharmacokinetic determinants. We reported the interaction of fractionated [^3H]heparin with several plasma proteins such as α -globulin and albumin by gel filtration

chromatography.¹¹⁾ We also reported the inhibitory effect of α -globulin on the uptake of fractionated [^3H]heparin in the liver perfusion¹²⁾ and the primary culture of parenchymal hepatocytes.^{13,14)} And those studies suggested the involvement of protein-mediated transport or the apparently enhanced dissociation of fractionated [^3H]heparin in the hepatic uptake. However, the effect of plasma proteins on the hepatic uptake of fractionated [^3H]heparin is yet to be clarified: the *in vitro* binding parameters have not been available and the effect of albumin, which is most abundant in plasma, has not been evaluated.

In the present study, we first determined *in vitro* binding parameters of fractionated [^3H]heparin to α -globulin and albumin by ultrafiltration, secondly, examined the effect of albumin as well as α -globulin on the uptake of fractionated [^3H]heparin in the perfused rat liver, and compared *in vitro* binding parameters with those estimated by the kinetic analysis of hepatic uptake, and finally, confirmed the involvement of protein-mediated transport for both α -globulin and albumin. The liver was perfused to evaluate the hepatic uptake of fractionated [^3H]heparin under a more physiologically controlled condition, where the organ architecture is maintained, more than in isolated or cultured cells.

Materials and Methods

Materials [^3H (G)]Heparin sodium salt of porcine mucosal origin (No. 2643-105, specific radioactivity of 0.29 mCi/mg; No. 2643-135, specific radioactivity of 0.2 mCi/mg; M.W. 6000–20000 Da) and Protosol (tissue

solubilizer) were purchased from New England Nuclear (Boston, MA). Unlabeled heparin sodium salt of porcine mucosal origin, bovine α -globulin (Cohn fraction IV-1) and bovine serum albumin (fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO). Scintisol EX-H (scintillation cocktail) was purchased from Dojindo Lab., Kumamoto, Japan. All other chemicals were commercially available and of the highest grade.

Fractionation of Heparin Commercially available [3 H]heparin and unlabeled heparin were fractionated by affinity chromatography on protamine-Sepharose followed by gel filtration chromatography on Sephadex G-100 as described in our previous reports.^{10,15} Fractionated [3 H]heparin and unlabeled heparin of 20000–23000 and 15000 Da were used for the liver perfusion experiments and *in vitro* binding experiments, respectively. The concentration of unlabeled heparin was determined by the carbazole reaction.¹⁶

In Vitro Binding Experiments Test solutions (1 ml) were prepared in phosphate buffered saline (pH 7.4) and contained physiological concentration of α -globulin (8 mg/ml) or albumin (40 mg/ml) and various concentrations of fractionated [3 H]heparin. After incubation for 5 min at 37°C, 400 μ l of each test solution was placed in an ultrafilter tube (Centricut® W-50, Kurashikibouseki Co., Ltd., Osaka, Japan). The samples were centrifuged at 1000 $\times g$ (Tomy MRX-150 rotor) for 5 min at room temperature. Forty-microliter aliquots of filtrate and test solution were taken for the measurement of radioactivity.

Isolated Rat Liver Perfusion The liver perfusion was conducted as described in our previous report, using male Wistar rats (6 or 7 weeks old, approximately 200 g), without fasting.¹⁷ Each rat was anesthetized with urethane (1 g/kg, i.p.), and cannulated into the bile duct, the portal vein and the superior vena cava. The liver was excised and placed in a Millar-type organ perfusion apparatus. The isolated liver was perfused with the Krebs–Ringer buffer (121 mM NaCl, 6 mM KCl, 0.74 mM KH_2PO_4 , 0.6 mM MgSO_4 , 12 mM NaHCO_3) supplemented with 5 mM glucose and constantly gassed with 95% oxygen and 5% carbon dioxide (pH 7.4) by a recirculating method at 37°C and the flow rate of 33 ml/min (hydrostatic pressure of 15–20 cm). The liver was preperfused for about 20 min prior to the addition of various concentrations of fractionated [3 H]heparin and α -globulin (1 or 8 mg/ml) or albumin (40 mg/ml). At the heparin concentrations of 180 and 70 nM, unlabeled fractionated heparin was added to adjust the concentration. One hundred microliter aliquots of perfusate were periodically taken and the bile was collected at 15-min intervals. Five milliliters of Scintisol was added to the samples and the radioactivity was determined by liquid scintillation spectrometry (LCS-1000, Aloka Co., Tokyo). At the end of perfusion, the whole liver was homogenized with 20 ml of saline by Polytron (homogenizer, PT20 “OD” S Kinematica GmbH, Switzerland). About 100 mg of homogenate was solubilized with 1 ml of Protosol (tissue solubilizer) at 55°C for about 30 min, decolorized with 0.2 ml of 30% H_2O_2 at 55°C for 30 min, and added with 5 ml of Scintisol and 0.25 ml of 1 N HCl to determine the radioactivity. As a measure of viability of the liver, glutamic-oxaloacetic transaminase (GOT) activity was measured, using a GOT activity measurement kit (Wako, Japan). Two hundred microliter aliquots of the perfusate were removed for the GOT activity measurement every 10 min after the initiation of perfusion. The GOT activity was in the normal range of 0 to 40 Karmen unit/ml in every perfusion.

Analysis of *in Vitro* Binding Data Assuming a single kind of protein binding for fractionated [3 H]heparin and using the Scatchard protein binding model, the unbound fraction (f) is described as follows:

$$f = \frac{K_d + C_f}{K_d + n \cdot P_t + C_f} \quad (1)$$

where K_d , n , P_t and C_f are the dissociation constant, the binding capacity of the protein, the total concentration of the protein and the free concentration of fractionated [3 H]heparin, respectively. Although α -globulin is heterogeneous, we can estimate the molar concentration (P_t) by assuming the effective molecular weight (M_{eff}) of 1.14×10^6 g/mol.¹⁴ Substituting $C_f = f \cdot C_t$, where C_t is the total concentration of fractionated [3 H]heparin, into Eq. 1, Eq. 1 is solved for f as follows:

$$f = \frac{A + \sqrt{A^2 + 4 \cdot K_d / C_t}}{2} \quad (2)$$

where

$$A = 1 - K_d / C_t - n \cdot P_t / C_t \quad (3)$$

In order to estimate the values of K_d and n in *in vitro*, Eqs. 2 and 3 were

fitted to f versus C_t profiles using a nonlinear regression program Pcnonlin (Statistical Consultants, Inc., Lexington, KY).¹⁸

Analysis of Perfusion Data The remaining fractionated [3 H]heparin in the perfusate (FR) versus time profiles were fitted to the 1-exponential equation, $FR(\%) = FR_0 \times e^{-k_{el} \cdot t}$, where FR_0 is the extrapolated remaining fraction at 0 min and k_{el} is the elimination rate constant, by using a nonlinear regression program, MULTI.¹⁹ The apparent hepatic uptake clearance (CL_h) was obtained by multiplying k_{el} by the distribution volume, $V_d = V / FR_0 \times 100$, where V is the volume of perfusate.

The concentration-dependent hepatic uptake of fractionated [3 H]heparin was analyzed using the Michaelis–Menten type transport model. Assuming that only unbound fractionated [3 H]heparin is taken up by the liver, the uptake rate (J_h) is described as follows:

$$J_h = \frac{V_{\max}}{K_m + C_f} \cdot C_f = CL_h \cdot C_t \quad (4)$$

where $V_{\max}$ and K_m are the maximum uptake rate and the Michaelis constant, respectively. Substituting $C_f = f \cdot C_t$ into Eq. 4 and dividing by C_t , CL_h is described as follows:

$$CL_h = \frac{V_{\max}}{K_m + f \cdot C_t} \cdot f \quad (5)$$

In the following analyses of the data of CL_h , the average concentration (C_{av}), given by dividing the area under the concentration versus time, was used for C_t , and Pcnonlin was used for model fitting. At first, $V_{\max}$ and K_m were estimated by fitting those $V_{\max}$ and K_m fixed, K_d and n were estimated by fitting Eq. 5, where f is described as Eq. 2, to CL_h versus C_t profiles in the presence of α -globulin.

Results and Discussion

In Vitro Binding of Fractionated [3 H]Heparin to α -Globulin and Albumin The unbound fraction of fractionated [3 H]heparin increased with concentration in the presence of α -globulin (8 mg/ml) or albumin (40 mg/ml), suggesting the saturable binding of fractionated [3 H]heparin to those proteins (Fig. 1). By fitting Eq. 2 (the Scatchard model) to the unbound fraction versus concentration profiles, the dissociation constants (K_d) were estimated to be 168 ± 13 nM and 1021 ± 88 nM, respectively, for α -globulin and albumin, and the binding capacity (n) to be 0.5 and 0.02, respectively (Table I). α -Globulin showed higher affinity and capacity than albumin, suggesting the stronger binding of fractionated [3 H]heparin to α -globulin than albumin, in agreement with our previous finding by gel filtration chromatography.¹¹ The n was less than 1 for both α -globulin and albumin. This suggests that more than one protein molecule may bind to one fractionated [3 H]heparin molecule.

Time Courses of the Disappearance of Fractionated [3 H]Heparin from the Perfusate in Liver Perfusion Figure 2 shows the typical profiles of remaining fractionated [3 H]heparin in the perfusate versus time. The disappearance of fractionated [3 H]heparin followed the first-order kinetics at each concentration and in the presence and absence of proteins. This disappearance of fractionated [3 H]heparin represents the hepatic uptake. The elimination rate constants were estimated by nonlinear regression analysis

TABLE I. Parameters of Protein Binding for Fractionated [3 H]Heparin in *in Vitro*

Protein	Concentration (mg/ml)	K_d (nM)	n
α -Globulin	8	168 ± 13	0.5 ± 0.03
Albumin	40	1021 ± 88	0.02 ± 0.001

Each value represents the computer-fitted parameter with S.E.

Fig. 1. Unbound Fraction *versus* Concentration Profiles for Fractionated [^3H]Heparin in the Presence of 8 mg/ml α -Globulin (A) and 40 mg/ml Albumin (B)

Each point with vertical bar represents the mean $\pm$ S.D. from three experiments. The solid lines represent the computer-fitted profiles.

Fig. 2. Time Courses of Remaining Fractionated [^3H]Heparin in the Perfusate in Liver Perfusion

○, in the absence of protein; ●, in the presence of 8 mg/ml α -globulin (A) or 40 mg/ml albumin (B). Initial concentrations of fractionated heparin were 10 nM (A) and 30 nM (B). Each point with vertical bar represents the mean $\pm$ S.D. from three rats. The solid lines represent computer-fitted profiles.

and multiplied by the distribution volume to estimate the hepatic uptake clearance. The disappearance of fractionated [^3H]heparin was delayed by 8 mg/ml α -globulin but not by 40 mg/ml albumin. The effect of proteins on the hepatic uptake clearance of fractionated [^3H]heparin is further examined in the following section.

The extrapolated remaining fraction of fractionated [^3H]heparin at 0 min, FR_0 , was 75 to 90%, giving about 20% or 20 ml larger distribution volume, V_d , than the perfusate volume of 100 ml. This increase in V_d cannot be accounted for by the dead volume of the perfusion apparatus, vascular space and Disse's space, and suggests the contribution of initial rapid association of fractionated [^3H]heparin to the liver as we reported in the primary culture of parenchymal hepatocytes.²⁰⁾ However, the effect of proteins on FR_0 was statistically insignificant.

Effect of Proteins on the Concentration-Dependent Hepatic Uptake of Fractionated [^3H]Heparin Figure 3 shows the hepatic uptake clearance *versus* concentration profiles for fractionated [^3H]heparin. In the absence of α -globulin, the hepatic uptake clearance decreased with concentration. This saturable uptake suggests the participation of a specialized transport system. By fitting Eq. 5, where f equals 1, to the uptake clearance *versus* concentration profile, the maximum uptake velocity ($V_{\max}$) and the Michaelis constant (K_m) were

estimated as 7.6 ± 2.4 pmol/min/g liver and 32.2 ± 11.5 nM, respectively (Table II). The $V_{\max}$ for the corresponding uptake phase of 60 min in the primary culture of rat parenchymal hepatocytes in our preceding report²⁰⁾ suggested an order of magnitude larger value of 109 ± 15 pmol/min/g liver, assuming the protein content of 4.2×10^5 cells/mg protein and 1.25×10^8 parenchymal hepatocytes in 1 g liver.²¹⁾ This discrepancy may suggest the stimulation of transport function in the cultured cells. Since not only parenchymal cells but also non-parenchymal cells are supposed to be involved in the uptake in the perfused liver, the discrepancy in $V_{\max}$ for parenchymal cells could be even larger. The K_m was comparable with that in cultured hepatocytes (21 ± 5 nM).²⁰⁾

The hepatic uptake clearance of fractionated [^3H]heparin was decreased at the lower concentrations of fractionated [^3H]heparin by 8 mg/ml α -globulin and minimally by 1 mg/ml α -globulin (Fig. 3A). The decrease in the uptake of fractionated [^3H]heparin could happen because fractionated [^3H]heparin bound to α -globulin cannot be taken up. And the slight increase in the uptake clearance at the medium concentration range may suggest an increase in the unbound fraction due to the saturable binding. Assuming that only unbound fractionated [^3H]heparin is taken up by the liver, Eq. 5, where f is described as Eq. 2,

Fig. 3. Concentration-Dependent Hepatic Uptake of Fractionated [^3H]Heparin and the Effect of α -Globulin (A) and Albumin (B) in the Recirculated Rat Liver Perfusion

○, in the absence of protein; ●, in the presence of 1 mg/ml α -globulin (A) or 40 mg/ml albumin (B); △, in the presence of 8 mg/ml α -globulin (A). Each point with vertical bar represents the mean $\pm$ S.D. from three rats. *a*) $n=4$. The solid lines and the dashed lines represent the profiles obtained by computer-fitting and predicted from *in vitro* binding parameters, respectively. The dashed line in panel A is for 8 mg/ml α -globulin.

TABLE II. Parameters of Hepatic Uptake and Protein Binding for Fractionated [^3H]Heparin in the Perfused Rat Liver

α -Globulin conc. (mg/ml)	$V_{\max}$ (pmol/min/g liver)	K_m (nM)	K_d (nM)	n
0	7.6 ± 2.4	32.2 ± 11.5	—	—
1	7.6^a	32.2	2.6 ± 4.6	0.002 ± 0.002
8	7.6^a	32.2	2.1 ± 1.2	0.002 ± 0.001

Each value represents the computer-fitted parameter with S.E. *a*) Parameters determined in the absence of α -globulin.

was fitted to the hepatic uptake clearance *versus* concentration profiles in the presence of α -globulin. The estimated binding parameters are shown in Table II. The K_d of 2.1 nM and n of 0.002 in the presence of 8 mg/ml of α -globulin were both far smaller than those in *in vitro* binding experiments, suggesting the higher affinity and lower capacity in perfusion. The result that the value of n is smaller than 1 supports the suggestion in *in vitro* binding experiments that more than one protein molecule may bind to one fractionated [^3H]heparin molecule. The binding parameters obtained in the presence of 1 mg/ml α -globulin were comparable to those in the presence of 8 mg/ml α -globulin, though they were accompanied by relatively large S.E.'s.

The predicted unbound fraction of fractionated [^3H]heparin based on those binding parameters in the presence of 8 mg/ml α -globulin was much larger than that in *in vitro* binding experiments, suggesting the apparently enhanced dissociation of fractionated [^3H]heparin in the perfused liver (Fig. 4). The unbound fraction of fractionated [^3H]heparin is below 0.05 up to 1000 nM *in vitro*, while the unbound fraction in perfusion was about 0.15 below 10 nM and increased to about 1 at 1000 nM. In the presence of 1 mg/ml α -globulin, the unbound fraction was predicted to be 0.56 at 1 nM to 0.68 at 150 nM from *in vitro* data, while the unbound fraction in perfusion was close to 1 through the range of fractionated heparin concentration examined, as suggested by the minimal effect of α -globulin on the hepatic uptake of fractionated [^3H]heparin. The apparently enhanced dissociation in perfusion suggests that the unbound fraction of fractionated [^3H]heparin may be increased on the surface of liver cells. This phenomenon is well known for low molecular weight compounds and is

Fig. 4. Unbound Fraction *versus* Concentration Profiles for Fractionated [^3H]Heparin in the Presence of 8 mg/ml α -Globulin: Comparison of Unbound Fraction *in Vitro* with That Estimated from the Kinetic Analysis of Hepatic Uptake

-----, liver perfusion; —, *in vitro*.

called protein-mediated transport.^{25,26} As reviewed by Pardridge,²⁴ proposed mechanisms for protein-mediated transport have been the enhanced dissociation of the ligand from proteins mediated by protein receptors on the cell surface, charge selective interaction of proteins with cell surface components such as sialic acids, and binding inhibitors from the cytosol. More recently, it has been suggested that the slow dissociation of the ligand and/or the diffusional barrier of the unstirred extracellular water layer could cause protein-mediated transport.^{25,26} The mechanism of this transport, however, is yet to be fully clarified. Although the present study showed the involvement of protein-mediated transport in the hepatic uptake of fractionated [^3H]heparin, a macromolecular drug, the mechanism for this particular case is also unknown. It may be noteworthy that the apparently enhanced dissociation of fractionated [^3H]heparin in perfusion is attributed to the decrease in the binding capacity, with even the dissociation constant being decreased or the affinity being increased (Tables I, II), while the reported enhanced dissociation of low molecular weight compounds has been attributed to the increased dissociation constant or the decreased affinity, with the binding capacity being unchanged. This difference may suggest the involvement of

a different manner of protein binding and a different mechanism of protein-mediated transport for fractionated [^3H]heparin than for low molecular weight compounds.

An alternative explanation for the apparently enhanced dissociation is the uptake of fractionated [^3H]heparin bound to α -globulin. The dashed line in Fig. 3A shows the uptake clearance of fractionated [^3H]heparin in the presence of 8 mg/ml α -globulin predicted from the binding parameters in *in vitro* binding experiments, assuming that only unbound fractionated [^3H]heparin is taken up. The predicted uptake clearance is much smaller than those observed and is concentration-independent. The difference between this observed and predicted clearance may represent the uptake of bound fractionated [^3H]heparin. However, it was unsuccessful to fit models incorporating the uptake of bound fractionated [^3H]heparin.

Albumin (40 mg/ml) did not affect the hepatic uptake of fractionated [^3H]heparin, though it was shown to bind to fractionated [^3H]heparin in *in vitro* binding experiments (Fig. 3B). This could also be interpreted as the result of the enhanced dissociation of fractionated [^3H]heparin in the perfusion.

The apparent enhanced dissociation of fractionated [^3H]heparin in perfusion is similar to phenomenon of "protein-mediated transport" reported for low molecular weight compounds. We previously suggested the involvement of protein-mediated transport in the hepatic uptake of fractionated [^3H]heparin in the presence of α -globulin.¹²⁻¹⁴ However, in those studies we used the semi-quantitative binding data of gel filtration chromatography¹¹ as *in vitro* reference, and binding parameters were not available. In the present study, employing *in vitro* binding parameters, we further confirmed the involvement of protein-mediated transport in the hepatic uptake of fractionated [^3H]heparin for albumin as well as α -globulin.

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