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Macromolecule–Macromolecule Interaction in Drug Distribution. II. Effect of α-Globulin on Saturable Uptake of Fractionated [^{3}H]Heparin by Rat Parenchymal Hepatocytes in Primary Culture

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The uptake of fractionated [^{3}H]heparin was investigated in rat parenchymal hepatocytes in primary culture. The initial uptake of fractionated [^{3}H]heparin was found to be saturable with the maximum uptake velocity (V_{max}) of 10.1 ± 1.46 pmol/min/mg protein and the Michaelis constant (K_m) of 284 ± 47.9 nM. The effect of α-globulin, the major protein binding to fractionated [^{3}H]heparin, on the saturable uptake profile of fractionated [^{3}H]heparin was also investigated. The uptake clearance was reduced, depending on the concentration of fractionated [^{3}H]heparin, by the addition of 1 mg/ml α-globulin. We assumed that fractionated ^{3}H-heparin bound to α-globulin was not available for uptake and that the reduction in the uptake clearance was solely attributable to the saturable binding of fractionated [^{3}H]heparin to α-globulin. The uptake clearance *versus* concentration profile was analyzed to obtain the dissociation constant (K_d) of 31.8 nM and the capacity (n) of 0.047 for the binding of fractionated [^{3}H]heparin to α-globulin. The saturable binding of fractionated [^{3}H]heparin to α-globulin was supported by *in vitro* binding experiments using gel chromatography, in which bound fractionated [^{3}H]heparin decreased with the concentration of fractionated [^{3}H]heparin in the presence of α-globulin. In conclusion, the present study demonstrated the saturable uptake of fractionated [^{3}H]heparin by rat parenchymal hepatocytes and the saturable binding of fractionated [^{3}H]heparin to α-globulin. The saturable uptake may suggest the involvement of a specific transport system such as receptor-mediated endocytosis.

Keywords rat parenchymal hepatocyte; primary culture; saturable uptake; fractionated [^{3}H]heparin; α-globulin; protein binding; macromolecule–macromolecule interaction; Michaelis constant

Heparin, a water soluble mucopolysaccharide,[1] has been widely used for more than fifty years, *e.g.*, as anticoagulant[2,3] for thrombolytic treatment. Although it was originally identified as an endogenous anticoagulant, heparin has recently been found to be involved in various biologically important phenomena, such as potentiation of angiogenesis,[4] inhibition of cell growth,[5,6] inhibition of pulmonary metastasis of tumor cells,[7,8] and interaction with endothelial cell growth factor[9] and fibroblast growth factors.[10] Such newly found biological properties of heparin might be exploited for clinical application as those of biologically active polypeptides. Heparin might also be applied as a drug carrier or modifier for drug delivery, as reported for several polysaccharides such as agar, pectin and chitin,[11] and as glycosylation materials for polypeptides.[12,13] In order to optimize heparin therapy and to expand the clinical application of heparin, exploiting the newly found biological properties, it is important to clarify its pharmacokinetic profile and to elucidate the mechanism of its disposition.

Macromolecular materials are generally heterogeneous with respect to biological and physicochemical properties, sometimes obscuring underlying mechanisms and rendering insufficient clinical outcomes. The homogenization of those materials could be an issue to be considered for basic research as well as clinical applications.

Commercial heparin, which is polydisperse with respect to molecular weight and heterogeneous with respect to biological and chemical properties, is a macromolecular material. We have been conducting pharmacokinetic studies in rats using fractionated [^{3}H]heparin, which has an affinity to protamine and of which the molecular weight is approximately 20000 Da. Our pharmacokinetic studies revealed that the drug is mainly taken up by the liver after intravenous injection.[14,15] α-Globulin was found to be the major protein binding to fractionated [^{3}H]heparin in plasma. α-Globulin was also found to decrease the hepatic uptake of fractionated [^{3}H]heparin in recirculated rat liver[16] and rat parenchymal hepatocytes in primary culture,[17] suggesting that fractionated [^{3}H]heparin bound to α-globulin is not, or is less, available to hepatic uptake.

Heparin has been considered to be taken up by the reticuloendothelial system (RES). The endocytotic uptake of heparin has been suggested in cultured endothelial cells which belong to RES.[18] On the other hand, a significant contribution of parenchymal hepatocytes in the hepatic uptake of heparin was also suggested by Praaning van Dalen *et al.*[19] The uptake mechanism of heparin by parenchymal hepatocytes is yet to be clarified.

In order to further elucidate the hepatic uptake mechanism of fractionated [^{3}H]heparin and the interaction of fractionated [^{3}H]heparin to α-globulin, we kinetically characterized the saturable uptake of fractionated [^{3}H]-heparin using a Michaelis–Menten type transport model and examined the effect of α-globulin on the saturable uptake profile to estimate the apparent binding parameters of fractionated [^{3}H]heparin to α-globulin.

Experimental

Materials [^{3}H(G)]Heparin sodium salt of porcine mucosal origin (#2275-254; 0.49 mCi/mg, #2643-105; 0.29 mCi/mg, #2643-135; 0.2 mCi/mg) and Biofluor (scintillation cocktail) were purchased from New England Nuclear (Boston, MA). Unlabeled heparin sodium salt of porcine mucosal origin, bovine α-globulin (Cohn fraction IV-1), and *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) were purchased from Sigma Chemical Co. (St. Louis, MO). Collagenase S-1 was purchased from Nitta Gelatin Co. (Osaka, Japan). Collagen-coated plastic dishes (35 mm in diameter) were purchased from Iwaki Glass Co. (Tokyo, Japan). All other chemicals used were commercially available and of the highest grade.

Fractionation of Heparin [^{3}H]Heparin and unlabeled heparin were fractionated by affinity chromatography followed by gel filtration chromatography as described in our preceding report.[14,15] Fractionated [^{3}H]heparin and unlabeled heparin which had an affinity to protamine

and of which the molecular weight was about 15000 Da were used for the uptake experiments. The amount of fractionated, unlabeled heparin was determined by the Carbazole reaction.[20]

Binding Study Test solutions, prepared in phosphate buffered saline (pH 7.4) contained 8 mg/ml α-globulin, and 8.7, 120 or 1040 nM fractionated [^{3}H]heparin. For the highest concentration (1040 nM), unlabeled fractionated heparin was added to adjust the concentration. One milliliter of each test solution was placed on a 1.6 × 100 cm column filled with Sephadex G-200 (Pharmacia, Sweden) and eluted with phosphate buffered saline at 3.3 ml/cm^2/h of flow rate into every 120-drop fraction. The experiment was performed at 4 °C. As a control, the elution profile for 25 nM of fractionated [^{3}H]heparin was determined in the absence of α-globulin.

Isolation and Culture of Pharenchymal Hepatocytes Male Wistar rats weighing 180 to 200 g were used without fasting. Each rat was anesthetized with urethane (1000 mg/kg, i.p.), and parenchymal hepatocytes were isolated by the collagenase perfusion technique of Seglen.[21] The viability of the hepatocytes was routinely checked by the trypan blue exclusion test, and was greater than 93%. The hepatocytes suspension was adjusted to 5×10^5 cells/ml in Williams' E medium containing 5% newborn calf serum (Whittaker M. A. Bioproducts, Inc., MD), 10^{-7} M insulin, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B, and seeded to 35-mm collagen-coated plastic dishes at 2 ml/dish. The cultures were maintained in 95% air–5% CO_2 at 37 °C. The medium was changed to Williams' E medium without newborn calf serum 20 h after plating. By that time, viable cells had attached to the bottom of the dishes and had formed a monolayer. Uptake experiments were carried out using the cultured cells 4 h after the medium was changed. The number of hepatocytes after a 24-h culture was 4.2×10^5 cells/mg protein.[19]

Uptake Experiments Test solutions, prepared in Hanks' basic salt solution containing 10 mM HEPES (pH 7.4), contained 7.5, 37.5, 70.0, 310 or 850 nM of fractionated [^{3}H]heparin. For the highest three concentrations, unlabeled fractionated heparin was added to adjusted the concentration. α-Globulin was added to the test solution at 1 or 8 mg/ml to examine the effect of α-globulin on the uptake of fractionated [^{3}H]heparin. The culture of parenchymal hepatocytes was washed once with Hanks' basic salt solution 24 h after plating the cells. Two milliliters of a test solution (pH 7.4) was added to the culture with stirring at 50 strokes/min at 37 °C, to start the experiment. The test solution was removed rapidly at a specified time (0.25, 0.5, 0.75 or 1.0 min) to stop the uptake. The culture was washed twice with 1 ml of ice-cold saline, detached from the dish by using a rubber policeman, and collected by suspending in 3 ml of ice-cold saline. The cells (in 0.5 ml of saline) were solubilized with 1 ml of Protosol added with 10 ml of Biofluor to determine radioactivity by liquid scintillation counting.

Data Analysis The initial uptake velocity was determined as slope of line for uptake *versus* time profile for 1 min. The uptake clearance was given by dividing the initial uptake velocity by concentration of fractionated [^{3}H]heparin. The uptake data were analyzed by using Michaelis–Menten equation. Assuming that only unbound fractionated heparin is taken up by the hepatocytes, the uptake clearance (CL_{app}) on the basis of total concentration is described as follows:

$$CL_{app} = \frac{V_{max}}{(K_m + C_f)} \cdot \frac{C_f}{C_t} \quad (1)$$

$$CL_{app} = \frac{V_{max}}{K_m/f + C_t} \quad (2)$$

where V_{max} is the maximum uptake velocity, K_m is Michaelis constant, C_t is the total concentration of fractionated heparin, C_f is the concentration of unbound fractionated [^{3}H]heparin, and f is fraction unbound (C_f/C_t). Then, using the fixed V_{max} and K_m value, dissociation constant (K_d) and binding capacity of the protein (n) were estimated in the similar way, using the data in the presence of α-globulin. The drug protein binding model,[22] which has been widely used to describe the protein binding of low molecular weight drugs, is tentatively applied to characterize the protein binding of fractionated [^{3}H]heparin by assuming a single kind of binding. The dissociation equilibrium of fractionated heparin to α-globulin is described as follows:

$$K_d = \frac{P \cdot C_f}{C_b} \quad (3)$$

where P is the concentration of unbound sites of protein and C_b is the concentration of bound fractionated heparin. Replacing P, C_f and C_b, respectively with $n \cdot P_t - (1-f) \cdot C_t$, $f \cdot C_t$ and $(1-f) \cdot C_t$, Eq. 3 is expressed in terms of n, f, C_t and concentration of α-globulin (P_t).

$$K_d = \frac{(n \cdot P_t - C_t + f \cdot C_t) \cdot f \cdot C_t}{(1-f) \cdot C_t} \quad (4)$$

Replacing P_t with P_t'/M_{eff}, where P_t' is the concentration of α-globulin (mg/ml) and M_{eff} is the constant for conversion of unit (1.14×10^{-3} g/nmol)[23] and solving Eq. 4 for f, f is described as follows:

$$f = \frac{A + A^2 + 4K_d/C_t}{2} \quad (5)$$

$$A = 1 - \frac{K_d}{C_t} - \frac{(n/M_{eff}) \cdot P_t'}{C_t} \quad (6)$$

Substituting Eqs. 5 and 6 into Eq. 2, CL_{app} is described as a function of P_t' and $C_t \cdot V_{max}$, K_m, n/M_{eff} and K_d are the parameters to be estimated. First, V_{max} and K_m were estimated by fitting Eq. 1 to the CL_{app} *versus* C_t profile in the absence of α-globulin, using a nonlinear regression program PCNONLIN (Statistical Consultants, Inc., Lexington, KY) and weights equal to the reciprocal of variance of the CL_{app} at individual C_t values. Secondly, using the V_{max} and K_m determined in the absence of α-globulin, K_d and n/M_{eff} were estimated by fitting Eq. 2 in combination with Eqs. 5 and 6 to CL_{app} *versus* concentration profile in the presence of α-globulin.

Results and Discussion

Uptake Study Figure 1 shows the time courses of the uptake of fractionated [^{3}H]heparin at three selected concentrations and with or without α-globulin. The uptake of fractionated heparin appeared to be linear for at least 1 min at any condition. The initial uptake velocity, determined as the slope, was decreased by the addition of α-globulin at the lowest concentration of fractionated heparin (7.5 nM), but was not changed at higher concentrations.

Figure 2 shows relations between the uptake clearance, given by dividing the initial uptake velocity by the concentration, and the concentration. The uptake clearance of fractionated heparin decreased with concentration in the absence of α-globulin, suggesting the contribution of a saturable transport. By fitting Eq. 2, with putting f at 1, to the data, the maximum uptake velocity (V_{max}) and Michaelis constant (K_m) were estimated to be 10.1 ± 1.5 pmol/min/mg protein and 284 ± 48 nM, respectively (Table I). This K_m value is comparable to those reported for the uptake of several polysaccharides and glycoproteins, which are known to be endocytosed *via* the receptor system, by isolated sinusoidal cells; mannan (74 nM),[24] hexosaminase (43 nM),[25] mannose-terminated orosomucoid (360 nM),[25] agalacto-orosomucoid (110 nM),[26] ribonuclease B (640 nM)[26] and ovalbumin glycopeptide (420 nM).[26] Since heparin is a high molecular weight compound, a receptor-mediated endocytotic uptake similar to those suggested for the uptake of these polysaccharides and glycoproteins rather than a carrier-mediated transport may be involved in the hepatic uptake of fractionated heparin. Endocytosis of heparin has been suggested in cultured human endothelial cells.[18] Receptor-mediated endocytosis has been reported for the transport of peptide hormones such as insulin,[27] epidermal growth factor,[28,29] parathyroid hormone[30] and human growth hormone,[31] asialoglycoproteins,[32] and lipoproteins.[33]

The uptake clearance was decreased in the presence of 1 mg/ml α-globulin, compared with that in the absence at the lower concentrations of fractionated heparin, suggesting that the uptake of protein-bound fractionated heparin is

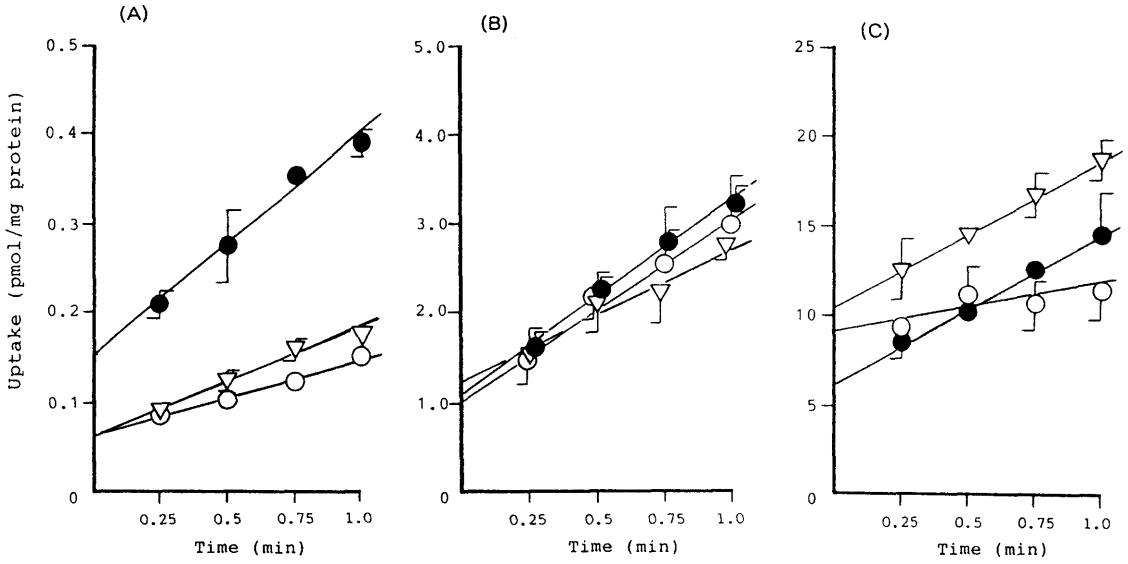

Fig. 1. Time Courses of Uptake of Fractionated [^{3}H]Heparin by Rat Parenchymal Hepatocytes in Primary Culture

Concentration of fractionated [^{3}H]heparin: (A) 7.5, (B) 70.0, (C) 850 nM. Concentration of α-globulin: ●, 0; ▽, 1.0; ○, 8.0 mg/ml. Each point with vertical bar represents the mean ± S.D. of three rats. The solid lines represent the results of linear regression.

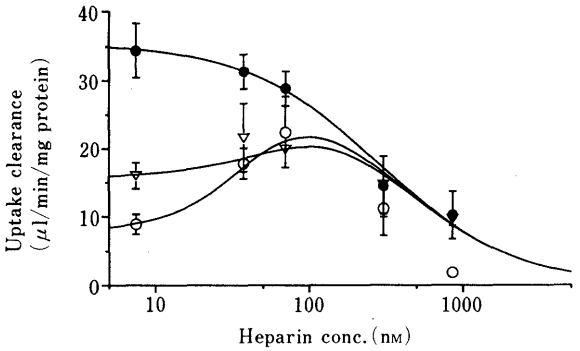

Fig. 2. Uptake Clearance *versus* Concentration of Fractionated [^{3}H]Heparin in Primary Culture of Rat Parenchymal Hepatocytes

Concentration of α-globulin: ●, 0; ▽, 1.0; ○, 8.0 mg/ml. Each point with vertical bar represents the mean ± S.D. of three rats. The solid curves represent the results of fitting by PCNONLIN, using Eq. 2 (upper curve) or Eqs. 2, 5 and 6 (lower two curves).

slower than that of unbound heparin. The uptake clearance was further decreased in the presence of 8 mg/ml α-globulin. The uptake clearance was found to increase up to about 100 nM of fractionated heparin in the presence of α-globulin (Fig. 2). Provided that the uptake of fractionated heparin is described by the Michaelis–Menten equation, and assuming that only unbound fractionated heparin is available for uptake, the increase in the uptake clearance with a concentration of heparin in the presence of α-globulin can be interpreted as the result of the increase in the fraction unbound as suggested by Eq. 2, suggesting the nonlinearity of the binding of fractionated heparin to α-globulin. Using the V_{max} of 10.1 pmol/min/mg protein and K_m of 284 nM determined in the absence of α-globulin, K_d and n/M_{eff} were obtained as 31.8 ± 10.1 nM and 41.3 ± 7.2 nmol/g, respectively, by fitting Eq. 2 in combination with Eqs. 5 and 6 to CL_{app} *versus* the concentration profile in the presence of 1 mg/ml α-globulin (Table I). This K_d value suggests relatively tight binding of heparin compared with that with antithrombin III[34] (K_d = 100 nM) or that with fibronectin[35] (two kinds of binding sites: K_d = 35, 1000 nM), which were

TABLE I. Parameters for Uptake of Fractionated Heparin and Binding of Fractionated Heparin to α-Globulin

α-Globulin conc. (mg/ml)	0	1.0	8.0
V_{max} (pmol/min/mg protein)	10.1 ± 1.5	10.1[a]	10.1[a]
K_m (nM)	284 ± 48	284[a]	284[a]
K_d (nM)	—	31.8 ± 10.1	6.4 ± 17.8
n/M_{eff}[b] (nmol/g)	—	41.3 ± 7.2	3.0 ± 5.7
n	—	0.047	0.003

Each value represents the parameter ± S.E. (n = 15), unless otherwise indicated. Parameters were obtained as described in the text. *a*) Parameters determined in the absence of α-globulin. *b*) $M_{eff} = 1.14 \times 10^{-3}$ g/nmol.

reported in *in vitro* binding studies. Although the parameters for the *in vitro* binding of fractionated heparin to α-globulin has not yet been determined, the K_d is comparable to those reported for the macromolecule–macromolecule interaction of several compounds *in vitro*. Reported K_d values are 36 nM (lipoprotein-elastin)[36] and 56, 550 nM (two kinds of binding sites, proteins from platelet lysate-fibrillar collagen).[37] Considering that M_{eff} is 1.14×10^{-3} g/nmol, n was estimated as 0.047. This may suggest that a molecules of fractionated heparin binds to about 20 of molecules of α-globulin. Since heparin is a long chain polysaccharide, it might be possible for a molecule of heparin to bind a number of α-globulin molecules. The CL_{app} *versus* the concentration profile in the presence of 8 mg/ml α-globulin was not satisfactorily described by the proposal models, giving the values of S.E. larger than the estimated parameters (Table I). In addition, the K_d and n/M_{eff} were an order magnitude smaller than those in the presence of 1 mg/ml α-globulin. This apparent protein-concentration dependency in the parameters for the protein binding of fractionated heparin is an issue to be investigated in the future.

***In Vitro* Binding Study** Figure 3 shows the gel chromatograms for three concentrations of fractionated heparin in the presence of 8 mg/ml α-globulin. Almost all fractionated heparin interacted with α-globulin at 8.7 nM fractionated heparin (Fig. 3A). The fraction unbound of

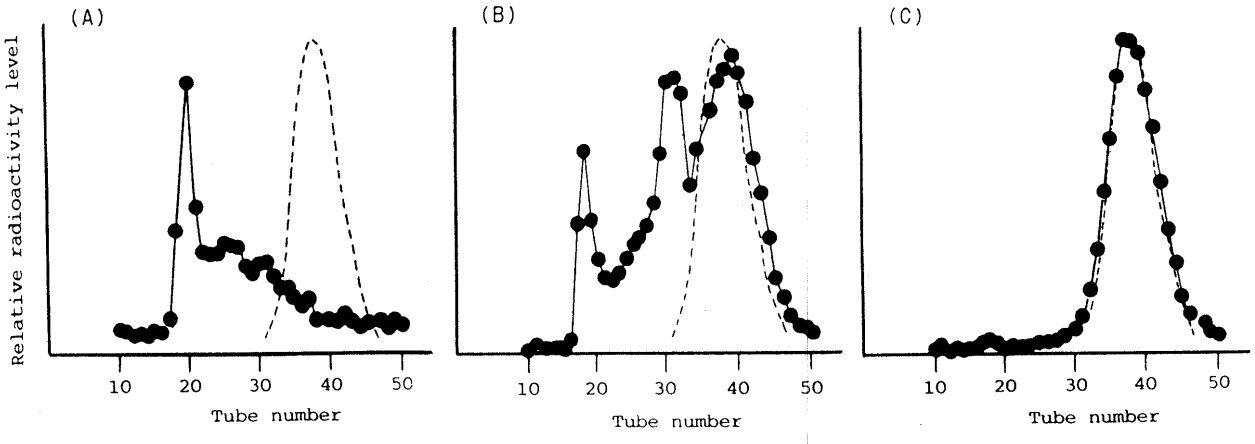

Fig. 3. Gel Filtration Chromatograms Obtained with Application of Fractionated [^{3}H]Heparin in α-Globulin Solutions (8 mg/ml)

Concentration of fractionated [^{3}H]heparin: (A) 8.7, (B) 120, (C) 1040 nM. The broken curves represent the elution profiles for 25 nM fractionated ^{3}H-heparin without α-globulin.

fractionated heparin increased with concentration. Increasing to 1040 nM fractionated heparin, most parts of the bound fraction disappeared and almost all fractionated heparin existed as free form (Fig. 3C). These results suggested that the unbound fraction increased with the concentration of fractionated heparin under the constant concentration level of α-globulin. This result supports the nonlinearity or saturation of the binding of fractionated heparin to α-globulin, suggested in the uptake study.

At 120 nM, the unbound fraction appeared to be about 50% and two sharp peaks were observed in addition to the peak which overlapped with that of free fractionated heparin. Considering that α-globulin is heterogeneous with respect to molecular weight, it was suggested that fractionated heparin bound preferentially to two specific molecular sizes of α-globulin or that one molecule of fractionated heparin might bind to more than two molecules of α-globulin (Fig. 3B).

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