Uptake Mechanism of Fractionated [^3H]Heparin in Isolated Rat Kupffer Cells: Involvement of Scavenger Receptors

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The uptake of fractionated [^3H]heparin was examined to elucidate the uptake mechanism in isolated rat Kupffer cells. The equilibrium binding of fractionated [^3H]heparin to Kupffer cells was concentration-dependent with the dissociation constant of 5.7 nM and the maximum binding capacity of 1.5 pmol/ 10^6 cells. Several ligands of scavenger receptors inhibited the binding of fractionated [^3H]heparin to Kupffer cells competitively and also the internalization of heparin, suggesting the involvement of scavenger receptors in the uptake of fractionated [^3H]heparin. Fractionated [^3H]heparin was also suggested to be internalized according to first order kinetics with the apparent internalization rate constant of 0.010 min^{-1} . Lowering temperature from 37 to 4°C reduced the fraction internalized from 33% to 6% without affecting the total association, while the fraction internalized at 25°C was comparable with that at 37°C. Metabolic inhibitors (2,4-dinitrophenol and rotenone), an inhibitor of receptor-mediated and adsorptive endocytosis of polypeptides (phenylarsine oxide) and phagocytosis inhibitors (cytochalasin B and colchicine) did not inhibit the internalization of fractionated [^3H]heparin. As known inhibitors of receptor-mediated and adsorptive endocytosis of polypeptides and phagocytosis did not affect the uptake of fractionated heparin, the scavenger receptor-mediated uptake is suggested to be ATP-independent and different from receptor-mediated and adsorptive endocytosis of polypeptides and phagocytosis, although for temperature dependency it showed the typical characteristics of receptor-mediated endocytosis.

Key words scavenger receptor; fractionated [^3H]heparin; Kupffer cell; rat; receptor-mediated endocytosis

Mucopolysaccharides such as heparin have recently been of pharmaceutical interest with regard to the clinical application of their newly found biological activities of anti-human immunodeficiency virus effect and inhibitory effect on the metastasis of cancer.^{1,2)} To establish the rational use of mucopolysaccharides in clinical therapy, we selected heparin, which has been widely used as an anticoagulant, as a typical mucopolysaccharide. We have been using fractionated heparin with a defined molecular weight and chemical properties, because heparin is heterogeneous with respect to these factors,¹⁾ which could be disadvantageous in controlling heparin disposition and establishing not only the rational use of mucopolysaccharides in clinical therapy but also efficient delivery to target tissues.

Our previous studies showed that fractionated [^3H]heparin with a molecular weight of 20000 Da is rapidly eliminated from plasma according to first order kinetics, and that dose-dependent hepatic uptake is mainly responsible for this.^{3,4)} Both parenchymal and non-parenchymal cells significantly contribute to the hepatic uptake of fractionated [^3H]heparin⁵⁾ and a specialized uptake mechanism is suggested to be involved in the uptake in cultured parenchymal cells.^{6–8)} Our recent study also suggested that scavenger receptors may be involved in the uptake of fractionated [^3H]heparin in isolated rat Kupffer cells as demonstrated by the inhibition of the binding by some ligands of scavenger receptors, and that the uptake is ATP-independent and different from receptor-mediated and adsorptive endocytosis of polypeptides.⁹⁾

To further examine the involvement of scavenger receptors in this study, we investigated the inhibition mechanism of the binding by some ligands of scavenger

receptors and the effects of those ligands of scavenger receptors on the internalization of fractionated [^3H]heparin in isolated rat Kupffer cells.

MATERIALS AND METHODS

Materials [$^3\text{H}(\text{G})$]Heparin sodium salt of porcine mucosal origin (#2643-177; 130.0 U/mg and 16 MBq/mg) was purchased from DuPont-NEN Co. (Boston, Mass., U.S.A.). Heparin sodium salt of porcine mucosal origin (#29F-0314; 178 U/mg) was obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). RPMI1640 medium and uncoated plastic dishes were purchased from Dainippon Pharmaceutical Co. (Osaka, Japan) and Sumitomo Bakelite Co. (Tokyo, Japan), respectively. All other chemicals were of analytical grade.

Fractionation of Heparin Tritiated and unlabeled heparin were fractionated as described^{3,4)} to obtain a heparin fraction with affinity for protamine-Sepharose and a molecular weight of 23000 Da.

Isolation of Kupffer Cells Non-parenchymal cells were isolated from non-fasting male Wistar rats weighing 180–230 g (6 to 7 weeks old), and Kupffer cells were purified from the non-parenchymal cells as described previously.⁹⁾ Briefly, non-parenchymal cells (more than 95% viable by the trypan blue exclusion test) were seeded in uncoated plastic dishes (2 ml/dish) at a density of 6.5×10^5 cells/ml in RPMI1640 medium and maintained in 95% air–5% CO_2 at 37°C for 15 min. The dishes were then washed thoroughly twice with 2 ml RPMI1640 medium to obtain Kupffer cells which attached to the dishes. Kupffer cells are known to attach to uncoated plastic dishes, while other subpopulations of non-parenchymal cells can not.¹⁰⁾

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Binding Experiments Test solutions were prepared in Hanks' basic salt solution supplemented with 10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) and adjusted to pH 7.4; the solutions contained 2.5, 5, 10, 25 and 50 nM of fractionated [^3H]heparin. For the two highest concentrations, unlabeled fractionated heparin was added to achieve the desired concentrations. Kupffer cells, which were attached to the plastic dishes, were washed twice with 2 ml Hanks' basic salt solution containing HEPES (pH 7.4). The binding was measured as the uptake at 4°C for 2 h. Binding experiments were initiated by adding 2 ml of test solution (pH 7.4) to each dish in the presence or absence of various compounds, and was stopped by rapid removal of the test solution at specified times and by washing the cells twice with ice-cold Hanks' basic salt solution. Kupffer cells were dissolved in 1 ml 3 N NaOH for 1 h at 37°C and neutralized with 1 ml 3 N HCl. Each sample (0.5 ml) was then mixed with 5 ml Scintisol EX-H (Dojindo Co., Kumamoto, Japan) to determine the radioactivity. Protein content was determined by the method of Lowry *et al.*¹¹⁾ at the end of each experiment and converted to cell numbers using the relationship of 6.7×10^6 cells/mg protein, which was determined in advance. Non-specific binding was estimated as the binding of fractionated [^3H]heparin in the presence of 100-fold concentration of unlabeled one and was subtracted from the apparent binding to estimate the specific binding.

Effects of Various Compounds on Internalization To examine the effects of transport inhibitors (phenylarsine oxide, monensin, chloroquine, cytochalasin B and colchicine) on the internalization of fractionated [^3H]heparin, after preincubation with an inhibitor for 30 min at 37°C, the cells were incubated with 5 nM fractionated [^3H]heparin at 37°C for 2 h in 95% air–5% CO₂ in the presence of the inhibitor. Phenylarsine oxide and cytochalasin B were dissolved in dimethyl sulfoxide and diluted 500-fold in test solution. When examining the effect of metabolic inhibitors (2,4-dinitrophenol and rotenone), cells were preincubated with an inhibitor for 15 min and then incubated in the absence of inhibitors. To examine the effects of ligands of scavenger receptors (dextran sulphate, fucoidan and polyguanylic acid and polycytidylic acid), the cells were incubated with 5 nM fractionated [^3H]heparin at 37°C for 2 h in 95% air–5% CO₂ in the presence of a ligand. Preincubation was not carried out for them.

To estimate the surface-bound and internalized amount of fractionated [^3H]heparin, Hanks' solution containing 1 μM dextran sulphate (500 kDa, 2 ml) was added to the cells after uptake experiments, and the cells were left for 20 min at 4°C. The dissociated radioactivity into the solution was assumed to represent surface-bound fractionated [^3H]heparin and undissociated radioactivity in the cells was assumed to represent the internalized amount. The radioactivity was determined as described in the section on "Binding Experiments". Non-specific uptake was estimated as the uptake of fractionated [^3H]heparin in the presence of 100-fold concentration of unlabeled one and subtracted from the apparent uptake to estimate the specific uptake.

Data Treatment The binding (B) versus concentration (C) profile was analyzed using the following equation:

$$B = \frac{B_{\max} C}{K_d + C} \quad (1)$$

where $B_{\max}$ and K_d represent the maximum binding capacity and dissociation constant, respectively. Computer-fitting was conducted using a nonlinear regression program, MULTI¹²⁾ to estimate $B_{\max}$ and K_d .

The binding of fractionated [^3H]heparin in the presence of competitive inhibitors is expressed as follows:

$$B = \frac{B_{\max} C}{K_d \left(1 + \frac{C_i}{K_i} \right) + C} \quad (2)$$

where C_i and K_i are the inhibitor concentration and inhibition constant, respectively. Inhibition constant was estimated by fitting Eq. 2 to the binding versus concentration profiles in the presence of an inhibitor, using MULTI. $B_{\max}$ and K_d were fixed by using the values in the absence of inhibitors.

Assuming that the release of radioactivity after internalization of fractionated [^3H]heparin is negligible, the internalization rate of fractionated [^3H]heparin can be described as follows:

$$\frac{dX_{\text{in}}}{dt} = k_{\text{int,app}} X_s \quad (3)$$

where X_{in} and X_s represent the internalized and cell surface-bound amount of fractionated [^3H]heparin, respectively, and $k_{\text{int,app}}$ is the apparent internalization rate constant. Integration of Eq. 3 from time 0 to t yields

$$X_{\text{in}} = k_{\text{int,app}} \int_0^t X_s dt \quad (4)$$

The apparent internalization rate constant was estimated by linear regression analysis of the internalized amount versus integrated surface binding plot.

Statistical Analysis Levels of statistical significance were assessed using Student's t -test.

RESULTS

Concentration Dependency in the Binding The binding of fractionated [^3H]heparin to isolated rat Kupffer cells was found to reach equilibrium within 2 h (Fig. 1). As

Fig. 1. Time Course of the Binding of Fractionated [^3H]Heparin to Isolated Rat Kupffer Cells

Each point and vertical bar represents the mean $\pm$ S.D. from three rats. The binding was measured at 4°C.

shown in Fig. 2, binding of fractionated [^3H]heparin at equilibrium was saturable with a dissociation constant of 5.7 nM and maximum binding capacity of 1.5 pmol/ 10^6 cells.

Inhibition Mechanism of the Binding As shown in Fig. 3, a heparin analog (heparan sulphate) and ligands of scavenger receptors (dextran sulphate and polyguanylic acid)¹³ inhibited the binding of fractionated [^3H]heparin competitively, suggesting the involvement of scavenger receptors in the binding of fractionated [^3H]heparin and

Fig. 2. Concentration-Dependent Binding of Fractionated [^3H]-Heparin to Isolated Rat Kupffer Cells

Each point and vertical bar represents the mean $\pm$ S.D. from three rats. Solid line represents the computer-fitted profile.

its analogs to Kupffer cells. The inhibition constants were 370, 2.6 and 71.9 nM, respectively, for heparan sulphate, dextran sulphate and polyguanylic acid. 4,4'-Diisothiocyanatostilben-2,2'-disulfonic acid (DIDS), an inhibitor of the anion transport system with a sulfuric group,¹⁴ also inhibited the binding competitively, suggesting that a sulfuric group may play an important role in the binding of fractionated heparin to scavenger receptors.

Effects of Ligands of Scavenger Receptors on the Internalization As shown in Fig. 4, internalization of fractionated [^3H]heparin as well as the surface binding was inhibited by ligands of scavenger receptors, such as dextran sulphate,¹³ fucoidan¹⁵ and polyguanylic acid,¹⁴ indicating the scavenger receptor-mediated uptake of fractionated [^3H]heparin in Kupffer cells; in contrast, polycytidylic acid, an analog of polyguanylic acid, affected neither the surface binding nor internalization of fractionated [^3H]heparin. The internalized amount of fractionated [^3H]heparin was estimated as undissociated fractionated heparin in Kupffer cells after washing with dextran sulphate at 4 °C for 20 min. The washing procedure was verified by the results that the undissociated amount of fractionated [^3H]heparin was unchanged after washing with dextran sulphate for more than 20 min or at a higher

Fig. 3. Competitive Inhibition of the Binding of Fractionated [^3H]Heparin to Isolated Rat Kupffer Cells by Various Compounds

Each point and vertical bar represents the mean $\pm$ S.D. from three rats. The binding was measured at 4 °C for 2 h in the absence (●) or presence (■) of 500 nM heparin sulphate (A), 50 μM DIDS (B), 5 nM dextran sulphate (C) and 100 nM polyguanylic acid (D).

Fig. 4. Effects of Ligands of Scavenger Receptors on the Surface-Binding and Internalization of Fractionated [^3H]Heparin in Isolated Rat Kupffer Cells

Each column and horizontal bar represents the mean $\pm$ S.D. from three rats. Internalization, surface-binding and total association were measured at 37 °C at 5 nM fractionated [^3H]heparin and at 2 h in the presence of the ligands. * Significantly different from the control at $p < 0.05$.

Fig. 5. Effects of Temperature on the Total Association and Fraction Internalized of Fractionated [³H]Heparin in Isolated Rat Kupffer Cells

Each column and horizontal bar represents the mean $\pm$ S.D. for three rats. Total association was measured at 5 nM fractionated [³H]heparin at 2 h. *Significantly different from the value for 37°C at $p < 0.05$.

Fig. 6. Effects of Transport Inhibitors on the Total Association and Internalization of Fractionated [³H]Heparin in Isolated Rat Kupffer Cells

After the preincubation of Kupffer cells in the presence of transport inhibitors, internalization and total association were measured at 37°C at 5 nM fractionated [³H]heparin and at 2 h in the presence or absence of inhibitors.

dextran sulphate concentration of 3 μ M. Washing with acetate buffer of pH 3 (acid wash technique), which is used to estimate the internalization of polypeptides in hepatocytes and endothelial cells,^{16,17} did not make surface-binding of fractionated [³H]heparin dissociate (data not shown).

Effect of Temperature on the Internalization Figure 5 shows the effect of temperature on the total association of fractionated [³H]heparin and the fraction internalized. Total association represents the sum of the internalized and surface-bound amount of fractionated [³H]heparin, and the fraction internalized represents a ratio of the internalized amount to total association. Lowering temperature from 37 to 4°C reduced the fraction internalized from 33 to 6% without affecting the total association, in agreement with our previous results.⁹ The fraction of internalization at 4°C may represent non-specific binding and/or internalization of fractionated [³H]heparin. The internalized fraction of 27% at 25°C was comparable with that at 37°C. Although the total association at 25°C was about 30% smaller than that at 37°C, this decrease did not seem to be meaningful, because the total association at 4°C was comparable with those at 25 and 37°C.

Effect of Transport Inhibitors on the Internalization Our previous study showed that the scavenger receptor-mediated uptake of fractionated [³H]heparin is ATP-independent and different from receptor-mediated and adsorptive endocytosis of polypeptides, based on the lack

of inhibitory effect of 2,4-dinitrophenol and phenylarsine oxide on the internalization.⁹ In addition to these transport inhibitors, effects of various transport inhibitors on the internalization were investigated (Fig. 6). Metabolic inhibitors (2,4-dinitrophenol and rotenone) and phenylarsine oxide, an inhibitor of receptor-mediated and adsorptive endocytosis of polypeptides,^{17,18} as well as chloroquine and monensine (an inhibitor of lysosomal degradation)¹⁹ inhibited neither the internalization nor total association of fractionated [³H]heparin. The effect of chloroquine was consistent with our earlier result.⁹ In addition, colchicine and cytochalasin B, which are known to inhibit phagocytosis by inhibiting polymerization of tubulin and actin,^{20,21} also affected neither the internalization nor total association.

Estimation of the Apparent Internalization Rate Constant Figure 7 shows time courses of surface binding and internalization of fractionated [³H]heparin in rat Kupffer cells. Figure 8 shows the plot of internalization versus integrated surface binding, based on the results in Fig. 7. A linear relationship with a correlation coefficient of 0.982 was obtained using the data points up to 30 min in Fig. 7B, giving the apparent internalization rate constant of 0.010 min⁻¹ according to Eq. 3. Data points after 30 min in Fig. 8 deviated from the linear relationship, probably due to the release of radioactivity derived from internalized fractionated [³H]heparin.

Fig. 7. Time Courses of Surface Binding and Internalization of Fractionated [^3H]Heparin in Isolated Rat Kupffer Cells

(A) Each point and vertical bar represents the mean $\pm$ S.D. from three rats. Surface binding and internalization were measured at 37°C at 5 nM fractionated [^3H]heparin. ●, surface binding; ○, internalization.

Fig. 8. Internalization versus Integrated Surface Binding of Fractionated [^3H]Heparin in Isolated Rat Kupffer Cells

The solid lines represent results of linear regression analysis. Integrated surface binding was estimated from the data points up to 120 min in Fig. 7 by the trapezoidal method. The insert represents a plot with a magnified scale.

DISCUSSION

Our earlier study showed that scavenger receptors may be involved in the binding of fractionated [^3H]heparin to isolated rat Kupffer cells as demonstrated by inhibition of the binding by the ligands of scavenger receptors, and that the uptake is ATP-independent and different from receptor-mediated and adsorptive endocytosis of polypeptides.⁹⁾ In this study, in order to further examine the involvement of scavenger receptors, we investigated the inhibition mechanisms of the binding by ligands of scavenger receptors and the effects of those ligands on the internalization. Ligands of scavenger receptors such as dextran sulphate,¹³⁾ fucoidan¹⁵⁾ and polyguanylic acid¹³⁾ inhibited the binding of fractionated [^3H]heparin to isolated rat Kupffer cells competitively as well as the internalization, suggesting the scavenger receptor-mediated uptake of fractionated [^3H]heparin in those cells. Although the dissociation constants for the binding of dextran sulphate and polyguanylic acid to scavenger receptors have not been reported, the inhibition constants of 2.1 and 65.9 nM for dextran sulphate and polyguanylic acid, respectively, were within the range of dissociation constants reported for other ligands of scavenger receptors such as maleylated bovine serum albumin in purified rat liver scavenger receptor protein (5–30 nM),²²⁾ acetylated low density lipoprotein in purified rat liver scavenger receptor protein (2–7 nM)²²⁾ and rat liver sinusoidal cells (14 nM),²³⁾ and advanced glycosylation end product of bovine serum albumin in rat liver sinusoidal cells (210 nM) and peritoneal macrophages (240 nM).²⁴⁾ These results

provide additional evidence for the involvement of scavenger receptors in the uptake of fractionated heparin in Kupffer cells. The selective inhibitory effect of polyguanylic acid on the surface binding and internalization of fractionated [^3H]heparin also supports the involvement of scavenger receptors. Polycytidylic acid, an analog of polyguanylic acid, can not bind to scavenger receptors, probably due to the difference in the steric configuration from polyguanylic acid.²⁵⁾

Although lowering the temperature from 37 to 4°C reduced the fraction internalized from 33 to 6% without affecting the total association, the internalized fraction of 27% at 25°C was comparable with that at 37°C. Thus, for temperature dependency, the internalization of fractionated [^3H]heparin by scavenger receptors showed typical characteristics of receptor-mediated endocytosis, where the binding of ligands to receptors on the cell surface and clustering can occur at temperature as low as 1–4°C, but the internalization of receptor-ligand complexes can occur at temperature higher than 23°C.²⁶⁾ An inhibitor of receptor-mediated endocytosis for polypeptides (phenylarsine oxide)^{17,18)} and phagocytosis inhibitors (cytochalasine B, colchicine)^{20,21)} did not inhibit the internalization of fractionated [^3H]heparin in Kupffer cells, suggesting a unique characteristic of scavenger receptor-mediated uptake of fractionated [^3H]heparin, which may be different from receptor-mediated and adsorptive endocytosis of polypeptides and phagocytosis. The effects of cytochalasine B on the internalization by scavenger receptors was consistent with the finding by Srinivasan *et al.* that internalization of acetylated low density lipoprotein by scavenger receptors has not been inhibited by cytochalasine B in mouse peritoneal macrophages.²⁷⁾ However, the effects of transport inhibitors other than cytochalasine B on the internalization of ligands for scavenger receptors have not been investigated, and, hence, this study is the first to suggest the unique internalization mechanism by scavenger receptors. An inhibitor of lysosomal degradation, such as chloroquine or monensin, affected neither internalization nor total association of fractionated [^3H]heparin in Kupffer cells. Kamps *et al.* have recently suggested the involvement of lysosomal proteolysis in degradations of acetylated and oxidized low density lipoprotein, ligands of scavenger receptors, by demonstrating inhibitory effects of chloroquine on degradations of those ligands in human Kupffer cells.²⁸⁾ In this study, degradations of fractionated [^3H]heparin were not determined in the presence of chloroquine and monensin, and the sorting processes of fractionated [^3H]heparin after the internalization by scavenger receptors thus remains to be clarified.

Our previous study showed that liver parenchymal and Kupffer cells comparably contribute to the hepatic uptake (64.4% of dose) 1 h after intravenous administration of fractionated [^3H]heparin at the dose of 0.3 U/kg to rats,⁵⁾ suggesting that 32.2% of administered radioactivity is taken up into those cells by scavenger receptors.

The apparent internalization rate constant ($k_{\text{int,app}}$) of 0.010 min⁻¹ was an order of magnitude smaller than that for succinylated bovine serum albumin (0.3 min⁻¹), a

ligand of scavenger receptors with a molecular weight of 70000 Da in the rat liver perfusion system.²⁹⁾ Considering that molecular weight of succinylated bovine serum albumin (70000 Da) is about three times larger than that of fractionated heparin, molecular weight might be one of the major factors determining the rate of internalization by scavenger receptors. The difference in chemical structures and/or charge density may also be involved in the difference in apparent internalization rate constants, though details are yet to be fully clarified.

Srinivasan *et al.* have suggested that a heparin fraction with affinity for low density lipoprotein enhances the uptake of that lipoprotein by scavenger receptors in mouse peritoneal macrophages by interacting with low density lipoprotein.²⁷⁾ Murata *et al.* reported that scavenger receptor-mediated uptake of maleylated bovine serum albumin and dextran sulphate regulate lipoprotein lipase secretion in mouse peritoneal macrophages.¹³⁾ Although heparin has been thought to regulate lipoprotein concentration in blood by releasing lipoprotein lipase on endothelial cells,³⁰⁾ these findings by Srinivansan²⁷⁾ and Murata *et al.*¹⁴⁾ raise the possibility that, in addition to the release of lipoprotein lipase in endothelial cells, scavenger receptor-mediated uptake of fractionated [³H]-heparin in Kupffer cells contributes to regulation of the concentration of lipoproteins such as low density lipoprotein in blood, and prevents the development of atherosclerosis.

In conclusion, several ligands of scavenger receptors inhibited the binding of fractionated heparin competitively and also the internalization, indicating the involvement of scavenger receptors in the uptake of fractionated [³H]heparin in isolated rat Kupffer cells. As known inhibitors of receptor-mediated and adsorptive endocytosis of polypeptides and phagocytosis did not affect the uptake of fractionated heparin, the scavenger receptor-mediated uptake appeared to be unique, and it may be different from that of receptor-mediated and adsorptive endocytosis of polypeptides and phagocytosis.

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REFERENCES

- 1) Jaques L. B., *Pharmacol. Rev.*, **33**, 99—166 (1980).
- 2) Ito M., Baba M., Sato A., Pauwels R., Clercq E. D., Shigeta S., *Antivir. Res.*, **7**, 361—367 (1987).
- 3) Watanabe J., Hori K., Iwamoto K., Ozeki S., *J. Pharmacobio-Dyn.*, **6**, 423—432 (1983).
- 4) Watanabe J., Hori K., Ozeki S., *J. Pharmacobio-Dyn.*, **8**, 468—476 (1985).
- 5) Watanabe J., Haba M., Muranishi H., Yuasa H., *Biol. Pharm. Bull.*, **16**, 1031—1034 (1993).
- 6) Watanabe J., Muranishi H., Yuasa H., Ozeki S., *Chem. Pharm. Bull.*, **40**, 3052—3055 (1992).
- 7) Watanabe J., Muranishi H., Yuasa H., Ozeki S., *J. Pharm. Sci.*, **81**, 513—517 (1992).
- 8) Watanabe J., Muranishi H., Yuasa H., *Biol. Pharm. Bull.*, **16**, 497—500 (1993).
- 9) Watanabe J., Haba M., Yuasa H., *Pharm. Res.*, **12**, 1092—1095 (1995).
- 10) Smedsrod B., Pertoft H., *J. Leuko. Biol.*, **38**, 213—230 (1985).
- 11) Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J., *J. Biol. Chem.*, **193**, 265—275 (1951).
- 12) Yamaoka K., Tanigawara Y., Nakagawa T., Uno T., *J. Pharmacobio-Dyn.*, **4**, 879—885 (1981).
- 13) Murata Y., Behr S. R., Kraemer F. B., *Biochim. Biophys. Acta*, **972**, 17—24 (1988).
- 14) Knauf P. A., Rothstein A., *J. Gen. Phys.*, **58**, 190—210 (1971).
- 15) Falcone D. J., *J. Cell. Physiol.*, **140**, 219—226 (1989).
- 16) Yanai S., Sugiyama Y., Kim D. C., Iga T., Fuwa T., Hanano M., *Am. J. Physiol.*, **260**, 457—467 (1991).
- 17) Terasaki T., Takakuwa S., Saheki A., Moritani S., Shimura T., Tabata S., Tsuji A., *Pharm. Res.*, **9**, 529—534 (1992).
- 18) Wiley H. S., Cunningham D. D., *J. Biol. Chem.*, **257**, 4222—4229 (1982).
- 19) Ohkuma S., Poole B., *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 3327—3331 (1978).
- 20) Sandvig K., Van Deurs B., *J. Biol. Chem.*, **265**, 6382—6388 (1990).
- 21) Pratten M. K., Lloyd J. B., *Biochem. J.*, **180**, 567—571 (1979).
- 22) Ottand E., Via D. P., Frubis J., Sinn H., Freidrich E., Ziegler R., Dresel H. A., *Biochem. J.*, **281**, 745—751 (1992).
- 23) Horiuchi S., Takata K., Maeda H., Morino Y., *J. Biol. Chem.*, **259**, 53—56 (1985).
- 24) Takata K., *J. Biol. Chem.*, **263**, 14819—14825 (1988).
- 25) Rohrer L., Freeman M., Kodama T., Penman M., Krieger M., *Nature (London)*, **343**, 570—572 (1990).
- 26) Barzu T., Molho P., Tobelem G., Petitou M., Caen J., *Biochim. Biophys. Acta*, **845**, 196—203 (1985).
- 27) Srinivasan S. R., Vijayagopal P., Eberle K., Radhakrishnamurthy B., Berenson G. S., *Biochim. Biophys. Acta*, **1081**, 188—196 (1991).
- 28) Kamps J. A. A. M., Kruijt J. K., Kuiper J., Van Berkel T. J. C., *Arterioscler. Thromb.*, **12**, 1079—1087 (1992).
- 29) Furitu H., Yamashita T., Takakura Y., Hashida M., *Yakubutsu Dotai*, **8**, 907 (1993).
- 30) Harenberg J., Stehle G., Augustin J., Zimmerman R., *Semin. Thromb. Hemostasis*, **15**, 414—423 (1989).