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Studies on the Uptake Mechanism of Liposomes by Perfused Rat Liver. I. An Investigation of Effluent Profiles with Perfusate Containing No Blood Component[1)]

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Uptake of liposomes by perfused rat liver was examined, and the effluent profiles are discussed. Reverse-phase evaporation vesicles (REV, about 0.1—0.2 μm in diameter) were able to pass through the liver without any interaction or interference and there was little uptake by the liver during single perfusion with phosphate-buffered saline. The transit time of REV was shorter than that of inulin simultaneously injected as a flow marker. These results suggest that the uptake of REV by the liver requires opsonization by blood components, and if REV can escape opsonization, they may be able to pass through the liver freely. It is also clear that the distribution volume of the REV is smaller than that of inulin.

On the other hand, small unilamellar vesicles (SUV, about 0.06 μm in diameter) showed uptake corresponding to about 0.25 μmol of total lipid without any participation of blood components. This result suggests that the uptake mechanism of SUV may be different from that of REV, and opsonization may not be essential for the uptake of SUV by the liver. A comparison of the results for REV and SUV suggests that the functional pore size of the fenestration of liver sinusoids is about 0.06 μm, and about half of the SUV prepared in this study could pass through the fenestration and reach the hepatocytes, while the other part of the SUV drained through the liver as did REV.

The uptake of SUV by the liver seemed to be limited in capacity, and it was influenced by temperature and inhibited by predosing with liposomes containing sodium azide or cytochalasin B.

Keywords——liposome; perfused liver; opsonization; uptake; liver sinusoid; metabolic inhibitor; mean transit time

Introduction

In recent years, many studies on the application of liposomes as drug carriers have been reported.[2)] For that purpose, it is important to understand the factors affecting the biological fate of liposomes. It is generally accepted that substantial fractions of intravenously injected liposomes are rapidly taken up by the liver.[3)] This uptake may be convenient if the liver is a target organ, but it is generally a problem to be overcome in the case of usage of liposomes for sustained release of a drug or targeting to other organs. Thus, it is necessary to control the uptake of liposomes by the liver. Blockade of the reticuloendothelial system (RES) by predosing of empty liposomes[4)] or other colloids[5-8)] may be one approach for depressing the liver uptake, but such attempts have not yet yielded satisfactory results.

On the other hand, attempts have been made to elucidate the mechanisms of the liver uptake of liposomes.[8,9)] Large liposomes are generally cleared more rapidly than small ones,[10,11)] and they are taken up by the Kupffer cells lining the liver sinusoids, whereas small unilamellar liposomes (SUV) are able to pass through the fenestration of the liver sinusoids and are taken up by the hepatocytes.[8,12)] Besides the size, lipid composition and surface charge were reported to affect the blood clearance or liver uptake of liposomes.[10,13,14)] It also

seems that the uptake is affected by the blood components.[15] However, the detailed mechanism of the liver uptake is still uncertain, because many complex biological factors may affect the uptake in an *in vivo* study.

When the mechanism becomes apparent, it should be possible to control the biological fate of liposomes for the purpose of development of effective drug carriers circulating throughout the whole body and having targeting capacity for other tissues or organs. In this study, in order to obtain information about the mechanisms, the effluent profiles of liposomes were examined during single perfusion of the rat liver. To simplify the system and to avoid interaction with the blood components, the perfusion was carried out with phosphate-buffered solution saturated with O_2–CO_2 (95 : 5) and containing no blood components.

Experimental

Materials——Egg L-α-phosphatidylcholine (PC), egg L-α-phosphatidic acid sodium salt (PA), D-α-tocopherol (α-T) and cytochalasin B were purchased from Sigma Chem. Co. (St. Louis, MO). PA was extracted from acid aqueous solution with chloroform and methanol (9 : 1) prior to use. Cholesterol (CH) and inulin were from Kanto Chem. Co. (Tokyo). Sodium azide and sodium iodoacetate were from Nakarai Chem. Ltd. (Kyoto). ^{3}H-Inulin and ^{14}C-cholesterol were from New England Nuclear (Boston, MA). All other chemicals were of reagent grade or better.

Preparation of Liposomes——The liposomes used in this experiment were composed of PC, PA, CH and α-T in a molar ratio of 4 : 1 : 3 : 0.1, and they contained appropriate radioactivity of ^{14}C-CH as a liposomal marker. Reverse-phase evaporation vesicles (REV) were prepared as described in a previous paper,[16] and sized by extrusion and dialysis as described in the previous paper.[17] Mean diameters of the REV extruded through polycarbonate membranes, having pore sizes of 0.2 μm and 0.1 μm (0.2-REV and 0.1-REV) were 0.223 ± 0.050 μm and 0.151 ± 0.063 μm, respectively (Coulter, model N4). SUV were prepared by the sonication method as described in a previous paper,[16] and their mean diameter was 0.057 ± 0.020 μm.

Liver Perfusion——The liver of a Wistar male rat (body weight 200 ± 10 g) was perfused *in situ* with phosphate-buffered saline (PBS, pH 7.4, 280 mOsm/kg) which was saturated with O_2–CO_2 (95 : 5), from the portal vein to the inferior *vena cava* according to the method of Tyrrell *et al.*[18] at 37 °C as shown in Fig. 1. The flow rate of the perfusate was 6.5 ml/min. Several minutes after the start of the perfusion, *i.e.* after the liver became pale due to loss of the red blood cells, 0.1 ml of liposome suspension (0.5 μmol of total lipid), to which 0.01 nmol of inulin and ^{3}H-inulin had been added as markers of channels and leakage in the liver, was rapidly injected at the portal vein side. Immediately after injection, the outflow from the *vena cava* was collected in fractions of 6 drops each (about 0.5 ml). The radioactivity of each fraction was counted with a liquid scintillation counter (Aloka LSC-673) after adding scintillation cocktail (Scintisol EX-H, Wako Pure Chem. Co., Osaka). The results obtained in experiments with a low recovery of total inulin in 20 fractions (below 80%) were omitted. In some experiments, the viability of the liver cells was checked by the Trypan-blue exclusion test after isolation of the cells without separation of the parenchymal and non-parenchymal cells[19] at the end of the experiment; it was always more than 85%.

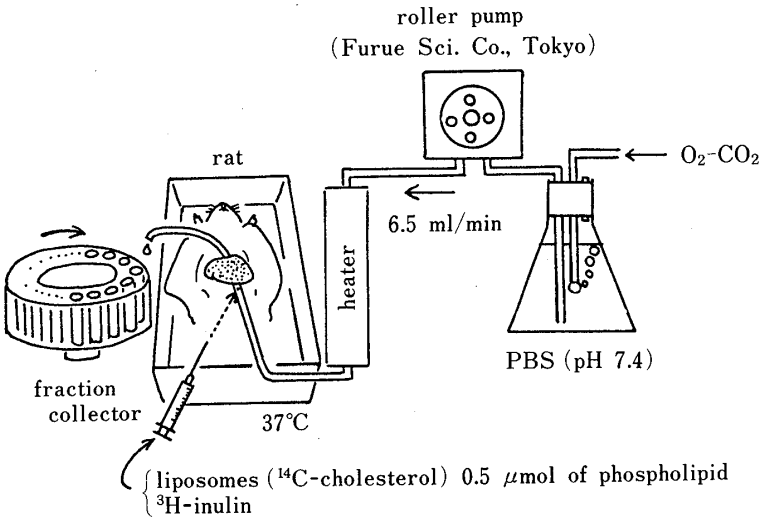

Fig. 1. Illustration of the Perfusion System Used in the Present Experiment

Opsonization——The liposomal suspension prepared as described above (0.2-REV) at the high lipid concentration (40 μmol/ml) was diluted 8 times with fresh rat serum to give the same lipid concentration as in the control experiment, and incubated at 20 °C for 1 h.[20] The opsonized liposomes were used for the experiment immediately. Release or transfer of the liposomal marker (^{14}C-CH) from the liposomes during the incubation was examined by gel filtration (Sephacryl S-1000; Pharmacia Fine Chem., Sweden) and no transfer of the marker was observed.

Effects of Temperature and Metabolic Inhibitor——The experiments at low temperature were carried out at 20 °C through cannulation and perfusion. Three types of metabolic inhibitor were used in the inhibition experiments, as reported by Hsu and Juliano.[15] SUV containing these inhibitors, sodium iodoacetate, sodium azide and cytochalasin B, were prepared at concentrations of 0.2, 0.04 and 0.04 mg/20 μmol total lipids, respectively. Two hours before the perfusion experiment, the liposomes containing the inhibitor were injected intravenously into the rat at the dose of 20 μmol of total lipid. In the control experiment, empty liposomes were injected at the same dose.

Evaluation of Data——The recovery and mean effluent fraction number were determined. The recovery was calculated as the ratio of the total liposomes (^{14}C-CH) passing through the liver to inulin (^{3}H-inulin) which was injected simultaneously as follows;

$$\text{recovery} = \frac{\sum_{i=1}^{20} F^{L}(i)}{\sum_{i=1} F^{I}(i)}$$

where i is the fraction number, and $F^{L}(i)$ and $F^{I}(i)$ are the percentages of injected liposomes and inulin in fraction i, respectively. Inulin is considered to be inert as regards biological interactions,[5,9] so a low recovery indicates the uptake of liposomes by the liver, corrected for leakage in the perfusion system. Mean transit time (Tr) was expressed as follows[21];

$$Tr = \frac{\int_0^{\infty} t \cdot C(t)dt}{\int_0^{\infty} C(t)dt}$$

where $C(t)$ is the concentration of the indicator in the outflow at time t. Thus, a large Tr value indicates a long residence time of the indicator in the system, and shows that the indicator has a large distribution volume in the system or an affinity for the system.[21]

In the present study, the time was expressed in terms of the fraction number, and concentration was expressed as percent of injected dose, using the mean effluent fraction number (MEF) calculated as follows;

$$MEF = \frac{\sum_{i=1}^{20} i \cdot F(i)}{\sum_{i=1} F(i)}$$

where $F(i)$ is the percent of the dose in fraction i. This is comparable to the Tr and corresponds to the mean effluent fraction number of the indicator (in the present case, ^{3}H-inulin and ^{14}C-labeled liposomes) effused from the liver, as well as the residence time of the indicator in the liver.

Results and Discussion

Effect of Liposomal Size

Three types of liposomes (0.2-REV, 0.1-REV and SUV) were injected into the perfused rat liver with inulin, and the effluent patterns are shown in Fig. 2. They show that liposomes can pass more rapidly through the liver than inulin. The calculated MEF and recovery of the liposomes *vs.* inulin are listed in Table I. A smaller MEF value of liposomes than inulin suggests that the distribution volume of liposomes in the liver is smaller than that of inulin. This in turn indicates that liposomes cannot pass through the fenestration of the liver sinusoids, whereas inulin can pass through the fenestration into the Disse's spaces of the liver. The mean diameter of the fenestration has been reported to be 0.1 μm,[22] though a larger diameter of more than 0.25 μm has also been reported.[23] However, the results obtained in the

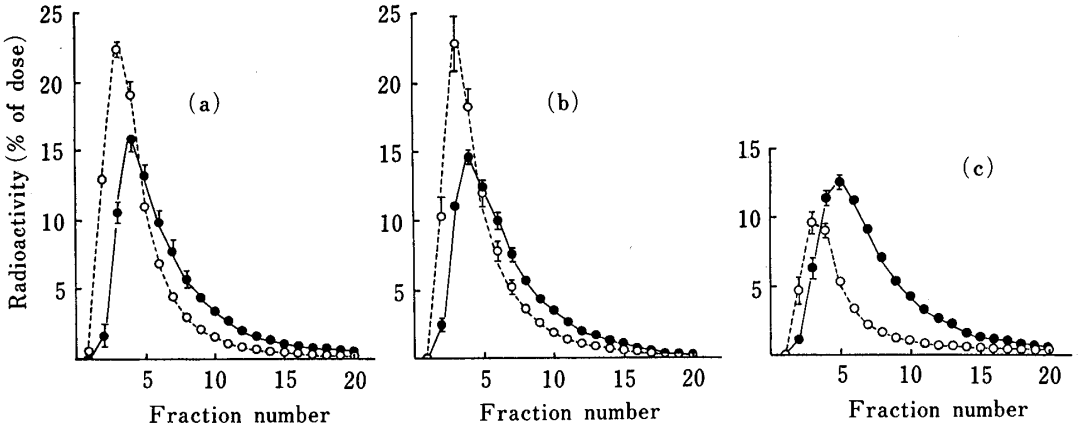

Fig. 2. Effluent Patterns of Various Liposomes from Perfused Rat Liver after Single Injection

(a) 0.2-REV, (b) 0.1-REV, (c) SUV.
Mean values and S.D. of three experiments.
---○---, liposomes; —●—, inulin.

TABLE I. Effects of Size or Opsonization on the Recovery and *MEF*

	Recovery (%)	*MEF* (Fraction)		Remaining activity (%)	
		Liposomes	Inulin	Liposomes	Inulin
0.2-REV	99.79± 1.54	4.99±0.56	6.82±0.16[a]	8.56±1.36	8.41±0.99
0.1-REV	101.44± 2.53	5.12±0.33	6.77±0.07	ND	ND
SUV	54.87± 5.15[b]	5.72±0.19	7.29±0.60	47.60±2.71	7.85±1.31
Ops-REV[c]	89.34±14.32	6.69±0.57[d]	6.78±0.50	ND	ND

a) Significantly different from *MEF* of liposomes ($p<0.01$). *b*) Significantly different from 0.2-REV ($p<0.01$). *c*) Opsonized 0.2-REV. *d*) Significantly different from 0.2-REV ($p<0.05$). Values are means ± S.D. of three experiments. ND: not determined.

present study suggest that the functional size of the fenestration of the liver sinusoids is smaller than the literature values, because 0.2-REV (mean diameter 0.223 μm), 0.1-REV (0.151 μm) and a part of SUV (0.057 μm) could not pass through it, as shown in Table I. Therefore, it is expected that SUV smaller than those used in this experiment might be taken up more effectively by the liver.

Recovery of SUV was about 50% as shown in Table I. In some experiments, we checked whether the residual activity remained in the liver by counting the activity after homogenization and digestion with KOH/isopropanol. Reasonable activity was found in the liver, as shown in Table I. This result shows that about a half of SUV was taken up by the liver, possibly by parenchymal cells as reported by Roerdink *et al.*[8] and Rahman *et al.*[12] In contrast, the recoveries of both types of REV were almost complete, as shown in Table I. This suggests that the REV are able to pass freely through the liver if no blood components interact with the liposomes, and about a half of SUV can also pass freely through the liver because of the small *MEF* value.

The most important observation obtained in this experiment is that REV may be able to pass unimpeded through the liver if they do not interact with blood components, whereas SUV smaller than 0.06 μm are taken up by the liver *per se* in the absence of any blood components.

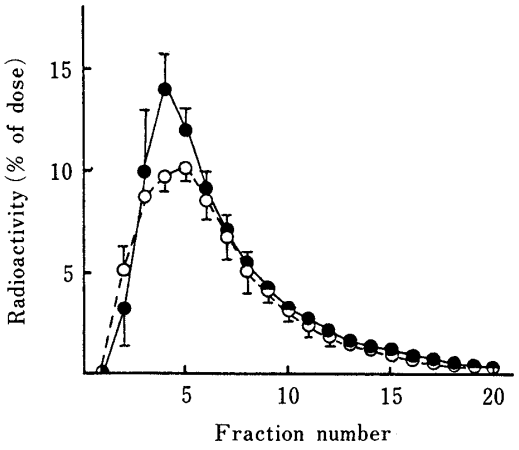

Fig. 3. Effect of Opsonization on the Passage of 0.2-REV through the Perfused Rat Liver

All symbols and expressions are the same as those in Fig. 2.

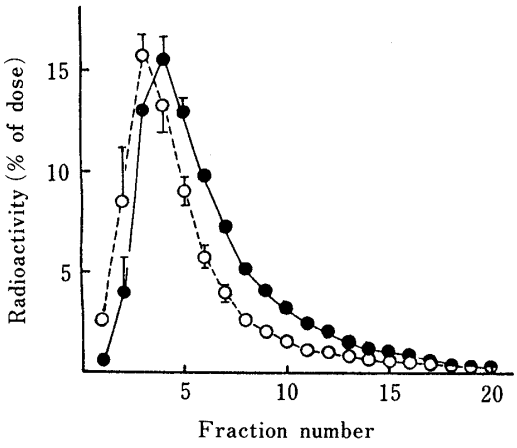

Fig. 4. Effluent Pattern of SUV at 20 °C

All symbols and expressions are the same as those in Fig. 2.

Effect of Opsonization

The effluent pattern of the opsonized 0.2-REV is shown in Fig. 3, and *MEF* value and recovery are given in Table I. The recovery of the opsonized REV was about 90%. However, the recovery did not show a statistically significant difference as compared with that of non-opsonized REV. Therefore the uptake mechanism of the opsonized liposomes seems to be very complex. It seemed difficult to reproduce the *in vivo* uptake in the present *in vitro* experiment; possibly some other factor(s) such as inorganic ions omitted from the present perfusate is required for the uptake by the liver.

On the other hand, the *MEF* value was significantly larger than those of non-opsonized liposomes, as shown in Table I. This indicates that the REV acquired affinity for the liver as a result of incubation with serum, because it seems unlikely that the opsonized liposomes had a larger distribution volume in the liver. It is considered that the duration of transit is not altered by any effect of the serum injected with liposomes on the liver or liver sinusoids, because the injected volume of the serum was only 0.1 ml, which is very small compared with the amount of perfusate. Therefore, the effect observed in this experiment seems to be caused by the opsonization at the surface of the liposomes by some blood component(s), though it is not yet clear what factor(s) in the blood contributes to this effect. Hsu and Juliano reported that plasma fibronectin increased the uptake of liposomes by mouse peritoneal macrophages.[15] Opsonizing ability of plasma fibronectin was reported with lipid emulsion.[24] Alving reported the occurrence of natural antibodies against phospholipid and liposomes in Humans.[25] Therefore, the plasma fibronectin and/or the antibodies might play a role as an opsonin in the present experiment.

Effect of Temperature

The uptake of SUV by the perfused liver decreased at lower temperature (20 °C) as shown in Fig. 4. The recovery and *MEF* are shown in Table II. The result shows that the uptake mechanism of SUV by the liver is influenced by temperature. This suggests that the uptake of SUV in the present system involves a biological mechanism, and is not due to a simple mechanical stoppage in the liver sinusoids.

Dose Dependency

As presented above, about 50% of SUV injected at the dose of 0.5 μmol/head (about 0.25 μmol) was trapped in the liver. However, it is uncertain whether the 0.25 μmol of uptake

TABLE II. Recovery and *MEF* of SUV under Various Experimental Conditions

Conditions	Recovery (%)	*MEF* (Fraction)	
		Liposomes	Inulin
Control[a]	54.87± 5.15	5.72±0.19	7.29±0.60
20 °C	81.00± 1.55[b]	5.37±0.35	6.43±0.29
Low dose	62.04±10.05	6.02±0.33	7.38±0.91
High dose	78.77± 9.22[c]	6.18±0.34	7.69±0.40
Empty[d]	50.57± 2.77	5.68±0.09	7.40±0.11
Sodium azide	81.94±10.18[e]	5.58±0.17	6.85±0.14
Iodoacetate	58.77± 6.65	5.47±0.10	6.84±0.10
Cytochalasin B	85.59±13.38[f]	5.37±0.82	6.53±0.98

a) Experimental conditions for the control were as follows: dose, medium (0.5 μmol/head); temperature, 37 °C; no predosing. *b*) Significantly different from the control ($p<0.01$). *c*) Significantly different from the control ($p<0.02$). *d*) Control for the effect of metabolic inhibitor; predosing of liposomes containing no inhibitor. *e*) Significantly different from *d* ($p<0.01$). *f*) Significantly different from *d* ($p<0.02$). Values are mean ± S.D. of three experiments.

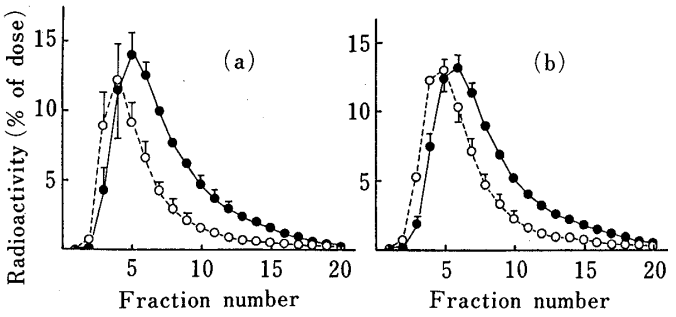

Fig. 5. Dose Dependency of the Passage of SUV through the Perfused Rat Liver

(a) low dose (0.2 μmol), (b) high dose (1.25 μmol). All symbols and expressions are the same as those in Fig. 2.

represents the capacity of the liver or whether a half of the SUV preparation can be taken up by the liver and the other half cannot, as mentioned above. If 0.25 μmol of lipid is the uptake capacity of the liver, complete or at least higher uptake would be expected after the injection of 0.2 μmol/head SUV. As shown in Fig. 5, there was no significant difference in the recovery and the *MEF* (Table II). This result suggests that a part of the larger SUV preparation (mean diameter 0.057 μm) in the present experiments may act as REV, and smaller SUV can pass unimpeded through the fenestration and be taken up by the hepatocytes. Therefore, the functional pore size of the fenestration of the liver sinusoids seems to be about 0.06 μm. This speculation agrees with the observations of Roerdink *et al.*[8] and Rhaman *et al.*[12] that larger liposomes were taken up by the Kupffer cells and SUV were taken up by both the Kupffer cells and hepatocytes. Berger *et al.*[26] indicated that particles of less than 0.05 μm can pass through the fenestrations of the liver endothelium and this also supports our speculation.

On the other hand, in the case of a high dose (1.25 μmol/head), lower uptake (recovery was about 80%) was observed. However, the trapped amount was about 0.25 μmol, and this amount was similar to that in the case of the medium dose (0.5 μmol/head). It seems likely that the uptake capacity of SUV by the liver is limited, and is about 0.25 μmol of liposomal lipid at least in this single perfusion system.

Effect of Metabolic Inhibitor

Predosing of empty SUV (20 μmol/head) at 2 h before the test injection did not affect the effluent pattern, as shown in Fig. 6a, and recovery and *MEF* also showed no significant difference from the control values, as shown in Table II. On the other hand, predosing of SUV containing various metabolic inhibitors changed the effluent patterns of SUV, as shown in

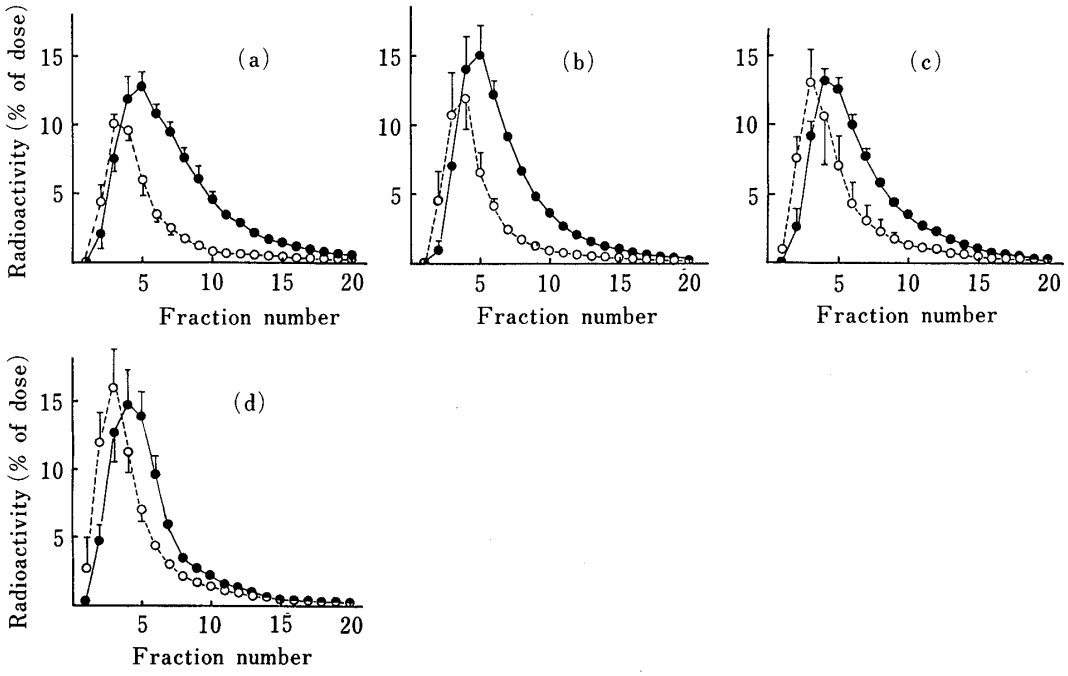

Fig. 6. Effects of Metabolic Inhibitors on the Passage of SUV through the Perfused Rat Liver

(a) control (empty liposomes), (b) iodoacetate, (c) sodium azide, and (d) cytochalasin B. All symbols and expressions are the same as those in Fig. 2.

Fig. 6, and recovery was increased (Table II). Only iodoacetate had little effect (no significant difference compared with the predosing of empty liposomes). Hsu and Juliano[14] reported on the effects of the same metabolic inhibitors on the uptake of liposomes by mouse peritoneal macrophages, and showed that iodoacetate and cytochalasin B inhibited the uptake but sodium azide did not. They commented that this pattern of inhibition is typical of 'classic' phagocytosis; these inhibitors would not be expected to affect the liposome binding to the cell surface or liposome-cell fusion. The results obtained in this study showed a different inhibitory pattern, *i.e.*, cytochalasin B and sodium azide were inhibitory and iodoacetate was not. We may speculate that the retention of SUV in the liver in the present perfusion system may not be due to phagocytosis but to binding on the cell surface of hepatocytes. However, the inhibitors were administered in encapsulated form in liposomes in this system, so the inhibitory effects might be different from the *in vitro* data in the literature.[15]

Conclusion

In a rat liver perfusion system, REV can pass unimpeded through the liver, whereas SUV are taken up by the liver in the absence of blood components. The results suggested that REV are taken up by the Kupffer cells after opsonization, whereas SUV, perhaps less than 0.06 μm in diameter, may be taken up by the hepatocytes directly without opsonization. This uptake process of SUV is saturable, is influenced by temperature, and is inhibited by some metabolic inhibitors.

It became apparent that the uptake mechanisms of REV and SUV (less than about 0.06 μm in diameter) are different, and the possibility arises that large liposomes might pass through the liver if they can escape the opsonization process *in vivo*. It is also suggested that small liposomes (less than about 0.06 μm) are more convenient for targeting to the hepatocytes. However, the REV incubated with serum did not show significant uptake, though they acquired affinity for the liver. It seems that complex factors govern the *in vivo* fate of liposomes.

References and Notes

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