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Application of Drug-Containing Liposomes to the Duration of the Intramuscular Absorption of Water-Soluble Drugs in Rats[1,2]

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An attempt was made to use liposomes as a carrier intended for prolonged intramuscular absorption of highly water-soluble drugs. ^{131}I-Insulin, ^{14}C-sucrose, ^{14}C-inulin, and ^{14}C-cefazolin sodium, a new derivative of cepharosporin antibiotics, were chosen as model compounds.

These model drugs were dissolved in pH 7.0 buffer solution and entrapped in liposomes composed of various ratios of phosphatidyl choline, cholesterol, and dicetylphosphate. After injection in the form of such liposomal suspensions, clearance of model drugs from the intramuscular injection site was delayed to a considerable extent in comparison with the control aqueous buffer injections. It was observed that the larger the amount of cholesterol incorporation into liposomes were, the slower the drug absorption became.

Although the intramuscular absorption of the drugs from the rat thigh muscle was delayed by their entrapment in liposomes, extent of the prolongation was less than that expected from their *in vitro* release characteristics determined by dialysis experiments. Physical stability of liposomes containing ^{14}C-cefazolin sodium was tested in the presence of serum and muscle homogenate. It appears that liposomes are gradually hydrolysed or affected by serum thereby losing their integrity and releasing the entrapped drugs.

Parenteral dosage forms with prolonged action are of pharmaceutical importance. Several ways to approach duration of parenteral absorption have been reported. Implants, oleaginous solution and suspensions are some examples. Recently, an attempt to inject subcutaneously trifluoperazine embonate entrapped in microcapsules has been reported.[4]

What is relatively lacking in these studies is an attempt to prolong the duration of parenteral absorption of drugs of high water solubility. The present investigation has been undertaken to develop a parenteral drug delivery device of prolonged release type suitable for such drugs using liposomes. Liposomes,[5] artificial lipid spherules, have been used as a biomembrane model. Since liposomes can entrap drugs of high water solubility and negligible membrane interaction, use of liposomes as a means of drug delivery intended for prolonged release would be of interest.

Experimental

1) Materials

^{14}C-Sucrose, ^{14}C-inulin, and ^{131}I-insulin were purchased from Japan Radioisotope Association (Tokyo, Japan). Sodium ^{14}C-cefazolin, sodium 7-[1-(1H)-tetrazolylacetamido]-3-[2-(5-methyl-1,3,4-thiadiazolyl)-thiomethyl-2-^{14}C-Δ^3-cepham]-4-carboxylate, was kindly donated by Fujisawa Pharmaceutical Co., Ltd.

1) *a*) This paper constitutes the 9th report in a series of "Biopharmaceutical Studies on the Parenteral Preparations." *b*) Preceding paper, Part VIII: T. Tanaka, H. Kobayashi, K. Okumura, S. Muranishi, and H. Sezaki, *Chem. Pharm. Bull.* (Tokyo), **22**, 1275 (1974).

2) Part of this work was presented at the 93rd Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April, 1973.

3) Location: *Yoshidashimoadachi-cho, Sakyo-ku, Kyoto, 606, Japan*; *a*) To whom inquiries should be directed.

4) A.T. Florence, A.W. Jenkins, and A.H. Loveless, *J. Pharm. Pharmacol.*, **25**, 120p. (1973).

5) A.D. Bangham, *Annu. Rev. Biochem.*, **41**, 753 (1972).

Cholesterol and dicetylphosphate were of analytical grade and used as received. Phosphatidylcholine was extracted from egg yolk with chloroform–methanol (1: 1) and chromatographed on neutral alumina (W 200, Woelm). Neutral lipids were removed with chloroform and phosphatidylcholine fraction was eluted with chloroform–methanol (3: 2) and evaporated under nitrogen. The residue was re-dissolved in chloroform and stored under nitrogen at −5°.

2) Preparation of Liposome Solutions

Ten μmoles of phosphatidylcholine was mixed with cholesterol and dicetylphosphate or stearylamine in various molar ratios and the mixture was dissolved in chloroform in a 10 ml round-bottomed flask. The flask was fitted in a rotary evaporator and a thin film of lipids was formed on the surface of the flask on evaporation. The trace of the solvent was completely removed under reduced pressure (5 mmHg) for over one hour. This dried thin film of lipids was re-suspended by mechanical shaking with a Vortex mixer in 1 ml of a drug solution (^{14}C-cefazolin, 20 μCi/ml; ^{14}C-inulin, 13 μCi/ml; ^{14}C-sucrose, 1 μCi/ml; and ^{131}I-insulin, 200 μCi/ml) in buffered saline (Na_2HPO_4–NaH_2PO_4 pH 7.0 buffer and 0.9% NaCl solution, 1/10 and 9/10 by volume, respectively) with about 20 glass beads of 0.5 mm in diameter at room temperature for 20 min. After mixing, free drug (non-entrapped drug) was removed by passing a Sephadex column (16 mm ID, 170 mm length) and eluting with the buffered saline. Sephadex G-50 was used for sucrose and cefazolin, and Sephadex G-75 for inulin and insulin.

3) *In Vitro* Experiments

a) Drug Release Experiments——The release of a drug from liposomal suspensions was determined by the modified method of dynamic dialysis system of Meyer and Guttman[6] with the apparatus shown in Fig. 1. Eight ml of a liposomal suspension containing a drug was added to a Visking dialysis tube (20/32) (inner solution) and a beaker containing 80 ml of buffered saline (outer solution) was maintained at 37° by circulating water. Solutions were stirred at the rates of 180 and 400—500 rpm for the inner and the outer solution respectively and 1 ml sample was withdrawn from the outer solution at fixed time intervals for analyses. Adsorption of drugs to Visking dialysis tube was negligible.

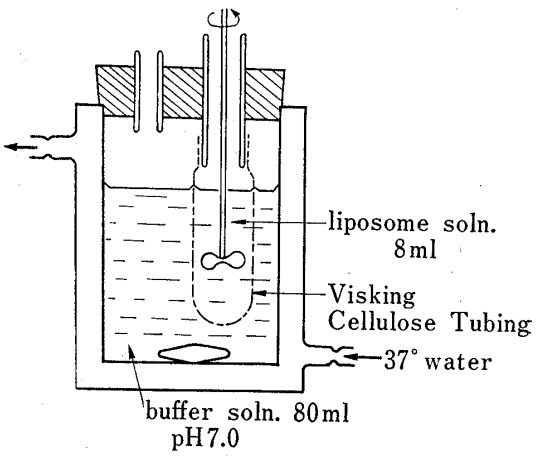

Fig. 1. Apparatus used to Study the Release of Drug from Liposome Suspensions

b) Effect of Serum and Muscle Homogenate on the Release of Drugs from Liposomes——Blood was collected from the carotid arteries of a rat killed by a blow on the head and centrifuged at 0°. Muscle of the hind legs of the bled rat was homogenized with the buffered saline and diluted to about 6 ml with the same buffer solution. Drug-containing liposome suspension was mixed with the serum or muscle homogenate in a volume ratio of 2:1, transferred into a Visking tube (20/32) and the release of a drug from liposomes was determined in a similar manner as mentioned elsewhere.

4) Animals

Male Wistar albino rats weighing 160—180 g were used in all the absorption experiments.

5) Procedure of Absorption Experiments

The absorption experiments were almost identical with those described in the previous paper from this laboratory.[7] The injection volume of 20 μl was delivered into the center of the *m. rectus femoris* and the amount of drug remaining in the muscle was determined.

6) Analytical Methods

a) ^{14}C-Sucrose, ^{14}C-Inulin, and ^{14}C-Cefazolin——(i) *In Vitro* Experiments: The radioactivity of the outer solution was determined according to the method described in the previous paper[1] with NT-scintillation medium.

(ii) Absorption Experiments: The radioactivity remained in the muscle was determined by the method described previously.[8]

b) ^{131}I-Insulin——(i) *In Vitro* Experiments: One milliliter of the outer solution was added into a polyethylene tube and ^{131}I activity was determined in a well-type scintillation counter (Model ATS-121).

(ii) Absorption Experiments: The muscle was excised, homogenized, and dissolved in distilled water to make a volume of 5 ml. The protein was precipitated by the addition of 2 ml of 10% $ZnSO_4$ solution. After centrifugation, 1 ml of the supernatant was analyzed in the same manner described elsewhere.

6) M.C. Meyer and D.E. Guttman, *J. Pharm. Sci.*, **59**, 33 (1970).
7) K. Kakemi, H. Sezaki, K. Okumura, and S. Ashida, *Chem. Pharm. Bull.* (Tokyo), **17**, 1332 (1969).
8) H. Kobayashi, T. Nishimura, K. Okumura, S. Muranishi, and H. Sezaki, *J. Pharm. Sci.*, **63**, 580 (1974).

Results and Discussion

Oleaginous solution and suspensions, emulsions, and aqueous suspensions are currently used as the parenteral preparations intended for delayed absorption. These preparations, however, are sometimes unsuitable for a drug or prodrug having high aqueous solubility. A method for ensuring delayed or prolonged parenteral absorption of such drugs is desirable.

Liposomes are biodegradable and consist of concentric lipid bilayers alternating with aqueous compartment within which water-soluble drugs can be entrapped.

Previous investigation in this field clarified that most of the drugs injected into the muscle in the form of aqueous injections are absorbed mainly through the pores of the capillary. It was considered therefore that the extravascular application of drug-containing liposomes should result in duration of the intramuscular absorption since drugs contained in liposomes were absorbed rapidly after being released slowly from liposomes for a prolonged period of time.

Figures 2 and 3 show the results of some of the *in vitro* drug release experiments. Figure 2 shows the release of ^{14}C-inulin from liposomes prepared with phosphatidylcholine, cholesterol, and dicetylphosphate in various molar ratios.

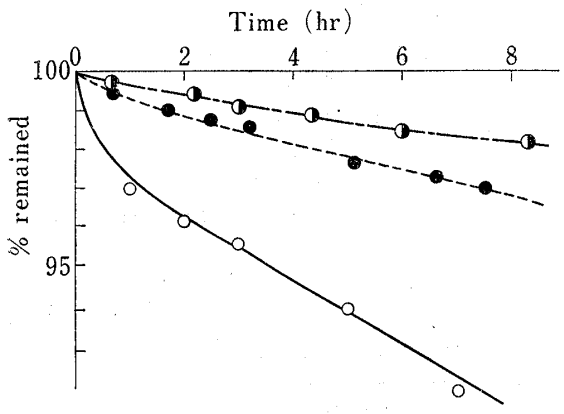

Fig. 2. Release of ^{14}C-Inulin from Liposome Suspensions

—○—○—: (L: C: D=6: 2: 1) L……lecithin
···●···●···: (L: C: D=6: 6: 1) C……cholesterol
——◑——: (L: C: D=3: 9: 1) D……dicetylphosphate
Each point represents the mean value of at least three experiments.

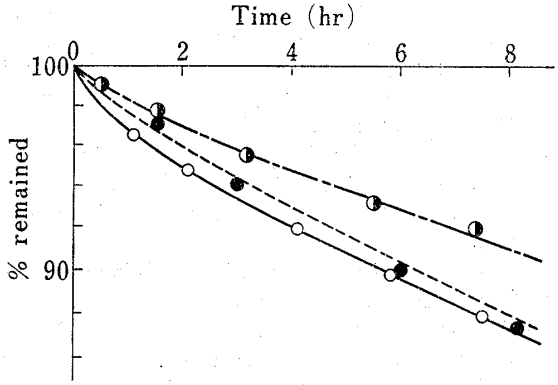

Fig. 3. Release of Sodium ^{14}C-Cefazolin from Liposome Suspensions

Each point represent the mean value of at least three experiments. Key: Refer to Fig. 2 for the points in this graph.

It is obvious from the figure that contrary to the rapid release of free ^{14}C-inulin from a Visking dialysis sac, the release of ^{14}C-inulin from liposomes and through Visking sac was considerably small. Since the half-release time of the drugs in buffer solution through Visking sac, $T_{50\%}$, usually fall within one hour, liposome entrapment resulted in a remarkable retardation of the release of inulin.

The release of ^{14}C-cefazolin, a new derivative of cephalosporin antibiotics, was likewise markedly reduced as shown in Fig. 3. Similar tendency was noticed in the cases of ^{131}I–insulin and ^{14}C-sucrose. It is worthy to note that the larger the amount of incorporation of cholesterol into the liposome compositions are, the slower the release of the drugs becomes. Such effect of cholesterol is more pronounced in the case of inulin than cefazolin. The role of sterol on the permeability and topography of the membrane has been studied extensively in mono and bi-layers. It is well established that a sterol like cholesterol reduces the mean molecular area of lecithin molecules when spread together with lecithin at the air-water interface.[9] Similar behavior of cholesterol may be expected in case of lipid bilayers. This is

9) L.L.M. Van Deenen, *Chem. Phys. Lipids*, **8**, 366 (1972).

indeed the case as demonstrated by the decrease in permeability of glycerol and glycol from liposomes in which cholesterol was inserted.[10] On the other hand, it seems likely that permeability of liposomal membranes depends not only on such details of the lipid compositions but also on the physicochemical nature of the drugs entrapped. There seems to be subtle differences in release rate among drugs in these experiments although same membrane compositions and same procedures of preparation were used. Many still unknown details such as drug-phospholipids interaction are needed to understand the permeability of drugs through liposomal membranes.

The absorption studies of drugs entrapped in liposomes were carried out in rats using previously established local clearance method. The results are shown in Figs. 4, 5, 6, and 7.

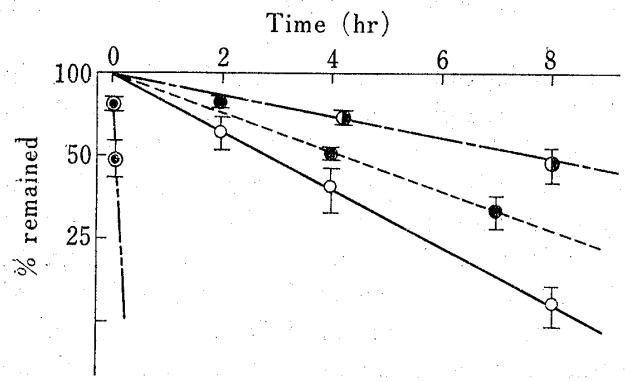

Fig. 4. Intramuscular Absorption of Sodium ^{14}C-Cefazolin

—○—○—: liposome (L: C: D=6: 2: 1)
···●···●···: liposome (L: C: D=6: 6: 1)
——◑—— : liposome (L: C: D=3: 9: 1)
——⊙——: aqueous solution
Each point represents the mean value of at least six experiments. Vertical bars indicate ± SD.

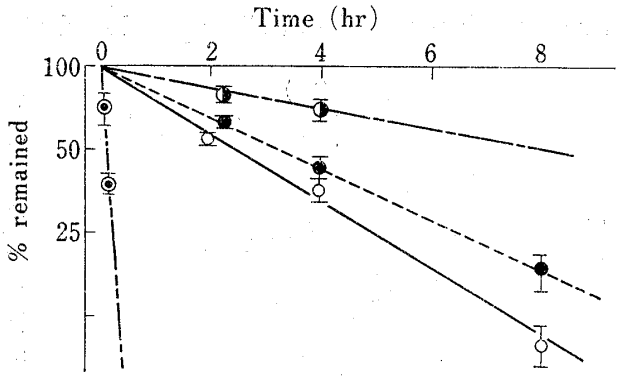

Fig. 5. Intramuscular Absorption of ^{131}I-Insulin

Each point represents the mean value of at least six experiments. Vertical bars indicate ± SD.
Key: Refer to Fig. 4 for the points in this graph.

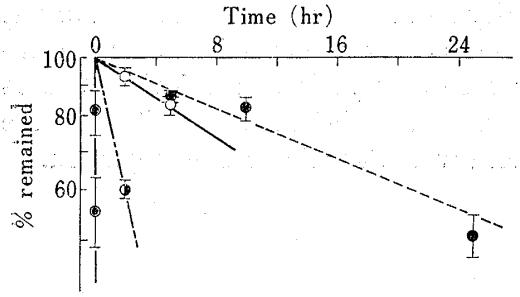

Fig. 6. Intramuscular Absorption of ^{14}C-Sucrose

—○—○—: liposome (L: C: D=4: 4: 1)
···●···●···: liposome (L: C: D=4: 12: 1)
——◑—— : liposome (L: C: D=5: 1: 0)
—⊙—⊙—: aqueous solution
Each point represents the mean value of at least six experiments. Vertical bars indicate ± SD.

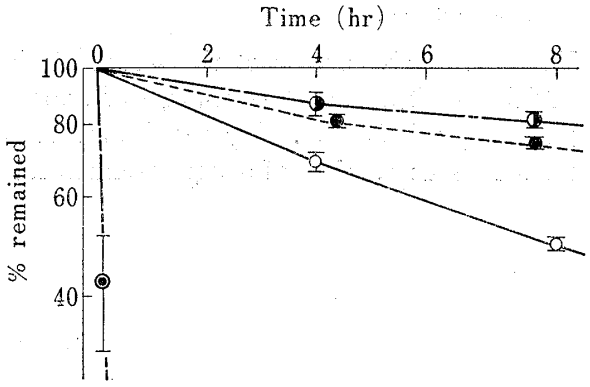

Fig. 7. Intramuscular Absorption of ^{14}C-Inulin

Each point represents the mean value of at least six experiments. Vertical bars indicate ± SD. Key: Refer to Fig. 4 for the points in this graph.

As shown in Fig. 4, intramuscular absorption of ^{14}C-cefazolin from liposomes was much slower than from aqueous buffer injection. The absorption half-life of the latter injection (av. 90 seconds) was prolonged to about 8 hrs in the case of liposomes composed of the molar ratio of 6: 2: 1 of phosphatidylcholine, cholesterol, and dicetylphosphate, respectively. As was

10) J. De Gier, J.G. Mandersloot, and L.L.M. Van Deenen, *Biochim. Biophys. Acta*, 173, 143 (1969).

observed in the *in vitro* release studies, the increase of cholesterol content augmented the liposomal effect of prolonged absorption.

Such effect was also evident in the intramuscular absorption of insulin, sucrose, and inulin from liposomal preparations. Absorption of ^{131}I-insulin entrapped in liposomes from the muscle was about 26 to 80 times slower than that of free insulin. In the cases of ^{14}C-sucrose and ^{14}C-inulin, more pronounced effect was noticed and the rate of absorption of the drugs entrapped in liposomes decreased to the extent of almost several hundred times slower than control aqueous buffer injections.

Although the intramuscular absorption of drugs were prolonged by their entrapment in liposomes, it should be noted that the extent of prolongation by liposomal preparations observed *in vivo* was much less than that expected from the results of *in vitro* release experiments. It is possible that the stability of liposomes injected into rat thigh muscle was affected by some biochemical as well as physiological factors.

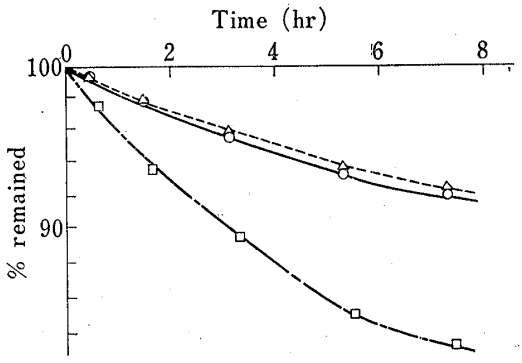

Fig. 8. Release of Sodium ^{14}C-Cefazolin from Liposome Suspensions

—○—○—: liposome only
···△···△···: with muscle homogenate
——□—— : with serum
lipid composition (L: C: D=3: 9: 1)
Each point represents the mean value of at least three experiments.

Stability of liposomes containing ^{14}C-cefazolin which has a lipid composition of phosphatidylcholine, cholesterol, and dicetylphosphate, 3:9:1, respectively in the presence of the muscle homogenate or the rat serum was investigated by *in vitro* release technique. As shown in Fig. 8, faster release of ^{14}C-cefazolin from the liposomal preparations was observed in the presence of serum, whereas in the presence of muscle tissue homogenate, almost equal release pattern with liposome alone was obtained. It appears therefore that gradual leakage of entrapped drug through liposomal membranes occurs from the intact liposome after the injection while liposomes remaining at the injection site are gradually hydrolyzed or affected by the serum thereby losing their integrity and releasing the entrapped drugs. The degradative action of the serum at the site of injection may be dependent on the composition of lipids, surface charges of liposomes, and on the other physico-chemical as well as physiological factors. By manipulating such characteristics, it should be possible to use liposomes as carriers of drugs intended for prolonged extravascular release after injection.