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Drug-carrier Property of Albumin Microspheres in Chemotherapy. II.[1] Preparation and Tissue Distribution in Mice of Microsphere-entrapped 5-Fluorouracil[2]

Kenji Sugibayashi, Yasunori Morimoto, Tanekazu Nadai, Yoshio Kato,[3a] Akihiko Hasegawa, and Takaichi Arita[3b]

Faculty of Pharmaceutical Sciences, Josai University,[3a] and Faculty of Pharmaceutical Sciences, Hokkaido University[3b]

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Bovine serum albumin microspheres containing 5-fluorouracil-6-^{3}H were prepared by heating at 180° (or 150°, 100°) of 25% albumin solution in cottonseed oil emulsion. The shape of this microsphere was invariably spherical, and the average diameter was 0.66 μ. After intravenous injection in mice, 5-fluorouracil-6-^{3}H entrapped in albumin microspheres localized mainly in the liver, and the disappearance rate of radioactivity in microspheres from the tissue was very slow in comparison with that of free drugs. The microsphere might be delivered into reticuloendothelial system in the liver because of its phagocytic activity, as well as the distribution following injection of albumin macroaggregates. Such preferential localization and sustained release of entrapped drugs suggested that albumin microspheres are useful as drug-carrier in chemotherapy.

Keywords——albumin microsphere; antitumor agent; drug-carrier; reticuloendothelial system; phagocytosis; drug distribution

After the drug is absorbed or injected into the blood stream, it may be distributed generally throughout the body in the medium of the body fluids. It is well known that the rate, extent, and pattern of the distribution are determined by the physicochemical characteristics of the drug. As antitumor agents frequently exert undesirable toxic effects, localization of the drugs to tumor sites would reduce the side effects. Then it is desirable that the antitumor drug selectively reaches its target tissues in drug therapy.

Past approaches to targeting of chemotherapeutic agents have been largely related to several drug carriers. Most tumor cells have been shown to phagocytize some spheres.[4,5] If a drug carrier containing an antitumor agent is phagocytized into tumor cell, the carrier may be digested with lysosomal enzymes in the cell and free active drug may be then released to the environments.

Trouet, *et al.*[6] reported reduced toxicity and increased effectiveness in the treatment of leukemia when DNA complexes of antitumor agent daunomycin were pinocytized from solution. Furthermore, they found that the free active drug could be released from DNA complex after digestion of the complex with lysosomal enzymes. The use of liposomes (phospholipid vesicles) as drug carriers has been noted recently. Gregoriadis and his coworkers[7]

1) Preceding paper, Part I: K. Sugibayashi, Y. Morimoto, T. Nadai, and Y. Kato, *Chem. Pharm. Bull.* (Tokyo), **25**, 3433 (1977).
2) Part of this work was presented at 98th Annual Meeting of Pharmaceutical Society of Japan, Okayama, April, 1978.
3) Location: *a) 1-1 Keyakidai, Sakado, Saitama, 350-02, Japan*; *b) Kita 12-jo, Nishi 6-chome, Kita-ku, Sapporo, 060, Japan.*
4) R.F. Gilfillian, *Cancer Res.*, **28**, 137 (1968).
5) I. Palyi, *Arch. Geswulstforsch*, **33**, 345 (1969).
6) *a)* A. Trouet, D.D. Campeneere, and C. de Duve, *Nature* (London), **239**, 110 (1972); *b)* A. Trouet, D.D. Campeneere, M.D. Smedt-Malengreaux, and G. Atassi, *Europ. J. Cancer*, **10**, 405 (1974).
7) *a)* G. Gregoriadis and B.E. Ryman, *Europ. J. Biochem.*, **24**, 485 (1972); *b)* G. Gregoriadis, *FEBS Letters*, **36**, 292 (1973).

reported that liposome-entrapped drugs intravenously injected into rats were concentrated in the liver and the spleen by phagocytosis in the reticuloendothelial systems. Gregoriadis and Neerunjun[8] investigated the possibility of homing liposomes to target cells by using liposomes associated with molecular probes which exhibit a specific affinity for the surface of a variety of normal and malignant cells. Radiologists utilized the phagocytic activity of the liver and the spleen to study and diagnose the function of reticuloendothelial system by radiolabeled albumin microspheres.[9] Kramer, *et al.*[10] reported that human serum albumin microspheres containing 6-mercaptopurine were phagocytized by HeLa and glioblastoma *in vitro*, and they suggested the possibility that the albumin microspheres could be utilized as drug carrier.

We have recently reported that 5-fluorouracil (5-FU) entrapped in bovine serum albumin microspheres localized in the liver after intravenous injection in mice.[1] In this paper we wish to report physicochemical properties of albumin microsphere and its usefulness as a drug carrier.

Experimental

Materials——5-Fluorouracil-6-^{3}H (^{3}H-5-FU) was purchased from Japan Radioisotope Association (Tokyo, Japan). Unlabeled 5-FU was supplied from Kyowa Hakko Co., Ltd. Bovine serum albumin was obtained from Seikagaku Kogyo Co., Ltd. and cottonseed oil was selected as a vegetable oil.

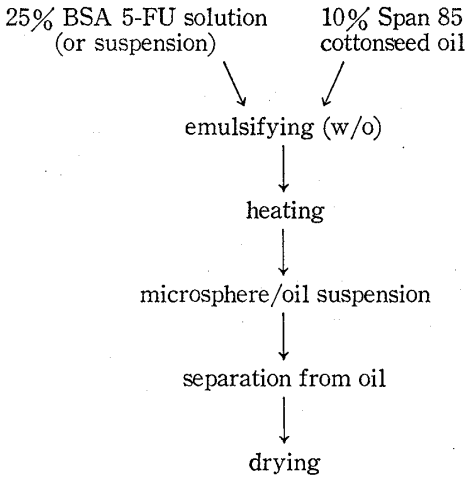

Chart 1. Schematic Diagram of Preparation of Albumin Microsphere Containing 5-Fluorouracil

Preparation of Albumin Microspheres——Bovine serum albumin microspheres containing antitumor agent ^{3}H-5-FU were prepared as follows by modification of the method of Scheffel, *et al.*[9b] (Chart 1). Two hundreds and fifty mg of bovine serum albumin were dissolved in 1 ml of radiolabeled 5-FU solution. The resulting solution was mixed with 100 ml of 10% span 85 in cottonseed oil, and homogenated with a motor driven glass stirrer (Tokyo Rikakiki, Model MS-75) at about 2500 rpm for 10 min, and was furthermore emulsified with an ultrasonic homogenizer (Nihonseiki Seisakusho, Model G50022-4) at 100 W for 30 min.

Another 100 ml of cottonseed oil were heated to 100°, 150°, or 180° in a 500 ml three necked round-bottomed flask under continuous stirring by a glass stirrer at 2500 rpm. The homogenated albumin-oil was gradually added to the heated oil, the temperature was adjusted to 100°, 150°, or 180°, and heating and stirring was maintained for 10 min. After the suspension was cooled to room temperature, each suspension was mixed with 200 ml of diethyl ether. The mixture was separated by centrifugation and the oil–ether phase was discarded. For complete removal of adhering oil, the preparate was washed in ether and in ethyl alcohol. After washing the spheres, the precipitates were stored in a desiccator. And just before experiments, the dried microsphere was dispersed with 0.2% polysorbate 80 and sonicated at 100 W for 10 min so as to remove the free drug loosely adhered to the surface of the microspheres. After centrifugation, the precipitate was dispersed with 0.2% polysorbate 80 in 0.9% NaCl solution and suspended well with an ultrasonicator.

Preparation of Albumin Macroaggregates——Bovine serum albumin macroaggregates containing the ^{3}H-5-FU were prepared by a slight modification of the method of Thomas, *et al.*[11] The macroaggregates were prepared on demand. Twenty five mg of bovine serum albumin were dissolved in 1 ml of radiolabeled 5-FU solution. The pH of the solution was adjusted to 5.7 ± 0.2 with 0.1 N HCl. And the solution was heated at 90° for 10 min under continuous stirring. In order to wash the macroaggregates and to remove

8) G. Gregoriadis and E.D. Neerunjun, *Biochem. Biophys. Res. Commun.*, **65**, 537 (1975).
9) *a*) I. Zolle, B.A. Rhodes, and H.N. Wagner, Jr., *Int. J. Appl. Radiat. Isotop.*, **21**, 155 (1970); *b*) U. Scheffel, B.A. Rhodes, T.K. Natarajan, and H.N. Wagner, Jr., *J. Nucl. Med.*, **13**, 498 (1972).
10) *a*) P.A. Kramer, *J. Pharm. Sci.*, **63**, 1646 (1974); *b*) P.A. Kramer and T. Burnstein, *Life Sci.*, **19**, 515 (1976).
11) J. Thomas and S.N. Wiener, *Int. J. Appl. Radiat. Isotop.*, **25**, 463 (1974).

free 5-FU, small amount of water was added to this macroaggregates and the suspension was sonicated at 100 W for 10 min. After centrifugation, 10 ml of 0.9% NaCl solution was added to precipitate. And the suspension was obtained by dispersing the mixture with an ultrasonic cleaner (Branson, Model 220) and used for animal experiments.

Drug Distribution in Mice——JCL-ICR mice weighing about 30 g were used in all experiments. ^{3}H-5-FU was injected into mice through tail vein, in 0.5 ml solution (0.9% NaCl solution), 0.5 ml suspension of albumin microspheres, or 0.25 ml suspension of albumin macroaggregates. Each mouse received 5 mg of microspheres or 0.625 mg of macroaggregates. The mice were sequentially killed by decapitation over a 3-day period. At the time of sacrifice, the liver, spleen, kidney, and lung were excised, and immediately weighed and burned on a sample oxidizer (Aloka, Model ASC-111). The tritium samples collected from the oxidizer were measured for radioactivity with a liquid scintillation counter (Aloka, Model LSC-651).

Experimental Tumor System in Mice——Ehrlich carcinoma ascites were transplanted intraperitoneally to ICR mice (both males and females, avg wt: 30 g) at every ten days. This treatment was the inoculation with Ehrlich ascites 2×10^{7} cells/0.25 ml. Ehrlich solid carcinoma was prepared with the method which Ehrlich ascites (2×10^{7} cells/0.25 ml) were injected subcutaneously into the scapular region of male mice. The mice were used for the experiment tenth day after the injection of Ehrlich ascites.

Results and Discussion

(1) Physicochemical Property of Drug-entrapped Microsphere

The shape of albumin microspheres was invariably spherical probably due to the preparation method which the inner albumin phase of w/o emulsion was immediately solidified at high temperature. Figure 1 shows the scanning electron microphotograph of microspheres. The photograph was taken after the metal coating was carried out on microspheres with an ion-coater. (Eiko Seiki, Model 1B-3)

The size distribution of albumin microspheres was determined with a Model Zb Coulter Counter. Figure 2 shows the size distribution of albumin microspheres prepared at 180°. The most frequently occurring diameter varied between 0.4 and 1.0 μ, and average diameter was 0.66 μ. The size distribution of microspheres was mainly depending on the particle size of emulsion in the first step of the preparation of microspheres, and that particle size was influenced by the concentration of emulsifier and mechanical agitation.[12]

The microsphere is physically and chemically stable. After 5-FU powder was heated at 180° for 10 min, we determined the weight changes of the powder, UV spectrum of 5-FU in an acetate buffer (pH 4.7), and absorbance of the 5-FU solution at 266 nm. The evidence

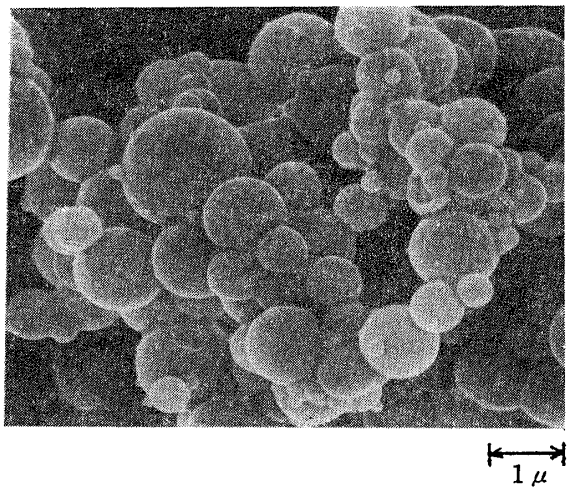

Fig. 1. Scanning Electron Microphotograph of Albumin Microspheres prepared at 180°

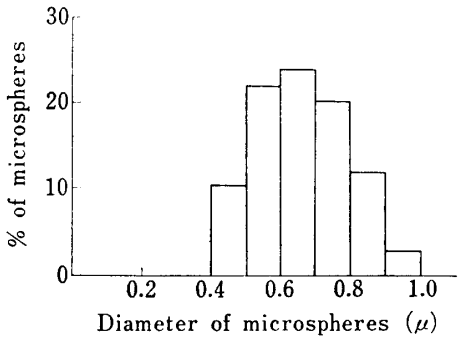

Fig. 2. Particle Size and Distribution of Albumin Microsphere prepared at 180°

12) M. Koishi, N. Fukuhara, and T. Kondo, *Chem. Pharm. Bull.* (Tokyo), **17**, 804 (1969).

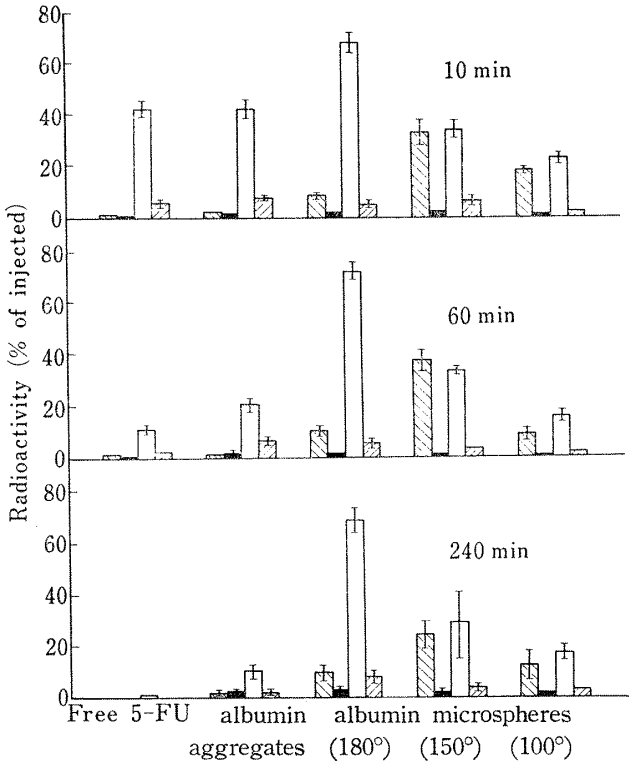

Fig. 3. Time Course of Tissue Distribution of Radioactivity after Intravenous Injection of Free 5-Fluorouracil, Albumin Macroaggregates, or Three Types of Albumin Microspheres

▨: lung, ■: spleen, □: liver, ▨: kidney
Each column represents the mean value of at least three measurements. Vertical bars indicate ±S.E.

of degradation of 5-FU by heating was not found in the results. When the albumin microspheres were prepared by the method described previously, about 4% of 5-FU present in original emulsion was associated with the microspheres. In contrast, entrapment of 5-FU in albumin macroaggregates was 6%.

(2) Tissue Distribution in Mice

Figure 3 shows the distribution of radioactivity to various organs at 10, 60, and 240 min after intravenous injection of free ^{3}H-5-FU, three types of albumin microspheres or albumin macroaggregates. In the figure, the distribution is represented as % of dose per whole tissue. Soon after injection of non-entrapped ^{3}H-5-FU, most radioactivity was removed from the circulation and some of it transiently was in the liver and kidney. But radioactivity in the liver decreased from 42.7% (10 min) to 1.0% (240 min). In contrast, albumin macroaggregates entrapped drug was accumulated mainly in the liver (about 40% at 10 min), and 10% of dose were measured in the liver at 4 hr. The macroaggregates have been utilized as a scanning agent, and they are phagocytized by reticuloendothelial system of the liver. In case of albumin microspheres solidified at 180°, about 70% of dose were localized in the liver. This value was much higher than the radioactivity in the liver following injection of free drug or macroaggregate albumin including drug. The microspheres prepared at 180° might be delivered into the reticuloendothelial system because of its high phagocytic activity, as well as the distribution following injection of the macroaggregates. The temperature at which the albumin microspheres were prepared effects bio-distribution of radioactivity after intravenous injection. The microspheres prepared at 150° or 100° were accumulated not only in the liver but also in the lung, as shown in Fig. 3. Zolle, *et al.*[9a] pointed out that when the microsphere was suspended in a solution, swelling occurred, and the degree of swelling depended on the temperature at which the microspheres were prepared. This swelling of microsphere reflected in the accumulation in the lung, and the liver as well. And the change, that is increasing in size of the microspheres, occasionally occurs to obliteration of pulmonary vessels and pulmonary embolism.[13,14] Such localization of entrapped drugs in the liver suggested that albumin microspheres are potential drug carriers in chemotherapy.

Furthermore, we studied the drug carrier property of microsphere. The time courses of the disappearance of radioactivity in the liver and lung from 10 min to 3 day following

13) H.N. Wagner, Jr., D.C. Sabiston, Jr., J.G. McAfee, D. Tow, and H.S. Stern, *New Engl. J. Med.*, **271**, 377 (1964).
14) J. Szymendera, O. Mioduszewska, I. Licinska, A. Czarnomska, and B. Luska, *J. Nucl. Med.*, **18**, 478 (1977).

administration of three types of microspheres in mice are shown in Fig. 4 and 5. The disappearance of radioactivity in the liver was very slow. And the elimination of radioactivity from the lung was faster than that from the liver. Amount of drug accumulated and the elimination rate depended on the temperature at which the microspheres were prepared. The elimination of radioactivity after injection of microsphere prepared at 180° was slower than that after injection of spheres fixed at low temperature. Other work from our labolatory has shown that *in vitro* drug release from the microsphere entrapped 5-FU continued over a week, although the release rate was slow.[15] Therefore, these microspheres in the body may be disintegrated gradually and 5-FU entrapped in microspheres may be released. These results suggest that the albumin microspheres are instructive drug carriers with sustained drug release property, and it is expected that the microspheres are useful for maintenance of clinical effectiveness or therapeutic concentration in the tissue. Furthermore the release rate might be controlled by combination of three types of microspheres.

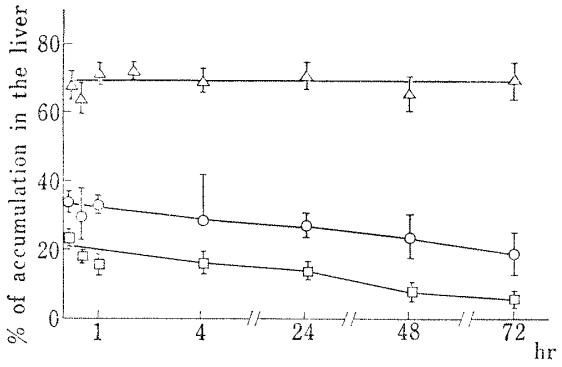

Fig. 4. Time Course of Drug Amount in the Liver after Injection of Microsphere prepared at 180° (△), 150° (○) 100° (□)

Each point represents the mean value of at least three measurements. Vertical bars indicate ±S.E.

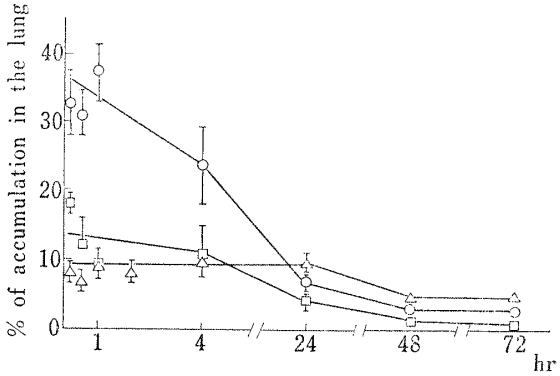

Fig. 5. Time Course of Drug Amount in the Lung after Injection of Microsphere prepared at 180° (△), 150° (○) or 100° (□)

Each point represents the mean value of at least three measurements. Vertical bars indicate ±S.E.

(3) Effect of Pretreatment with Non-entrapped Microspheres on the Tissue Distribution of Drug-entrapped Microsphere

We studied the effect of pretreatment with non-entrapped albumin microsphere on the tissue distribution of drug-entrapped microsphere. Non-entrapped microspheres and drug-entrapped spheres were prepared at 180°. After the mice were pretreated with administration of non-entrapped microspheres (5 mg/0.5 ml), drug-entrapped microspheres (5 mg/0.5 ml) were injected at 5 min. Figure 6 shows the tissue distribution at 30 min following injection of drug-entrapped microspheres. Amount of drug accumulated in the lung of microsphere-pretreated mice increased and the uptake in the liver and spleen decreased, compared with a control (without pretreatment). Because the phagocytosis of the microspheres in the liver and spleen might be saturated after first injection of the microspheres (non-entrapped microspheres), the amount of drug accumulated decreased following second injection of the microspheres (5-FU entrapped spheres). In contrast, since the phagocytosis in the liver and the spleen was saturated, the uptake in the lung might gradually proceed. As the phagocytic activity in the lung may be low, it is doubtful that uptake in the lung occurs only by phagocytosis. In order to clarify the uptake mechanism, we are now investigating the histological study in some tissues after injection of the microspheres in mice.

15) K. Sugibayashi, M. Akimoto, Y. Morimoto, T. Nadai, and Y. Kato, *Life Sci.*, in preparation.

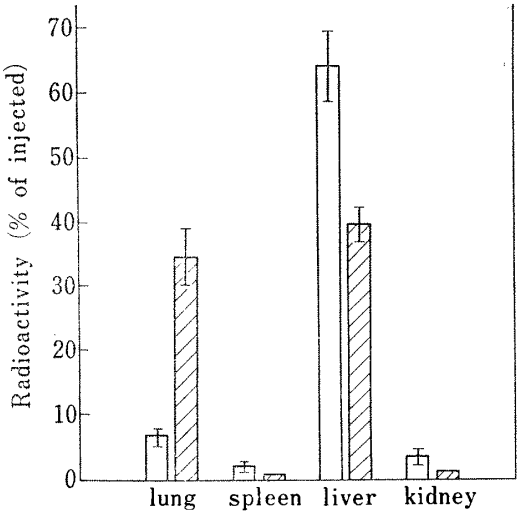

Fig. 6. Effect of Pretreatment with Non-entrapped Microsphere on the Tissue Distribution of Drug-entrapped Microsphere

□: control
▨: pretreated with non-entrapped microsphere
Each column represents the mean value of at least three measurements. Vertical bars indicate ±S.E.

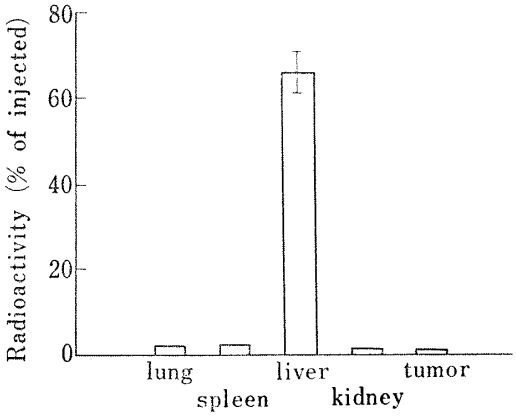

Fig. 7. Tissue Distribution of ^{3}H-5-Fluorouracil entrapped in Microspheres at 30 min after Injection in Tumor Bearing Mice

Each column represents the mean values of four measurements. Vertical bars indicate ±S.E.

(4) Application to Tumor Bearing Mice

In order to study the effect of the albumin microspheres on Ehrlich solid carcinoma in mice, we measured the radioactivity in the tumor tissues after injection of ^{3}H-5-FU entrapped in albumin microsphere prepared at 180° (5 mg/0.5 ml) into tumor bearing mice. Figure 7 shows the tissue distribution of tumor bearing mice. The microspheres were not preferentially localized in the tumor cells, and most of the radioactivity was accumulated in the liver.

Gregoriadis, *et al.*[16] found that a degree of preferential uptake of liposome was observed in malignant deposits in most organ. Such preferential localization of liposome could be explained by an increased accessibility of the diseased tissue to liposomes due to extensive local vascularization or to an increased endocytic activity of tumor cells, or both. But Newton[17] suggested that access of dye penetration to tumor was poor. The uptake mechanism of drug or drug-entrapped carrier to tumor cells should not be a simple process, but a complex process which varied with the different tumor cells. We will study whether the albumin microspheres are valid to several experimental tumor systems, especially hepatoma cells. But if target tissue is in the liver, the albumin microsphere is useful drug carrier with its selectivity for the liver and prolonged action. We will make a study of possibility to direct drug-entrapped albumin microspheres to target tissues other than the liver through specific manipulations of the microsphere surface.

16) G. Gregoriadis, C.P. Swain, E.J. Wills, and A.S. Tavill, *Lancet*, **1**, 1313 (1974).
17) K.A. Newton, *Brit. J. Radiol.*, **38**, 224 (1965).