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Dosage Form Characteristics of Microsphere-in-Oil Emulsion. II: Examination of Some Factors Affecting Lymphotropicity

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The relation between the physico-chemical properties and lymphotropicity of microsphere-in-oil (S/O) emulsion was investigated in comparison with water-in-oil (W/O) emulsion and sesame oil without any pharmaceutical modification. S/O emulsion gave the best lymph node accumulation of tripalmitin (^{14}C), used as an oil tracer, after intramuscular injection. W/O emulsion was next most effective. Increase of injection volume and massage of the injection site accelerated the lymphatic transport of S/O emulsion, suggesting that hydrostatic tissue pressure plays a role in lymphatic delivery. Sesame oil solution of tripalmitin (^{14}C) injected without any pharmaceutical modification was not effectively transferred to the lymph nodes, but addition of surfactants to the oil resulted in an increased accumulation of tripalmitin (^{14}C) in the lymph nodes. Measurements of surface and interfacial tension of various formulations revealed that addition of surfactants caused a marked decrease of interfacial tension against water, which would promote dispersion of the formulations into smaller oil droplets. Microscopic observations of the thigh muscle after intramuscular injection of various oil formulations revealed a correlation between their extent of dispersion and their lymphotropicity. These results suggst that S/O emulsions have physico-chemical properties suitable for effective lymphotropic transport.

Keywords——microsphere-in-oil emulsion; drug delivery system; lymphotropicity; physico-chemical properties; water-in-oil emulsion; sesame oil; injection volume; massage; surface-active agent; interfacial tension

An important prerequisite for success in the application of pharmacologically active agents is site specificity. This is particularly applicable to cancer chemotherapy in which the supply of cytotoxic drugs into nondiseased tissue leads to serious side effects. To improve cancer chemotherapy, therefore, it is desirable to develop drug delivery systems which can control the transfer of anticancer agents and direct them to the tissues where the tumors are located, and many efforts have been directed to this end through drug latentiation[2] or the utilization of various types of dosage form.[3]

1) Location: *Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto.*

2) M. Hashida, T. Kojima, S. Muranishi, and H. Sezaki, *Gann*, **69**, 839 (1978); T. Kojima, M. Hashida, S. Muranishi, and H. Sezaki, *Chem. Pharm. Bull.*, **26**, 1818 (1978); B. Chu and J. Whiteley, *Mol. Pharmacol.*, **13**, 80 (1977); A. Bernstein, E. Hurwitz, R. Maron, R. Arnon, M. Sela, and M. Wilchek, *J. Natl. Cancer Inst.*, **60**, 379 (1978).

3) G. Gregoliadis, C. Swain, E. Wills, and A. Tavill, *Lancet*, **1**, 1313 (1974); P. Kramer, *J. Pharm. Sci.*, **63**, 1646 (1974).

In previous studies,[4] it has been shown that the use of emulsion formulations is very promising in the targeting of anticancer agents to lymphatics and in preventing metastasis. In these studies, water-in-oil (W/O) emulsion was found to be more effective in enhancing the lymphatic transport of anticancer agents than oil-in-water (O/W) emulsion.[4b,c] The largest enhancement was exhibited by a microsphere-in-oil (S/O) emulsion, in which W/O emulsion was improved through replacement of its inner water droplets by gelled gelatin microspheres about 1.6 μm in diameter.[4c,f] By quantitative, kinetic, and histological studies of the transport of the emulsion and the drug, it became clear that both W/O and S/O emulsions were converted into multiple emulsions after injection, and the resulting oil droplets acted as the lymphotropic carrier, incorporating the drug in the innermost aqueous globules.[4c-f] It was suggested that there is a close relation between the degree of accumulation of oil in the lymph nodes, determined by employing ^{14}C-tripalmitin as a tracer, and the extent of enhancement of the drug localization, both in various emulsion formulations and in oily suspensions.[4c,d]

The present investigation was undertaken to clarify in detail the characteristics of S/O emulsion as a lymphotropic drug delivery system, and the relation of these characteristics to its physicochemical properties. Some considerations on the underlying physiological factors which participate in lymphatic delivery from the interstitial spaces are also presented.

Experimental

Materials——Sesame oil, gelatin and Sudan blue were obtained from Nakarai Chemicals Co. Nonionic surfactants, polyoxyethylene derivative of hydrogenated castor oil (HCO-60) and sorbitan sesquioleate (SO-15), were supplied by Nikko Chemicals Co. Radiolabelled tripalmitin (carboxyl-^{14}C) was purchased from New England Nuclear, U.S.A., with a specific radioactivity of 0.05 mCi/mg. All other chemicals were reagent grade products.

Preparation of Injection Formulation——Both S/O and W/O emulsions were prepared with 40 volumes of oily phase and 7 volumes of aqueous phase. Sesame oil incorporating 6.7% (v/v) SO-15 and 1.7% (v/v) HCO-60 was used as the oily phase. The aqueous phase was 20% (w/v) gelatin solution (S/O emulsion) or distilled water (W/O emulsion).

After heating at about 50°, the phases were mixed and emulsified by ultrasonification. Sonification was carried out in a water bath maintained at 70° followed by rapid cooling to about 0°.

In the experiments on the disappearance and lymphatic transfer of oil after intramuscular injection, radiolabelled tripalmitin (^{14}C) was dissolved into sesame oil at a concentration of 10 μCi/ml as a tracer before preparing each formulation. For determining the whole-body distribution of sesame oil, 1.5 μCi of tripalmitin was dissolved per 50 μl of sesame oil. Sudan blue was dissolved in sesame oil at a concentration of 3 mg/ml and used for injection. This solution was also used for preparing emulsions for use in the histological experiments.

Procedure of Animal Experiments——Male Wistar albino rats weighing between 200 and 230 g were used in all animal experiments. The animals were anesthetized with ether, injected with aliquots of various formulations into the center of the right thigh muscle using a microliter syringe and then housed in standard animal cages. At various times after injection, rats were sacrificed and the muscle and the regional lymph node (right iliac lymph node) were excised. Massage of the muscle tissue was carried out for 5 min immediately after injection.

In order to determine the tissue distribution of sesame oil, rats were housed in metabolic cages and respiratory carbon dioxide was collected in a sodium hydroxide (2 N) trap. At 6 hr after injection, rats were sacrificed. The muscle and organs such as the liver, spleen, lung, and kidney were excised, and blood samples were withdrawn from the heart.

Measurement of Radioactivity——The procedure employed for the determination of ^{14}C-radioactivity was modified from the method of Mahin and Loftberg.[5] The muscle was solubilized in 5 ml of 1 N NaOH–ethyl alcohol solution by shaking overnight at 37°, then diluted to 10 ml with the same medium, and 0.2 ml

4) *a*) T. Takahashi, M. Mizuno, Y. Fujita, S. Ueda, B. Nishioka, and S. Majima, *Gann*, **64**, 345 (1973); *b*) Y. Nakamoto, M. Hashida, S. Muranishi, and H. Sezaki, *Chem. Pharm. Bull.*, **23**, 3125 (1975); *c*) M. Hashida, M. Egawa, S. Muranishi, and H. Sezaki, *J. Pharmacokin. Biopharm.*, **5**, 225 (1977); *d*) M. Hashida, Y. Takahashi, S. Muranishi, and H. Sezaki, *ibid.*, **5**, 241 (1977); *e*) M. Hashida, S. Muranishi, and H. Sezaki, *Chem. Pharm. Bull.*, **25**, 2410 (1977); *f*) M. Hashida, S. Muranishi, H. Sezaki, N. Tanigawa, K. Satomura, and Y. Hikasa, *Int. J. Pharm.*, **2**, 245 (1979).

5) D.T. Mahin and R.T. Loftberg, *Anal. Biochem.*, **16**, 500 (1966).

of sample solution was put in a counting vial, together with 0.2 ml of perchloric acid (60%) and 0.2 ml of hydrogen peroxide (35%). The resulting mixture was heated at 70° for 90 min with occasional agitation. After cooling to room temperature, 15 ml of scintillation medium consisting of a mixture of ethylene glycol-monoethyl ether (500 ml), toluene (1000 ml), and 2,5-diphenyloxazole (6 g) was added, and the radioactivities were determined in a liquid scintillation system (Beckman LS-232). The radioactivities in the liver, spleen, lung, and kidney were measured in the same manner.

The weighed lymph node or 0.2 ml of plasma sample was put into the vial, and the radioactivities were measured in the same manner described above. The radioactivities of respiratory CO_2 (^{14}C) were measured by putting 0.5 ml of trap solution into the vial with scintillation medium. The counts obtained were corrected with the aid of external standards.

Determination of Amount of Sudan Blue——The muscle was homogenized and diluted to 20 ml with distilled water. The Sudan blue was extracted with 10 ml of benzene from homogenate containing 7 g of NaCl. The optical density of the benzene phase was determined at 645 nm after centrifugation.

Measurement of Surface Tension and Interfacial Tension——Both surface tension and interfacial tension were measured using a Du Nouy tensiometer (Shimadzu) without correction. Interfacial tension was measured by laying oil or an oily formulation on the water layer. All measurements were carried out at 25°.

Microscopic Observation——Frozen sections of the thigh muscle at 1 hr after injection were prepared and observed microphotometrically. Lipophilic dye (Sudan blue) was dissolved in sesame oil prior to preparing the formulations so that the oil could be seen easily.

Results

Disappearance of Sesame Oil from the Injection Site

In order to understand the transfer characteristics of sesame oil employed as a base in various formulations, it is desirable to examine its movement after injection. Fig. 1 shows the time courses of disappearance of tripalmitin(^{14}C) following intramuscular injection with three oil formulations, *i.e.* S/O emulsion, W/O emulsion, and an oily solution of tripalmitin(^{14}C). In the case of the oily solution, results with various injection volumes are also listed. In this figure, the results obtained in the present work are plotted together with those given in a previous report.[4c)]

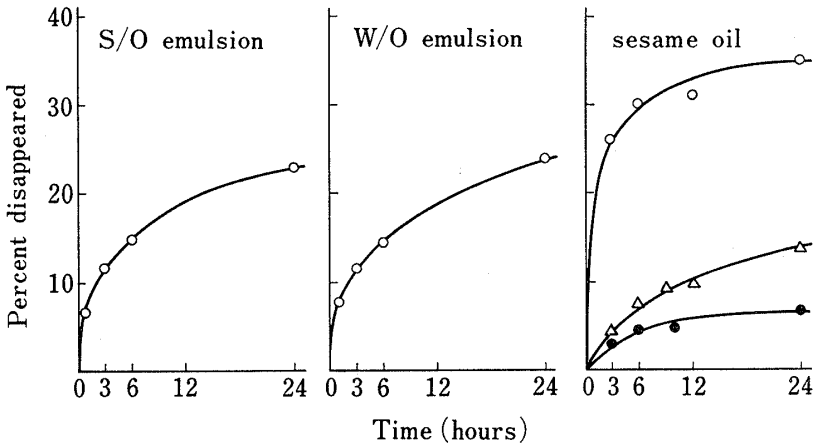


Fig. 1. Disappearance Patterns of Tripalmitin (^{14}C) from the Thigh Muscle after Intramuscular Injection of Various Oil Formulations

Injection volume; ○, 50 μl; △, 20 μl; ●, 10 μl.
Results are expressed as the mean values of at least five animals.

In general, tripalmitin(^{14}C) disappeared from the injection site slowly, but in the case of oily solution the percentage of tripalmitin(^{14}C) disappearance increased with increase of injection volume. Disappearance patterns were similar under all injection conditions, appearing to be biphasic; an initial rapidly disappearing phase and a subsequent slow phase. In the following examinations, therefore, the transfer characteristics of various formulations were compared in terms of the results at 6 hr after injection, since the faster disappearing phases had essentially gone at this time.

Effect of Injection Volume on the Transfer of Oil Injected with Various Formulations

Fig. 2 shows the disappearance (percent) of tripalmitin(^{14}C) at 6 hr after intramuscular injection of various injection volumes of three formulations. Following injection of S/O emulsion and W/O emulsion, disappearance proceeded slowly, and did not exceed 15% of the injected dose at any injection volume. No marked effect of injection volume on the disappearance was seen. Oily solution give a similar disappearance rate at the injection volume of 10 μl, but the percentage disappearance of tripalmitin(^{14}C) increased with increase in the injection volume, and at an injection volume of 50 μl, about 35% of the initial dose was lost from the thigh muscle within 6 hr. These data indicate that the disappearance process of oil injected without any pharmaceutical modification is influenced by injection volume, whereas that of S/O emulsion and W/O emulsion is not affected.

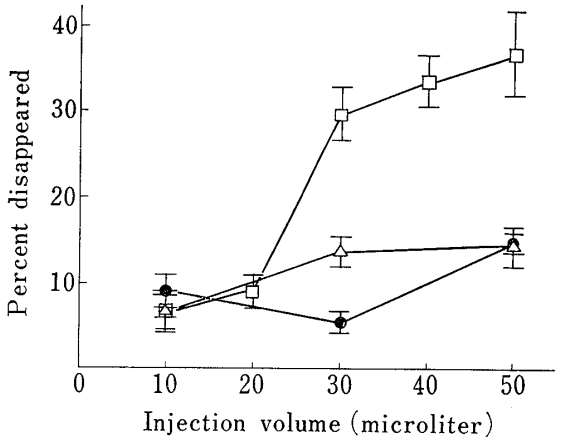


Fig. 2. Effect of Injection Volume on the Disappearance of Tripalmitin (^{14}C) from the Thigh Muscle at Six Hours after Intramuscular Injection of Various Oil Formulations

□, oily solution; △, W/O emulsion; ●, S/O emulsion. Results are expressed as the means ± SE of at least five animals.

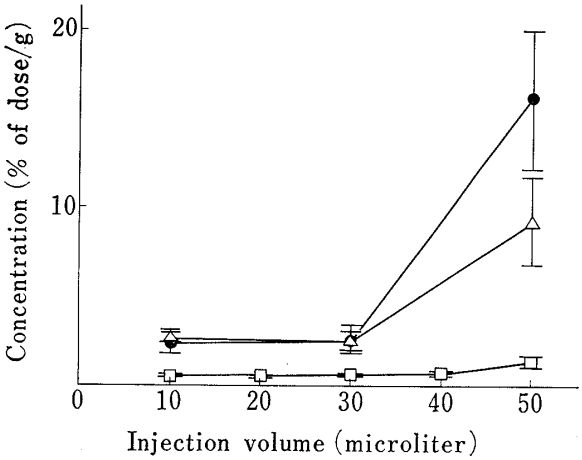


Fig. 3. Effect of Injection Volume on the Lymphatic Transfer of Tripalmitin (^{14}C) from the Thigh Muscle at Six Hours after Intramuscular Injection of Various Oil Formulations

□, oily solution; △, W/O emulsion; ●, S/O emulsion. Results are expressed as the means ± SE of at least five animals.

Fig. 3 shows the regional lymph node concentrations of tripalmitin(^{14}C) after intramuscular injection of three formulations using various injection volumes. It is clear that tripalmitin(^{14}C) injected in the form of oily solution was transferred to the regional lymph node to only a small extent, which was not related to the injection volume. In contrast, S/O emulsion and W/O emulsion produced large accumulations. At an injection volume of 50 μl, S/O emulsion showed the highest concentration of about 20% of dose/g lymph node. The W/O emulsion gave 12% of dose/g, whereas the oily solution gave only 1.2% of dose/g. The specific transport of the emulsion itself to the lymphatics was thus demonstrated.

Effect of Massage on the Transfer of Oil Injected with Various Formulations

Zelman[6] has demonstrated that deep and firm massage of the muscle tissue following an intramuscular injection favors the spread of the medication through a wide region of tissue, thus increasing the volume over which absorption can take place. It is also well known that a massage increases regional lymph flow.[7] Consequently, the effect of massage treatment

6) S. Zelman, *Am. J. Med. Sci.*, **241**, 563 (1961).
7) J.M. Yoffey and F.C. Courtice, "Lymphatics, Lymph, and Lymphomyeloid Complex," Academic Press, London, 1971.

on the disappearance and lymphatic transport of oil after intramuscular injection was investigated for three formulations. In this experiment, an injection volume of 10 μl was selected since it showed the smallest difference in the transfer of oil among the three formulations.

As shown in Fig. 4, disappearance rates were increased to almost the same extent by massage treatment with every formulation and no significant difference could be seen between the effects on the disappearance of the three formulations. In contrast, the lymph node concentration of oil after injection of S/O emulsion increased markedly after massage treatment, reaching about six times that obtained with sesame oil. From these data, it can be concluded that the effects of massage treatment on the disappearance and lymphatic transport were rather different.

Detailed Examination of the Transport of Oil Injected in the Form of Oil Itself

As shown in Figs. 2, 3, and 4, sesame oil injected without any pharmaceutical modification was not significantly transported into the lymphatics, although it disappeared from the injection site very rapidly. It appears that the transfer route of injected sesame oil may be rather different from that of emulsion. In order to clarify the fate of injected sesame oil and the underlying physiological process, therefore, the movement of sesame oil was traced by visual observation and radioactivity measurement throughout the whole body.

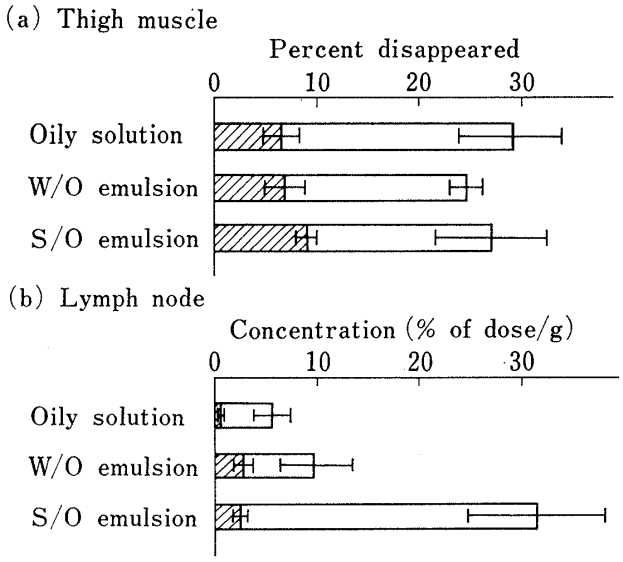
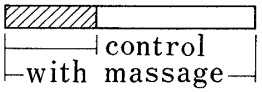


Fig. 4. Effect of Massage on the Disappearance (a) and Lymphatic Transfer (b) of Tripalmitin (^{14}C) at Six Hours after Intramuscular Injection of Various Oil Formulations



Results are expressed as the means ± SE at least five animals.
Injection volume; 10 μl.

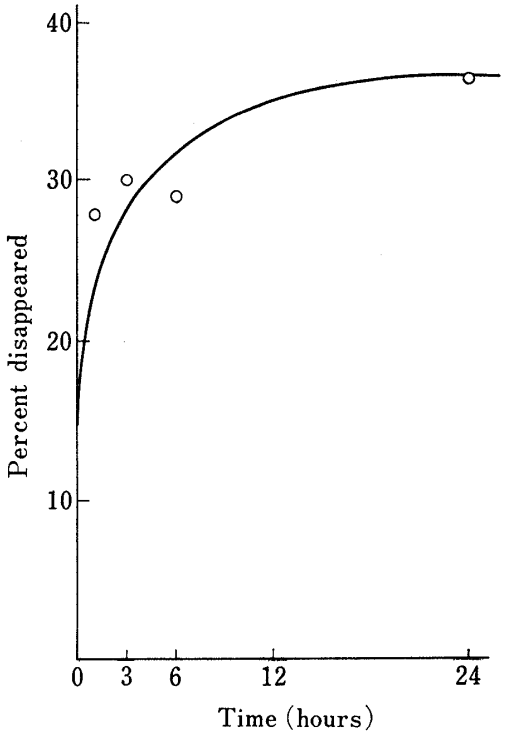


Fig. 5. Disappearance Pattern of Sudan Blue from the Thigh Muscle after Intramuscular Injection of Sesame Oil Solution

Concentration; 3 mg/ml.
Injection volume; 50 μl.
Results are expressed as the means of five animals.

Fig. 5 shows the disappearance rate of Sudan blue dissolved in sesame oil from the injection site after intramuscular injection. There is no significant difference between the disappearance pattern of Sudan blue shown in this figure and that of tripalmitin (^{14}C) shown in Fig. 1, so the movement of Sudan blue may correspond well to that of oil. To confirm this, the movement of dye was followed by visual observation after injection of 50 μl of sesame oil. It was shown

that some part of the injected oil moved to the gluteal area along the fascial planes, whereas such direct movement was not noted in the case of emulsions containing Sudan blue. Little leakage of oil from the needle hole was noted; this made only a minor contribution to the rapid disappearance of oil from the injection site.

Table I. Tissue Distribution of Tripalmitin (^{14}C) at Six Hours after Intramuscular Injection of Sesame Oil

Tissue	Concentration (% of dose/g)	Total recovery (% of dose)
Muscle (Site of administration)	—	69.68±5.23
Plasma	0.04±0.01	—
Liver	0.28±0.04	2.49±0.26
Spleen	0.10±0.02	0.06±0.02
Lung	0.10±0.01	0.13±0.03
Kidney	0.12±0.03	0.22±0.06
Recovery in respired CO_2	—	1.28±0.32

Results are expressed as the means±SE of five animals.

Table I summarized the distribution of radioactivity of tripalmitin (^{14}C) at 6 hr after intramuscular injection of 50 μl of sesame oil, together with the amount of radioactivity recovered from the respiratory CO_2. The liver showed the largest accumulation of tripalmitin (^{14}C), but radioactivity was also detected in other tissues including respired air. This distribution pattern is in reasonably good accord with that after intravenous injection of tripalmitin (^{14}C) reported by Goldman *et al.*[8] It can be concluded from these data that some portion of the sesame oil was absorbed through blood capillaries and distributed throughout the body.

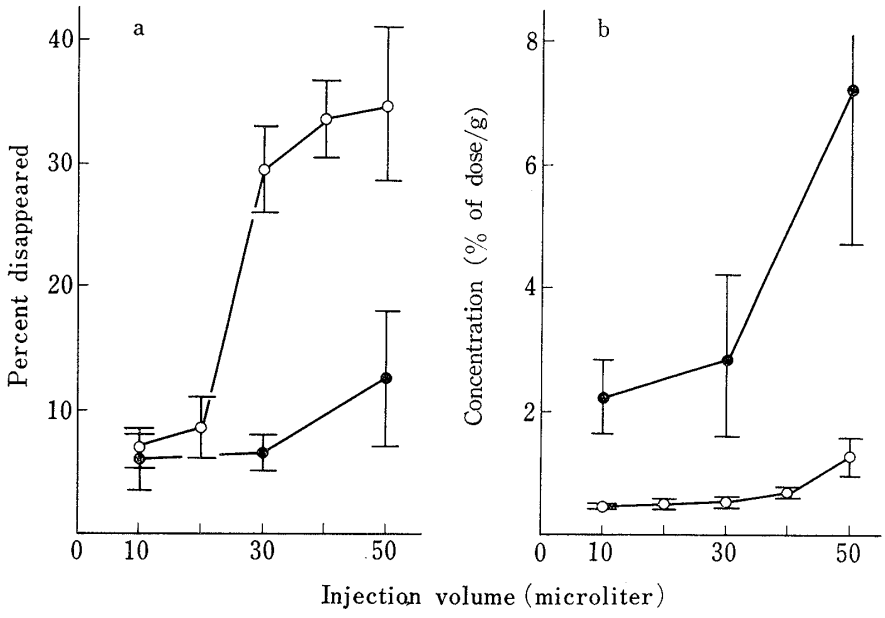


Fig. 6. Effects of the Addition of Surface-active Agents on the Disappearance (a) and the Lymphatic Transfer (b) of Tripalmitin (^{14}C) at Six Hours after Intramuscular Injection

○, sesame oil; ●, sesame oil+surface-active agents.
Results are expressed as the mean±SE of at least five animals.
Surface-active agents; SO-15 (6.7%)+HCO-60 (1.7%).

8) D. Goldman, I. Chaikoff, W. Reinhardt, C. Entenman, and W. Dauben, *J. Biol. Chem.*, **184**, 727 (1950).

Effect of Surfactant on the Transfer of Oil

Among the components of S/O emulsion and W/O emulsion, surfactants employed as an emulsifier are common to both formulations and may be partly responsible for the different trantsport patterns of emulsions and native oil described above. Consequently, the effect of surfactants on the transfer of oil was investigated by dissolving HCO-60 and SO-15 into sesame oil at the same concentration as that employed for emulsion preparation. The results are shown in Fig. 6 together with those for native sesame oil, for comparison.

The addition of surfactants caused the disappearance rate of tripalmitin (^{14}C) to decrease markedly and even at an injection volume of 50 μl, the disappearance did not exceed 13% of the dose. In contrast, the lymphatic transport of sesame oil was clearly increased by the addition of surfactants to sesame oil. This increment was particularly marked at the injection volume of 50 μl. These results show that the transfer pattern of sesame oil became similar to that of emulsion on adding surfactants, and it can be concluded that the presence of surfactants plays an important role in the delivery of oil formulations.

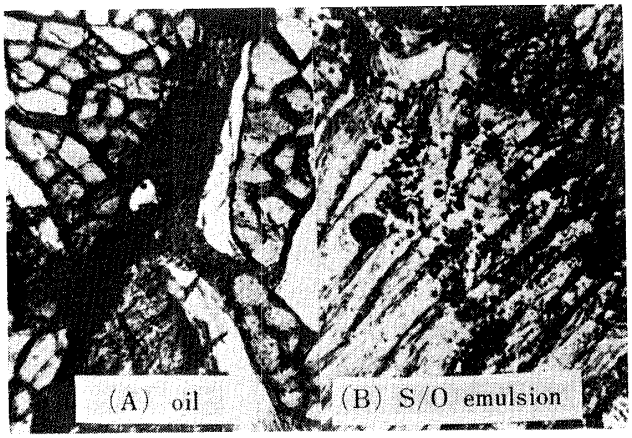


Fig. 7. Microphotographs of the Thigh Muscle at One Hour after Intramuscular Injection of Sesame Oil (A) and S/O Emulsion (B)

The oil phase was colored by dissolving Sudan blue prior to injection. Injection volume; 50 μl. Photo. A, B; 100×.

Table II. Surface Tension and Interfacial Tension against Water of Various Oily Vehicles at 25°

Substance	Surface tension[a] (dyne/cm)	Interfacial tension against water[a] (dyne/cm)
Sesame oil	34.9	26.2
Sesame oil+ Surface-active agent (SO-15, HCO-60)[b]	34.9	2.5
W/O emulsion	34.7	1.5
S/O emulsion	35.0	1.5
Water	71.3	—

a) Instrument; Du Nouy tensiometer.
b) SO-15; 6.7% (v/v), HCO-60; 1.7% (v/v).

In order to clarify in detail the mechanism of enhanced lymphatic transport caused by the addition of surfactants, the interfacial properties of these formulations were investigated. The results are summarized in Table II. As regards surface tension, there was no significant difference between native sesame oil and three formulations containing surfactants. However, the interfacial tensions of formulations containing surfactants against water were very small in comparison with that of native sesame oil.

Fig. 7 shows microphotographs of frozen sections of the thigh muscle after injection of sesame oil or S/O emulsion. Sesame oil formed a large deposit of oil which extended along the fascial planes between muscle fibers without dispersion. S/O emulsion, in contrast, dispersed into fine oily droplets which migrated between the muscle fibers. In the case of W/O emulsion and sesame oil incorporating HCO-60 and SO-15, uniform dispersion of oily droplets was also observed by microscopic examination. Consequently, it can be concluded that the presence of surfactants facilitates the dispersion of a formulation, which may be the first step in lymphatic delivery.

Discussion

In order to deliver anticancer agents to the lymphatics with an emulsion system, the oil droplets (carriers) formed on injection of the emulsion must show lymphotropicity. The

present results show that the S/O emulsion which was shown to give the largest delivery of bleomycin into the lymph node[4f)] exhibited the largest accumulation of tripalmitin (^{14}C) in the lymph node, irrespective of the injection volume (Fig. 3). The finding that the accumulation of tripalmitin (^{14}C) was smallest when injected in the form of an oily solution is in accord with this work, since the oily suspension of bleomycin also did not show any significant delivery of the drug into the lymphatics.

It is well known that various materials such as dyes, india ink, chylomicrons, proteins, bacteria, and red cells are readily taken up from the tissues by the lymphatic vessels.[7)] Concerning water–immiscible oil, however, it became clear from the present results that the oil forms depots or large pools in the injection site and is transported to the whole body through the blood vessels without any significant uptake by the lymphatic systems. Consequently, the enhanced lymphatic transfer of S/O emulsion suggests the existance of some particular physical properties responsible for its effectiveness. The different effects of injection volume and massage treatment on the transfer patterns of native oil and S/O emulsion (Figs. 2, 3, and 4) also the operation of different transfer mechanism.

The results in Fig. 6 show that addition of surfactants causes a marked increase of the lymphatic transport and a decrease of the disappearance of tripalmitin (^{14}C) injected in the form of sesame oil solution. Further, a decrease of interfacial tension against water was observed with formulations containing surfactants (Table II). In order to explain this correlation between lymphotropicity and decreased interfacial tension, we hypothesized that dispersion of oil formulations in the tissues following injection is the first step in their intrusion into the lymphatic vesseles; the results of microscopic observations supported this hypothesis in part (Fig. 7). Thus the significance of interfacial properties in the lymphatic delivery of oil formulations seems clear.

Rheological examinations of S/O emulsion and W/O emulsion have shown previously that both emulsions exhibit non-Newtonean plastic flow curve, and that S/O emulsion has a larger viscosity than W/O emulsion, especially at low shear rate.[9)] During injection, i.*e.* under conditions of high shear rate, both emulsions can be considered to show adequate syringeability and fine dispersion into small particles in the interstitial space because of their relatively low viscosities. In contrast, after dispersion into small particles, these particles would have high viscosity with the decrease of shear rate. Some properties such as solidity and interfacial fluidity of particles are considered to be closely related to the rheological characteristics of emulsions, and all these factors must affect the lymphotropicity of emulsions. From this point of view, the difference of viscosities between S/O emulsion and W/O emulsion may be important, and requires further study.

Concerning physiological factors, the present results show that the lymphatic transport of S/O emulsion droplets was enhanced by an increase of the injection volume and by massage treatment. Both should lead to an increase of the hydrostatic tissue pressure, so it can be considered that tissue pressure is a driving force in the lymphatic delivery of oil droplets.

On the basis of the evidence presented here, it can be concluded that emulsion formulations, especially S/O emulsion, have properties promoting their lymphatic transport. This lymphotropicity may make possible the preferential lymphatic delivery of anticancer agents in the view of the stable incorporation of the drug into carrier droplets reported previously.[9)]

9) M. Hashida, T. Yoshioka, S. Muranishi, and H. Sezaki, *Chem. Pharm. Bull.*, 28, 1009 (1980).