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Mechanism of the Inhibitory Effect of Surfactants on Intramuscular Absorption of Drugs. (3)[1]

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The mechanism of the absorption inhibitory effect of surfactants on the intramuscular absorption of drugs was investigated. The following observations were demonstrated.

1) All surfactants used in this study whether nonionic or ionic caused solubilization of muscle protein. A good correlation between the solubilization of muscle protein and the absorption inhibitory effect was also found except in the case of the nonionic surfactant HCO-120.

2) The effect of hyaluronidase in the absence or presence of various concentrations of polysorbate 80 on the intramuscular absorption of drugs of different molecular weight was examined. The coexistence of hyaluronidase and polysorbate 80 greatly affected the absorption of drugs. This was depending upon the molecular size of diffused drug and the concentration of surfactant. It was found that increasing the concentrations of polysorbate 80 in the presence of hyaluronidase was accompanied by an inhibition in the absorption of isonicotinamide and abolishment of the accelerating effect of hyaluronidase on the absorption of ^{14}C-sucrose, but it could not show any effect on the absorption of ^{14}C-inulin.

3) The permeability coefficient of gelatin-sodium chondroitin sulfate mixed membrane in the presence of polysorbate 80 was decreased significantly.

From these results, it can be concluded that the surfactants interacted with proteins and mucopolysaccharides which are the main components of connective tissue, consequently the intramuscular absorption of drugs was extremely inhibited.

Keywords——inhibitory effect of surfactants; intramuscular absorption; rat thigh muscle; solubilization of muscle protein; hyaluronidase; permeation; gelatin-sodium chondroitin sulfate membrane

In previous papers,[3] the effect of surfactants on the intramuscular absorption of water-soluble drugs from the rat thigh muscle was investigated. It was found that the presence of a low concentration of surfactants caused a pronounced decrease in the absorption rate of water-soluble drugs. The inhibitory effect of surfactants was ascribed to an inhibition in drug permeation through extracellular spaces and connective tissue.

Several reviews[4] concerned with the physico-chemical properties and structural functions of connective tissue have been published. Day[5] showed that hyaluronic acid plays

1) *a*) This paper constitutes the 13th report in a series of "Biopharmaceutical Studies on Parenteral Preparations"; *b*) Preceding paper, Part XII: H. Kobayashi, T. Peng, R. Kawamura, S. Muranishi, and H. Sezaki, *Chem. Pharm. Bull.* (Tokyo), **25**, 569 (1977).

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3) *a*) H. Kobayashi, T. Nishimura, K. Okumura, S. Muranishi, and H. Sezaki, *J. Pharm. Sci.*, **63**, 580 (1974); *b*) H. Kobayashi, T. Peng, A. Kagayama, K. Okumura, S. Muranishi, and H. Sezaki, *Chem. Pharm. Bull.* (Tokyo), **23**, 42 (1975); *c*) H. Kobayashi, T. Peng, M. Fujikawa, S. Muranishi, and H. Sezaki, *Chem. Pharm. Bull.* (Tokyo), **24**, 2383 (1976); *d*) H. Kobayashi, T. Peng, R. Kawamura, S. Muranishi, and H. Sezaki, *Chem. Pharm. Bull.* (Tokyo), **25**, 569 (1977).

4) *a*) T.C. Laurent, *Pflügers Arch.*, **336** (Suppl.), S21 (1972); *b*) S.F. Jackson, R.D. Harkness, S.M. Partridge, and G.R. Tristram, "Structure and Function of Connective and Skeletal Tissue," Butterworths and Co., London, 1965; *c*) F. Egami and Oshima, "Biochemistry and Medicine of Mucopolysaccharides," Research Association of Mucopolysaccharides, The University of Tokyo, 1962.

5) T.D. Day, *J. Physiol.*, **117**, 1 (1952).

an important role in the resistance of connective tissue to the flow of fluid through it. Also, the enzyme hyaluronidase increases connective tissue permeability by degradation of mucopolysaccharide substances present in it. Furthermore, many workers[4a,6] have demonstrated that a network of mucopolysaccharides act as a filter and affect the bulk passage of water and the diffusion of various solutes.

It has also been shown that hyaluronic acid in its native state is always associated with proteins. Using model connective tissue systems, the interactions between mucopolysaccharides and polypeptides[7] and diffusional behavior of small non-electrolytes were investigated.[8]

On the other hand, previous works on the solubilization of membranes by detergents showed a different mechanism of action which depend mainly on the nature of the surfactant used.[9] Although Suzuki[10] demonstrated a difference in the mechanism of inflammation caused by intracutaneous injection of anionic and nonionic surfactants, and mentioned that the active site of the former is protein and the latter is lipid, our previous studies showed that the absorption inhibitory effect is not specific to nonionic surfactants but it was also demomstrated when the ionic surfactants were used, and the extent of inhibition was almost equal in both cases.[3b]

Kakemi, *et al.*[11] have shown that the contribution of lipid layer to the intramuscular absorption of water-soluble drugs is relatively small. Moreover, in a previous publication,[3a] we have illustrated that the magnitude of the absorption inhibitory effect in the presence of surfactants is not different irrespective of the lipophilicity of drugs.

Consequently, in order to elucidate the mechanism of the inhibitory effect of surfactants on the intramuscular absorption of drugs, our researches were directed to investigate the influence of polysorbate 80 on proteins and mucopolysaccharides which are the main components of the connective tissue. Furthermore, permeation studies using an artificial mixed membranes were also performed.

Experimental

Materials——Isonicotinamide was of analytical grade and obtained commercially. Bovine testis hyaluronidase 360 NF units/mg (Sigma Co., U.S.A.), sodium chondroitin sulfate (Kakenyakukako Co., Japan) and gelatin (Difco Lab.) were used without further purification. ^{14}C-sucrose and inulin-(carboxylic acid-^{14}C) were purchased from Japan Radio Isotope Association. All other chemicals were reagent grade.

Preparation of Injectable Solutions——Solutions of Isonicotinamide, ^{14}C-sucrose and ^{14}C-inulin with or without hyaluronidase for intramuscular injection were prepared in an isotonic pH 7.0 phosphate buffer solution in the following concentrations: 50 mM, 5 μCi/ml and 5 μCi/ml respectively.

Absorption Experiments——Male Wistar albino rats weighing 180—220 g were used. Preparation of animals and injection technique were mentioned previously.[3a]

Determination of the Degree of Solubilization of Muscle Protein——Rats were anesthetized with pentobarbital and the thigh muscles removed, excised and then homogenized in 10 ml isotonic phosphate buffer (pH 7.0) per g tissue weight using Potter-Elvehjem glass homogenizer. Five ml of tissue homogenates were mixed with an equal volume of surfactants solutions. After 30 minutes incubation at room temperature, the mixtures were centrifuged at $140000 \times g$ for 60 minutes. Materials not sedimenting under these conditions was defined as soluble.[9a] The supernatant was carefully removed for analysis

6) *a*) A.G. Ogston and T.F. Sherman, *J. Physiol.*, **156**, 67 (1961); *b*) A.G. Ogston and P. Silpananta, *J. Biochem.*, **116**, 171 (1970); *c*) B.O. Hedbys and S. Mishima, *Exp. Eye Res.*, **1**, 262 (1963); *d*) T.C. Laurent and A. Pietruszliewicz, *Biochem. Biophys. Acta*, **49**, 258 (1961).
7) F.A. Bettelhem and D.E. Phrilpott, *Biochem. Biophys. Acta*, **34**, 124 (1959); R.A. Gelman and J. Blackwell, *Biopolymers*, **12**, 1959 (1973).
8) B.N. Preston and J.M. Snowden, *Biopolymers*, **11**, 1627 (1972).
9) *a*) F.H. Kirkpatrick, S.E. Gordesky, and G.V. Marinetti, *Biochem. Biophys. Acta*, **345**, 154 (1974); *b*) A. Helenius and K. Simons, *ibid.*, **415**, 29 (1975).
10) M. Suzuki, *Hyomen*, **6**, 392 (1968).
11) K. Kakemi, H. Sezaki, K. Okumura, and S. Ashida, *Chem. Pharm. Bull.* (Tokyo), **17**, 1332 (1969).

of protein content. The protein solubilization ratio was defined as the ratio of the concentration of protein in the presence of surfactant to that in its absence.

Hyaluronidase Activity Measurement——Various amounts of hyaluronidase were added to 2.0% (w/v) sodium chondroitin sulfate either in the presence or absence of polysorbate 80 and mixed well. Then, the reduction in their viscosities were measured at 25° using Ostwald Viscometer. Solutions were prepared in phosphate buffer of pH 7.0 and ionic strength 0.1.

Permeation Study through a Mixed Membrane——Preparation of gelatin-sodium chondroitin sulfate mixed membranes and procedures of permeation study were mentioned elsewhere.[12]

The apparatus used for diffusion study is shown in Fig. 1, and it was used for measuring the permeability constant in an earlier report from this laboratory.[13] It consists of 2 glass cells A, A′, the volume of each cell is approximately 7.0 ml. The mixed membrane C is fixed between the edges B, B′. Membranes were incubated in pH 7.0 phosphate buffer of ionic strength 0.1 either for 90 minutes (control), or for 30 minutes then transfered to a solution of 1.0% polysorbate 80 in the same buffer and incubated for further 60 minutes. Membranes were removed from the incubation medium, blotted on filter papers, weighed and their thickness measured by Peacock Dial Gauge (Ozaki Seisakujo, Japan), then fixed between the diffusion cells. Isonicotinamide (1 mM) in either surfactant-free phosphate buffer solution (control) or the same buffer containing 1.0% polysorbate 80 was introduced into cell A. The other cell A′ was filled with buffer solution alone (control), or containing 1.0% polysorbate 80. Temperature of all solutions was maintained at 37° before starting the experiments. The entire apparatus was water jacketed to maintain constant temperature through the whole experimental work. The permeation experiments were run for 90 minutes, then the concentration of isonicotinamide in both cells A, A′ were determined and the apparent permeability constant was calculated.[14]

Analytical Methods——Protein was determined by the method of Lowry, *et al.*[15] Isonicotinamide was determined spectrophotometrically as described previously.[3a]

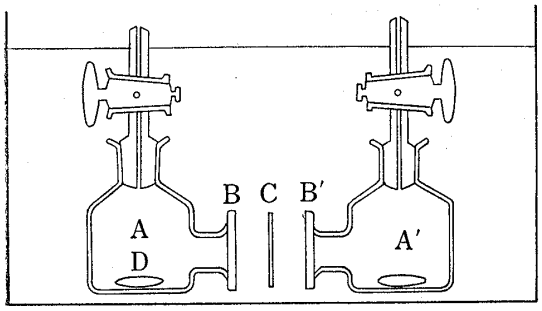

Fig. 1. Schematic Diagram of the Apparatus Used for the Permeation Studies through the Gelatin Sodium Chondroitin Sulfate Mixed Membrane

Key: A, A′, cell; B, B′, ground-glass end; C, mixed membrane; D, magnetic stirrer.

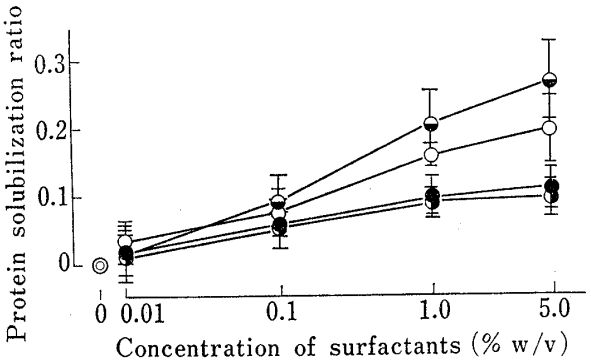

Fig. 2. Effect of Nonionic Surfactants on the Solubilization of Muscle Protein

Each point represents the mean value of 4 experiments. Vertical bars indicate standard deviation.
Key: ○: polysorbate 80; ●: HCO-60; ◑: HCO-120; ◒: Triton X-100.

Results and Discussion

Effect of Surfactants on the Solubilization of Muscle Protein

Figures 2 and 3 show the solubilization of muscle protein by nonionic and ionic surfactants respectively. It is clear from the figures that the muscle protein was solubilized by both kinds of surfactants and the solubilizing effect of ionic surfactants (Fig. 3) was greater than that of nonionic surfactants (Fig. 2).

Furthermore, the relationship between the inhibitory effect of surfactants on intramuscular absorption of isonicotinamide and solubilization of muscle protein is shown in Figures

12) M. Yonese and M. Nakagaki, *Yakugaku Zasshi*, **95**, 75 (1975).
13) K. Kakemi, H. Sezaki, K. Okumura, H. Kobayashi, and S. Furusawa, *Chem. Pharm. Bull.* (Tokyo), **20**, 443 (1972).
14) M. Nakagaki, N. Koga, and S. Iwata, *Yakugaku Zasshi*, **82**, 1138 (1962); M. Nakagaki, "Yakubutu no Seitainai Ikou," Nanko-do, Tokyo, 1969, p. 22.
15) O.H. Lowry, N.J. Roseborough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

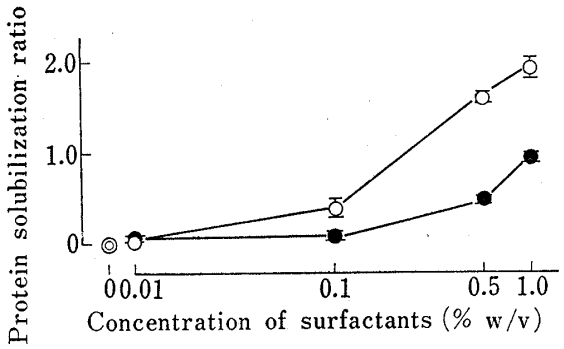

Fig. 3. Effect of Ionic Surfactants on the Solubilization of Muscle Protein

Each point represents the mean value of 4 experiments. Vertical bars indicate standard deviation.
Key: ○: sodium lauryl sulfate;
●: cetyl trimethyl ammonium bromide.

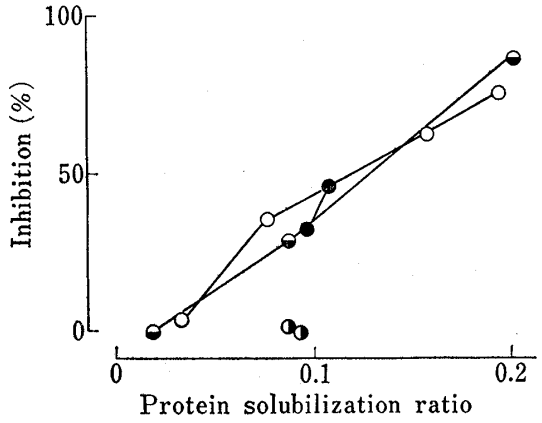

Fig. 4. Relationship between the Inhibitory Effect of Nonionic Surfactants on the Absorption of Isonicotinamide[3a] and the Solubilization of Muscle Protein

Key: ○: polysorbate 80; ●: HCO-60;
◑: HCO-120; ◒: Triton X-100.

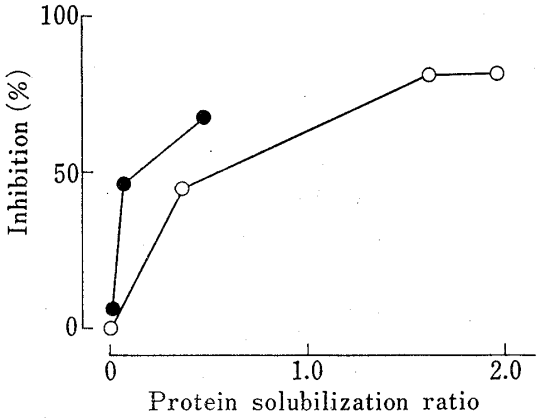

Fig. 5. Relationship between the Inhibitory Effect of Ionic Surfactants on the Absorption of Isonicotinamide[3a] and the Solubilization of Muscle Protein

Key: ○: sodium lauryl sulfate;
●: cetyl trimethyl ammonium bromide.

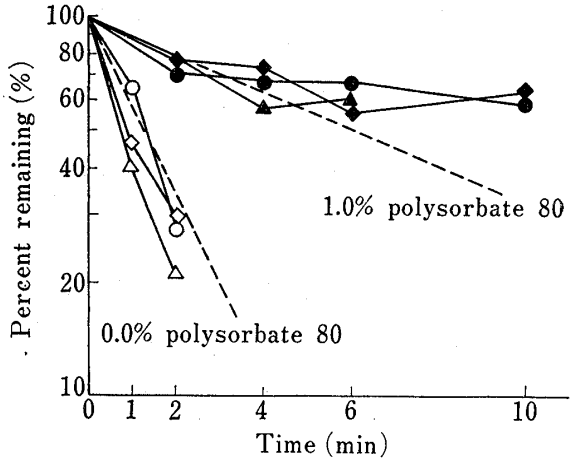

Fig. 6. Effect of Hyaluronidase on the Absorption of Isonicotinamide

The concentration of isonicotinamide was 50 mM. Each point represents the mean value of at least 4 animals.
Key (hyaluronidase concentration): ▲△: 5 units/ml; ●○: 300 units/ml; ◆◇ :1500 units/ml.
Open symbols, in the absence of polysorbate 80; solid symbols, in the presence of 1.0% polysorbate 80. Broken lines were taken from a previous report[3a] (hyaluronidase was not included).

4 and 5. Nonionic surfactants with the exception of HCO-120 showed good correlation between the solubilization of muscle protein and the absorption inhibitory effect (Fig. 4). HCO-120 has a solubilizing effect on muscle protein, but no inhibition on the intramuscular absorption of isonicotinamide could be observed. The deviation of HCO-120 from the other nonionic surfactants suggests involvement of factors other than protein solubilization which affect the absorption process. In the case of ionic surfactants a similar correlation between the solubilization of muscle protein and the absorption inhibitory effect was also demonstrated. Figures 4 and 5 also illustrate that the extent of inhibition was not specific to nonionic surfactants, and was not proportional to the greater solubilizing effect of ionic surfactants, but it was more or less the same in both cases.

These results suggest that solubilization of muscle protein is not the sole explanation of absorption inhibitory effect caused by surfactants, but is one of some other factors which are involved in the same process.

Hence, studies were proceeded to examine the effect of surfactant on some of the other constituents of the muscles. Though it has been known that hyaluronidase promotes connective tissue permeability,[5] subcutaneous[16] and intramuscular absorption,[17] little has been known of the influence of surfactants on its function.

Effect of Hyaluronidase on the Absorption of Drugs in the Presence of Polysorbate 80

In regard to the absorption inhibitory effect of surfactants, if the active site is the mucopolysaccharides which is present in the connective tissue, the influence of the coexistence of hyaluronidase with polysorbate 80 on the intramuscular absorption of drugs can be expected.

Fig. 6 shows the intramuscular absorption of isonicotinamide from a solution containing different concentrations of hyaluronidase (5, 300 or 1500 units/ml) in the absence or presence of 1.0% polysorbate 80. As is evident in the Figure, the coexistence of polysorbate 80 and hyaluronidase inhibited the absorption of isonicotinamide. Also, irrespective of the presence or absence of polysorbate 80, the influence of the hyaluronidase concentration on the absorption process was not significant.

Furthermore, the effect of ploysorbate 80 on the enzymatic activity of hyaluronidase was examined by the viscosity reduction method[18] using sodium chondroitin sulfate as a substrate. It was found that irrespective of the presence or absence of polysorbate 80, increasing the concentration of hyaluronidase was accompanied by a direct decrease in the viscosity of chondroitin sulfate solution (Fig. 7). The pattern of the relative viscosity

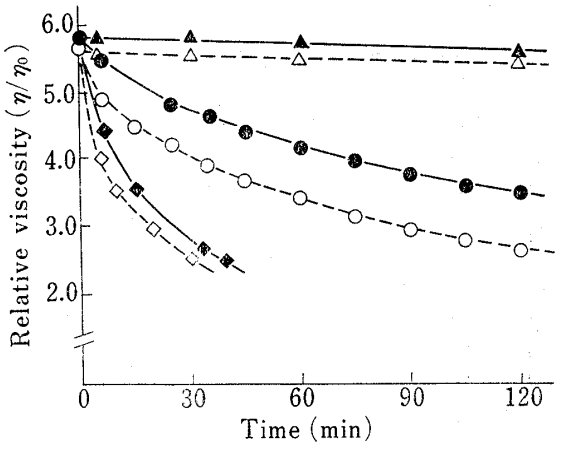

Fig. 7. Effect of polysorbate 80 on the Hyaluronidase Activity Measured by Viscosity Reduction of Sodium Chondroitin Sulfate Solution

The concentration of sodium chondroitin sulfate was 2.0% (w/v).
Key (hyaluronidase concentration): ▲△: 5 units/ml; ●○: 300 units/ml; ◆◇: 1500 units/ml.
Solid lines and solid symbols, in the presence of 1.0% polysorbate 80; broken lines and open symbols, in the absence of polysorbate 80.

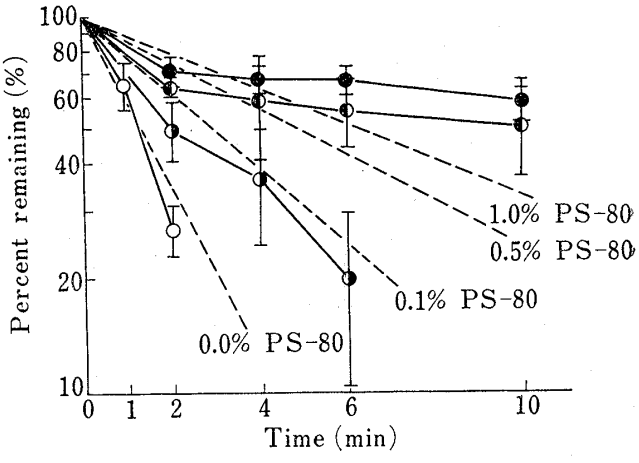

Fig. 8. Effect of Hyaluronidase on the Absorption of Isonicotinamide in the Presence of Polysorbate 80

Concentrations of hyaluronidase and isonicotinamide were 300 units/ml and 50 mM respectively. Each point represents the mean value of at least 4 animals. Vertical bars indicate standard deviation.
Key (polysorbate 80 concentration): ○: 0.0%; ◑: 0.1%; ◐: 0.5%; ●: 1.0%.
Solid lines, in the presence of hyaluronidase; broken lines, in the absence of hyaluronidase (values were taken from previous report[3a]).

16) E. Secher-Hansen, H. Langgard, and J. Schou, *Acta Pharmacol. et Toxicol.*, **25**, 290 (1967); *idem, ibid.*, **26**, 9 (1968); E. Secher-Hansen, *ibid.*, **26**, 229 (1968); *idem, ibid.*, **26**, 316 (1968).
17) R.B. Sund and J. Schou, *Acta Pharmacol. et Toxicol.*, **23**, 194 (1965).
18) M.B. Mathewa and A. Dorfman, *Arch. Biochem. Biophys.*, **42**, 41 (1953).

curves in the presence of polysorbate 80 was more or less similar to these in its absence. These results (Fig. 6, 7) suggest that the effect of hyaluronidase on the absorption of isonicotinamide (Fig. 6) can not be attributed to a change of hyaluronidase activity due to its coexistence with the surfactants.

In general, it has been known that the transport of molecules through the ground substance takes place partly by diffusion which is influenced by hyaluronidase to an extent determined by the molecular size of the diffused compound, and partly by filtration which is likely to be of greater importance the greater the molecule is, and is not affected by hyaluronidase.[17] Furthermore, Goodford, *et al.*,[19] demonstrated that the extracellular space of *taenia coli* smooth muscles was increased after the tissue had been pretreated with hyaluronidase. However, little has been known about the effect of the coexistence of hyaluronidase and surfactants.

Therefore, the effect of hyaluronidase (300 units/ml) in the absence or presence of various concentrations of polysorbate 80 on the intramuscular absorption of drugs of different molecular weights was examined. Isonicotinamide, ^{14}C-sucrose and ^{14}C-inulin which have molecular weights of 122.13, 342.3 and approx. 5000 respectively were used. Figure 8 demonstrates little effect of hyaluronidase on the intramuscular absorption of isonicotinamide in the absence or presence of a low concentration of polysorbate 80. But, when the concentration of polysorbate 80 was increased to 0.5% and 1.0%, a marked inhibition in the absorption of isonicotinamide after 2 minutes was observed. The intramuscular absorption of ^{14}C-sucrose, a compound of moderate molecular weight, (Fig. 9) and ^{14}C-inulin, a compound of large molecular weight, (Fig. 10) were accelerated in the presence of hyaluronidase.

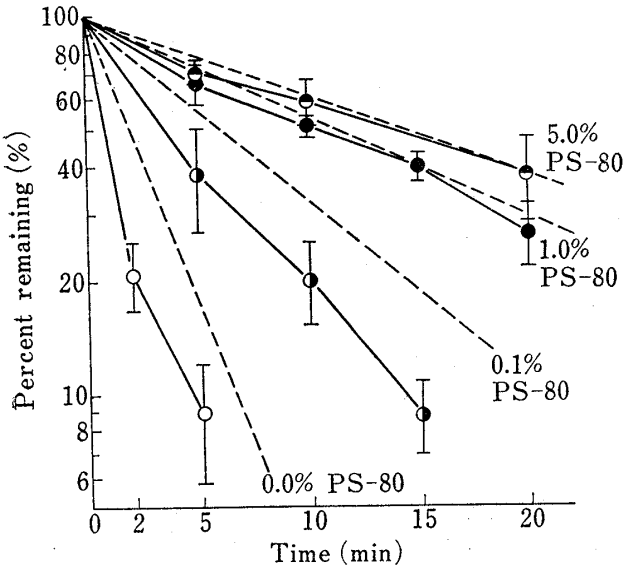

Fig. 9. Effect of Hyaluronidase on the Absorption of ^{14}C-Sucrose in the Presence of Polysorbate 80

The concentrations of hyaluronidase and ^{14}C-sucrose were 300 units/ml and 5 μCi/ml respectively. Each point represents the mean value of at least 4 animals. Vertical bars indicate standard deviation.

Key (polysorbate 80 concentration): ○: 0.0%; ◑: 0.1%; ●: 1.0%; ◓: 5.0%.

Solid lines, in the presence of hyaluronidase; broken lines, in the absence of hyaluronidase (values were taken from previous report[3a]).

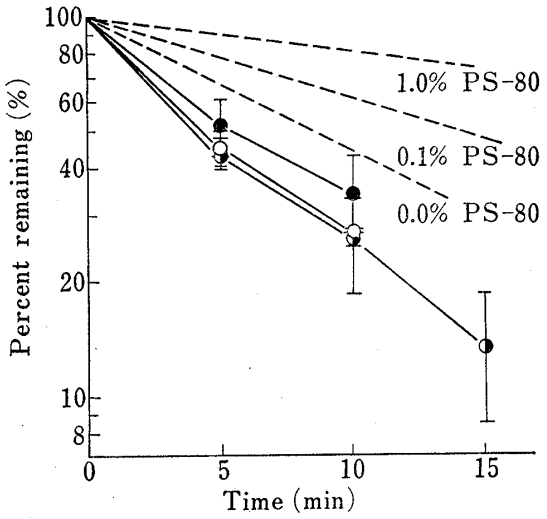

Fig. 10. Effect of Hyaluronidase on the Absorption of ^{14}C-Inulin in the Presence of Polysorbate 80

The concentrations of hyaluronidase and ^{14}C-inulin were 300 units/ml and 5 μCi/ml respectively. Each point represents the mean value of at least 4 animals. Vertical bars indicate standard deviation.

Key (polysorbate 80 concentration): ○: 0.0%, ◑: 0.1%; ●: 1.0%.

Solid lines, in the presence of hyaluronidase; broken lines, in the absence of hyaluronidase (values were taken from previous report[3a]).

19) P.J. Goodford and E.H. Leach, *J. Physiol.*, **186**, 1 (1966).

This coincides with Schou's observations,[17,20] where he mentioned that hyaluronidase depolymerizes the hyaluronic acid of the connective tissue ground substance that is the mucopolysaccharide barrier to diffusion, reduces the water-binding capacity and increases the absorption rate of drugs. The enhancing effect of hyaluronidase on the absorption of ^{14}C-sucrose was recognized at low concentrations of polysorbate 80, while at higher concentrations no significant difference could be noticed. But, the remarkable enhancing effect of hyaluronidase on the absorption of ^{14}C-inulin was demonstrated at both the lower and higher concentrations of polysorbate 80. Consequently, increasing the concentration of polysorbate 80 (in the presence of hyaluronidase) was accompanied by an inhibition in the absorption of isonicotinamide (m.w. 122.13) and abolishment of the accelerating effect of hyaluronidase on the absorption of ^{14}C-sucrose (m.w. 342.3), but had no effect on the absorption of ^{14}C-inulin (m.w. approx. 5000). These results suggest that surfactants as well as hyaluronidase, have an influence on hyaluronic acid of the mucopolysaccharides. Also it shows that the intramuscular absorption of drugs in the presence of hyaluronidase and surfactants is controlled by the concentration of surfactants, hyaluronidase and the molecular weight of drugs.

Effect of Polysorbate 80 on the Permeation of Drug through Gelatin-Sodium Chondroitin Sulfate Mixed Membrane

As it has been known that the main components of connective tissues are proteins and mucopolysaccharides,[4] mixed membrane of gelatin-sodium chondroitin sulfate was used to examine the effect of polysorbate 80 on the permeability of this membrane to isonicotinamide.

Table I. Effect of Polysorbate 80 on the Permeation of Isonicotinamide through Gelatin and Sodium Chondroitin Sulfate Mixed Membrane

Cell No.	Permeability coefficient ($\times 10^{-6}$ cm^2/sec) concentration of polysorbate 80		Ratio
	0.0%	1.0%	
Mixed membrane (I)			
1	8.074	7.130	0.883
3	6.635	5.793	0.873
4	5.243	4.388	0.837
Mixed membrane (II)			
2	7.597	6.198	0.816
3	8.765	7.850	0.896
4	6.303	5.578	0.885

The concentration of isonicotinamide was 1.0mM.
a) Gel/ChsNa=2.92, and 2.0 mM formaldehyde.
b) Gel/ChsNa=2.33, and 2.0 mM formaldehyde.

Results in Table I clarify that the permeability coefficient of the mixed membrane was decreased significantly by the presence of polysorbate 80. The permeability coefficient P, according to Nakagaki, *et al.*,[14,21] as far as the case in which the membrane permeation process is the rate limiting one, is represented by the equation (1)

$$P = f \cdot b \cdot \rho \qquad (1)$$

where f is constant and influenced by both the porosity of the membrane and tortuosity of its pores, b stands for the partition coefficient of the solute between the membrane surface and the solvent, and ρ for the interaction coefficient.

20) J. Schou, *Pharmacol. Rev.*, **13**, 441 (1961); *idem*, "Handbook of Experimental Pharmacology," Vol. 28 (part I), Springer-Verlog, Berlin, 1971, p. 48.
21) M. Nakagaki and M. Yonese, *Yakugaku Zasshi*, **91**, 274 (1971).

The interaction between isonicotinamide and polysorbate 80 is almost negligible,[3a] hence the partition coefficient of isonicotinamide is not affected in the presence of polysorbate 80, therefore ρ and b are considered almost constant. Furthermore, preliminary experiments showed that the water content of the membrane was not affected by the presence of polysorbate 80. Therefore, the decrease of the permeability coefficient (P) of the mixed membrane is attributed to f. This result demonstrates that the surfactants had affected the permeability of the membrane by reducing the porosity and tortuosity, consequently, the passage of drug molecules was hindered.

The present study demonstrates the influence of polysorbate 80 on proteins and mucopolysaccharides of the connective tissue which resulted in an inhibition in the intramuscular absorption of drugs. Although it was clarified that gelatin-sodium chondroitin sulfate membrane was useful in studying the mechanism of the absorption inhibitory effect of surfactants, it remained a point of discussion whether the effect of polysorbate 80 on this artificial membrane is directly reflects its effect *in vivo*. Further studies are required for more clarification.