Chem. Pharm. Bull. **41**(9) 1616—1620 (1993) Vol. 41, No. 9

The Freezing Point Depression Method for the Determination of Binding Parameters of Drugs by Polyvinylpyrrolidone and Polyoxyethylene *n*-Dodecylether[1,2]

Masahiko SUZUKI,*[,a] Kiyoko ITO,[a] Chigusa FUSHIMI,[a] and Tamotsu KONDO[b]

Product Development Laboratories, Sankyo Co., Ltd.,[a] Hiromachi, Shinagawa-ku, Tokyo 140, Japan and Faculty of Pharmaceutical Sciences, Science University of Tokyo,[b] 12 Funagawaramachi, Shinjuku-ku, Tokyo 162, Japan.

Received February 17, 1993

The interaction of sodium salicylate and naphazoline hydrochloride with polyvinylpyrrolidones having four different molecular weights (10, 25, 40, and 360 kDa), and with the micelles of non-ionic surfactant polyoxyethylene *n*-dodecylethers having various numbers of oxyethylene units (5, 6, 7, and 8) were investigated using the freezing point depression method. The advantages of this method are that the binding parameters can be rapidly obtained by a simple procedure using a commercially available osmometer. Based on the colligative properties, the binding parameters were calculated. The data were expressed in the form of nonlinear Scatchard plots. The results suggested that salicylate and naphazoline were bound to two kinds of binding sites on a molecule of polyvinylpyrrolidone or to a micelle of polyoxyethylene *n*-dodecylether; the primary binding site exhibited a high affinity but a low capacity for the drug, while the second binding site has a lower affinity and a high capacity; that is, the interaction on the second binding site could be described as non-specific binding. For the interactions of drugs with polyvinylpyrrolidone, the number of *N*-vinyl-2-pyrrolidone units (monomer) that interacted with one molecule of salicylate and naphazoline were fairly constant, and from 10 to 14 and from 21 to 30, respectively. For the interactions of drugs with a micelle of polyoxyethylene *n*-dodecylether, the number of polyoxyethylene *n*-dodecylether molecules which interacted was also nearly constant at 3 to 4 and 5 to 6, respectively.

Keywords freezing point depression; association constant; Scatchard plot; polyvinylpyrrolidone; polyoxyethylene *n*-dodecylether

In the pharmaceutical field, many water soluble polymers and non-ionic surfactants are used as excipients, solubilizing agents, and stabilizing agents. Very recently, these compounds have come to be used as carriers for drug delivery system application. Under these circumstances, however, these polymers and surfactants lead to the unstabilization and precipitation of drugs.[3] For this reason, it is important to elucidate the interaction of drugs with the polymers and micelles of surfactants.

Polyvinylpyrrolidones (PVPs) having mean molecular weights ranging from about 10 to 700 kDa, are in widespread use in pharmaceuticals,[3] and polyoxyethylene *n*-dodecylethers (PDs) are used as model surfactants in the basic research of surfactants and micelles.[4] Many methods have been proposed to investigate interactions. These methods can be summarized into two broad categories: (1) the equilibrium dialysis method,[5] based on measuring changes in ligand concentration, and (2) the direct measurement of the property of a complex, such as the solubility method[6] or the potentiometric titration method.[7]

In our previous study, the freezing point depression method[2] for measuring a decrease in osmotic concentration depending on host–guest complex formation was presented, and it was concluded that this method was useful for determining of the stability constants for complex formation in aqueous solutions. In this paper, we report that the freezing point depression method is utilized to determine binding parameters in interactions between drugs (sodium salicylate and naphazoline hydrochloride) and macromolecules (PVPs and micelles of PD) in an aqueous solution. Moreover, the binding characteristics of the drugs on the polymer and the micelle are discussed.

Experimental

Materials Sodium salicylate, naphazoline hydrochloride, and *N*-vinyl-2-pyrrolidone (VP) were obtained from Tokyo Kasei Kogyo Co., Ltd. PVPs with K-numbers of K-15, K-25, K-30, and K-90 were from G. A. F. Corp., and they were purified by ultrafiltration through Millipore filters [molecular weight (M.W.) cut-offs: 5, 10, 30, and 100 kDa] to remove low-molecular-weight impurities prior to use. Polyoxyethylene *n*-dodecylethers (PD-5, PD-6, PD-7, and PD-8: the number denotes the nominal number of oxyethylene units in the molecule) were of commercial grade and were obtained from Nikko Chemicals Co., Ltd. All other reagents were of reagent grade and were used without further purification.

Freezing Point Depression Method Theories and practices were reported previously.[2] The freezing point depression method is a suitable for determining the stability constant for complex formation in an aqueous solution. Based on their colligative properties, the stability constants can be calculated from the change in osmotic concentration obtained in terms of freezing point depression using a commercially available osmometer.

Apparatus An osmometer (Osmette model 2007, Precision Systems, Inc.) was employed for the determination of osmotic concentration based on the freezing point depression.[8] The instrument was calibrated with standard solutions of dextrose (100, 500 mOsm/kg) supplied by the company.

Measurement of Osmotic Concentration All solutions were prepared with distilled water. In dilute solutions, the molality is proportional to the molarity of the solute.[2] Therefore, the preparation of samples was carried out according to molarity, for convenience. Measurements were performed by varying the amounts of sodium salicylate and naphazoline hydrochloride (20—100 mM) while maintaining the PVP or PD concentration at 2.5% and 5%, except for PVP K-90. The concentration of PVP K-90 was 1%. The pH values were observed to be constant at 8.3—8.5 and 6.9—7.1 for the salicylate complex systems and naphazoine complex systems, respectively. The osmotic concentration was measured with 2 ml of a sample solution and was replicated three times for each solution. The reproducibility of the measurement of osmotic concentration was reported previously to be within ± 1 mOsm/kg.

Degree of Polymerization of PVP and Association of PD The degree of polymerization (*dp*) of PVP, or, in other words, the number of VPs per PVP molecule, was calculated from the molecular weight of PVP and its constituent unit, VP, and the values obtained are summarized in Table I, *dp* = PVP M.W./monomer M.W. (111.14).

TABLE I. Degree of Polymerization of PVP

PVP	M.W. (kDa)[a]	dp[b]
PVP K-15	10	90
PVP K-25	25	225
PVP K-30	40	360
PVP K-90	360	3239

a) Average molecular weight. *b*) The number of vinylpyrrolidones (M.W. 111.14) per PVP.

TABLE II. Degree of Association of PD

PD	HLB	Monomer M.W.	Micellar M.W.[a]	da[b]
PD-5	10.8	406.6	1.70×10^6	4181
PD-6	11.7	450.7	6.81×10^4	151
PD-7	12.5	494.7	9.06×10^4	183
PD-8	13.1	538.8	6.79×10^4	126

a) Average molecular weight determined by light scattering measurement. *b*) The number of PD molecules per micelle.

The degree of association (da) of PD (the number of PD molecules per micelle) was calculated from the molecular weights of PD (monomer) and micelle, da=micellar M.W./PD M.W. The micellar molecular weight was determined by light scattering measurement using a photogoniodiffusometer (model DLS-700, Otsuka Electronics Co., Ltd.) with 5 ml of a solution containing 1, 2.5, and 5 mg/ml of PD. Scattering angles were varied from 30° to 150°, in 10° increments. The data were processed by computer according to Zimm plots,[9] and the values obtained are listed in Table II. The micellar molecular weights, shown, are the average of three measurements.

Results and Discussion

Theoretical Procedure It can be observed that the experimentally determined osmotic concentration of the mixture containing either salicylate (50 mM) or naphazoline (50 mM) and PVP K-25 (2.5 and 5%) was smaller than the sum of their respective values measured separately. In Table III, Δ represents these diminutions. It can be said that these diminutions arise from the interaction between these two compounds. Similar results were obtained in other systems: mixtures of each macromolecule (PVP K-15, -30, -90, and PD-5, -6, -7, -8) and either salicylate or naphazoline. Based on their colligative properties, we developed a calculation to determine the binding parameters for interaction in these systems.

Analysis of the binding phenomenon in low-molecular weight drug/macromolecule systems is well established and can be considered to obey the law of mass action[10]:

$$r=\frac{[A_b]}{S_0}=\frac{nK[A_f]}{1+K[A_f]} \tag{1}$$

where r is the molar ratio of bound drug $[A_b]$ to total macromolecule S_0, and n and K are the number of independent equivalent binding sites and the association (binding) constant, respectively. $[A_f]$ is the concentration of free (unbound) drug.

In order to apply the experimentally obtained osmotic concentration to the above equation (Eq. 1), the following theoretical treatments were evolved. For the interaction between drug A and macromolecule S with n binding sites, the total molar concentration M and osmotic concentration $\bar{M}$ of the mixture are written:

TABLE III. Experimentally Determined Osmotic Concentration and Calculated Δ Values of Aqueous Solutions of PVP K-25, Salicylate and Naphazoline Individually, and Salicylate/PVP K-25 and Naphazoline/PVP K-25 in Combination, Measured by Freezing Point Depression

System	Compound		Osmotic concentration (mOsm/kg)	Δ[a] (mOsm/kg)
Individual	Sodium salicylate	50 mM	100	
	Naphazoline hydrochloride	50 mM	100	
	PVP K-25	2.5%	2	
		5.0%	4	
Mixture	Salicylate 50 mM + PVP K-25	2.5%	90	12
		5.0%	83	21
	Naphazoline 50 mM + PVP K-25	2.5%	98	4
		5.0%	95	9

a) Δ represents the difference between stoichiometric concentration and experimentally determined osmotic concentration.

$$M=[A_f]+[S_f]+(n+1)[A_nS] \tag{2}$$

$$\bar{M}=[A_f]+[S_f]+[A_nS] \tag{3}$$

where $[S_f]$ and $[A_nS]$ represent either the concentration of macromolecule not binding with or binding with the drug, respectively.

The concentration of bound drug $[A_b]$, which is the same as $n[A_nS]$, is deduced from M and $\bar{M}$.

$$\Delta=[A_b]=n[A_nS]=M-\bar{M} \tag{4}$$

where the difference between M and $\bar{M}$ is replaced by Δ. Therefore,

$$r=\frac{\Delta}{S_0}=\frac{M-\bar{M}}{S_0} \tag{5}$$

The concentration of free drug $[A_f]$ is taken as the difference between $\bar{M}$ and $S_0(=[S_f]+[A_nS])$.

$$[A_f]=\bar{M}-S_0 \tag{6}$$

Therefore, r and $[A_f]$ can be obtained experimentally. Using these values, the binding parameters (n and K) can be calculated according to Eq. 1.

For the interaction of the drug with a micelle of PD (surfactant), theoretically, S_0 in terms of PD concentration should be the concentration of micelles. In analyzing the data, the molecular weights of micelles of PDs were determined using the light scattering technique as shown in Table II. Furthermore, since the critical micell concentrations (cmc) of PDs are sufficiently low (the cmcs of PD-5, -6, -7, and -8 are about 0.008% each), the monomer concentration may be neglected.

Data Treatment for Binding Phenomenon Many authors have suggested that Eq. 1, which has the same form as the Langmuir isotherm, involved a mechanism in which the interaction is an adsorption phenomena on the surface of a macromolecule (or micelle) or at some other site within it. However, in many cases, the binding and adsorption did not follow an identical process and involved more than one class of binding sites. In this study, when the Scatchard equation[11] (Eq. 7) was applied to all experimental data, the Scatchard $r/[A_f]$ $v/s.$ r plots produced a non-linear relationship, which was taken as evidence of the existence of more than one type of binding site.

$$\frac{r}{[A_f]}=nK-rK \tag{7}$$

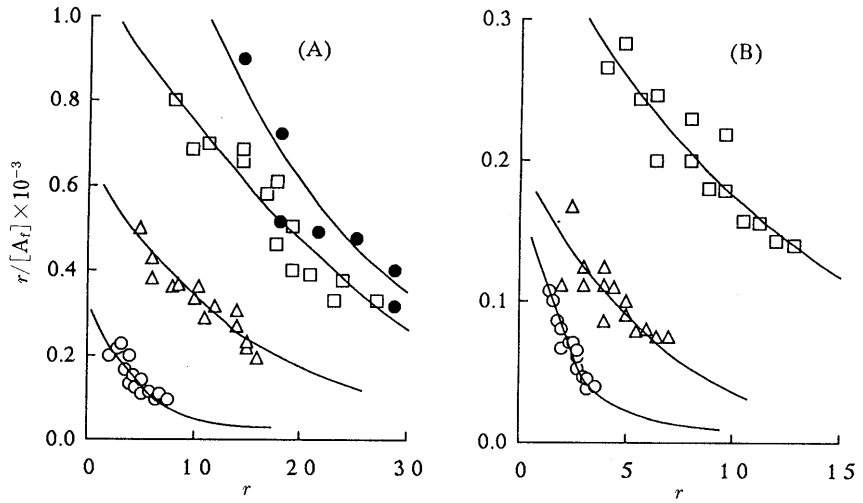

Fig. 1. Scatchard Plots for the Interaction of Salicylate (A) and Naphazoline (B) with PVP
○,PVP K-15; △, PVP K-25; □, PVP K-30; ●, PVP K-90 ($r/[Af]\times10^{-1}$, $r\times10^{-1}$).

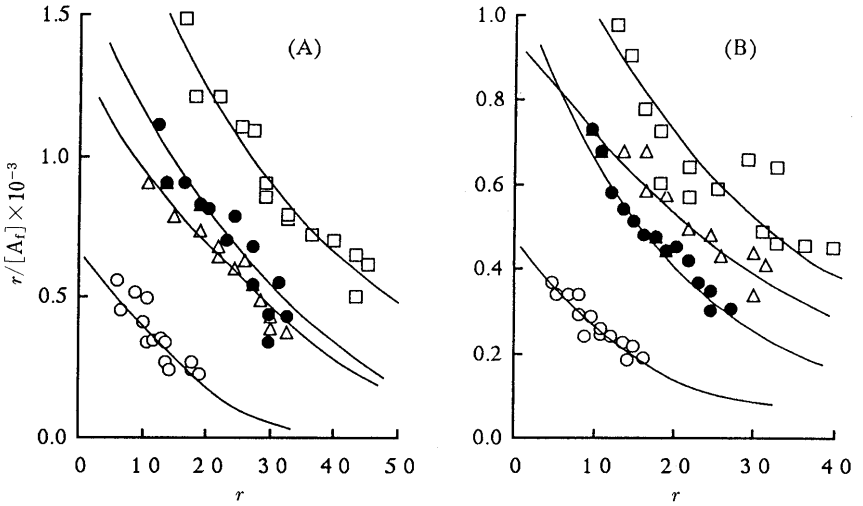

Fig. 2. Scatchard Plots for the Interaction of Salicylate (A) and Naphazoline (B) with PD Micelles
○, PD-5 ($r/[Af]\times50^{-1}$, $r\times50^{-1}$); △, PD-6; □, PD-7; ●, PD-8.

The plot for each drug system (salicylate or naphazoline)/macromolecule (PVP and PD) shown in Figs. 1 and 2 had a definite curvature.

If there are m classes of independent sites, with each class i, having n_i sites with an intrinsic association constant K_i, Eq. 1 is then written as:

$$r=\sum_{i=1}^{m}\frac{n_iK_i[A_f]}{1+K_i[A_f]} \tag{8}$$

To describe the more complex binding processes above, we assumed that the binding process involved two classes of sites ($m=2$). This model has been most commonly used to describe a binding phenomenon having a nonlinear Scatchard plot.[6,12] The experimental osmotic data also fitted best with this model. Association parameters are calculated according to Eq. 9 by means of a nonlinear least-square procedure (MULTI)[13] and are summarized in Tables IV and V.

$$\frac{r}{[A_f]}=\frac{\Delta}{S_0[A_f]}=\frac{n_1K_1}{1+K_1[A_f]}+\frac{n_2K_2}{1+K_2[A_f]} \tag{9}$$

Table IV. Binding Parameters for the Interaction of Salicylate and Naphazoline with PVP

Drug	PVP	n_1	K_1 (M^{-1})	n_2	K_2 (M^{-1})
Salicylate	PVP K-15	7.0± 0.50	41±3.3	50± 3.8	0.44±0.06
	PVP K-25	16 ± 2.2	34±2.8	67± 3.5	0.94±0.17
	PVP K-30	30 ± 2.8	33±2.4	82± 5.7	0.60±0.06
	PVP K-90	339 ±32	44±2.4	915±74	0.05±0.01
Naphazoline	PVP K-15	3.2± 0.54	50±3.9	91± 4.1	0.12±0.02
	PVP K-25	7.4± 1.4	23±2.3	66± 3.9	0.26±0.03
	PVP K-30	17 ± 2.0	20±1.0	22± 2.6	0.87±0.13
	PVP K-90	—	0	—	0

The binding parameters shown in Tables IV and V suggested that the primary binding site within PVP and PD exhibited a high affinity but a low capacity for salicylate and naphazoline, while the second binding site conversely had a lower affinity but a high capacity. That is to say, the binding phenomenon on the second binding site is characterized as non-specific and nonsaturable binding, in contrast with the primary binding site which has specific binding. Therefore, Eq. 9 reduces to Eq. 10 by means of

TABLE V. Binding Parameters for the Interaction of Salicylate and Naphazoline with PD

Drug	PD	n_1	K_1 (M^{-1})	n_2	K_2 (M^{-1})
Salicylate	PD-5	1344±55	24±3.0	5108±371	0.01±0.002
	PD-6	48± 5.3	26±2.7	86± 5.6	0.03±0.002
	PD-7	58± 3.3	23±2.6	115± 5.8	0.07±0.004
	PD-8	46± 1.4	31±2.6	84± 3.5	0.06±0.005
Naphazoline	PD-5	814±21	26±2.5	7096±169	0.41±0.029
	PD-6	28± 1.2	29±1.5	102± 5.6	1.4 ±0.17
	PD-7	35± 1.7	32±2.1	119± 13	1.2 ±0.11
	PD-8	21± 2.4	57±4.5	48± 4.5	1.1 ±0.14

Parameters were calculated using micellar molecular weight.

$1 \gg K_2[A_f]$, since $[A_f] \ll 0.1$ M ($=[A_f]+[A_b]$) and $K_2 \leqq 1$.

$$\frac{r}{[A_f]} = \frac{n_1 K_1}{1+K_1[A_f]} + C \tag{10}$$

where $C=n_2K_2$. The Scatchard plots exhibit a horizontal asymptote (constant value of $r/[A_f]$) at high values of r.

Interactions of Salicylate and Naphazoline with PVP It is well known that the conformation of dissolved linear polymers, such as PVP, is quite flexible, due to of a relatively large freedom of rotation around the bonds between carbon and other atoms forming their backbone, so that the Brownian motion of chain segments tends to produce roughly spherical random coils that interpenetrate one another and mechanically trap large amounts of solvent.[14] Salicylate and naphazoline molecules also seem to be trapped into random coils of PVP chain segments, similarly to solvent molecules. The polymer conformation is also known to be transformed by the viscosity of a solution and by the presence of electrolytes; furthermore, the viscosity also alters the osmotic data. In the present study, however, the viscosity of solutions containing 10% or less PVP was essentially the same as that of water.[15] In addition, no conformational change of an uncharged polymer (*i.e.* PVP) was usually observed at concentrations <0.1 M of electrolytes.[16]

As outlined above, PVP has two kinds of binding sites and the primary and the second binding sites were characterized as involving specific and non-specific binding, respectively. It therefore appears that the primary binding site is probably located in the interior region of random coils and that the binding form can be thought of as a type of inclusion complex; that is, the drug trapped into PVP is surrounded by several VPs (monomer) that have united to form a molecule of PVP (polymer). On the other hand, the second site binding seems to involve non-specific adsorption on the surface of random coils of PVP chain.

For the primary binding, the number of VPs that interacted with one drug molecule was calculated according to dp/n_1. From the results shown in Table VI, it can be considered that salicylate and naphazoline are trapped into PVP as though being surrounded by 10 to 14 VP units or 21 to 30 VP units, respectively, and that the number of VPs seems to remain constant for the various sizes of PVP. To confirm this surrounding conformation, the interactions of a drug with VP corresponding to the constituent unit of PVP were investigated. As shown in Table VII, no difference between the stoichiometric concentration and osmotic concentration was recognized. This result is evidence in support of their binding conformation.

TABLE VI. The Number of VPs That Interacted with Salicylate and Naphazoline

PVP	Salicylate dp/n_1	Naphazoline dp/n_1
PVP K-15	13 ±0.98	28±5.2
PVP K-25	14 ±1.8	30±6.7
PVP K-30	12 ±1.0	21±2.7
PVP K-90	9.6±0.97	—

n_1: the number of primary binding sites on PVP.

TABLE VII. Interaction of Salicylate and Naphazoline with VP

System (mM)			Osmotic concentration (mOsm/kg)	$\Delta^{a)}$ (mOsm/kg)
Vinylpyrrolidone	25		25	
	50		50	
Sodium salicylate	50		98	
Naphazoline hydrochloride	50		98	
Vinylpyrrolidone	25 + salicylate	50	123	0
	50 + salicylate	50	148	0
Vinylpyrrolidone	25 + naphazoline	50	123	0
	50 + naphazoline	50	148	0

a) Δ represents the difference between stoichiometric concentration and experimentally determined osmotic concentration.

The binding data shown in Table IV illustrate that K_1 for the primary binding of salicylate with PVP has almost the same value (30—40 M^{-1}) for each system, but the binding of naphazoline with PVP has a tendency to decrease with increasing PVP M.W., and hence there would be no appreciable interaction between naphazoline and PVP K-90. These results reveal that the binding mechanism of naphazoline with PVP is not necessarily the same among different types of PVP.

Interactions of Salicylate and Naphazoline with PD The binding parameters shown in Fig. 2 and Table V illustrate that salicylate and naphazoline are bound to two kinds of binding sites on the micelles of PDs: these are a high affinity-low capacity site, and a low affinity-high capacity (nonspecific) site. For the primary (high affinity-low capacity) binding site, K_1 is almost the same, ranging from 20 to 30 M^{-1}, regardless of the size of micelles, except for in the naphazoline/PD-8 system, which has a somewhat large K_1 value. Shimamoto and Ogawa[4] studied the interaction of methylparaben with polyoxyethylene dodecylethers using NMR, and concluded that preservative molecules were located at the interface of the hydrocarbon core and the polyoxyethylene mantle of a micelle, and also in the polyoxyethylene mantle of a micelle itself. Blanchard *et al.*[17] reported that the interaction of phenolic preservatives with polysorbate 80 might present a conformation similar to polyoxyethylene dodecylether. For the interaction of salicylate and naphazoline with a micelle of PD, a similar binding phenomenon can be considered: that the first site is a specific site, probably at the hydrocarbon core and in the polyoxyethylene chains of a micelle, while the second site involves the non-specific attraction of drugs in the polyoxyethylene region of a micelle.

The numbers of PDs that interacted with salicylate and naphazoline were calculated according to da/n_1. As shown in Table VIII, da/n_1 values remained constant in the ranges

TABLE VIII. The Number of PDs That Interacted with Salicylate and Naphazoline

PD	Salicylate da/n_1	Naphazoline da/n_1
PD-5	3.1±0.12	5.1±0.13
PD-6	3.2±0.34	5.4±0.24
PD-7	3.2±0.18	5.2±0.26
PD-8	2.7±0.08	6.1±0.70

n_1: the number of primary binding sites on micelles.

of 3 to 4 and 5 to 6, respectively. These results suggested that a possible primary binding site in the polyoxyethylene region may consist of several PD molecules in association with a regular number of PDs, regardless of PD varieties.

In conclusion, the most remarkable features of interactions such as salicylate with PVP and a micelle of PD, or of naphazoline with a micelle of PD, are the constancy of K_1 and the dp or da/n_1 values. According to these results, the interaction process at the primary binding site can be considered to be caused by a similar mechanism for both interactions, and also, a drug molecule is probably surrounded by several constituent units of PVP and the micelle with a constant unit number. The interaction of naphazoline with PVP has various K_1 depending on PVP molecular weight; however, the number of its constituent units of VP that interacted with a drug is constant. This indicates that the binding mechanism may be different, but its manner of appearance seems to be similar to that of other systems.

References and Notes

1) A part of this work was presented at the 110th Annual Meeting of the Pharmaceutical Society of Japan, Sapporo, August 1990.
2) This paper constitutes Part IV of a series of studies entitled "Application of Freezing Point Depression to Drug Interaction Studies," Part I: M. Suzuki, S. Ueda, A. Kusai, *Chem. Pharm. Bull.*, **36**, 720 (1988).
3) B. V. Robinson, F. M. Sullivan, J. F. Borzelleca, S. L. Schwartz, "PVP: A Critical Review of the Kinetics and Toxicology of Polyvinylpyrrolidone," Lewis Publishers, Inc., Michigan, 1990; "Polyvinylpyrrolidone: Physical, Chemical, Physiological and Functional Properties," GAF Technical Bulletin, 9642-079.
4) T. Shimamoto, Y. Ogawa, *Chem. Pharm. Bull.*, **23**, 3088 (1975).
5) N. K. Patel, H. B. Kostenbauder, *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 289 (1958); S. M. Balaug, A. G. Rich, *ibid.*, **54**, 30 (1965).
6) S. J. A. Kazmi, A. G. Mitchell, *J. Pharm. Pharmacol.*, **23**, 482 (1971).
7) A. G. Mitchell, K. F. Brown, *J. Pharm. Pharmacol.*, **18**, 115 (1966).
8) T. Ota, S. Kikuchi, K. Eyoshi, K. Ota, *J. Med. Tech.*, **17**, 1061, (1973).
9) B. Zimm, *J. Chem. Phys.*, **16**, 1099 (1948).
10) E. J. Garrett, *J. Pharm. Pharmacol.*, **18**, 589 (1966).
11) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).
12) M. J. Crooks, K. F. Brown, *J. Pharm. Pharmacol.*, **26**, 235 (1974).
13) K. Yamaoka, Y. Tanigawa, T. Nakagawa, T. Uno, *J. Pharmacobio-Dyn.*, **4**, 879 (1981).
14) A. Martin, J. Swarbrich, A. Cammarata, "Physical Pharmacy," 3rd ed., Lea and Febiger, Philadelphia, 1983, Chapter XXII.
15) D. M. Patel, W. D. Walkling, Z. Chowhan, "Handbook of Pharmaceutical Excipients," American Pharmaceutical Association, Washington, D. C., 1986, pp. 234—239.
16) I. D. Robb, "Chemistry and Technology of Water Soluble Polymers," ed. by C. Finch, Plenum Press, New York, 1985, pp. 193—202.
17) J. Blanchard, W. T. Fink, J. P. Duffy, *J. Pharm. Sci.*, **66**, 1470 (1970).