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Effect of SDS on Retention of Nucleic Acid Components in High-Performance Liquid Chromatography

Yong-Nam Kim*, Kyeung-Soo Choi, Dai-Woon Lee†, and Phyllis R. Brown‡

Department of Chemistry, Kyungnam University, Masan 631-701
†Department of Chemistry, Yonsei University, Seoul 120-749
‡Department of Chemistry, University of Rhode Island, Kingston, Rhode Island 02881, U.S.A.
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The effect of the addition of sodium dodecyl sulfate (SDS) to a buffered mobile phase (pH 3.4) on the retention of nucleotides, nucleosides and bases was investigated with a polyvinyl alcohol (PVA) column. Depending on the concentration of SDS, two different trends in the retention of nucleosides and bases containing an NH_2 group were observed. If the concentration of SDS was less than 5.5 mM, the retention of compounds containing an NH_2 group increased as the concentration of SDS in the mobile phase increased. In contrast, if the concentration was greater than 5.5 mM, the retention of compounds containing an NH_2 group decreased. Thus, the SDS acted as an ion-pairing reagent at lower concentration but formed micelles at higher concentrations. The retention behavior of the nucleosides and bases in the presence of a micellar concentration of SDS in the mobile phase on the PVA column was compared to the retention behavior on other types of columns.

Introduction

During the past decade, high-performance liquid chromatography (HPLC) has been extensively used for the separation of nucleotides, nucleosides and bases[1-14]. These HPLC methods include ion exchange[1-4], reversed-phase[5-10] and ion pair[11-14] liquid chromatography. Among these methods, the effects of small amount of surfactants in the mobile phase have been investigated in ion pair chromatography[15-21]. Kraak *et al.*[20] investigated the retention behavior of nucleosides and bases on a reversed-phase C_{18} column using water-ethanol solutions containing small amount of SDS as the mobile phase. In 1980, Armstrong and Henry reported the use of an aqueous micellar solution as a selective mobile phase in reversed-phase liquid chromatography[22]. Since then, micellar liquid chromatography has been investigated for the separation of many types of compounds[23-32].

Most of the separations of nucleic acid components reported have been performed on silica-based columns. Recently, the retention behavior of nucleotides, nucleosides and their bases on a polymeric polyvinyl alcohol (PVA) column with several different buffer and salt solutions have been reported[33-37]. However, detailed information on the characteristics of PVA columns is not available.

We previously reported the use of micellar mobile phases for the separation of nucleosides and bases on a column[29]. Effect of pH, temperature, and concentration of SDS and the counter ion on retention behavior were investigated. In this paper, we extended the studies on the retention behavior of nucleotides, nucleosides and bases on a PVA column using SDS mobile phases. Effect of different stationary phases on the separation of nucleosides and bases was also

Table 1. List of Columns Used

Name	Column Dimensions (mm)	Type	Particle Size (μm)	Manufacturer
PartiSphere C_{18}	110×4.7	C_{18}	5	Whatman Inc.
PartiSphere C_8	110×4.7	C_8	5	Whatman Inc.
Supelcosil LC-CN	150×4.6	Cyanopropyl	5	Supelco Inc.
Supelcosil LC-1	150×4.6	C_1	5	Supelco Inc.
PartiSphere Silica	110×4.7	Silica	5	Whatman Inc.
Protesil 300 Octyl	250×4.6	C_8	10	Whatman Inc.
PRP-1	150×4.1	Poly (styrene divinylbenzene)	10	Hamilton Co.
Asahipak GS 320H	250×7.6	Polyvinyl alcohol	9	Asahi Chemical Industry Co.

investigated using a micellar SDS mobile phase.

Experimental

Apparatus. An ICI LC 1500 pump equipped with a Rheodyne 7125 injector connected to an ICI LC 1200 UV/VIS detector was used. The eight different columns used in this study are listed in Table 1. Retention times were measured using a Hewlett-Packard Model 3394A integrator. A Millipore Milli-Q system was used for purification of water. The pH measurements were carried out with an Orion Model SA 720 pH meter equipped with a ROSS combination pH electrode.

Reagents. Nucleotide, nucleosides and bases were obtained from Sigma. Their abbreviations are listed in Table 2. Stock solutions were prepared in water and the pH was adjusted to 7.3 with phosphate buffer. All stock solutions were stored at −20℃. Electrophoresis grade SDS was obtained from Bio-Rad and used as received. Analytical grade sodium dihydrogen phosphate and HPLC grade phosphoric acid were used for the preparation of the mobile phases.

Procedure. SDS mobile phases were prepared by adding the appropriate amount of SDS to water. In all cases, the pH of the mobile phases was adjusted to 3.4 with phosphoric acid and the solutions were filtered through 0.45 μm Nylon-66 membrane filters. The separations were performed isocratically at a flow-rate of 2 ml/min at ambient temperature.

Results and Discussion

Effect of Mobile Phase. The best separation of the nucleic acid components using SDS in the mobile phase was found with a PVA column. Thus, this column was used in the studies of SDS concentration. Addition of SDS to the phosphate mobile phase changes the elution order of nucleotides. The effect of the surfactant on the retention of cytosine nucleotides is shown in Figure 1. Because of the electrostatic repulsion between negatively charged SDS molecules and negatively charged CTP, not only was the total elution time faster (less than 5 min) but also the CTP eluted faster thanCDP or CMP. The same result was observed with the majority of the other nucleotides listed in Table 2. It was found that decrease in retention of the nucleotides was not dependent on the concentration of SDS (Figure 2).

With nucleosides and bases, the major change in elution order on the addition of SDS to the mobile phase was in

Table 2. List of Abbreviations of Bases, Nucleosides and Nucleotides

Bases	
Ade	Adenine
Cyt	Cytosine
Hyp	Hypoxanthine
Thy	Thymine
UA	Uric acid
Ura	Uracil
Xan	Xanthine
Nucleosides	
Ado	Adenosine
Guo	Guanosine
Ino	Inosine
Thd	2′-Deoxythymidine
Urd	Uridine
Nucleotides	
AMP	Adenosine 5′-monophosphate
ADP	Adenosine 5′-diphosphate
ATP	Adenosine 5′-triphosphate
CMP	Cytidine 5′-monophosphate
CDP	Cytidine 5′-diphosphate
CTP	Cytidine 5′-triphosphate
GMP	Guanosine 5′-monophosphate
GDP	Guanosine 5′-diphosphate
GTP	Guanosine 5′-triphosphate
IMP	Inosine 5′-monophosphate
IDP	Inosine 5′-diphosphate
ITP	Inosine 5′-triphosphate
TMP	Thymidine 5′-monophosphate
TDP	Thymidine 5′-diphosphate
TTP	Thymidine 5′-triphosphate
UMP	Uridine 5′-monophosphate
UDP	Uridine 5′-diphosphate
UTP	Uridine 5′-triphosphate

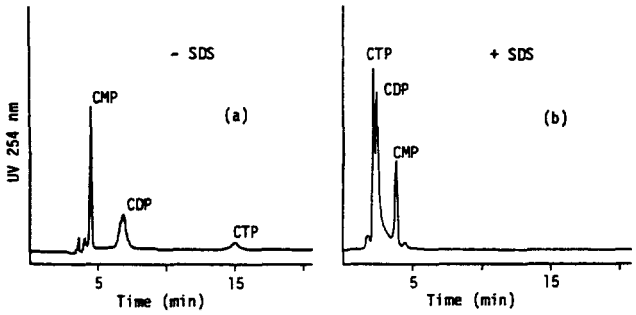

Figure 1. Effect of SDS surfactant on the retention of cytosine nucleotides. Column, 9 μm Asahipak GS 320H; mobile phase, (a) 1 mM NaH_2PO_4 (pH 3.4), (b) 1 mM SDS (pH 3.4); flow-rate, 2 ml/min; sensitivity, 0.01 a.u.f.s.; temperature, ambient.

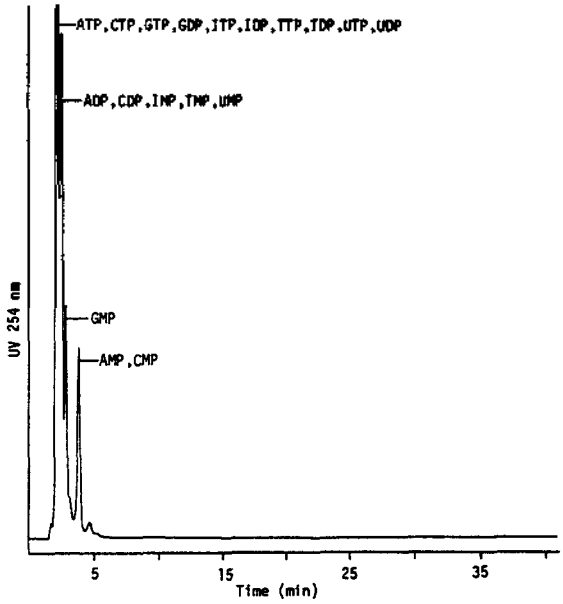

Figure 2. Isocratic elution profiles of a mixture of nucleotides. Mobile phase, 10 mM SDS (pH 3.4). Other chromatographic conditions are the same as in Figure 1.

the increase in retention of the nucleosides and bases which have an NH_2 group on the pyrimidine ring (Cyt) and in the 6 position on purine (Ado, Ade) (Figure 3). The retention of these Cyt, Ado and Ade significantly increased as the concentration of SDS increased and then decreased after reaching a maximum (Figure 4a). A similar result was observed by Knox and Hartwick[21] who investigated the effect of the mobile phase concentration of SDS on the capacity factor of tyrosine amide using a silica-based C_{18} column. Kraak *et al.*[20] also reported a similar effect on the retention of nucleosides and bases on a silica-bonded C_{18} column when SDS was added to the mobile phase. This maximum is considered as the critical micellar concentration (CMC). The reported CMC value of SDS in pure water is 8×10^{-3} M[37]. Because the addition of a salt to the SDS solution increases the surface activity of the SDS solution, micelles can be formed at lower concentration of SDS than in pure water; as the concentration of added salt increases, the CMC value of SDS decreases[27,37]. Kasturi and Gilpin[28] reported that at

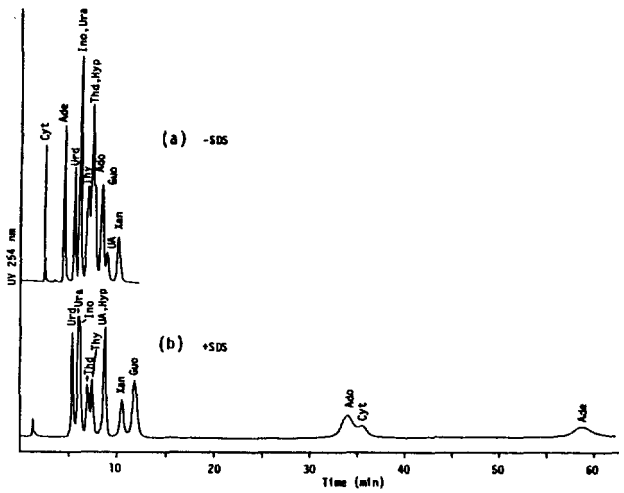

Figure 3. Isocratic elution profiles of a mixture of nucleosides and bases. Mobile phase, (a) aqueous solution (pH 3.4), (b) 1 mM SDS (pH 3.4). Other chromatographic conditions are the same as in Figure 1.

elevated pressure, the CMC of SDS is higher than at ambient pressure values. Therefore, the CMC value of SDS varies with physical and chemical parameters.

The fact that slopes for Ado, Cyt and Ade are different from those of other nucleosides and bases in the plot of log k′ *vs.* SDS concentration (Figure 4a) indicates that two different retention mechanisms are operative for compounds containing an NH_2 group. In the region of ion pairing concentration (<5.5 mM), as the concentration of SDS increases, the capacity factors of the compounds containing an NH_2 group increase. This result indicates that the adsorption of SDS monomers on the PVA stationary phase increases, as the concentration of SDS increases. Since compounds containing an NH_2 group are positively charged at pH 3.4, electrostatic attraction occurs with negatively charged SDS monomers adsorbed on the PVA stationary phase. Thus, compounds containing an NH_2 group experience more interactions with increased number of SDS monomers adsorbed on the PVA stationary phase and are retained longer than other nucleosides and bases.

On the contrary, in the region of micelle forming concentration (>5.5 mM), the capacity factors of compounds containing an NH_2 group decrease as the concentration of SDS increases. This decrease in retention can be explained by the electrostatic attraction of the positively charged compounds with increased number of SDS micelles in the mobile phase. Because SDS micelles are more negatively charged than SDS monomers, the positively charged compounds are more attracted to SDS micelles in the mobile phase than to SDS monomers adsorbed on the PVA stationary phase. Thus, the positively charged compounds containing an NH_2 group are less retained as the number of SDS micelles increases.

The retention of compounds which do not contain an NH_2 group were not significantly influenced by the addition of SDS to the mobile phase. However, the retention times of Thy and Thd are reversed in the presence of SDS (Figure 4b). Without SDS, the retention of Thd is longer than that

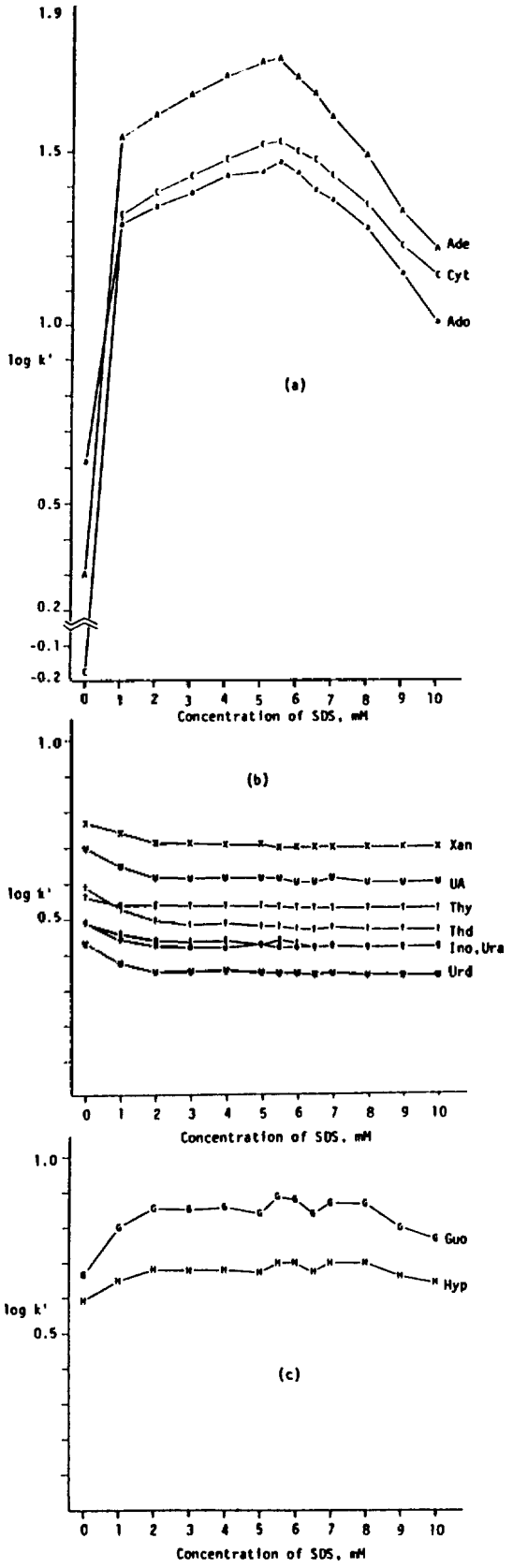

Figure 4. Effect of SDS concentration on the capacity factors of (a) Ado, Cyt and Ade, (b) Xan, UA, Thy, Thd, Ino, Ura and Urd, (c) Hyp and Guo. Mobile phase, aqueous SDS solutions (pH 3.4). Other chromatographic conditions are the same as in Figure 1.

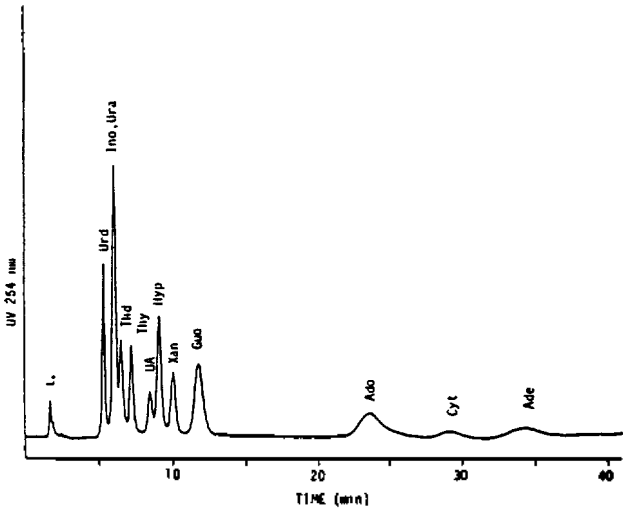

Figure 5. Isocratic elution profiles of a mixture of nucleosides and bases on a PVA column. Column, 9 μm Asahipak GS 320H; mobile phase, 10 mM SDS (pH 3.4). Other Chromatographic conditions are the same as in Figure 1.

of Thy but when SDS is in the mobile phase, Thy has a longer retention time, Thy is more hydrophobic than other pyrimidine bases because it has a methyl group; however, Thy's nucleoside, Thd is more hydrophilic than Thy because of the presence of the ribose. Thus, Thd is eluted earlier than Thy in the presence of SDS.

The retention behavior of Hyp is similar to that of Guo (Figure 4). In the region of lower SDS concentration (<2 mM), the retention times of Hyp an Guo increase as the SDS concentration increases. In the region of higher SDS concentration (>8 mM), the retention times of Hyp and Guo decrease as the SDS concentration increases. At present, we have no explanation for these results.

Effect of Stationary Phase. The retention behavior of nucleosides and bases was investigated with various columns using a micellar mobile phase. Because of the long retention times of Ado, Cyt and Ade at low concentration of SDS, a 10 mM SDS solution was used as the mobile phase.

On the PVA column, all compounds except Ino and Ura were separated and all compounds had good peak shapes except Ado, Cyt and Ade which had long retention times (Figure 5). The silica-based packing and PRP-1 column gave poor retention and resolution of a mixture of nucleosides and bases as can be seen in Figure 6. In addition, for the silica-based C_{18} and the C_8 columns, Ado, Cyt and Ade were not eluted in 50 min.

Ado, Cyt and Ade had lower retention on the silica-bonded cyano and the silica-bonded C_1 column than on the silica-bonded C_{18} and the silica-bonded C_8 columns (Figure 6c, d).

However, compounds containing an NH_2 group were retained longer than other species on bonded phase silica-based columns. This result can also be explained by the electrostatic attraction of positively charged compounds and the negatively charged SDS molecules adsorbed on the stationary phase. As the hydrophobicity of the stationary phase increases, the adsorption of SDS molecules on the stationary

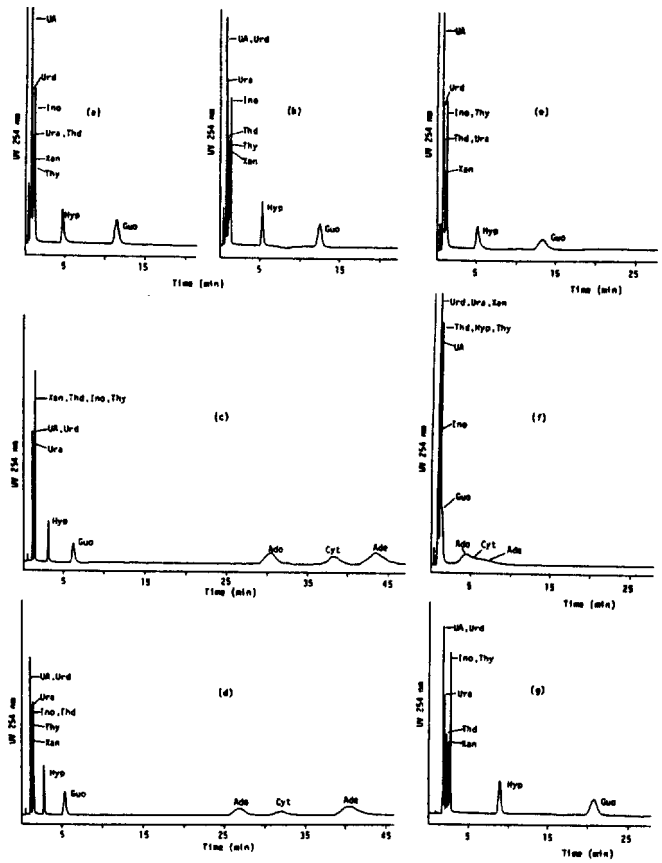

Figure 6. Isocratic elution profiles of a mixture of nucleosides and bases on various silica-based columns. Columns, (a) 5 μm PartiSphere C_{18}, (b) 5 μm PartiSphere C_8, (c) 5 μm Supelcosil LC-CN, (d) 5 μm Sulpelcosil LC-1, (e) 10 μm PRP-1, (f) 5 μm PartiSphere Silica, (g) 10 μm Protesil 300 Octyl. Other chromatographic conditions are the same as in Figure 5.

phase increases. Thus, increased number of negatively charged sites on the stationary phase are available for the positively charged compounds to interact. A similar result was also observed on the polymeric PRP-1 column (Figure 6e).

The retention of compounds containing an NH_2 group on the untreated silica column was much shorter than those on the bonded phase columns (Figure 6f). This result indicates that the SDS molecules are rarely adsorbed on the silica surface. A similar result was reported by Berthod *et al.*[16].

Similar trends in the retention behavior of Hyp and Guo were observed with all bonded phase silica-based and polymeric columns; however, Hyp and Guo were not separated from the other nucleosides and bases on the untreated silica column.

To determine the effect of pore size, a large pore size C_8 column was tested. The poor resolution obtained (Figure 6g) indicates that the pore size of the column packing is not a significant factor in the micellar separation of nucleosides and bases.

For Ado, Cyt and Ade, the retention on the silica-bonded C_1 column was similar to that on the PVA column. Thus, the retention characteristics of the PVA column is closer to this column than to any of the other columns studied.

Conclusions

Good separation of a mixture of nucleosides and bases using micellar liquid chromatography, was obtained only when a polyvinyl alcohol column was used. The micellar mobile phase made possible the separation of the majority of nucleosides and bases tested but affected mainly the nucleosides and bases containing an NH_2 group on the ring of the pyrimidine or in the 6 position of the purine. However, with bonded phase silica-based and other polymeric columns, a micellar mobile phase can be used to quantify Hyp and Guo concentrations in biological samples. These compounds which are important in metabolic studies, cannot be readily quantified when using reversed-phase or ion exchange liquid chromatographic methods because they cannot be separated clearly from other components in serum. Thus, micellar liquid chromatography can be a useful technique in studies of normal metabolism and disease processes.

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The Steric Repulsion Properties of Polyethylene Oxide

Sang Il Jeon* and Joseph D. Andrade†

**Department of Chemistry, Kangnung National University, Kangnung 210-702*
†*Departments of Bioengineering and Materials Science, University of Utah, Salt Lake City, Utah 84112, U.S.A.*
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Polyethylene oxide (PEO) surfaces are recognized as having an effective steric stabilization character. A theoretical scaling analysis involves the osmotic and elastic coefficients of the polymer as a function of molecular weight, in a good solvent. The calculated results show that PEO in water may exhibit the greatest flexibility among water soluble polymers, probably due to its lowest elastic contribution.

Introduction

Polymers adsorbed on solid surfaces immersed in a liquid medium are considerably protected against aggregation, a phenomenon termed steric stabilization. There exist long-range repulsion forces between two surfaces bearing such adsorbed layers, and these repulsive forces overcome the attractive van der Waals forces acting between the bare surfaces.

Polyethylene oxide (PEO) adsorbed surfaces are recognized as effective in minimizing protein adsorption[1-5], probably due to a steric stabilization effect[6,7]. Direct force measurements[8-10] between two adsorbed PEO surfaces onto mica in a good aqueous 0.1 M KNO_3 solvent by the Israelachvili force method[11] show that the repulsion forces develop at certain separation distances due to the steric repulsion phenomenon.

A scaling model of chains adsorbed onto a surface in a good solvent was proposed by Alexander[12] and further extended by de Gennes[13] to give a form for the sterc repulsion force profile. The force is analyzed in terms of a repulsive osmotic term, which comes from the increased polymer concentration in the intersurface gap as the surfaces approach, and an elastic term in which the reduction in free energy, on compression of the over-extended chains, is taken into account. The Alexander-de Gennes model has been developed into a theory of the forces between two such adsorbed layers by Patel *et al*[14]. Their result is that the force *vs* separation distance between two adsorbed surfaces can be expressed as a universal dimensionless function which contains two unknown proportionality constants resulting from the osmotic and elastic contributions.

In this paper, the more effective character of PEO for protein-resistant surfaces was studied by comparison of the osmotic and elastic coefficients of PEO of several molecular weights in good aqueous electrolyte and toluene solvents. The osmotic and elastic coefficients of PEO in aqueous electrolyte and toluene solvents were estimated by the universal curve-fitting method of Patel *et al.*[14] adsorbed on mica surfaces in 0.1 M aqueous KNO_3[8,9,15,16] and toluene solvents[10,16,17], using a least-squares curve fitting method. The data for polystyrene (PS) adsorbed on mica surface in toluene[7,18] is also