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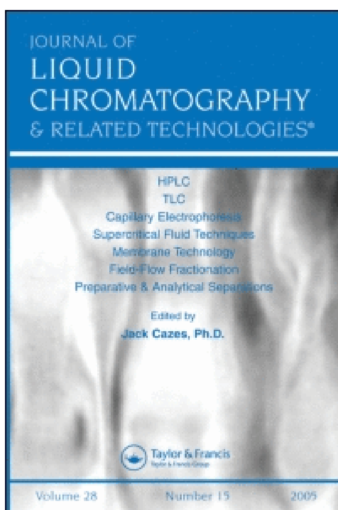

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HIGH-SPEED GEL FILTRATION OF POLYPEPTIDES IN SOME DENATURANTS

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ABSTRACT

The separation and analysis of proteins and polypeptides by use of a silica-based gel packing, G3000SW, for high-speed gel filtration are investigated. The peaks of bovine serum albumin, pepsin, trypsinogen, myoglobin and cytochrome c were completely separated in the presence of 0.2% SDS and 0.2 M sodium phosphate buffer (pH 7.0). The elution positions of native proteins, polypeptides in 8 M urea and polypeptide-SDS complexes were influenced by the concentration of sodium phosphate in eluents, though those of polypeptides in 6 M guanidine hydrochloride were little. These facts suggest the presence of the electrostatic interactions between negatively charged gel surfaces of the packing and polypeptides. Taking into account of the interactions, it is shown that the high-speed gel filtration by use of this column is available to the rapid estimation of molecular weight of polypeptides in both systems of SDS and 6 M guanidine hydrochloride.

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INTRODUCTION

In recent year, high-speed liquid chromatography has been frequently applied for the separation of proteins and polypeptides. In particular, the appearance of porous glass and silica has advanced the application of high-speed gel filtration to the field of protein separation. The packing, G3000SW, for high-speed gel filtration developed by Toyo Soda Manufacturing Company (Tokyo) is a particulate silica gel covalently bonded with hydrophilic compounds and its utility for the separation of proteins has been appreciated by some authors (1-6). In previous paper, we

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reported on the separation of polypeptide-SDS complexes by use of this column packing (2) and Ui showed also that this packing was applicable to rapid estimation of molecular weight of protein polypeptides in 6 M guanidine hydrochloride (3).

In this paper, we report on the separation of polypeptides in denaturants such as SDS, urea and guanidine hydrochloride. Taking into consideration of the electrostatic interaction between gel surfaces and polypeptides, the molecular weight estimation of polypeptides in some denaturants is also discussed.

MATERIALS

Bovine serum albumin, catalase, aldolase, carboxypeptidase A, chymotrypsinogen A, trypsinogen, α-chymotrypsin, trypsin, β-lactoglobulin, myoglobin, ribonuclease A, trypsin inhibitor (lima bean), glucagon and bacitracin were obtained from Sigma; ovomucoid, cytochrome c, aprotinin and insulin B and A chains (S-carboxymethylated) from Boehlinger Mannheim; ovalbumin from ICN Pharmaceuticals and pepsin from Nutrition Biochemicals. Guanidine hydrochloride and urea were purchased as specially prepared reagents from Nakarai Chemicals (Kyoto) and sodium dodecyl sulfate (SDS) was from Wako Chemicals (Osaka) as a reagent for biochemical research. These reagents were used without further purification. All other chemicals were of pure grade.

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METHODS

Preparation of Cyanogen Bromide Fragments

Some polypeptides and oligopeptides were prepared by cyanogen bromide (CNBr) treatments of bovine serum albumin, aldolase, carboxypeptidase A, pepsin, chymotrypsinogen A, α-chymotrypsin, trypsin, myoglobin, lysozyme and cytochrome c. Each protein dissolved in 70% formic acid to a concentration of 10 mg/ml was reacted with 50 molar quantities of CNBr for 20 hours at room temperature, then 10 volumes of distilled water was added and the solution was lyophilized. The values of molecular weight of CNBr fragments are listed in TABLE.

TABLE
Cyanogen Bromide Fragments Used in Gel Filtration Experiments

CNBr fragment		Molecular weight	Reference
Serum albumin	CNBr I	9,860	(7)
	CNBr III	29,520	
Aldolase	CNBr I	17,710	(7)
	CNBr II	7,370	
	CNBr III	2,040	
	CNBr IV	11,900	
Carboxypeptidase A	CNBr I	2,660	(7)
	CNBr II	9,230	
	CNBr III	22,120	
Pepsin	CNBr I	8,630	(7)
	CNBr II	12,640	
Chymotrypsinogen A	CNBr I	19,180	(8)
	CNBr II	1,060	
	CNBr III	5,560	
Trypsinogen	CNBr I	9,900	(8)
α-Chymotrypsin	B chain	13,920	(8)
	CNBr I	3,580	
	CNBr III	5,560	
Trypsin	CNBr I	9,100	(8)
Myoglobin	CNBr I	6,230	(7)
	CNBr III	2,510	
Lysozyme	CNBr I	1,270	(7)
	CNBr II	10,160	
	CNBr III	2,860	
Cytochrome c	CNBr I	7,030	(7)
	CNBr II	1,780	
	CNBr III	2,780	

CNBr fragments were prepared as described in METHODS. Each fragment was numbered in the order of amino acid sequence of the protein from N-terminal. Only the fragments which observed as chromatographically distinguishable peaks were listed. The values of molecular weight of fragments were calculated from amino acid sequence data of proteins appeared in references.

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Preparation of the Sample Solution for High-Speed Gel Filtration

The samples of proteins, polypeptides and CNBr fragments were dissolved in the buffers described in figure legends at the concentration of 3 to 5 mg/ml. For the chromatographic experiments in denaturants, proteins and polypeptides possessing disulfide bonds were reduced by 0.3% 2-mercaptoethanol. Since no significant differnces in chromatographic behaviours between poly-

peptides treated with 0.3% 2-mercaptoethanol and S-carboxymethylated polypeptides were detected in SDS for some dozen species of proteins, the samples were used without modification of SH groups in later experiments.

High-Speed Gel Filtration

High-speed gel filtration was carried out using a Model HLC 803 A liquid chromatograph (Toyo Soda) connected to a TSK-GEL G3000SW column (7.5 x 600 mm) with a pre-column (7.5 x 75 mm). The samples (20 μl) were injected in the column which was previously equilibrated with each eluent and eluted at the flow rate of 0.5 to 1.0 ml/min. Ultraviolet absorption (apparatus: Schoeffer Spectro Flow Monitor SF770) at 220 or 280 nm was employed for the detection of elution peaks of the samples. The distribution coefficients, K_d, were calculated from the equation of $K_d=(V_e-V_0)/(V_i-V_0)$, where V_e, V_0 and V_i were the elution volumes of the sample, Blue Dextran 2000 and 2-mercaptoethanol, respectively.

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RESULTS

Chromatographic Behaviours of Native Proteins in the Absence of the Denaturants

The values of distribution coefficients, K_d, for some native proteins were plotted against logarithm of sodium phosphate concentrations as shown in Fig. 1. The salt concentration dependence of K_d values was different between acidic and basic proteins. In acidic proteins such as serum albumin, ovalbumin, β-lactoglobulin and insulin A chain, the values of K_d increased with the rise of the concentration of sodium phosphate. Basic proteins such as chymotrypsinogen A, lysozyme and cytochrome c were adsorbed with the resin and symmetries of the elution peaks were lost in low salt concentration conditions of eluents. The K_d value of myoglobin, a neutral protein, was unaltered except in extensively low salt concentration. It is obvious that the low concentration conditions of sodium phosphate below 10 mM are unsuitable for the size separation of proteins. High concentration conditions of

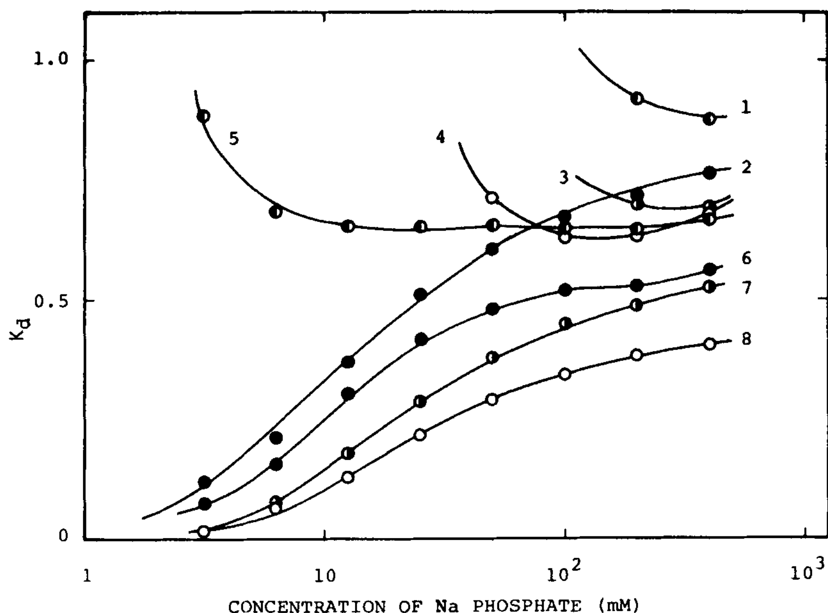

FIGURE 1 Relationships between distribution coefficients, K_d, of native proteins and sodium phosphate concentrations in the eluents. Protein samples (20 μl) dissolved in 0.05 M sodium phosphate buffer (pH 7.0) were charged in the column and eluted under various concentration conditions of sodium phosphate buffer (pH 7.0). The flow rate was 1.0 ml/min. Proteins are: 1, lysozyme; 2, insulin A chain; 3, cytochrome c; 4, chymotrypsinogen A; 5, myoglobin; 6, β-lactoglobulin; 7, ovalbumin; 8, serum albumin.

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sodium phosphate such as 0.2 M may be required for the elution of highly basic proteins such as lysozyme and cytochrome c. However, the concentration conditions above 0.4 M tended to make the elution peaks broadened.

8 M Urea System

Figure 2 shows the relationships between log molecular weight and K_d of polypeptides reduced on disulfide bonds in 8 M urea. The elution condition of 0.2 M sodium phosphate gave a relatively good linearity. The plots for both highly acidic and basic polypeptides, however, slightly deviated from the line and the extent of the deviation was enhanced in lower salt concen-

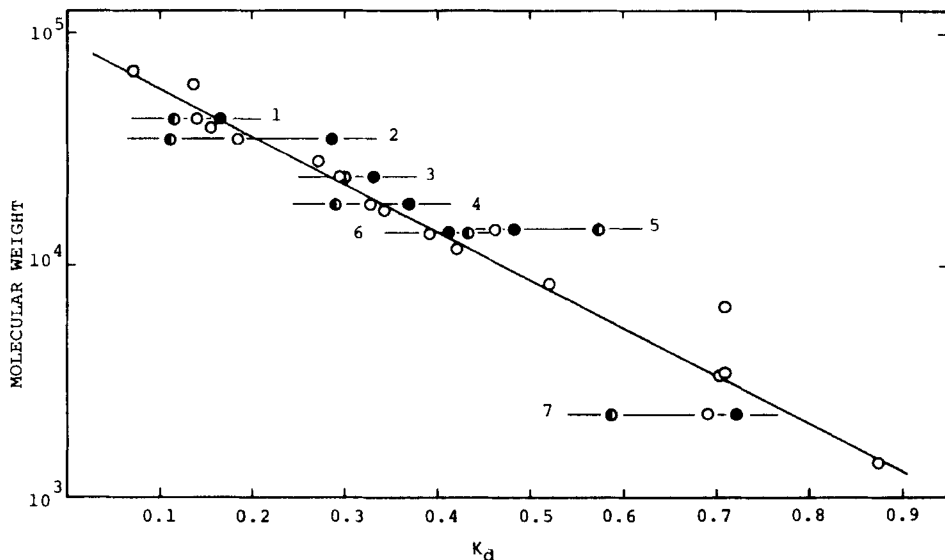

FIGURE 2 Plots for log molecular weight *versus* K_d of polypeptides in 8 M urea at various concentrations of sodium phosphate buffer (pH 7.0). Proteins dissolved in 8 M urea and 0.05 M sodium phosphate buffer (pH 7.0) containing 0.3% 2-mercaptoethanol were charged in the column and eluted at a flow rate of 1.0 ml/min. Proteins are: 1, ovalbumin; 2, pepsin; 3, trypsinogen; 4, β-lactoglobulin; 5, lysozyme; 6, ribonuclease; 7, insulin A chain. The concentrations of sodium phosphate buffer (pH 7.0) in the eluents were 0.05 M (◐), 0.20 M (○) and 0.40 M (●).

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tration. At higher sodium phosphate concentration such as 0.4 M, K_d values were larger than those expected on the basis of the electric properties of polypeptides and the resolution of the peaks was lost.

SDS System

Semi-logarithmic plots of molecular weight and K_d of polypeptides in 0.1% SDS are shown in Fig. 3. The plots of log molecular weight *versus* K_d of polypeptides were significantly influenced by the concentration of sodium phosphate as previously described (2) and there was the difference in the manner of the influence of salt concentration between polypeptides having the molecular weight values higher than about 15,000 daltons and

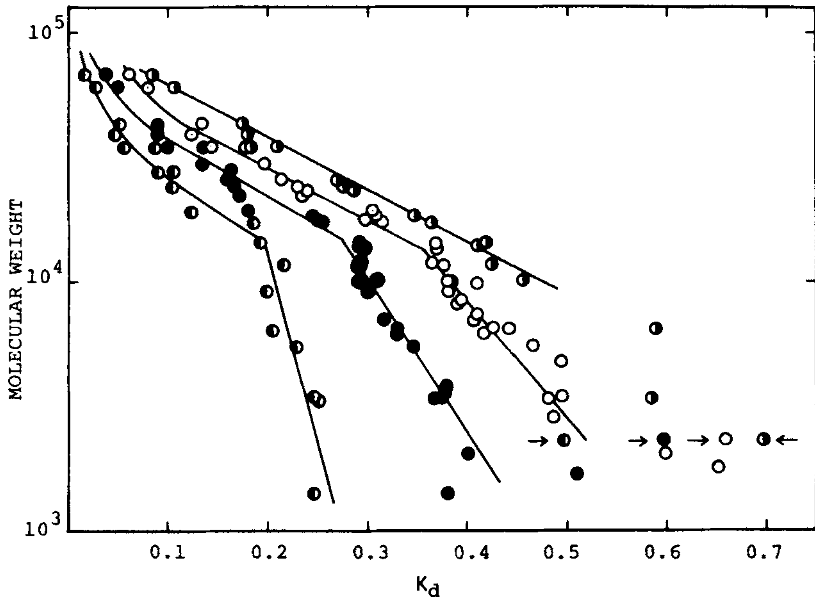

FIGURE 3 Sodium phosphate concentration dependence of the plots of log molecular weight *versus* K_d of polypeptides in SDS. The samples of proteins and polypeptides dissolved in 2% SDS and 0.05 M sodium phosphate buffer (pH 7.0) containing 0.3% 2-mercaptoethanol and incubated at 60°C for 1 hr were charged in the column and eluted at a flow rate of 1.0 ml/min. Sodium phosphate concentrations in the eluents illustrated were 0.025 M (◐), 0.05 M (●), 0.10 M (○) and 0.20 M (◑). The solid arrows represent the data for the sample of insulin A chain (S-carboxymethylated) which is probably a highly acidic oligopeptide. Only the points for insulin A chain were extremely deviated from the curves in relatively low sodium phosphate concentration.

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those lower than about 15,000 daltons, *i.e.*, oligopeptides. Detailed examination for many speacies of polypeptides showed that the slopes of the plots did not change with the variation of salt concentration but only the values of K_d increased with the rise of the salt concentration. The increments of K_d values were practically suppressed at about 0.2 M and higher sodium phosphate concentration gave broadened peaks.

In oligopeptides, the slopes of the plots were steeper than those for polypeptides in low salt concentration. Thus the plots

had an inflection around about 15,000 daltons. The magnitudes of the slopes for oligopeptides approached those for polypeptides with the rise of sodium phosphate concentration. For oligopeptides, the concentration condition of 0.2 M sodium phosphate, however, gave broadened peaks and linearity of the plots was lost. In 0.2 M sodium phosphate, a higher SDS concentration than 0.1% was required for the maintenance of the linearity of the plots. The concentration condition of 0.2 M sodium phosphate containing 0.2% SDS was selected for the preparation of a calibulation curve. The plot of log molecular weight *versus* K_d of different fortyfive polypeptides and oligopeptides is shown in Fig. 4. The plot gave a good linearity, particulary for polypeptides and an

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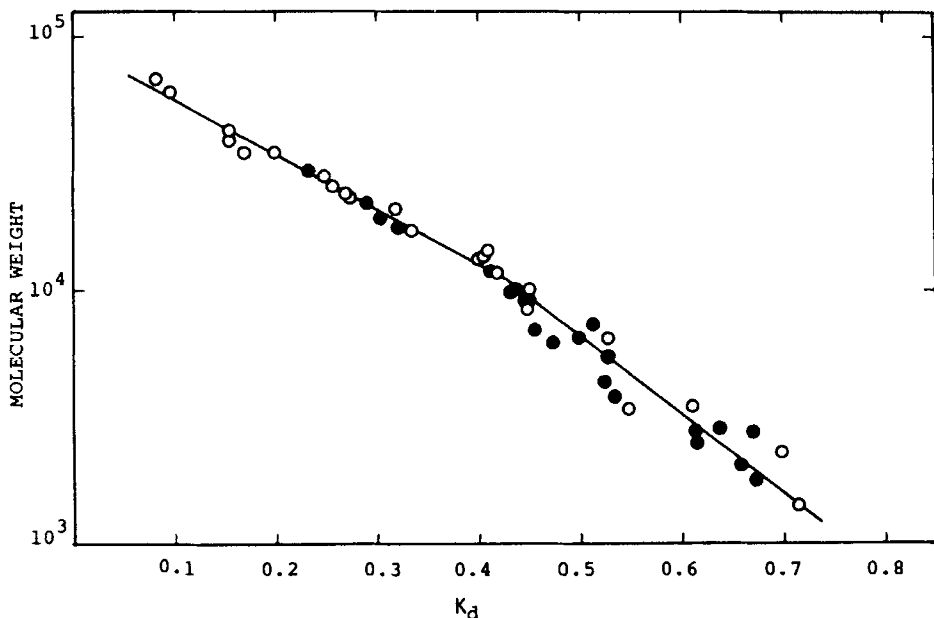

FIGURE 4 Plot of log molecular weight *versus* K_d of forty-five polypeptides in 0.2% SDS and 0.2 M sodium phosphate buffer (pH 7.0). The polypeptide samples were incubated and charged in a similar manner as described in Fig. 3. Filled circles represent the data for CNBr fragments.

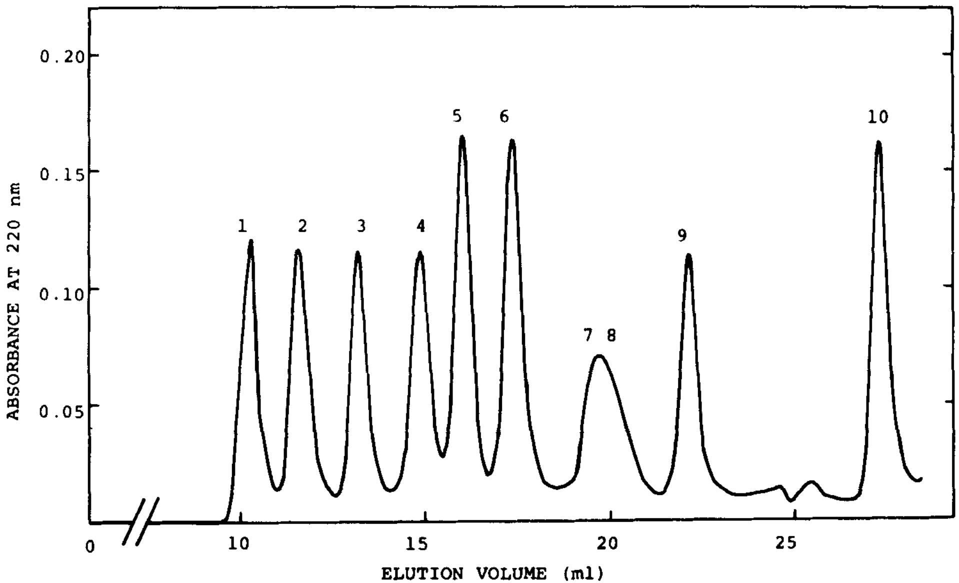

FIGURE 5 An elution profile of a mixture of polypeptides and oligopeptides from a G3000SW column. The elution was carried out in 0.2% SDS and 0.2 M sodium phosphate buffer (pH 7.0) and at a flow rate of 0.5 ml/min. Peaks are: 1, blue dextran; 2, serum albumin; 3, pepsin; 4, trypsinogen; 5, myoglobin; 6, cytochrome c; 7, aprotinin; 8, insulin B chain; 9, insulin A chain; 10, 2-mercaptoethanol. Aprotinin and insulin B chain were eluted as undistinguishable peaks under this condition. However, the peaks of both oligopeptides were completely separated under the condition of 0.1% SDS and 0.1 M sodium phosphate buffer (pH 7.0).

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inflection on the curve appeared around 15,000 daltons was moderated.

The examples for the separation of a mixture of polypeptides and oligopeptides and CNBr cleavage products from rabbit muscle aldolase under the elution condition of 0.2 M sodium phosphate (pH 7.0) containing 0.2% SDS are shown in Fig. 5 and 6, respectively. The peaks of serum albumin, pepsin, trypsinogen, myoglobin, cytochrome c and insulin A chain were completely separated. Four fragments produced by CNBr treatment of aldolase were ob-

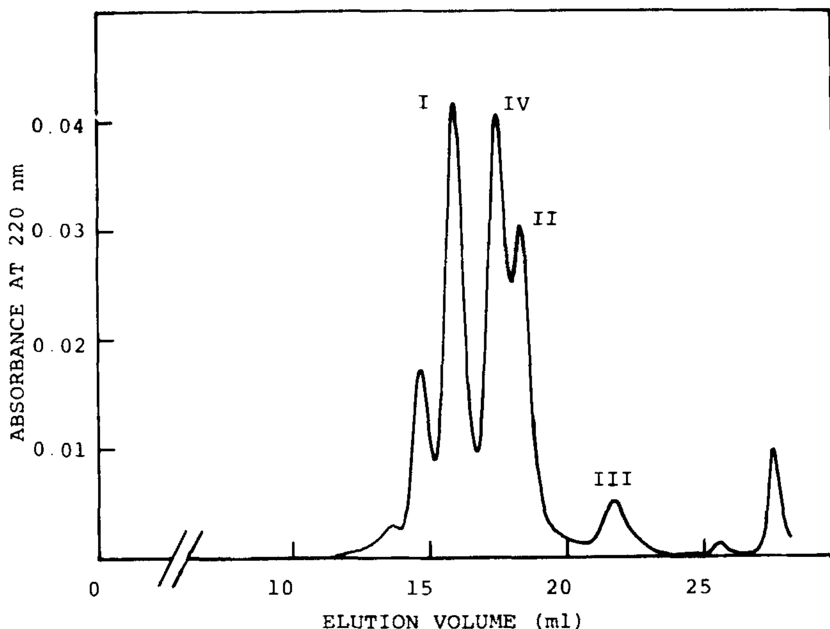

FIGURE 6 An elution profile of CNBr fragments from rabbit muscle aldolase in 0.2% SDS and 0.2 M sodium phosphate buffer (pH 7.0). The CNBr fragment sample was treated and charged in a similar manner as described in Fig. 3. The flow rate of elution was 1.0 ml/min. The values of molecular weight of CNBr fragments, I, II, III and IV are 17,710, 7,370, 2,040 and 11,900, respectively.

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served as distinguishable peaks and the peak of CNBr III was completely separated from other fragments.

6 M Guanidine Hydrochloride System

Figure 7 shows the relationships between log molecular weight and K_d of polypepetides and oligopeptides in 6 M guanidine hydrochloride and 0.05 M sodium phosphate buffer (pH 7.0). A linearity was kept in the molecular weight range of 2,000 to 70,000 daltons. It was the important characteristic of 6 M guanidine hydrochloride system that the K_d values of polypeptides were little influenced by the concentration of sodium phosphate.

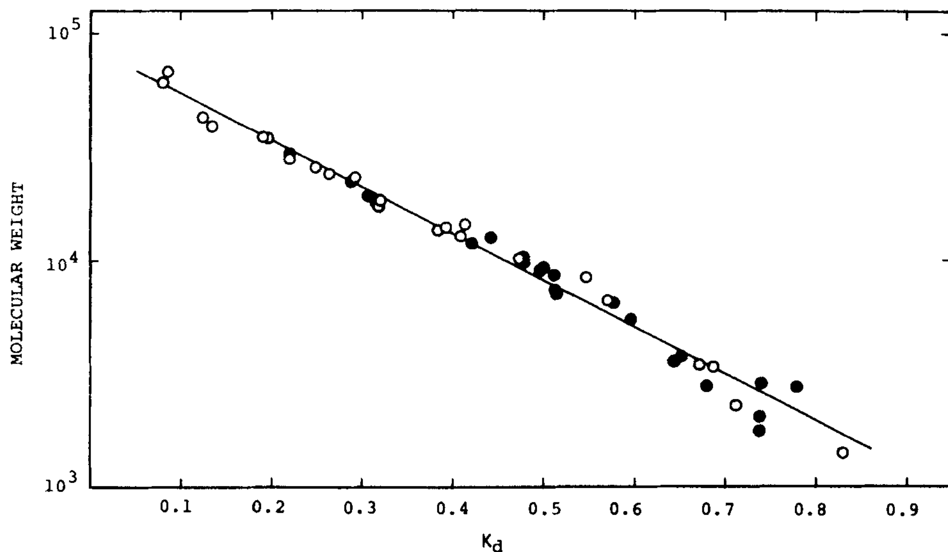

FIGURE 7 Plot of log molecular weight *versus* K_d of forty-five polypeptides and oligopeptides in 6 M guanidine hydrochloride and 0.05 M sodium phosphate buffer (pH 7.0). Charged samples were treated with 6 M guanidine hydrochloride and 0.05 M sodium phosphate buffer (pH 7.0) containing 0.3% 2-mercaptoethanol. The flow rate was 1.0 ml/min. Filled circles represent the data for CNBr fragments.

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DISCUSSION

The chromatographic behaviours of native proteins in G3000SW column were influenced by the variation of sodium phosphate concentration. The K_d values of acidic proteins increased with the rise of sodium phosphate concentration. The basic proteins were adsorbed on gel surfaces in low sodium phosphate concentration. The polypeptides in 8 M urea were also similar in chromatographic behaviours to native proteins, but the effects of sodium phosphate concentrations were not so drastic as those to native proteins. The polypeptide-SDS complexes which had highly negative charges behaved in a similar manner as acidic proteins. The K_d values of polypeptides in 6 M guanidine hydrochloride, however, were little affected by the variation of sodium phosphate con-

centration. It seems that these phenomena are attributable to the interaction between polypeptides and negatively charged gel surfaces. That is to say, the electric diffuse double layers on gel surfaces and polypeptides will be compressed by high salt concentration in eluents and acidic polypeptides and polypeptide-SDS complexes may be consequently capable to penetrate into gel interior. The elution positions of basic polypeptides may be retarded by attractive interaction between positively charged polypeptides and gel surfaces. High concentration of electrolytes such as 6 M guanidine hydrochloride may virtually eliminate the electrostatic interaction between polypeptides and gel surfaces. However, high concentration of sodium phosphate above 0.4 M will not be recommended because it was observed for native proteins, polypeptides in 8 M urea and polypeptide-SDS complexes that such a high sodium phosphate concentration resulted in low resolution owing to the broadening of the peaks.

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In order to systematically consider the chromatographic behaviours of native proteins and polypeptides in some denaturants, the G3000SW column may be necessary to be calibulated with the method based on the K_d values and Stokes radii of proteins and polypeptides. The method proposed by Ackers was used for this purpose (9-11). The relationships between Stokes radii, R_s, calculated from intrinsic viscosity data and $Erf^{-1}(1-K_d)$ of some proteins and polypeptides in various eluents are shown in Fig. 8. The concentration condition of 0.2 M sodium phosphate (pH 7.0) which was suitable for the separation of proteins and polypeptides was selected for the calibulation in spite of the presence or absence of the denaturants except for 6 M guanidine hydrochloride. The buffer concentration of 0.05 M sodium phosphate was used only for data in 6 M guanidine hydrochloride, since the elution positions of polypeptides in 6 M guanidine hydrochloride were independent on the sodium phosphate concentration. The plots for some native proteins and for polypeptides in 8 M urea and 6 M guanidine hydrochloride followed a common curve except for native lysozyme. The position of native lysozyme was deviated upward from

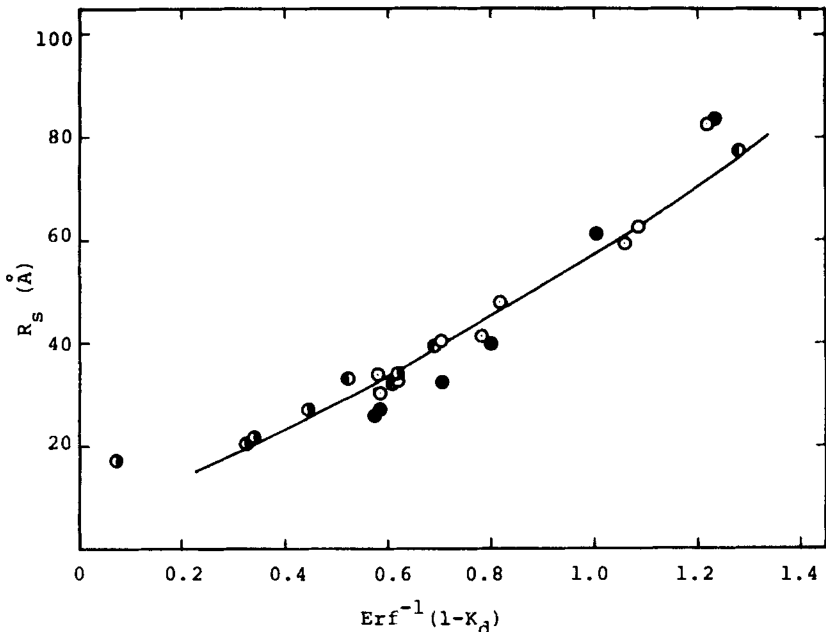

FIGURE 8 The relationships between R_s and $Erf^{-1}(1-K_d)$ of some polypeptides in various eluents. The eluents were: 0.20 M sodium phosphate (◑); 6 M guanidine hydrochloride and 0.05 M sodium phosphate (○); 8 M urea and 0.20 M sodium phosphate (◐); 0.2% SDS and 0.20 M sodium phosphate (●). The values of Stokes radii, R_s, were calculated from the intrinsic viscosity data according to the equation $[\eta]=2.5L/M(4\pi R_s^3/3)$, where L was Avogadro's constant, M was the molecular weight of polypeptide and $[\eta]$ was the intrinsic viscosity (12-14).

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the curve. This fact may be due to the attractive interaction of positively charged lysozyme with gel surfaces. The plots for some polypeptide-SDS complexes which had highly negative charges substantially followed the common curve. The repulsive interaction between negatively charged polypeptide-SDS complexes and gel surfaces may be taken as negligible small in 0.2 M sodium phosphate.

When this column is tried appling for the estimation of molecular weight of polypeptides, it is necessary to be taken into account of the interaction between polypeptides and gel surfaces. There may be two ways available for this purpose; one is to make

the interaction negligible small with high electrolyte concentration such as 6 M guanidine hydrochloride, the other is to bury the intrinsic charges of the polypeptides by formation of polypeptide-detergent complexes. It had been shown with Ui that the former method was available for the rapid estimation of molecular weight of polypeptides (3). Our results examined for fortyfive polypeptides and oligopeptides also showed that the relationship between log molecular weight and K_d was linear in the range of about 2,000 to 70,000 daltons. For the latter method, the use of cationic detergent, cetyltrimethyl ammonium bromide, resulted in extremely poor resolution of the peaks. Therefore, an anionic detergent, SDS was selected. Under the condition of 0.2 M sodium phosphate buffer (pH 7.0) containing 0.2% SDS, the relationship between log molecular weight and K_d of polypeptides exhibited a good linearity in a molecular weight range of 15,000 to 70,000 daltons and the resolution of elution peaks of polypeptide-SDS complexes was satisfactory (Fig. 5). For oligopeptides less than about 15,000 daltons, on the other hand, the elution profiles and positions were affected by SDS concentration and the properties of oligopeptide-SDS complexes are so far unclear. Therefore, the application of G3000SW column to the molecular weight estimation of oligopeptides in SDS system may be accompanied by some dangers, though the plots of log molecular weight *versus* K_d of oligopeptides showed a rough linearity in the range of 2,000 to 15,000 daltons. For the molecular weight estimation of oligopeptides less than 15,000 daltons, 6 M guanidine hydrochloride system may be rather recommended.

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