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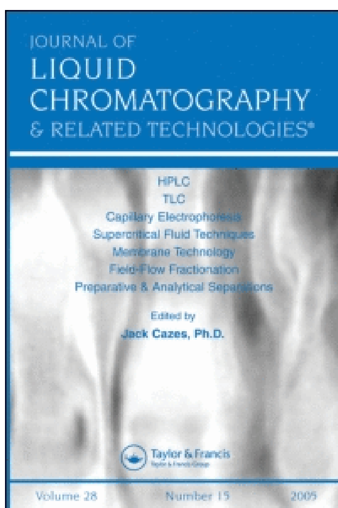

Journal of Liquid Chromatography & Related Technologies
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To cite this Article Mori, Sadao and Kato, Masatoshi(1987) 'High-Performance Aqueous Size-Exclusion Chromatography Using Diol-Bonded Porous Glass Packing Materials. Retention Behavior of Some Proteins', Journal of Liquid Chromatography & Related Technologies, 10: 14, 3113 — 3126

To link to this Article: DOI: 10.1080/01483918708068301

URL: http://dx.doi.org/10.1080/01483918708068301

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JOURNAL OF LIQUID CHROMATOGRAPHY, 10(14), 3113-3126 (1987)

HIGH-PERFORMANCE AQUEOUS SIZE-EXCLUSION CHROMATOGRAPHY USING DIOL-BONDED POROUS GLASS PACKING MATERIALS. RETENTION BEHAVIOR OF SOME PROTEINS

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ABSTRACT

Retention volume of proteins increased or decreased with increasing phosphate buffer or neutral electrolyte concentrations in the mobile phase. This variation suppressed or accelerated by changing pH values in the mobile phase. The behavior of proteins can be interpreted by knowing isoelectric points (pI) of proteins and pKa value of the residual silanol groups on the surface of diol-bonded porous glasses. Positively charged surface of proteins below pH 8.0 (cytochrome c, lysozyme) retarded the elution by the ion-adsorption effects and negatively charged proteins around pH 7.0 (egg albumin, bovin serum albumin) eluted earlier than expected by the ion-exclusion effects. These effects suppressed by increasing phosphate buffer and neutral electrolyte concentrations in the mobile phase. Size-exclusion separation was attained in the mobile phase over 0.1 M phosphates and 0.1 M NaCl concentrations at pH 7.0. McIlvaine buffer and Gomori buffer showed opposite action to proteins for retention comparing with Soerensen phosphate buffer. Potassium thiocyanate showed the different action for retention of proteins comparing with other neutral electrolytes and acted like sodium dodecyl sulphonate.

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INTRODUCTION

Hydrophilic polymer gels and hydrophilic group-bonded silica gels are currently available as packing materials for aqueous size exclusion chromatography(ASEC) of proteins and ionic water soluble synthetic polymers. Mobile phases used for the protein separation are mostly phosphate buffer solutions and the examples of the concentrations and the compositions of the buffer solutions appeared in the literature were as follows: 0.1 M potassium phosphate, monobasic (KH_2PO_4) (pH 6) on glycophase G-CPG (1), 0.067 M KH_2PO_4 (pH 6.8) + 0.1 M NaCl on TSK-GEL SW (2,3), 0.008 M potassium phosphate, dibasic (K_2HPO_4) + 0.042 M sodium phosphate, dibasic (Na_2HPO_4) (pH 7.5) + 0.1 M NaCl on LiChrosorb DIOL (4). These mobile phases were not always suitable for all the types of protein separations, because some secondary interactions between proteins and packing materials, i.e., adsorption effects caused by residual silanol groups on the surface of the diol-bonded porous glass packing materials, may not diminish for some types of proteins and when plotting the values of the retention volume (V_R) obtained on log MW vs. V_R diagram, a large scattering was observed.

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Schmidt et al.(5) used a mixed solution of sodium acetate and sodium sulfate at pH 5.0 as the mobile phase on LiChrosorb DIOL packing materials and examined the variation of the retention volume of representative proteins as a function of ionic strength of the mobile phase. They found that the residual ionic charge on the surface of LiChrosorb DIOL influenced the retention of proteins below an ionic strength of 0.20 M and the ionic interactions were neutralized above 0.2 M, but some proteins exhibited hydrophobic interaction. Pfannkoch et al. (6) studied elution behavior with several commercially available SEC columns and found that under certain conditions they exhibited ion exclusion, cation exchange, and hydrophobic partitioning. They mentioned that these nonpermeation effects were minimized by using mobile phases with ionic strengths between 0.1 and 0.60M at pH 7.05. Roumeliotis and Unger (7) discussed the effects of pH, ionic type,

ionic strength of the mobile phase on the retention volume of proteins.

The effect of changing electrolyte concentration in the mobile phase on the retention volume of several proteins in SEC has been studied extensinvely by several workers. Selection of the mobile phase having appropriate ionic strength seems to be very important for better SEC. Therefore, observation of retention behavior of several proteins at different concentration of mobile phases will be required on different SEC columns. Studies on the effect of changing the types of buffer or neutral salt are also necessary.

In the previous paper (8), high performance ASEC with diol - bonded porous glass packing materials was reported and the packing procedure and the construction of calibration graphs were discussed. This paper is concerned with the observation of the effects of the concentration, the type and pH of buffer solutions used as the mobile phase and the concentration of added neutral electrolytes on the retention volume. Elucidation of the various contributions from non-size-exclusion effects to the retention volume of proteins was attempted.

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EXPERIMENTAL

The porous glass materials as in the previous pape (8) were FPG 170 and 500 (Fuji Photo Film Co.), which had average pore diameters of 170 Å and 500 Å, respectively. The average particle diameter was 5 um and the surface of the porous glass was covered with glyceropropyl groups. These materials were packed in stainless-steel tubes of 7.2 mm i.d. x 25 cm length and connected in series. The number of theoretical plates (N) was 13000 plates per column (25 cm) by injecting 25 µl of a 1% ethylene glycol solution at a flow rate of 0.5 ml/min.

SEC measurements were performed on a Jasco TRIROTAR high - performance liquid chromatograph (Japan Spectroscopic Co., Tokyo,

TABLE I

Proteis Used In This Study

Sample number	Protein	Molecular weight	pI
1	Cytochrome c	13000	10.6*
2	Lysozyme (egg white)	14400	10.5**,11.0*
3	Myoglobin	17000	7.1*
4	Trypsin	23000	10.4 - 10.8*
5	Egg Albumin	45000	4.6*
6	Bovin Serum Albumin	69000	4.4 - 4.8*,5.1**
7	Human Serum -Globulin	160000	6.3 - 8.4

* Ref. 5

** Ref. 4

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Japan). A UVIDEC-100 ultraviolet absorption detector (UV) (Jasco) was operated at a wavelength of 280 nm. A sample solution was injected with a variable loop injector Model VL-611.

Sample proteins studied here were listed in Table I with molecular weight and isoelectric point (pI). Samples No. 2 and 3 were obtained from Nakarai Chemical Co., Japan, samples No. 1, 4, and 7 from Miles Laboratories Ltd., USA, and samples No. 5 and 6 from Seikagaku Kogyo Co., Japan. These samples were dissolved in the mobile phase in the concentration of 0.1% and 25 ul of the solutions were injected.

Mobile phases used in this study were aqueous solutions of mixtures of Na_2HPO_4, KH_2PO_4 and NaCl at several concentrations and different pH values. Besides these Soerensen phosphate buffer solutions, two different buffer solutions, Mcllvain buffer(0.2M Na_2HPO_4 + 0.1 M $C_6H_8O_7$ + 0.2 M NaCl (pH 7.0)) and Gomori buffer(0.2 M $NH_2C(CH_3OH)_3$ + 0.1 N HCl + 0.2 M NaCl (pH 7.0)), were

also tested. Besides NaCl added as neutral salt, KCl and KSCN were also used for cheking the elution behavior of proteins due to different cations and anions. A flow rate of the mobile phase was 0.5 ml/min in all experiments.

RESULTS AND DISCUSSION

Elution profiles of cytochrome c

SEC chromatograms of cytochrome c obtained in the mobile phases of phosphate buffers plus NaCl at different concentrations of the buffers and NaCl are shown in Figures 1 and 2. In Figure 1, concentrations of phosphates were between 0.01 and 0.4 M and the concentration of NaCl was constant at 0.2 M in all cases. The value of pH was also constant at 7.0. Cytochrome c eluted at the total permeation limit (V_R = 15.5 ml) and the shape of the peak was sharp when the concentration of phosphates in the mobile phase was between 0.1 and 0.4 M. The retention volume V_R increased and the peak became broad with decreasing the concentration of phosphates in the mobile phase below 0.1 M. Broadening of the peak and the increase of retention volume may be the proof of the secondary interactions such as the adsorption effects between proteins and the surface of porous glass. Though the porous glass used in this study was covered with glyceropropyl groups, some residual silanol groups on the surface of the porous glass packing materials may give rise to adsorption effects (4). These adsorption effects increased with decreasing the concentration of phosphates in the mobile phase.

Similar effects have been obtained with changing the concentration of NaCl in the mobile phase as shown in Figure 2. Concentration of phosphates was 0.1 M and the pH value was 7.0. Size exclusion separation was attained over 0.2 M concentration of NaCl. Peaks of cytochrome c became broad and the retention volume increased below 0.1 M NaCl, showing adsorption effects which increased with decreasing the concentration of NaCl.

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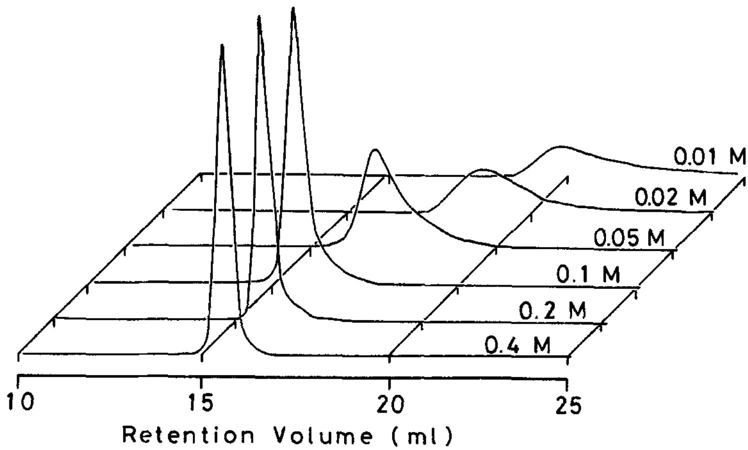

FIGURE 1. Size-exclusion chromatograms of cytochrome c obtadined in the different concentrations of phosphate buffer used as the mobile phase. Mobile phase: 0.01 M - 0.4 M (Na_2HPO_4 + KH_2PO_4) + 0.2 M NaCl at pH 7.0.

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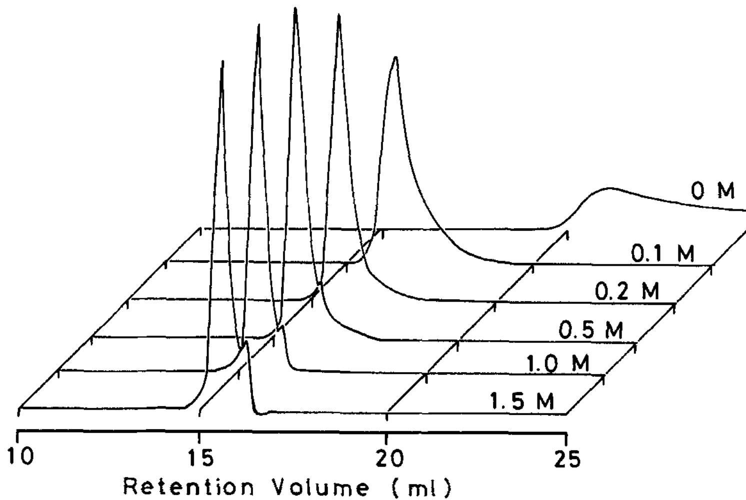

FIGURE 2. Size exclusion chromatograms of cytochrome c obtained in the different concentrations of NaCl in the mobile phase of phosphates. Mobile phase: 0.1 M (Na_2HPO_4 + KH_2PO_4) + 0 - 1.5 M NaCl at pH 7.0.

Considering the elution profiles in Figures 1 and 2, it can be said that preferable concentration of the mobile phase was over 0.1 M phosphates and 0.2 M NaCl.

<u>Retention volume vs. concentration of phosphates or NaCl in the mobile phase</u>

Figure 3 shows the difference in the retention volume of several proteins with changing the concentration of phosphates in the mobile phase (pH 7.0 and 0.2 M NaCl) as in Figure 1. They can be divided into three groups: the first group is a group of which retention volume decreased with increasing the concentration of phosphates (samples No. 1, 2, 4, and 7), the second is independent on the concentration of phosphates (sample No. 3), and the third is a group where the retention volume increased with increasing the concentration of phosphates (samples No. 5 and 6).

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Similar experiment was made with phosphate buffers of various pH ranging from 4.0 to 8.0 and constant NaCl concentration. Figure 4 summarizes the retention behavior as a function of the concentration of phosphates at different pH values for samples No. 1, 3, 5, and 7. Retention volume of cytochrome c (sample No. 1) decreased with increasing the concentration of phosphates and the variation of retention volume was greater at lower concentrations of phosphates in the mobile phase and at higher pH values as 6 and 7. The change in retention volume of myoglobin (sample No. 3) with the concentration of phosphates was small or negligible over 0.05 M except at pH 8.0. Variation of retention volume of egg albumin (sample No. 5) with the concentration of phosphates was smaller at lower pH values. Retention volume of egg albumin increased and that of human serum -globulin (sample No. 7) decreased with increasing the concentration of phosphates in the mobile phase except in the case of sample No. 7 at pH 8.0.

These behavior is easy to understand if isoelectric points of proteins and pKa value (= 7.0 ± 0.5) (4) of the residual silanol groups on the surface of diol-bonded porous glass packing materials are taken into condiseration (8). Residual silanol groups

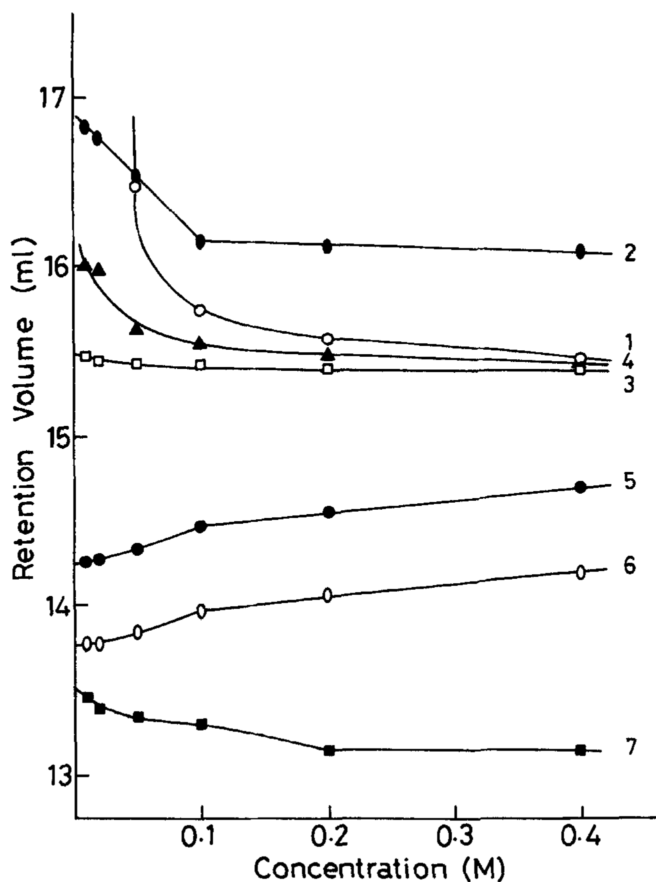

FIGURE 3. Variation of retention volume of proteins as a function of concentration of phosphates in the mobile phase. Other conditions as same as in Figure 1. Figures in the graph correspond to those in Table I.

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are mostly dissociated over pH 8.0 (negatively charged surface) and the dissociation was mostly suppressed below pH 5.0. Cytochrome c is supposed to be positively charged below pH 8.0 and ion adsorption of cytochrome c with the dissociated silanol groups on the surface of packing materials might be considered. Therefore, the elution of cytochrome c is retarded by secondary

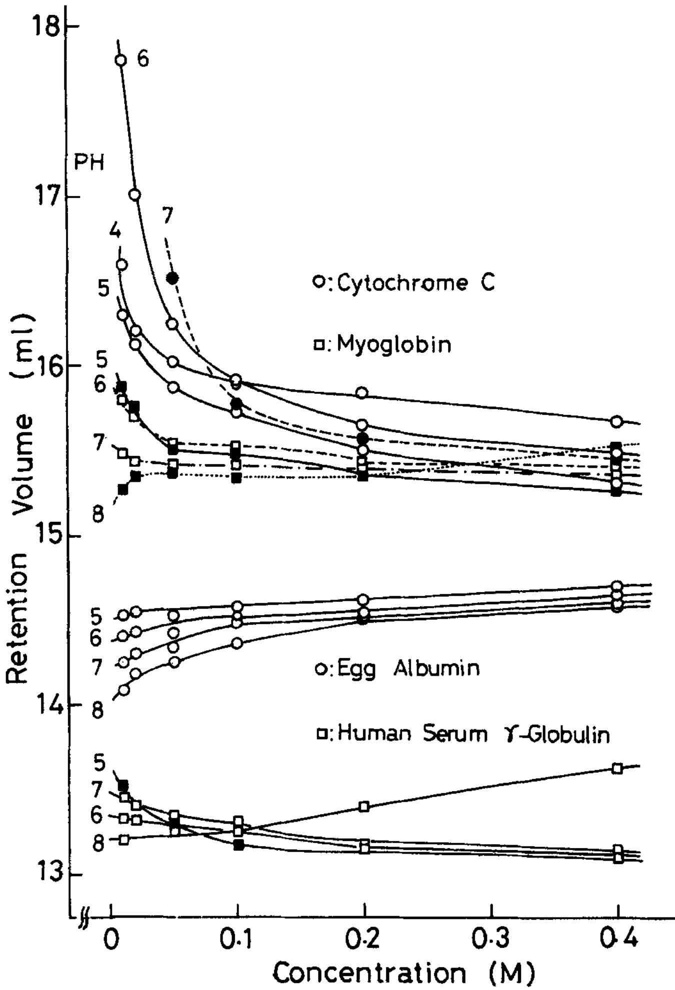

FIGURE 4. Effects of pH for the relationship between retention volume and the concentration of phosphate buffers. 0.2 M NaCl was included.

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effects (probably ionic adsorption) other than size -exclusion effect. As myoglobin is electrically neutralized at pH 7.0, the concentration dependence of the buffer solution is small. However, ion adsorption of positively charged myoglobin with the surface of packing materials retarded the elution at low ionic strength and low pH values, though silanol groups are undissociated at low pH values.

Egg albumin has pI value of 4.6 and is assumed to be negatively charged in the experimental pH range. Ion -exclusion of egg albumin with the packing materials might be considered and this effect is higher at higher pH values. Both ion adsorption and ion exclusion effects are suppressed by the increase of buffer concentration (4) and retention volume may converge to some elution point where proteins are separated mostly by the size-exclusion effect. Elution behavior of myoglobin and human serum γ-globulin at pH 8.0 is hard to understand from this elucidation and from the observation explained in next paragraph.

The effects of addition of a neutral electrolyte such as NaCl in the mobile phase to the retention volume were also examined. The concentration of the buffer solution was fixed to 0.1 M phosphates at pH 7.0 and that of NaCl was vaied from 0 to 1.5 M. The results are shown in Figure 5. Effects of the variation of the NaCl concentration to the retention volume were similar to those of the phosphate concentration. Such neutral electrolyte seems also to have the suppression action to the secondary interactions such as ion adsorption and ion exclusion. Only the difference in the behavior of proteins with changing the concentration of phosphates or NaCl was that the retention volume of most proteins examined decreased consecutively with increasing the concentration of phosphates in the mobile phase and in the case of NaCl, the retention volume attained to the constant values over 0.2M. These experimental results proved that the elution conditions reported previously (9), that was, 0.1 M phosphates + 0.2 M NaCl (pH 7.0), was adequate to have an optimum linear fit of the calibration curve (Figure 6, mark o). The increase of the concentra-

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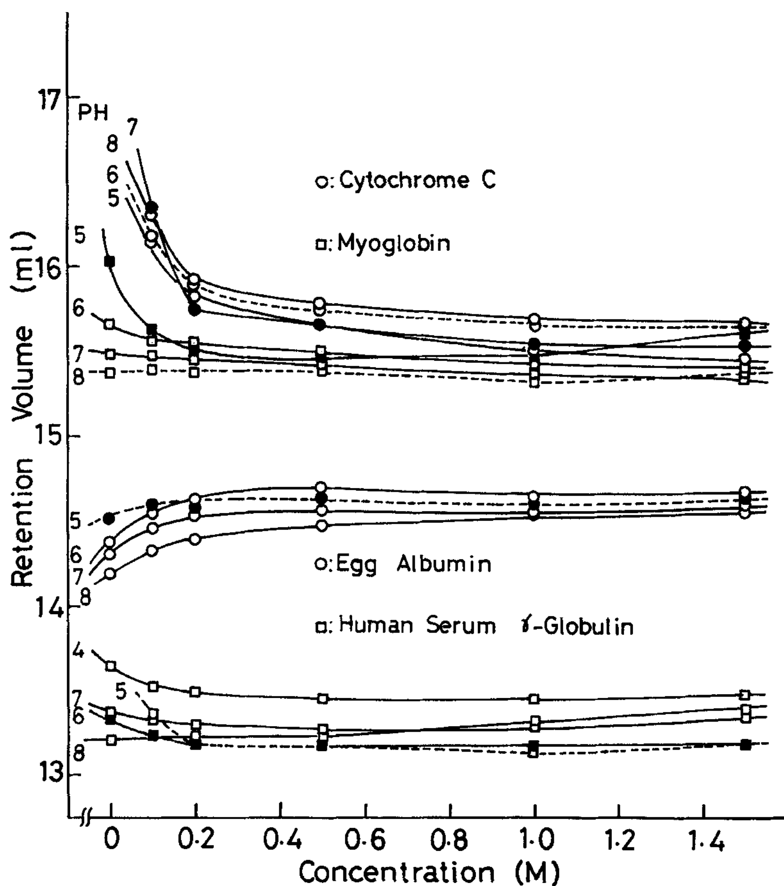

FIGURE 5. Effects of pH for the relationship between retention volume and concentration of NaCl. 0.1 M Phosphates were included.

tion of phosphates and NaCl was effective to suppress the secondary effects and to make a peak sharp. The change of pH values was also effective to increase the resolution between proteins. For example, in the mobile phase of 0.1 M phosphates + 0.2 M NaCl, the change of pH from above 5.0 to 4.0 resulted in the increase of the retention volume difference more than three times

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between samples 5 and 6, samples 3 and 4, and samples 1 and 2 or 3, respectively as shown in Figure 6, mark x.

Effects of different types of buffers and cations or anions of neutral electrolytes to the retention volume of proteins

Phosphates commonly used as buffers are sodium and potassium salts and the buffer solution is prepared by properly mixing these monobasic and dibasic phosphates. Therefore, four combinations of the same or different cations (potassium and sodium ions) can be considered when a phosphate buffer solution is prepared. However, variation of the retention volume of proteins was not observed in four different phosphate buffer solutions.

Retention volume of proteins was affected by the type of buffers. Three different buffers, Soerensen phosphate buffer, Mcllvaine buffer, and Gomori buffer (Tris-buffer) were examined in this study. These buffer solutions included 0.2 M NaCl and pH value was adjusted to 7.0. The results are shown in Figure 6. Though the close comparison is not possible, because of different ionic strength in the three different buffer solutions, the tendency of elution of proteins can be made clear. In a Mcllvaine buffer solution , elution of acidic proteins was retarded and that of basic proteins was accelerated, compared to a Soerensen buffer solution. Neutral proteins had the same retention volume in both mobile phases. A Gomori buffer solution had different tendency from Mcllvaine buffer. Acidic proteins eluted earlier and basic proteins later than in a Soerensen buffer solution. No difference was also observed in neutral proteins. Elucidation of these phenomena is not possible with the limited data. Datailed experiment should be done for the understanding these phenomena. However, from the point of view of mutual separation, it can be said that Gomori buffer may be better than others.

The effects of cations and anions of neutral salts were examined in next four combinations.

(a) 0.1 M Na_2HPO_4 + 0.1 M KH_2PO_4 + 0.2 M NaCl (pH 7.0)
(b) 0.1 M Na_2HPO_4 + 0.1 M NaH_2PO_4 + 0.2 M NaCl (pH 7.0)
(c) 0.1 M K_2HPO_4 + 0.1 M KH_2PO_4 + 0.2 M KCl (pH7.0)
(d) 0.1 M K_2HPO_4 + 0.1 M KH_2PO_4 + 0.2 M KSCN (pH7.0)

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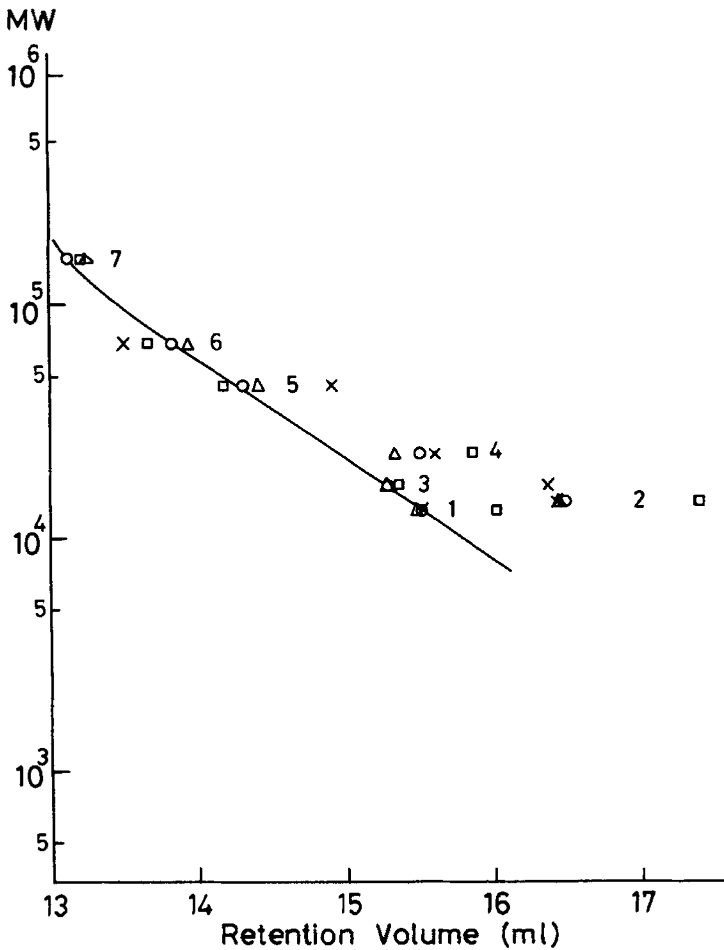

FIGURE 6. Calibration plots of proteins at various buffer solutions used as the mobile phase.

Soerensen phosphate buffer

(o) 0.1 M (Na_2HPO_4 + KH_2PO_4) + 0.2 M NaCl (pH 7.0)

(x) 0.1 M (Na_2HPO_4 + KH_2PO_4) + 0.2 M NaCl (pH 7.0)

Mcllvaine buffer

(△) 0.2 M Na_2HPO_4 + 0.1 M $C_6H_8O_7$ + 0.2 M NaCl (pH 7.0)

Gomori buffer (Tris-buffer)

(□) 0.2 M $NH_2C(CH_3OH)_3$ + 0.1 N HCl + 0.2 M NaCl (pH 7.0)

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There were no differences in retention volume of proteins in three combinations (a), (b), and (c). On the other hand, in the mobile phase of (d), all proteins eluted 0.4 ml earlier than in other three mobile phases. SCN^- ion might act like sodium dodecyl sulphonate (SDS) which denaturalizes proteins and accelerates the elution of them.

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