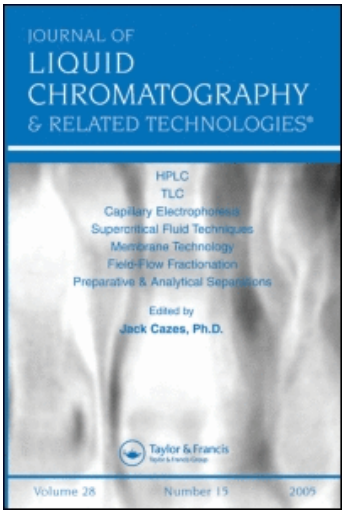


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# Adsorption Chromatography of Biopolymers on Porous Glass

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# ADSORPTION CHROMATOGRAPHY OF BIOPOLYMERS ON POROUS GLASS

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ABSTRACT

Biopolymers, such as proteins and nucleic acids, were separated by adsorption chromatography on porous glass or siliconized porous glass. The adsorption of proteins on porous glass was caused by two factors; one is amine-silanol ionic bonding and the other is a cooperative aggregative force between silica and proteins. The amount of proteins and the constituents was approximately 5μmol/g porous glass. Proteins adsorbed on porous glass were separated by exchange of buffers different in kind. Several examples of protein separation on porous glass were shown and the eluting conditions were discussed. Proteins, adsorbed on siliconized porous glass in a high salt solution, were eluted with a low salt solution or with detergents. Nucleic acids, such as tRNA, rRNA and DNA were adsorbed at high concentrations of salt on porous glass or siliconized glass, and separated with a gradient from a high salt solution to a low salt solution with good recovery. Siliconized porous glass was further coated with Adogen 464 and used an identical adsorbent with RPC-5 to separate tRNA species.



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## INTRODUCTION

Adsorption of proteins on the surface of glass is well known in the many fields of biochemistry (1-3). For example, blood clotting is prompted on glass surfaces, so siliconization is necessary to prevent this influence. The clotting starts when the XII factor of blood coagulation (Hageman Factor) is activated by the anionic charge of the silanol groups on the glass surfaces (4-5). Meanwhile, macrophages adhere well on the glass surface and this adhesiveness allows separation of macrophages from other cells. The adhesive substance was isolated from macrophages (6). The adhesion of blood platelets to glass surfaces is known in the field of clinical chemistry and blood platelets adhere more on surfaces coated with fibrinogen (7). Another general phenomenon of ad hesion to glass surfaces is the difference in adhesiveness between cancer cells and normal cells in culture dishes (8). The membranolytic potential of several crystalline and amorphous silicas were measured using human erythrocytes (9).

The amounts of proteins adsorbed on the surface of glassware have a significant effect on the quantitative analysis of very small amounts of proteins in the case of radioimmunoassay or enzymeimmunoassay (10-11). Columns of glass beads binding with antigens were used for preparation of lymphocytes to prove the clonal selection theory (12). Flagellin of flagella protein of Salmonella was found to be adsorbed on glass surface (13). Taking note of the adhesiveness, Boone (14) and Ito et al.(15) separated cells or proteins with glass beads or Quso microgranular silica. Also adsorption of proteins on the surface of plastics such as biomedical polymers has been studied and the details of this problem are described later.

As before, adsorption of proteins on glass surface is not a special phenomenon but a general phenomenon which is neglected because of the low surface area of typical glassware. However, in the case of porous glass which has a surface area of some hundreds of square meters per 1 g (2 ml), adsorption can not be neglected (16). Meanwhile, porous glass having a large pore size has also been developed for exclusion chromatography and is useful for isolation cell organella after the treatment with Carbowax 20M to prevent adsorption (17). In the first half of this review, the author describes adsorption of proteins on porous glass surfaces, the amount of proteins adsorbed, and applications to adsorption chromatography.



## WASHING AND PROPERTIES OF POROUS GLASS

### Washing

The porous glass used in our experiments was CPG-10, 240Å (surface area 97 $m^2/g$; 10 μm particle size) obtained from Electro-Nucleonics (Fairfield, NJ). It was composed of 96% silica made from boro-silica glass. After being suspended in water and deaerated, with an aspirator, in a desiccator, porous glass was packed in a column. The column was then washed with more than ten column volumes of 0.1% sodium dodecylsulfate (SDS), 2 column volumes of chromic acid mixture (10% dichromate and 30% sulfuric acid) and subsequently with more than 20 column volumes of distilled water (18). In this instance, the surface of the glass is of the hydrogen type. For some purposes, the column was bufferized before use. Since diluted salt solutions took much time to be bufferized, they had to be used for adsorption experiments after checks of pH and conductance were made. The porous glass column which had been used for adsorption experiments of proteins was repeatedly used

after being washed with 0.1% SDS, water, a chromic acid mixture, and water. When protein was bonded strongly on the porous glass, the column was filled with a chromic acid mixture or concentrated nitric acid and left for one or two nights. Bock et al.(19) also regenerated the column with sulfuric acid or nitric acid.

The advantage of porous glass is that it is stable in strong acid and autoclavable. The disadvantage is that porous glass is more labile in alkaline environments than other gel filtration matrixes (20). When 0.01N NaOH is applied to the porous glass column, its volume declines. But this is not a serious problem since normal biological materials are not treated in such strong alkaline solutions.



Amounts of Materials Adsorbed

By the modified frontal analysis method, the amounts of substances adsorbed were estimated from the loss of substances in the eluate from the column (21). Amino acids were dissolved in distilled water at a concentration of 0.2mM and the solution was loaded on the column. Table 1 shows the amount of amino acids adsorbed on porous glass (22). The amount of basic amino acids such as lysine, histidine, and arginine adsorbed on 97 $m^2$ of glass surface was 5 μmol. This result showed that the surface of the glass had an anionic silanol group which adsorbed 5 μmol of basic amino acids per 97 $m^2$ of glass surface. This adsorption must be due to ionic bonding between positively charged ammonium ions of amino acids and negetively charged silanol residues on the glass surface. In addition, Table 1 shows that neutral and acidic amino acids were also adsorbed, although the amount of those amino acids adsorbed was lower than the amount of basic amino acids adsorbed. This adsorption was confirmed by using glycine and leucine at

TABLE 1
Adsorption of Amino Acids on Porous Glass in Water

| Amino acids | μmoles / g of porous glass(97 $m^2$) |
|---|---|
| Lysine | 5.95 |
| | 0.16[a] |
| | 0.74[b] |
| Histidine | 4.62 |
| Arginine | 5.73 |
| Aspartic acid | 0.38 |
| Threonine | 0.44 |
| Serine | 0.20 |
| Glutamic acid | 0.73 |
| Proline | 0.42 |
| Glycine | 0.76 |
| Alanine | 0.69 |
| Cysteine | 0.49 |
| Valine | 0.15 |
| Methionine | 0.60 |
| Isoleucine | 0.19 |
| Leucine | 0.64 |
| Tyrosine | 0.18 |
| Phenylalanine | 0.50 |
| Tryptophan | 0.22 |

a The value in 0.15M NaCl.
b The value in 0.1M Tris-HCl, pH 8.6.



various pH levels (23). One g (97 $m^2$) of porous glass adsorbed 0.2 μmol of leucine and glycine at pH 2.6 and 6.0 but none at pH 10.3. The results suggested two states of adsorption. One is the adsorption of 5 μmol/ 100 $m^2$ of basic amino acid on a glass surface due to ionic bonding, and the other is the adsorption of about 0.2 μmol of neutral and acidic amino acid. The latter state could not be fully explained by ionic bonding.

The amount of nucleosides and nucleotides adsorbed on porous glass in distilled water was estimated (22). As for sugars, 5 μmol of hexosamine was adsorbed on 100 $m^2$ of porous glass in distilled water, but the amount of hexosamine adsorbed on the glass in NaCl or Tris-HCl

solution was low. Other carbohydrates such as neutral-sugars and acetylhexosamines were hardly adsorbed. From the point of view that the adsorption of amino sugar on porous glass was specific, the separation of glucose and glucosamine on a column of porous glass was investigated as shown in Figure 1 (22). Glucose was hardly adsorbed and eluted with distilled water. Glucosamine, however, was retained on the column in a distilled water medium and was later eluted with 0.15M NaCl. The results indicated that the adsorption of basic materials on glass was more specific and therefore presented the possibility of porous glass as a cation exchanger. Adsorption chromatography of serum protein also showed that protein of high hexosamine content was eluted later (22). As for components of nucleic acid, acidic necleotides were not adsorbed, but basic nucleosides such as cytidine were adsorbed, though the amount of adenosine adsorbed was lower (0.51 μmol/97 $m^2$). The adsorption of inorganic ions was also studied and the amounts were similar with that (5 μmol/97 $m^2$) of basic amino acids (24). Since inorganic cations were adsorbed easily on the surface, the ion concentration of 4nM must be a marginal point not affected by adsorption on the surface of glass beakers (50 ml) when glassware is used for the determination of inorganic ions. When anions such as sulfate ions were applied to a porous glass column, they were hardly adsorbed. The estimation of drug adsorption on glass surfaces with porous glass as a reference was carried out (23). The amounts of those basic drugs adsorbed in water were similar to the amounts of basic amino acids adsorbed. Meanwhile, in physiological saline those amount adsorbed on glass surfaces was negligible.

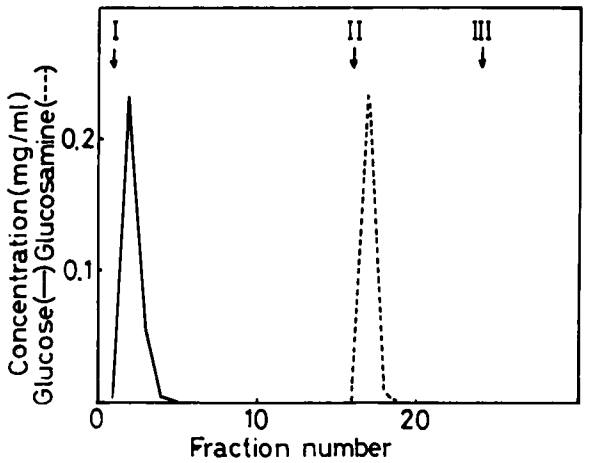


FIGURE 1
Separation of glucose and glucosamine on porous glass. Each 0.5 mg of glucose and glucosamine was used. The column size was 0.63 cm x 10 cm and the fraction volume was 2 ml. Eluant I, water; II, 0.15M NaCl; III, 0.1M Tris-HCl at pH 8.6. The concentrations of glucose and glucosamine were determined by the phenol-sulfuric method and the Elson-Morgan method, respectively. Reproduced with permission from Anal. Biochem., 83, 216 (1977).



Property of Protein Adsorption

Adsorption of serum albumin (10 mg sample) on columns (1.1 cm x 20 cm) of porous glass in various buffers was studied (25). As shown in Table 2, the yields were low in sodium chloride, sodium carbonate, phosphate, borate, acetate, citrate and barbital buffer, because of the adsorption of protein on porous glass,but were higher (30%) in Tris-HCl buffer. The adsorption of protein and basic amino acids depends much on the fact that the former is not inhibited by inorganic salts but the latter is inhibited by sodium chloride as described in the previous section. Elution with solutions of such amino acid buffers as glycine and alanine at pH 8-8.9 resulted in about 70% recovery of the protein. Although

TABLE 2
Yields of Bovine Serum Albumin from Porous Glass Column with Various Buffers

| Buffer | pH | Yield | Kav |
|---|---|---|---|
| 0.05M phosphate | 7.4 | 0 | - |
| 0.05M phosphate-0.2M NaCl | 7.4 | 0 | - |
| 0.05M phosphate-1M NaCl | 7.4 | 0 | - |
| 0.05M phosphate-3M NaCl | 7.4 | 0 | - |
| 0.05M phosphate-1M NaCl-2% ethanol | 7.4 | 3 | 0.3 |
| 0.05M phosphate-1M NaCl-5% ethanol | 7.4 | 4 | 0.3 |
| 0.05M $NaHCO_3$ | 8.7 | 7 | 0.8 |
| 0.05M $Na_2CO_3$ | 11.4 | 8 | 0.3 |
| 0.01N NaOH | 12 | 3 | 0.1 |
| 0.05M borate | 8.7 | 21 | 0.6 |
| 0.035M barbital | 8.6 | 16 | 0.5 |
| 0.5M Tris-HCl | 8.6 | 33 | 0.3 |
| 0.5M Tris-acetate | 8.6 | 27 | 0.2 |
| 0.1M ammonium acetate | 8.3 | 5 | 0.5 |
| 0.5M pottasium acetate | 8.3 | 17 | 0.9 |
| 0.1M citrate | 8.0 | 15 | 0.7 |
| 0.05M glycine | 6.0 | 17 | 0.2 |
| 0.05M glycine | 8.0 | 68 | 0.3 |
| 0.038M glycine-0.005M Tris | 8.9 | 76 | 0.2 |
| 0.38M glycine-0.05M Tris | 8.9 | 76 | 0.2 |
| 0.056M dl-alanine | 8.0 | 68 | 0.1 |
| 0.056M dl-alanine-0.2M Tris-HCl | 8.6 | 13 | 0.7 |
| 0.056M β-alanine | 8.0 | 64 | 0.1 |
| 0.056M β-alanine-0.2M Tris-HCl | 8.6 | 21 | 0.4 |

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ammonium acetate has carboxyl groups and amino groups, it could not be substituted for glycine of ampholytes. This suggested the significance of Zwitter ions. As the yield with β-alanine shows, the adjacency of amino groups to carboxyl groups is not always necessary. Such prevention of protein adsorption with amino acid buffer is believed to have its origin in the occupation of the protein-binding site on the glass surface by amino acids (the components of protein). But the prevention of adsorption by glycine is more effective by basic amino acid such as lysine.

The adsorption properties of protein on porous glass were studied at various pH. Figure 2 shows the adsorption patterns of bovine serum albumin, lysozyme, and chymotrypsin adsorbed on porous glass in a small column (23). The amount of these three kinds of proteins adsorbed varied with pH and the maximum amounts of proteins adsorbed on 1 g of porous glass were 136 mg (2 μmol) for serum albumin, 233 mg (9 μmol) for chymotrypsin and 84 mg (6 μmol) for lysozyme. These values (2-9 μmol) of proteins agreed with the value (5 μmol) of basic materials as described in the preceding section. The amount of 5 μmol/97 $m^2$ is equal to 2-3 molecules per 100Å x 100Å, and this value is comparable to the size of albumin molecule (30Å x 120Å). Therefore, protein adsorbs on the surfaces in a monolayer. From the value of chymotrypsin (233 mg/g), the amount of proteins adsorbed on a glass syringe and a glass ampule of 20 ml (inner area about 50 $cm^2$) was estimated to be more than 12 μg, so the protein concentration of 0.5 ug/ml must be a marginal point affected by adsorption. Actually, some kinds of drugs, such as PPD of tuberculin, are used almost at this concentration.

Figure 2 also shows that the amounts of protein adsorbed on porous glass varied with pH. Albumin was adsorbed the most at pH 5, lysozyme at pH 11, chymotrypsin at pH 8; these values are their isoelectric points. These results indicated that a protein was adsorbed most extensively to a glass surface at its isoelectric point. Considering the negative charge of the silanol groups on the glass surface, proteins should be adsorbed at a more acidic pH, at which proteins would have a positive charge. But for the adsorption of proteins, the aggregative action between silanol and proteins and the aggregative property of proteins would be

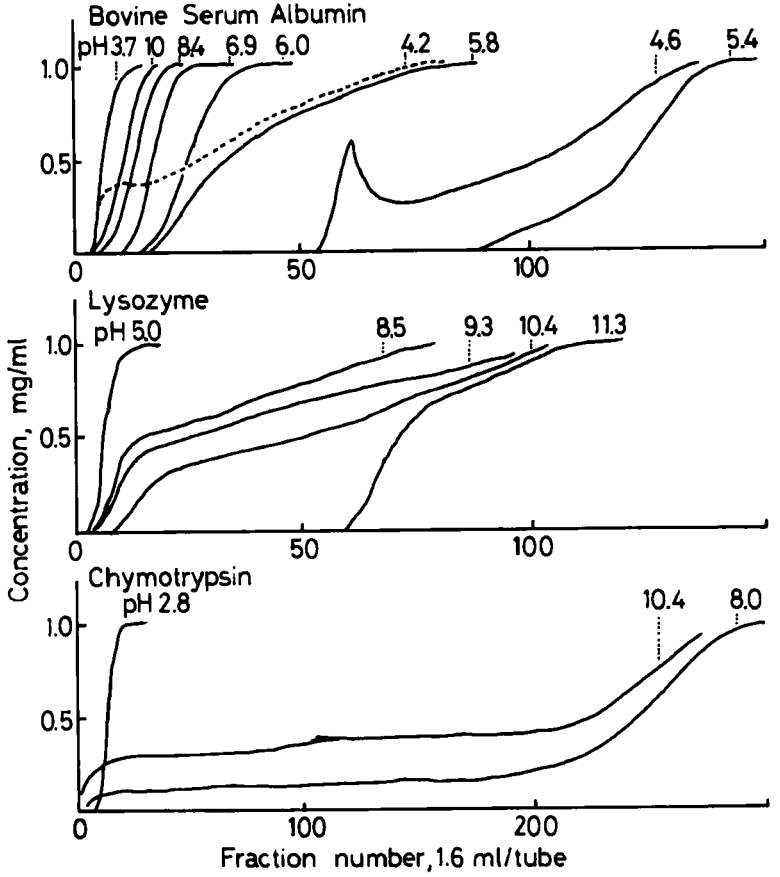


FIGURE 2
Adsorption patterns of bovine serum albumin, lysozyme, and chymotrypsin on a porous glass column (0.65 cm x 11 cm) in distilled water. The fraction volume was 1.6 ml. Reproduced with permission from J. Pharm. Sci., 67, 1102 (1978).

another important factor. Therefore it is concluded that the adsorption of proteins on porous glass was caused by two factors; one is amine-silanol ionic bonding and the other is a cooperative aggregative force between silica and proteins.

Messing has suggested that the forces involved in the reaction between proteins and glass surfaces were ionic bonding and hydrogen bonding (21). Meanwhile, Bresler et al. have suggested the participation of hydrophobic bonding based upon thermodynamic studies (26). To probe into this point, the adsorption of albumin on porous glass in various detergent solutions was studied. Albumin was adsorbed well on porous glass even in 8M urea and 6M guanidine HCl which affects hydrogen bonding. In urea, the amount of albumin adsorbed was much the same as it was in water. The amount adsorbed in guanidine-HCl was slightly lower than that in urea, and this phenomenon is not due to the inhibition of hydrogen bonding but to a high salt concentration, such as 6M of guanidine HCl. Since α-helix of protein is denatured in about 2-4M guanidine (27), hydrogen bonding does not seem to be present in protein molecules in 6M guanidine-HCl. But under this condition, proteins do bond to porous glass; therefore hydrogen bonding does not seem to be related to the protein adsorption on glass.

As for ionic bonding, another main factor of adsorption, shows that albumin modified with succinate was hardly adsorbed and the amount adsorbed was about 3 mg/g of porous glass (about 2% of that in distilled water). This indicated that ionic amine-silanol bonding between amino groups on protein and negative silanol on glass suraces played an important role. This phenomenon was confirmed on glass surfaces with the use of glyceryl porous glass. The amount adsorbed on 1 g (62 $m^2$) of modified porous glass was 8.7 mg (28). This was 9% of

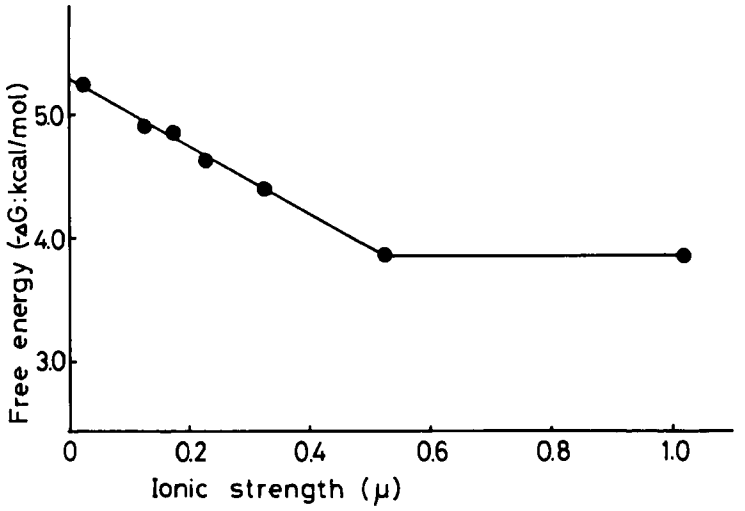


FIGURE 3
Relationship of ionic strength and ΔG to albumin adsorption on glass surfaces.



that on unmodified porous glass, which suggested that silanol on glass surfaces was essential for protein adsorption. Figure 3 shows the relation between the ΔG values and the ionic strength (29). The results support the conclusion of that ionic bonding is a major factor of adsorption, because the ΔG value decreased at high concentration of NaCl. Meanwhile, glass surfaces are hydrophilic because the contact angle is 10.7° for glass surfaces. Study with a hydrophobic probe (30) shows that the probe binds more to the proteins of low affinity to glass surfaces; this result suggests that hydrophobic interaction is not major factor of protein adsorption

Adsorption patterns of albumin on porous glass in various detergents were studied (31). Albumin was not adsorbed on glass in SDS and a hard soap solution, this reaction being caused by repulsion between terminal

silanol groups on porous glass and negatively charged dodecyl residues hydrophobically bound on protein molecules. In sodium valerate, this inhibition of adsorption was not observed, suggesting that a sodium salt of a fatty acid having detergent activity was a factor in inhibiting adsorption. In contrast, albumin was adsorbed more in alcoholic detergent solutions (Triton X-100 and Brij 35) and a cationic soap solution (benzalkonium chloride); therefore proteins adsorbed on glassware must not be completely washed with such detergent solutions. From the results that protein in ethylalcohol, octylalcohol, and alcoholic detergent solutions adsorbed well on porous glass, and that carbohydrates having alcoholic OH groups were not adsorbed on porous glass, it is concluded that alcoholic OH does not affect the adsorption of proteins.



## ADSORPTION CHROMATOGRAPHY OF PROTEINS ON POROUS GLASS

### Serum Proteins

Adsorption of proteins on porous glass differed with the kinds of proteins, buffers and the pH values of the buffers (25). From this difference, porous glass can be used as an adsorbent for the separation of proteins by adsorption chromatography (18, 32). Since porous glass (1 ml, 0.5 g) adsorbed 50-100 mg of proteins and this amount is much the same as the amount of protein adsorbed on a cellulose ion exchanger or other chromatographic supports as compared in Table 3 (33-34). Porous glass is a very favorable adsorbent for use in adsorption chromatography.

Figure 4 shows the elution pattern of rabbit serum (100 mg), which was dialyzed against distilled water, lyophilized, and used as a reference for a protein mix-

TABLE 3
Average Amount of Proteins to be Loaded on Supports

| Support | mg Protein/ml support |
|---|---|
| Porous glass | 3 |
| DEAE-cellulose | 3 |
| Sephadex, Sephacryl, AcA | 0.5 |
| Hydroxyapatite | 2 |
| Blue-Sepharose | 0.2 |
| Matrex gel redA | 2 |
| Octyl-Sepharose | 6 |

TABLE 4
Percentage Yields of Fractions Obtained on Chromatography of Serum at pH 8.0

| Fractions in Fig. 4 | CPG 100 mg 4° | CPG 20 mg 4° | CPG 100 mg 37° | CM-cellulose 100 mg 4° |
|---|---|---|---|---|
| I | 15.6 | 16.5 | 22.4 | 19.7 |
| II | 21.6 | 25.0 | 21.6 | 44.2 |
| III | 6.3 | 7.5 | 0 | 7.3 |
| IV | 9.3 | 14.1 | 8.8 | 3.0 |
| V | 19.0 | 21.2 | 22.0 | 1.8 |
| Other minor fractions | 3.4 | 2.5 | 4.0 | 0.8 |
| Total | 75.7 | 86.8 | 78.8 | 76.8 |

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ture. The patterns at pH 6.0, pH 7.0, pH 7.5, pH 8.0 and pH 8.5 on large columns of porous glass were obtained by elution with the following buffers: sodium chloride, Tris-HCl, glycine, and 0.1% SDS (18). At pH 6.0, almost all proteins were well adsorbed and separation was difficult. Better separation and elution were

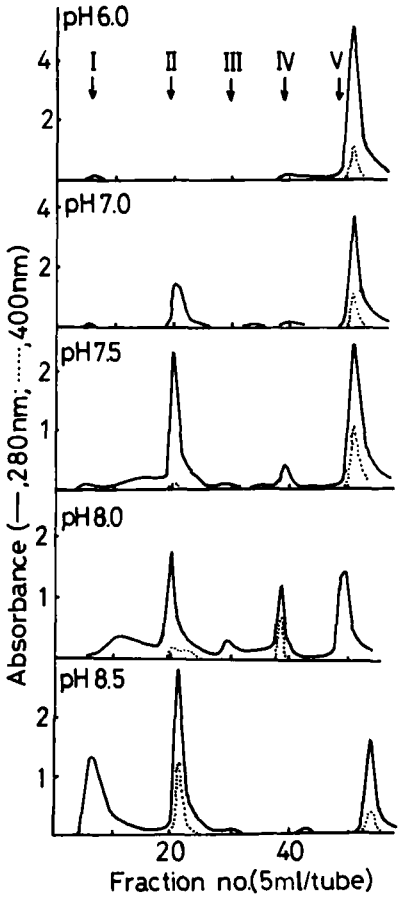


FIGURE 4
Elution patterns of rabbit serum (100 mg protein) on a porous glass column (1.2 cm x 20 cm) at 4°C and various pH values. The fraction volumes collected were 5 ml. Eluants: I, 0.2M NaCl-0.01M phsophate; II, 0.01M Tris-HCl; III, 0.2M Tris-HCl; IV, 0.2M glycine; V, 0.1% SDS in 0.05M phosphate. Reproduced with permission from J. Chromatogr., 168, 143 (1979).

observed at pH 7.5-8.5. Therefore the pH range suitable for chromatography with porous glass is 7.0-8.5. A 10 g amount of porous glass in the column should be capable of adsorbing 1-2 g of proteins. Therefore the amount (100 mg) of rabbit serum applied to the column was one-tenth to one-twentieth of the adsorption capacity of the porous glass. Yields of the fractions eluted at 4°C and 37°C using 20 mg and 100 mg of rabbit serum (one fiftieth to one hundredth of the adsorption capacity of porous glass) at pH 8.0 are shown in Table 4. Fractions I-V in Table 4 correspond to fractions I-V in Figure 4, respectively. Since yields of the fractions using 100 mg of rabbit serum were similar to those using 20 mg as in Table 4, it is concluded that the reproducibility of the elution pattern of proteins is good for protein amounts in the range of one tenth to one hundredth of the adsorption capacity of porous glass (2-10 mg of protein per gram (2 ml) of porous glass). The result using half of the adsorption capacity has been reported, but was not reproducible for some of the fractions eluted (32). Yields at 37°C were all similar to those at 4°C, suggesting that the influence of temperature was not so strong.

Next, let us consider the subject from the following point of view: How have proteins in each fraction really been separated? Disc gel patterns of separated fractions of rabbit serum at pH 8.0 are shown in Figure 5 (18). In fraction I, albumin with a relative mobility of 0.7 to BPB was found. α-Globulin (transferin) with a relative mobility of 0.4 was found in fraction II and γ-globulin with a low mobility was found in fraction IV. Most of the γ-globulin in fraction IV was of high mobility with a relative small size. γ-Globulin of large molecular weight, which did not move on the gel, was not absent in fraction IV. These globulin found in fraction

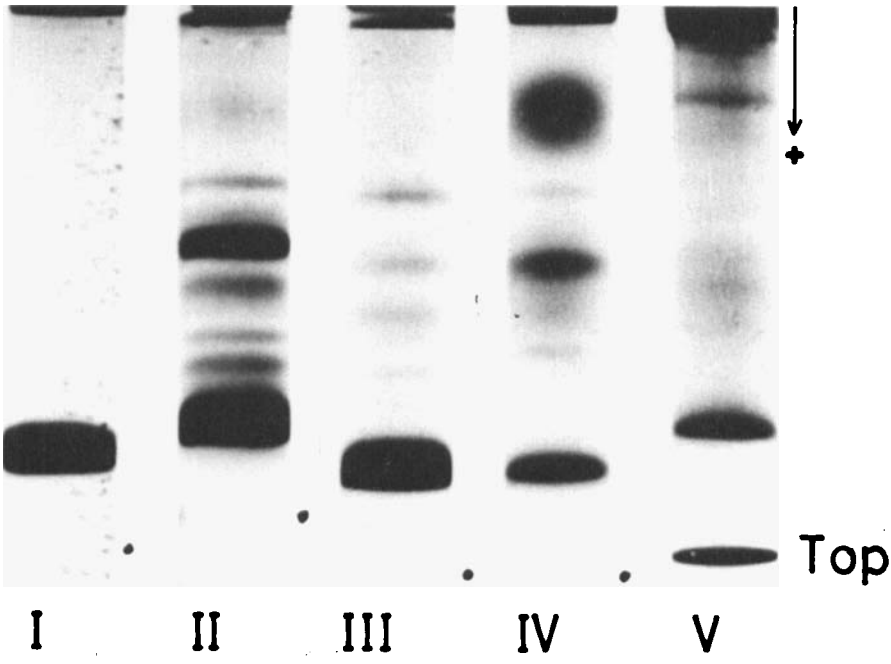


FIGURE 5
Disc gel patterns of separated fractions on prous glass. Fractions I-V are identical with those at pH 8 in Figure 4. Reproduced with permission from J. Chromatogr., 168, 143 (1979).



V eluted with 0.1% SDS and the recovery with solvent not containing SDS was low.

To investigate the adsorptivity of antibody, rabbit antiserum containing antibody (IgG class) was used. It was eluted with glycine in fraction IV and was not found in fraction I. But the approximate examination of yields by the Ouchterlony method revealed that the recovery of the IgG class was about 12%, suggesting that a part of the antibody was adsorbed on the porous glass and was not eluted. Antibody of the IgM class against sheep erythrocyte was eluted in fraction I with a recovery of 12% and with a total recovery of 16%. From

these results, it was found that IgG was adsorbed onto porous glass more than IgM (35). The adsorption pattern of the sheep globulin fraction showed that $\alpha_1$-globulin was adsorbed more than $\alpha_2$-globulin (35). Thus, in separating serum protein on porous glass, albumin was eluted first, followed by $\alpha$-globulin than $\gamma$-globulin. Meanwhile, separation of rabbit serum protein on CM-cellulose showed that albumin was eluted first and globulin next. The separation of proteins on porous glass was better than on CM-cellulose, as shown by disc gel patterns (18). Finally, adsorption chromatography of proteins on porous glass was effective, and the elution pattern showed that porous glass behaved like a cation exchanger such as CM-cellulose and not like DEAE-cellulose



Standard Proteins

By stepwise elution with a change of solvent, the pH values of solvent varied from the initial pH values caused by the properties of porous glass as an ion exchanger. The gradient elution method in a glycine buffer was carried out to prove this point. Figure 6 shows elution patterns of standard proteins on porous glass columns by this method (37). Each 5 mg of standard protein having similar molecular weight was dissolved in distilled water and loaded on a column. The column was washed with saline and eluted with a pH gradient (pH 6.6-9.0) in 0.2M glycine (total volume 200 ml). Peroxidase was eluted first with saline, bovine serum albumin came next with glycine and hemoglobin and catalase were eluted at a pH of nearly 8.0. Aldolase, lysozyme, chymotrypsinogen A, malatedehydrogenase, and cytochrome c were not eluted under this condition, but were eluted with 0.1% SDS. Those strongly bound proteins may be separable with some detergents, such as cholate, deoxycholate, and Triton X-100. As reported by

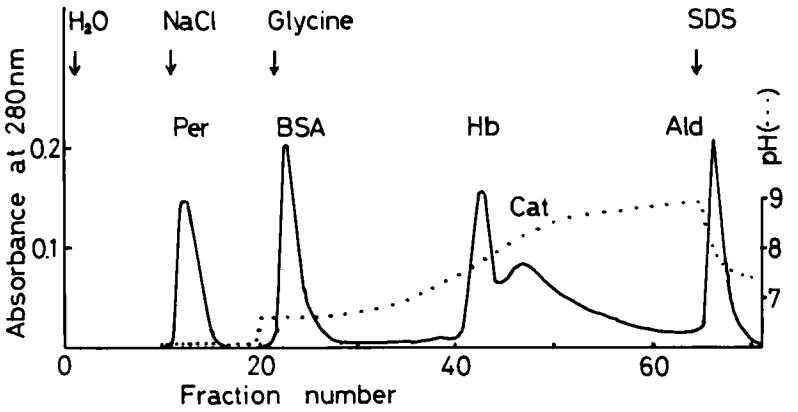


FIGURE 6
Elution pattern of standard proteins on a porous glass column (1.2 cm x 20 cm). Per, peroxidase; BSA, Bovine serum albumin; Hb, hemoglobin; Cat, catalase; Ald, aldolase. Fraction volumes were 5 ml. Reproduced with permission from J. Colloid Interface Sci., 79, 284 (1981).



Bresler et al. (26), protein was eluted well at a pH of nearly 8.0, which suggested a relationship to the pK value of silanol (8.2). The pH value of glycine buffer was not used beyond 9.0, taking the denaturation of protein and the solubilization of glass into consideration. The system of pH 6.6-9.0 was used as stated above, but the use of a buffer of whose pH was 6.0-8.0 for separation of serum protein resulted low yields. The elution order of standard protein from a porous glass column has been shown above but the order from a CM-cellulose column was a little different; albumin, chymotrypsinogen A, hemoglobin, and cytochrome c. The order of elution on porous glass and CM-cellulose had similarities to some extent, but apparently differed from each other with regard to chymotrypsinogen A and cytochrome c (18). This might depend on the fact that adsorption of protein on porous glass was caused not

only by ionic bonding but also by the cooperative aggregative forces between protein and silanol. Meanwhile, the pH range used during chromatography on CM-cellulose is usually acidic, but the range on porous glass is neutral, and this pH range might be valuable for maintaining the native conformations of some proteins

Peroxidase-coupled antibody is used for staining in the histochemical field. Peroxidase-coupled antibody was purified on porous glass (our unpublished results). As shown in Figure 6, peroxidase has a low affinity for glass surfaces. Globulin has a high affinity. From these properties of peroxidase and globulin, peroxidase-coupled antibody is separable from free peroxidase and globulin. The results are shown in Figure 7. Free peroxidase was passed through the column and peroxidase-coupled antibody was eluted later in tubes 37-39.

To investigate what determines the adsorption order of standard proteins with similar molecular weights which can be separated on porous glass columns (Figure 6), the properties of such proteins are compared (38). The comparison suggests that the adsorption order does not depend on the isoelectric point or molecular weight, but on helix content. The contents of the acidic amino acids or basic amino acids are not related to the adsorption of protein, but to the high content of aliphatic amino acid. In general, aliphatic amino acids tend to form helixes (39). The adsorption of protein on glass is too complicated to be explained only by ionic bonding, and might be partially caused by another factor due to the aggregative forces in aqueous solution.

Chromatographic Conditions

A high flow rate may be obtained during chromatography on porous glass. Figure 8 shows the results of elution at a high flow rate. Rabbit serum protein (100 mg) was eluted with a glycine buffer system (pH

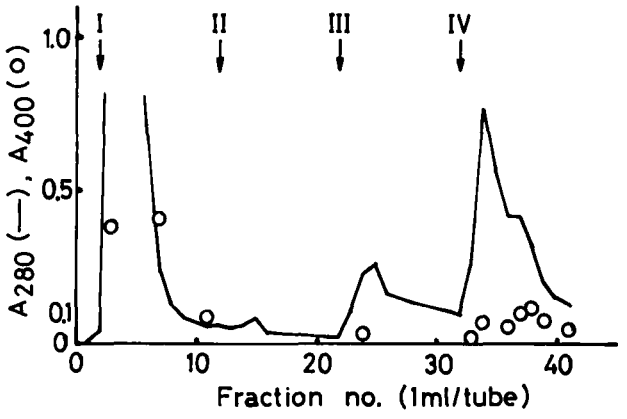


FIGURE 7
Separation of peroxidase and peroxidase-antibody complex on porous glass. I, Sample and 0.1M phosphate at pH 6.8; II, 0.2M glycine at pH 6.8; III, 0.2M glycine at pH 8; IV, 0.2M glycine at pH 9. $A_{400}$ indicates peroxidase.



6.6-9.0) on a column and the elution patterns were compared (35). Comparing the upper column, which shows the elution pattern at a flow rate of 0.5 ml/$cm^2$/min that is used for general chromatography, with the middle column at a flow rate of 5 ml/$cm^2$/min, the peak near pH 8.0 in the middle column was gentler and appeared later than in the upper column. Separation was less effective in the middle column as shown in the disc pattern of each fraction. Elution in the lower column was carried out at a flow rate of 20 ml/$cm^2$/min, and the amount of protein which passed through the column with albumin increased. Separation was less effective. This result of slow desorption of proteins is consistent with the results of physicochemical study of Brash (40). They showed that protein exchange for new protein reached equilibrium after 3 hr. So, slow flow rate such as 0.5 ml/$cm^2$/min is suitable for fine separation. In the case of large scale preparation of proteins, high flow rate may be recommended.

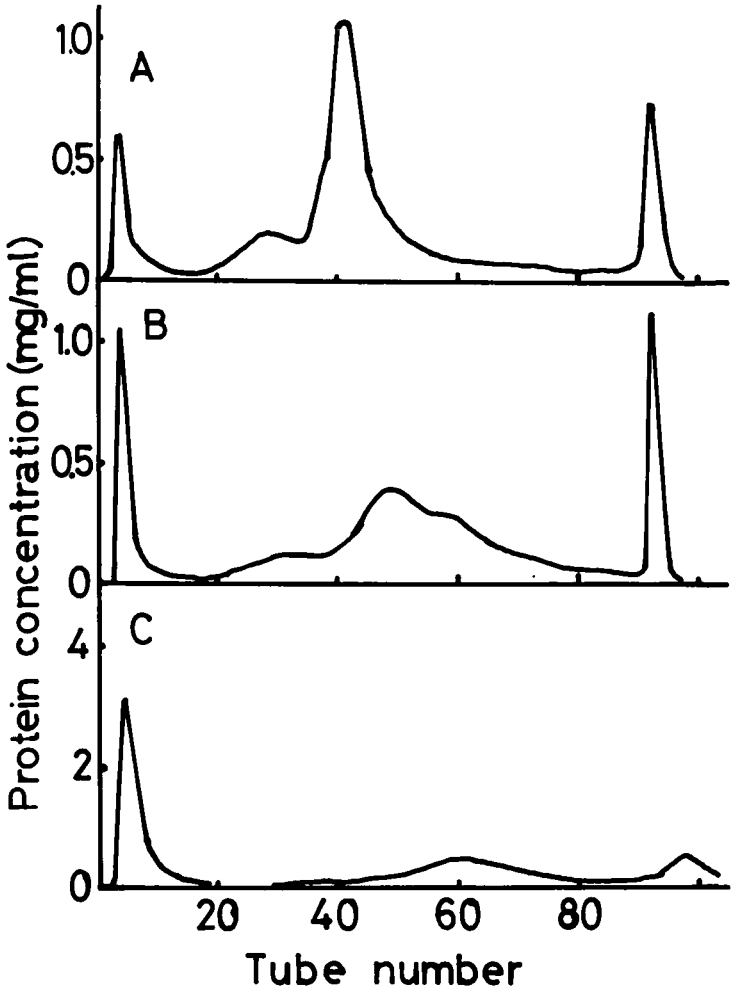


FIGURE 8
Elution patterns of rabbit serum (100 mg protein) on porous glass at various flow rates. The column size was 1.2 cm x 20 cm. Flow rate in A, 0.5 ml/cm$^2$/min; B, 5 ml/cm$^2$/min; C, 20 ml/cm$^2$/min. Reproduced with permission from J. Polymer Sci. Part C, 68, 129 (1980).

The forces of some solutes that cause elution of proteins from glass surfaces are described. Proteins are most easily adsorbed on hydrogen-type glass surfaces in distilled water, followed in order by NaCl, $Na_2SO_4$, sodium acetate, and phosphate. These salts have similar eluting forces as does carbonate. Tris-HCl buffer has a stronger force for eluting proteins, followed by thiocyanate of chaotropic ions described by Bock et al. (19). Glycine has a stronger eluting force than thiocyanate. Sodium deoxycholate has a force similar to glycine. Triethanolamine-HCl buffer has a slightly stronger force than glycine. The addition of ethanol in a buffer at concentrations of 5-10% increases the eluting force. Inorganic divalent cations and valeric acid show weak eluting forces.



## PRACTICAL EXAMPLES OF CHROMATOGRAPHY OF PROTEINS ON POROUS GLASS

### Hydroxybutyrate Apodehydrogenase and Staphylococcal α-Toxin

Bock et al. (19) reported the chromatography with the use of chaotropic buffers, which are known as solutes that decompose water environment. The mixture of bovine serum albumin, chymotrypsinogen A, lysozyme, and myoglobin (2 mg of each in 0.01M potassium phosphate buffer, pH 6.5) was chromatographed on a column of 3 ml of CPG. The serum albumin passed through the column with this buffer. Chymotrypsinogen A was eluted with 0.1M potassium phosphate at pH 6.5 and then lysozyme with 1M KI-0.01M potassium phosphate at pH 8.15. β-Hydroxybutyrate apodehydrogenase (0.4-0.6 mg) in 0.2 ml of 1mM Hepes buffer (pH 7.0) containing 0.4M LiBr and 5mM DTT was applied to a CPG column (0.56 cm x 2 cm) at 4°C. The column was washed with 1mM Hepes buffer at pH 7 containing 2mM DTT and then bufferized with 0.1M Tris-

acetate buffer at pH 8.15. The enzyme was eluted with 1M chaotropic salts, such as NaSCN, NaI, $NaNO_3$, KBr, and LiBr, in 0.1M Tris-acetate at pH 8.15. The recovery of enzymatic activity from the column was 80%. Among these salts, NaSCN and NaI have the strongest forces for eluting enzyme, and the activity on a column was almost completely recovered. KCl and NaCl show weak forces for elution and sodium acetate and phosphate did not have the appropriate characteristics to elute the enzyme. Staphylococcal α-toxin also showed a similar adsorption pattern with the enzyme.

Influenza Virus

Bresler et al. (26) reported adsorption chromatography of viruses on porous glass. Porous glass of large pore size was useful for purification of viruses as was exclusion chromatography. However, non-coated porous glass adsorbed viruses. They studied the adsorption isotherm of the influenza virus on porous glass. The adsorption isotherm of influenza viruses on wide-pore glass was measured and was found to conform to Langumuir's equation. The transition from adsorption to elution of influenza virus on porous glass proceeds under critical conditions of pH near the pK of the SiOH groups. Adsorption experiments were done by application of a virus suspension of 1 ml ($10^9$-$10^{10}$ virions) on a porous glass column (pore size, 850Å; 1.1 cm x 20 cm) and the column was washed at a flow rate of 1 ml/$cm^2$/min. The isoelectric points of viruses are in the range of 4-5. The influenza virus $A_2/2226/61$ was desorbed at pH 8.2, which is the pK value of silanol. The adsorption was partially dependent on the ionic bonding between silanol and protein, because the desorption was increased by addition of salt. Figure 9 shows the adsorption and desorption patterns of influenza virus $A_2$ Singapore (750 ml, 5 x $10^9$/ml) on a

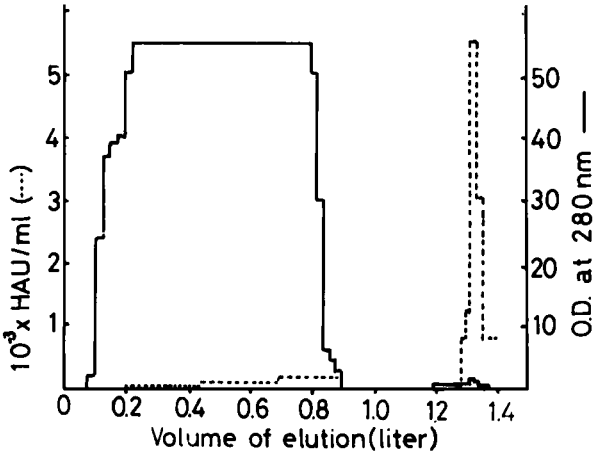


FIGURE 9
Chromatographic profiles during the adsorption (left) and elution (right) of influenza virus $A_2$ Singapore from a porous glass. HAV, hemagglutination units. Reproduced with permission from Bresler, J. Chromatogr., 130, 275 (1977).



porous glass column (2.6 cm x 20 cm). The virus was adsorbed at pH 7 and eluted with Tris-HCl at pH 8.5. The virus was purified 20-fold and the recovery was 40%. They also concluded that protein adsorption on glass surfaces was dependent on hydrophobic forces and hydrogen bonding as well as ionic bonding.

Collagenase

Huang et al. purified collagenase from epidermoid carcinoma of rat prostate on porous glass (41). A culture medium (2.55 g protein) of rat tumor was applied to a CPG column (2.6 cm x 25 cm) and collagenase was retained on the column in the buffer. The column was then eluted stepwise with the following buffers:

(I) 0.05M Tris-HCl at pH 7.6
(II) 1M NaCl in 0.05M Tris-HCl at pH 7.6
(III) 1M Tris-HCl at pH 7.6
(IV) 1M LiBr in 0.05M Tris-HCl at pH 8.0
(V) 2M LiBr in 0.05M Tris-HCl at pH 8.2.

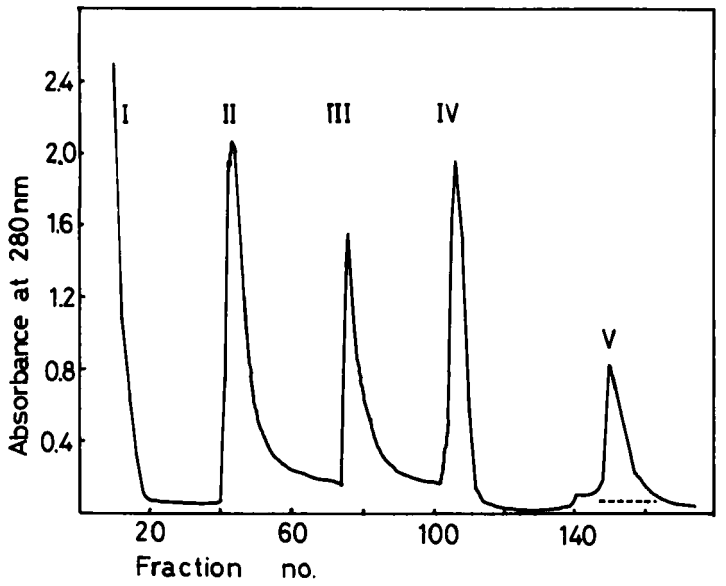


FIGURE 10
Adsorption chromatography of collagenase on porous glass. Culture medium (2.55 g protein) of epidermoid carcinoma of prostate in rats was applied on a column (2.6 cm x 25 cm). The column was then eluted stepwise with the buffers as shown (10 ml fractions; flow rate, 40 ml/hr). The dashed bars on the figure indicate fractions having collagenase activity. Fractions I, II, III, IV, and V were eluted with 0.05M Tris-HCl at pH 7.6, 1M NaCl at pH 7.6, 1M Tris-HCl at pH 7.6, 1M LiBr at pH 8.0 and 2M LiBr at pH 8.2, respectively. Reproduced with permission from Huang et al., Biochim. Biophys. Acta, 570, 149 (1979).

The results are shown in Figure 10. The collagenase was eluted with buffer (V) with a recovery of 80%. The specific activity increased 20-fold.

Interferon

Interferon was found by Isaacs and Linderman (42, 43) and has been used for cancer therapy (44, 45). Braude et al. purified mouse interferon by adsorption chromatography on porous glass (46, 47). A column (0.9 x 12 cm) of the glass was treated with concentrated

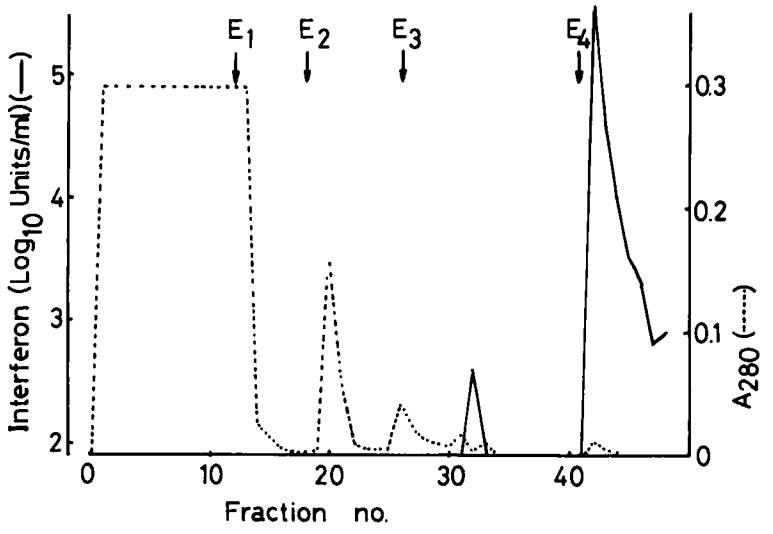


FIGURE 11
Adsorption chromatography of interferon with alkali and acid eluant on porous glass. Reproduced with permission from Braude et al., Biochim. Biophys. Acta, 580, 15 (1979).



nitric acid and washed fully with phosphate-buffered saline. Crude interferon solution (100 ml; 6.3 x $10^4$ units/ml) was loaded on the column and then the column was eluted with the following buffers: phosphate buffered saline (E1 in Figure 11), 0.01M Tris-HCl at pH 8.0-8.5 (E2 in Figure 11), 0.01M glycine-HCl at pH 2.5 (E3 in Figure 11), and 0.1M KCl-HCl at pH 2.0 (E4 in Figure 11). The flow rate was 10 ml/hr. 6.7 ml fractions were collected. Dotted and solid lines in Figure 11 show the concentrations of proteins and of interferon, respectively. Interferon was eluted with 0.1M KCl-HCl at pH 2 with a recovery of 64% and the recovery of protein in E4 in Figure 11 was 0.3% of the proteins applied on the column. Therefore, interferon was purified 200-fold by adsorption chromatography on

porous glass. For purification of interferon, adsorption chromatography on porous glass has become one of the standard methods.

Yip et al. recently purified human γ-interferon (immune interferon) on porous glass (CPG-10, 350Å)(48). The crude γ-interferon contained in a culture was mixed with CPG beads for several hrs and then packed into a column. The column was washed with phosphate buffered saline and eluted with 10 column volumes of 20mM sodium phosphate (pH 7.4)-0.15M NaCl-20% of ethyleneglycol. Chadha and Sulkowski reported that efficient elution of human α-interferon was accomplished with tetramethyl- or tetraethyl-ammonium chloride after protein impurities in the interferon preparation were washed from the column with phosphate or 0.1M Tris-buffer at pH 8.0 (49).



Chlorophyll-Protein Complex

Chlorophyll-protein complex was purified from Barley thylakoid membrane on a CPG-10 (350Å) column (0.9 cm x 14 cm)(50). Thylakoid membranes containing 0.5 mg chlorophyll, prepared from normal barley, were dissolved in 2.5 ml of 1% Triton X-100 in 50mM Tris-HCl at pH 8.2 and centrifuged at 10,000 x g for 10 min. The supernatant was applied to the column and the column was eluted with several buffers shown in Figure 12 at a flow rate of 0.5 ml/min and a temperature of 4°C. Arrows in Figure 12 indicate the point eluted with 1M KSCN, by which a tightly bound fraction was eluted. Absorbance at 670 nm in Figure 12 shows chlorophyll. In column B in Figure 12, 20-35% of the total protein was eluted as an unbound fraction. 50-60% appeared as a partially bound fraction and 10-15% was in a tightly bound fraction. Thus, the chlorophyll-protein complex was separated on a CPG column from barley thylakoid membranes.

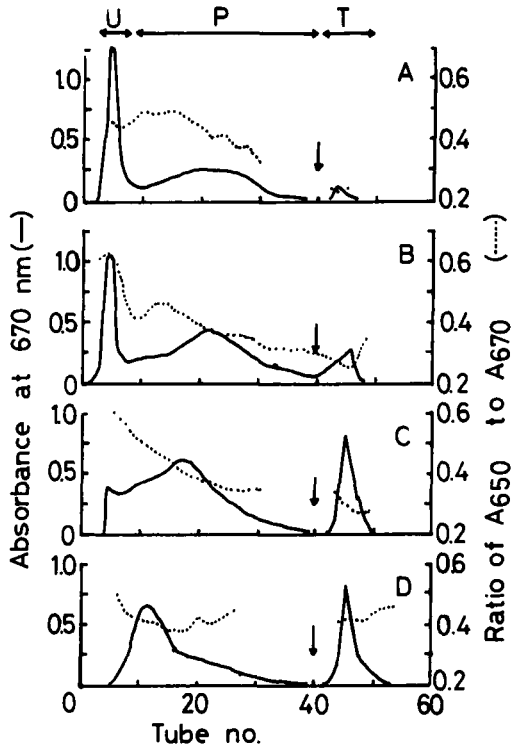


FIGURE 12
The effect of Tris buffer concentration on chlorophyll (——) elution from porous glass columns. Thylakoid membranes from normal barley were solubilized in 10(A), 50 (B), 100 (C), or 200(D) mM Tris buffers at pH 8 containing 1% Triton X-100. There distinct chlorophyll-containing fractions were eluted; an unbound fraction (U), a partially bound fraction (P), and a tightly bound fraction (T). Reproduced with permission from Dunkley and Anderson, Arch. Biochem. Biophys., 193, 469 (1979).

## Glass-Adhering Protein of Macrophages

In this section and the next, the adsorbent discussed is not porous glass but simple glass beads. Lehtinen et al. purified glass-adhering protein from plasma membrane of rat peritoneal macrophage (6), which has the property of adhering on glass surfaces. A fraction (2 mg protein) obtained by discontinuous sucrose

density gradient centrifugation (28/38%, w/v) was applied to a glass-bead (diameter 1 mm) column (1 cm x 18 cm) and allowed to standing for 2 hr. Then, the protein was purified by elution with 8M urea-2% SDS-2% mercaptoethanol.

Hageman Factor (Blood Clotting Factor XII)

Schoenmakers et al. purified blood clotting factor XII on glass beads (4). The blood clotting reaction proceeds by a change of the factor on the glass surfaces. Plasma was incubated with 0.1 volume of almina $C_r$ gel at 37°C for 10 min and then allowed to stand overnight at 4°C. After the almina gel was centrifuged off, the supernatant was loaded on a glass powder column (100-350 mesh) and the column was eluted with glycine-saline buffer at pH 9.6. The Hageman factor was purified by ion exchange chromatography, alcohol precipitation and gel filtration.

Phospholipid-Sensitive $Ca^{2+}$-dependent Protein Kinase

Phospholipid-sensitive $Ca^{2+}$-dependent protein kinase was purified on porous glass from bovine heart (51), as follows. The concentrated enzyme (5.25 g protein/30 ml) from the chromatography on DEAE-cellulose was mixed with 20 ml of packed glass beads previously washed and equilibrated with 20mM Tris buffer, pH 7.5, 2mM EDTA and 50mM mercaptoethanol. After the beads had settled, the remaining solution was poured off. The beads were washed once with 200 ml of the above buffer and then washed five times with 200 ml each of 20mM Tris-HCl at pH 8.3, containing 2mM EDTA, 50mM mercaptoethanol, and 2M NaCl. The enzyme was then eluted eight times with 100 ml each of 250mM Tris-HCl at pH 8.3 containing 50mM mercaptoethanol and 1M $MgCl_2$. The fractions were pooled, centrifuged at 30,000 x g for 10 min, and concentrated to about 150 ml using the Amicon con-

centrating system (YM 30 membrane). The concentrate was dialyzed overnight against 10 liters of the extraction buffer. Specific activity was increased ten-fold.

## STABILITY OF PROTEINS ON POROUS GLASS

Blood clotting starts after changing the Hageman factor on glass surfaces having a negative charge (52). The change of protein adsorbed on glass surfaces is of interest. Table 5 shows the remaining activity of some proteins after keeping them in the state to be adsorbed at 4°C on a small column for a week, a month and three months (53). Those proteins in Table 5 are stable and of commercial standard proteins. The recovery after adsorption for three months is 48% for peroxidase, about 14% for alkaline phosphatase, and 2% for catalase. This order of recovery coincides with that of elution from porous glass (Figure 6). The recovery of the proteins of weak affinity was better than that of strong affinity. A low activity recovered after adsorption and a low recovery of protein due to denaturation during adsorption on porous glass. Since catalase shows a brown color at the top of the porous glass column, the recovery is found by the color on the column. The recovery of aldolase and lysozyme can not be examined because neither can be eluted with any solvents except for those including SDS. Insulin is well adsorbed on porous glass, but it retains almost full activity as determined by the measurement of the glucose-lowering rate in blood, after adsorption on porous glass for three months as shown in Table 5.

The dissociation of alkaline phosphatase into subunits from E. coli after being adsorbed for some period was observed by SDS gel electrophoresis. When it was eluted immediately after being adsorbed, dissociation could not be found. But after being adsorbed for a week or more, it dissociated into subunits. The sub-

TABLE 5

Protein Activity in Eluates After Adsorption onto Porous Glass Column

| Protein | Total activity recovered | | |
|---|---|---|---|
| | 1 week | 1 month | 3 months |
| Horse-radish peroxidase | 97% | 77% | 48% |
| Alkaline phosphatase | 88% | 62% | 14% |
| Catalase | 63% | 26% | 2% |
| Insulin | - | - | 89% |

Reproduced with permission from Mizutani, J. Pharm. Sci., 69, 279 (1980).



unit did not dissociate by the decomposition of peptide bonding even after three months. Thus it has been proven that some changes were found on both the points of activity and the dissociation into subunits on porous glass.

The proteins adsorbed on porous glass have their activity (53). When the substrate solution flows into a porous glass column on which malate dehydrogenase, alkaline phosphatase, peroxidase, or catalase binds, the substrate in the eluate is broken down. But malate dehydrogenase on the glass showed 1/600 the reaction rate of that of the free enzyme. Phosphatase showed 1/5 the reaction rate of that of the free state (53). This low value seems to indicate a change in the high ordered structure of the active sites during adsorption. Drug binding on albumin, which plays a role in drug transfer in blood, was examined using the albumin bound on the glass (54). Aspirin and sulfamethoxazole, which do not bind on glass, bind about 0.4-0.6 mol/mol of albumin initially. After a week, however, it is lowered to 0.16-0.35 mol. This shows the changing of the ordered structure of albumin on glass.

In order to study the state of proteins on glass surfaces, the turbidity of the protein bound on porous glass was measured at 660 nm using a 0.1 cm light path cell, with a double beam spectrophotometer (38). Measurement of solutions containing the precipitated proteins with ammonium sulfate or with 40% ethanol were taken in controlled experiments according to the Cohn et al. method (55). The turbidity of the solution containing the precipitate of albumin with ammonium sulfate at a protein concentration of 5 mg/ml was 0.664 at 660 nm and that in 40% ethanol was 0.465. The turbidity of albumin on glass at the same concentration in a distilled water medium was 0.074 at 660 nm. This value was lower than the value obtained with ammonium sulfate, but the turbidity was distinctly measured at this value. Aldolase and lysozyme on the porous glass also showed similar value of turbidity at 660 nm. This result shows that proteins in the precipitated state clearly bind on glass surfaces (38). CD spectra of proteins adsorbed on glass surfaces also changed (38, 56, 57) and their molar ellipsity decreased to half.



## PROTEIN ADSORPTION ON SILICONIZED POROUS GLASS

### General Consideration

Glass surfaces having interactive properties are siliconized to prevent a blood clotting reaction. Therefore, it is obvious that siliconized surfaces do not adsorb proteins and some silicone polymers and siliconized materials are used for medical purposes (58). In the case of cell separation operations, it is recommended that glassware be siliconized. However, some siliconized surfaces as well as plastic surfaces adsorb proteins as a matter of course and the author shows some results showing protein adsorption on siliconized surfaces.

In the study of clinical chemistry, silicone rubber pieces, whose chemical structure is similar to that of silicone oil, were loaded with antibody and used for antigen estimation (59), including the rhematoid arthritis test, C-reactive protein test and anti-thyroglobulin test. These silicone rubber pieces were used instead of polystyrene latex (60, 61). Brash reported adsorption of proteins on siliconized glass surfaces (62). Proteins were adsorbed on polymers, such as polystyrene (60-64), polyethylene (65-68), polyhydroxymethacrylate (69-70) and block copolymer (62, 71), and those amounts are summarized in Table 6 (62). The adsorption of secretin was prevented by serum albumin(72). Packham et al. reported that platelets bind more strongly on silicone-coated glass surfaces than on non-coated glass surfaces (73).



Preparation and Properties of Siliconized Porous Glass

Siliconized porous glass was prepared as follows (28). After being washed thoroughly with distilled water, the glass was dried at 180°C in an evaporating dish. The silicone oil used was KF 96 (dimethylpolysiloxane), obtained from Shinetsu Chemical Tokyo. One g of the glass was added to 3 ml of carbon tetrachloride containing 0.05 ml of silicone oil and mixed. After evaporation of the carbon tetrachloride, the glass was tightly coated with silicone by heating at 300°C for 5 min. Silicone-coated porous glass floats on water. Therefore, the coated glass was deposited in 0.05M phosphate buffer at pH 7.3 containing 0.7% SDS.

In order to remove SDS from the glass, the deposited glass was packed in a column and washed with about 100 column volumes of degassed hot water. If the washing is carried out by decantation, part of the deposited glass floats, and the glass lacking SDS or not binding

TABLE 6
Adsorption of Albumin to Various Surfaces

| Surface | Steady State Surface Concentration ($\mu g/cm^2 \pm$S.D.) |
|---|---|
| Polyethylene | 0.18±0.005 |
| Polystyrene | 0.18±0.04 |
| Siliconized glass | 0.18±0.004 |
| Glass | 0.04±0.037 |
| Segemented polyurethane | 0.60±0.17 |
| Hydrophilic polyurethane | 0.02±0.009 |
| Collagen-coated glass | 0.09±0.007 |

Reproduced with permission of Brash, Ann. N.Y. Acad. Sci. 283, 356 (1977).



SDS also floats on contact with air. Therefore, glass in the deposited state should be washed in a column used for exclusion chromatography. After washing with distilled water, it was confirmed that SDS did not remain on the glass by elution with 0.2% barium chloride solution. After adsorption saturation of SDS on the glass surfaces, SDS bound on the glass was completely washed by elution with distilled water (74). But other detergents including Triton X-100, Brij 35, benzalkonium chloride and hard soap were not removed from the columns by washing with hot distilled water.

Protein adsorption on a siliconized porous glass column was determined by the modified frontal analysis method. Protein solutions at a concentration of 1 mg/ml were applied on the column in a distilled water medium or in phosphate-buffered saline (75). Albumin, globulin, lysozyme, hemoglobin and peroxidase were well adsorbed in phosphate-buffered saline with 83-132 mg/100 $m^2$ of siliconized surfaces. Meanwhile, those proteins

TABLE 7
Amounts of Bovine Serum Albumin Adsorbed on Siliconized Porous Glass in Buffers

| Buffer | Ionic Strength | pH | Amount (mg/40 $m^2$) |
|---|---|---|---|
| Distilled water | 0 | - | 1.2 |
| Phosphate buffered saline | 0.16 | 7.2 | 42 |
| 0.1M Citrate | 0.20 | 3.0 | 32 |
| 0.5M Tris-HCl | 0.05 | 9.0 | 31 |
| 0.01M Phosphate | 0.026 | 7.3 | 1.0 |
| 0.5M Phosphate | 1.3 | 7.3 | 32 |
| 0.01M Phosphate-0.05M NaCl | 0.076 | 7.3 | 20 |
| 0.01M Phosphate+2M NaCl | 2.026 | 7.3 | 43 |
| 0.1M Tris-HCl | 0.09 | 7.6 | 41 |
| 0.1M Acetate | 0.1 | 7.0 | 19 |
| 0.5M Acetate | 0.45 | 5.6 | 36 |
| 0.2M Glycine | 0.01 | 8.0 | 2.4 |
| 0.5M Glycine | 0.1 | 9.0 | 28 |
| 20% Sucrose | - | - | 10 |

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except peroxidase were not adsorbed on siliconized surfaces in a distilled water medium. Protein adsorption on siliconized surfaces in salt solutions must depend on hydrophobic interactions between aliphatic and aromatic amino acids and silicone residues. The observed adsorption coincides with micelle formation of SDS at high salt concentrations (76).

Protein adsorption on siliconized surfaces was not influenced by pH changes in the solutions as shown in Table 7. Albumin also adsorbed on siliconized surfaces in a urea solution (76). Figure 13 shows the relationship between the amount of albumin adsorbed and the salt con-

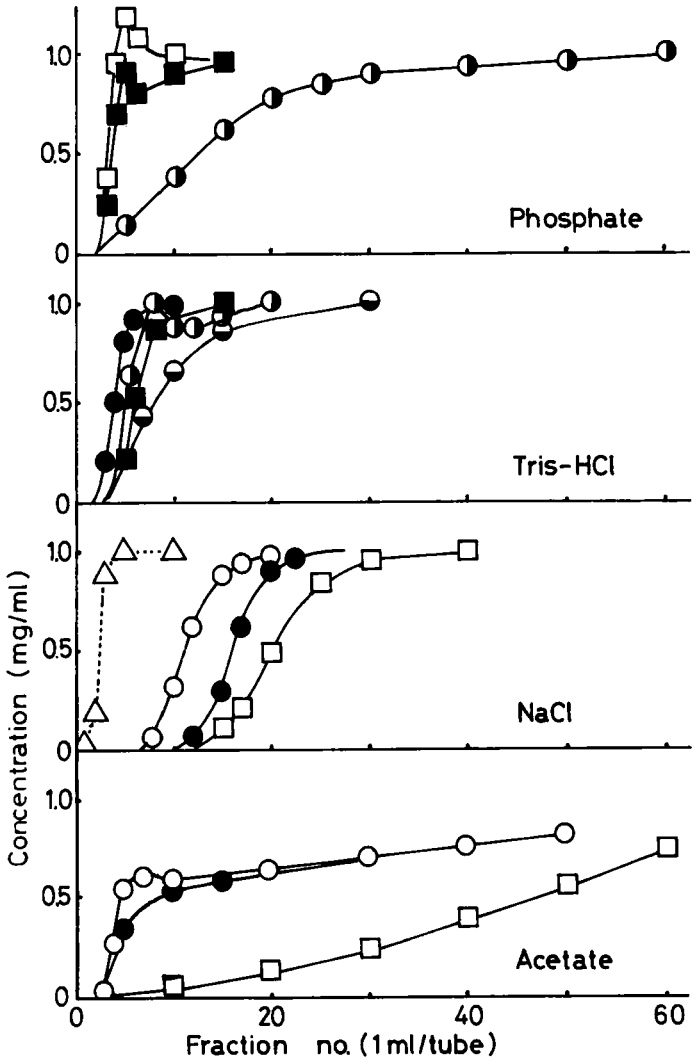


FIGURE 13
Adsorption patterns of BSA (1 mg/ml) on a siliconized porous glass column (0.75 cm x 4.5 cm) at room temperature. The concentration of salts are: ○, 2mM; ●, 5mM; □, 10mM; ■, 20mM; ◑, 50mM; ◒, 100mM; △, distilled water. Reproduced with permission from J. Colloid Interface Sci., 82, 162 (1981).

centration. The albumin adsorbed on siliconized surfaces at high salt concentrations, but the amount differed depending on the kind of salt.

The amounts of insulin, atropine sulfate, physostigmine salicylate and diazepam adsorbed on the siliconized surfaces were more than those on non-coated glass surfaces (75, 77). The amounts of atropine (0.81 mg/100 $m^2$) and physostigmine (3.88 mg/100 $m^2$) on siliconized surfaces in a saline medium are greater than those of atropine (0.05 mg/100 $m^2$) and physostigmine (0.08 mg/100 $m^2$) on non-coated glass surfaces. Insulin adsorbed well (71 mg/100 $m^2$) onto the siliconized surfaces in a pH 2.6 isotonic glycerin solution and (6.14 mg/100 $m^2$) on a non-coated glass surface in isotonic solution (78). Thus, the siliconized surface adsorbs more drugs than the non-coated glass surface. Diazepam, a most useful minor tranquilizer, adsorbs well on the surfaces of plastic bags made of cellulose propionate (79, 80).

Proteins have different affinities for siliconized surfaces as shown in Figure 14 (75). The upper column in Figure 14 shows the results of an adsorption pattern of a mixture of albumin and peroxidase (indicated by $A_{403}$). Peroxidase had weaker affinity than albumin. The lower column included a mixture of hemoglobin (indicated by $A_{541}$) and albumin. The results showed that albumin had a weaker affinity than hemoglobin. The order of affinity of the three proteins is peroxidase< albumin<hemoglobin.

In order to study the mechanism of protein adsorption on siliconized surfaces, the inhibition of adsorption with amino acids was studied and the results are shown in Table 8 (76). Acidic amino acids, such as aspartic acid and glutamic acid, prevented protein adsorption on silicone-coated surfaces. The reason for this is not clear. Aliphatic and aromatic amino acids were

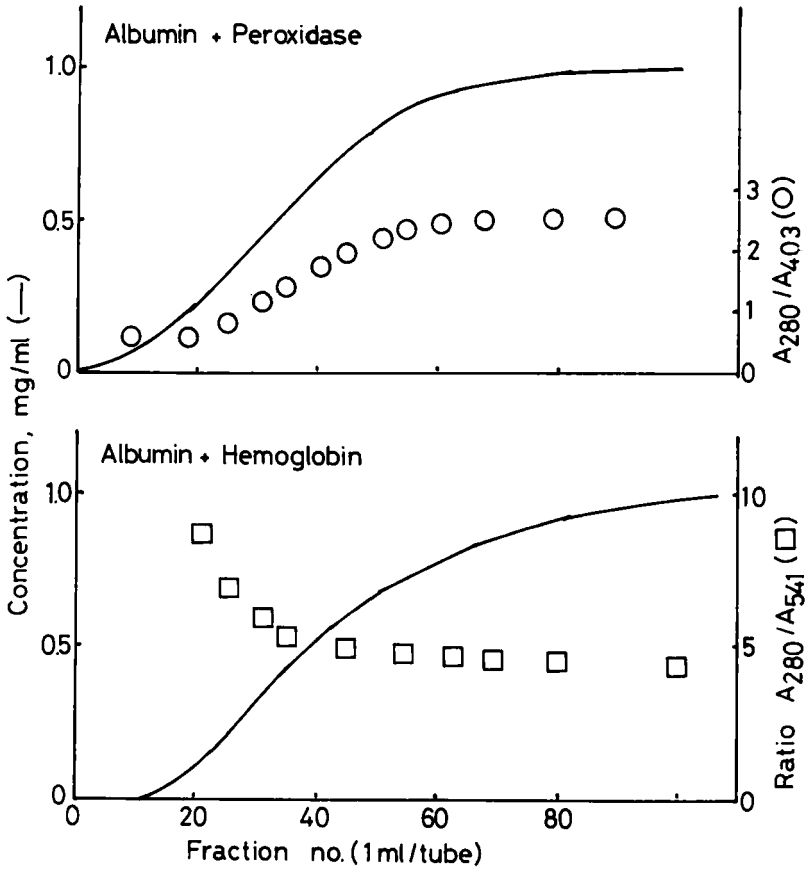


FIGURE 14
Adsorption patterns of mixtures on siliconized glass column in phosphate-bufferd saline at pH 7.2. Each protein of 0.5 mg was used at a concentration 0.5 mg/ml. Peproduced with permission from J. Pharm. Sci., 70, 493 (1981).

TABLE 8
Amino Acid-Induced Prevention of Protein Adsorption to Silicone-coated Glass

| Amino acid | Amount (mg/40 $m^2$) adsorbed | | |
|---|---|---|---|
| | Hemoglobin | Lysozyme | Albumin |
| Glu | 1.3 | 1.3 | 0.7 |
| Arg | 6.8 | 3.0 | 1.1 |
| Ile | 6.7 | 2.8 | 1.3 |
| Leu | 6.3 | 2.9 | 1.3 |
| Met | 6.0 | 2.7 | 1.2 |
| Ser | 6.3 | 3.2 | 1.1 |
| Phe | 6.8 | 3.0 | 1.2 |
| Trp | 5.0 | - | - |
| Asp | 0.5 | 1.4 | 0.9 |
| Asn | 5.5 | 3.2 | 1.0 |

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not effective enough to prevent protein adsorption as shown in Table 8.

The comparison of protein adsorption properties on siliconized surfaces and non-coated surfaces is shown in Table 9. (76). There are some differences between them, depending on affinity in solutions and stability of proteins. Proteins adsorbed more on siliconized surfaces in the solution containing salt than in distilled water. Proteins bind on non-coated glass surfaces more in pure water than on coated surfaces. These differences between protein adsorption on siliconized surfaces and non-coated surfaces in Table 9 is mainly dependent on the bonding between the proteins and surfaces and on the properties of the proteins themselves in solution.

TABLE 9
Protein Adsorption to Silicone-Coated and Non-Coated Porous Glass

| Property | Coated glass | Non-coated glass |
|---|---|---|
| Bonding | Hydrophobic interactions | Ionic bonding and cohesive force |
| Affinity in solutions | water<salt | water>salt |
| Maximum proteins adsorbed to 100 $m^2$ | 153 mg | 240 mg |
| Effect of urea | None | None |
| Stability of proteins adsorbed | Stable | Labile |
| Adsorption-inhibiting amino acid | Glu and Asp | None |

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## PROTEIN SEPARATION ON SILICONIZED POROUS GLASS

Protein separation on siliconized porous glass was attempted as shown in Figure 15 (81). Figure 15 the result of the elution of a mixture of albumin (5 mg) and hemoglobin (5 mg) on a silicone-coated porous glass column. Dashed lines in Figure 15 show the absorbance at 541 nm of hemoglobin. The upper column (A in Figure 15) is the result of elution with 0.01M NaCl, the middle (B in Figure 15) is that with 5mM Tris-HCl, and the lower (C) is that with 5mM Tris-HCl-10mM glutamic acid. Albumin was separated from hemoglobin in the middle column even though the yield was lower. Proteins adsorbed were eluted with SDS solution. Protein adsorbed at a high

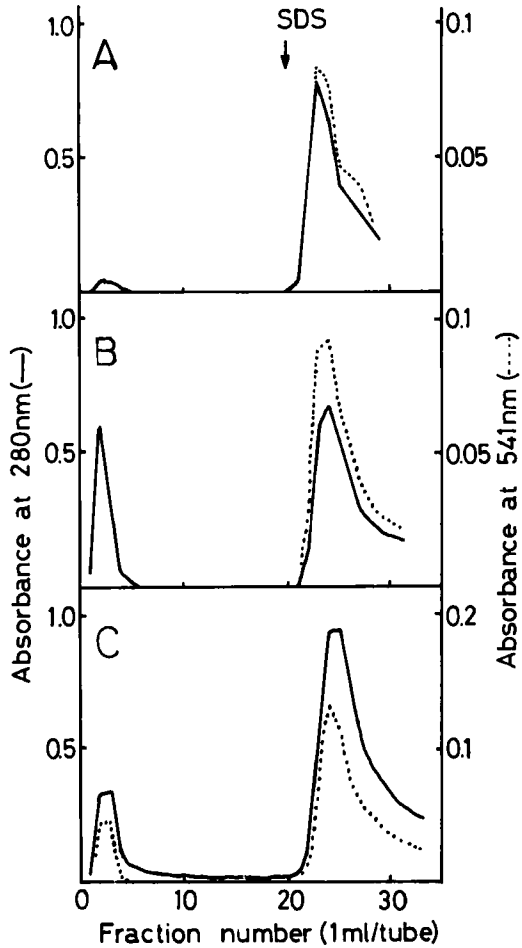


FIGURE 15
Elution profiles of a mixture of albumin and hemoglobin. At fraction 20 of each chromatogram, the columns were eluted with 1% SDS-0.2M phosphate at pH 7.4. Reproduced with permission from J. Chromatogr., 207, 276 (1981).

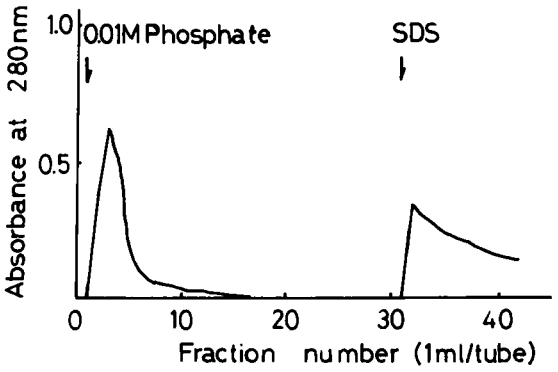


FIGURE 16
An elution profile of globulin (10 mg) on a siliconized porous glass column (0.75 cm x 4.5 cm) in 0.01M phosphate at pH 7.4. Reproduced with permission from J. Chromatogr., 207, 276 (1981).



salt concentration was not eluted with the solution at a low salt concentration.

Figure 16 shows the result of adsorption chromatography of bovine globulin (81). Densitometric protein tracing (electrophoresis) on cellulose acetate showed that $\alpha_2$-globulin in the globulin fraction did not specifically adsorb on siliconized surfaces and was eluted from the column. $\beta$- and $\gamma$-globulins had a stronger affinity on siliconized surfaces. The Fc portion of the surface immunoglobulin had an affinity to hydrophobic residues (71). These results show that some proteins are separable on siliconized surfaces after careful buffer selection. However, the method does not give a complete elution of proteins adsorbed on the glass with natural buffers.

Figure 17 shows an elution pattern of microsomal proteins on siliconized porous glass. Rat liver microsomes, precipitated at 105,000 x g, were diluted ten-fold with 1M NaCl. One ml of the solution (1.1 mg) was applied to a siliconized porous glass column. The

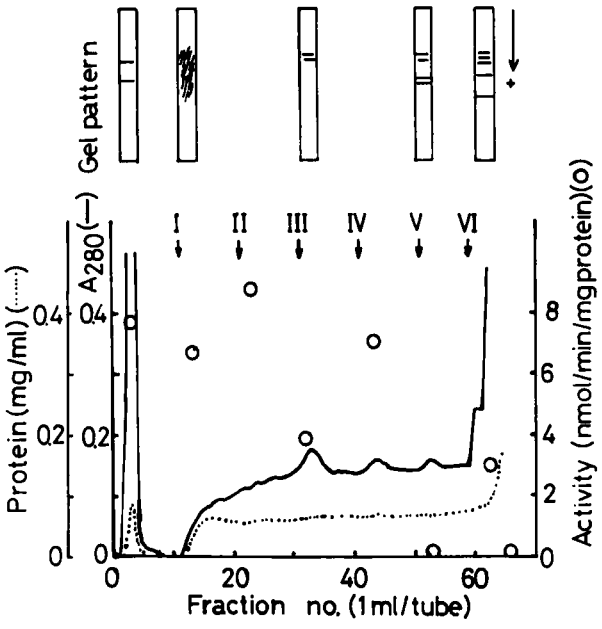


FIGURE 17
Adsorption chromatography and disc gel patterns of microsomal proteins on siliconized porous glass. Buffers for elution are as follows: I, 0.05M Tris-HCl-20% glycerol (pH 7.6); II, 0.125% sodium cholate in I; III, 0.25% sodium cholate in I; IV, 0.375% sodium cholate in I; V, 0.5% sodium cholate in I; VI, 1% Triton X-100 in I. Activity is that of 1-acylglycerophosphate acyltransferase. Reproduced with permission from J. Chromatogr., 239, 755 (1982).

column was eluted by gradually increasing the concentration of sodium cholate (0.125-0.5%) in 0.05M Tris-HCl-20% glycerol at 0°C. The protein recovery was about 80%. Pattern of disc electrophoresis are also shown in Figure 17. The gel patterns show that proteins in microsomes were separated on siliconized porous glass.

Mcrobial lipases were purified 1000-fold on columns of siliconized glass beads with 58% recovery (83). Adsorbed lipase was eluted from the beads with 0.1% Triton X-100 solution.

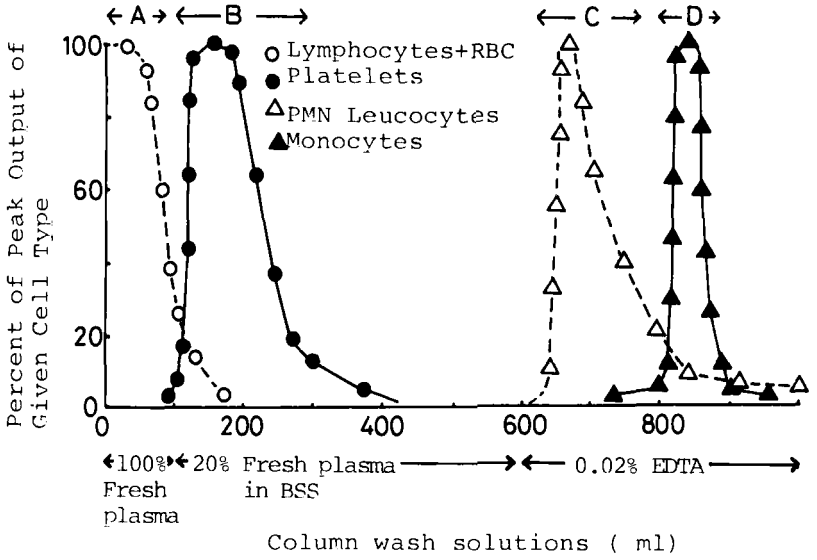


FIGURE 18
Separation of blood cells on a siliconized glass bead column (2.5 cm x 40 cm). Reproduced with permission from Rabinowitz, Blood, 23, 811 (1964).



Cells in blood have phagocytic or adhesive properties. Using these properties, separation of cells was achieved on siliconized beads (84-86). Figure 18 shows one pattern of cell separation (84). Siliconized glass beads of about 100 mesh were packed in a column (2.5 cm x 40 cm). Blood clotting was inhibited by the addition of heparin (0.1 ml of 1% solution per 10 ml of blood). The blood was centrifuged to prepare the cell suspension. A cell solution (75 ml) prepared from 500 ml of blood was applied on a column. Column operation was carried out at 37°C with water jackets for temperature control. Lymphocytes, red blood cells, platelets, polymorphonuclear leucocytes and monocytes were separated on the column as shown in Figure 18.

## ADSORPTION CHROMATOGRAPHY OF NUCLEIC ACIDS ON POROUS GLASS

Many kinds of mRNA were purified on oligo(dT)-cellulose or with similar materials. Globin mRNA in rats was also purified by oligo(dT)-cellulose chromatography (87). Adsorption chromatography of nucleic acids, a technique which is not based on ionic bonding, has been developed on Kel-F (88), $C_{18}$ column (89, 90) and Sepharose (91).

High molecular RNAs, such as rRNA or mRNA, were also separated on porous glass (92, 93) or siliconized porous glass (94-96) with good recovery. RNAs were well adsorbed on those porous glass in 5M NaCl (92). Therefore RNA was loaded on a column in 5M NaCl and then eluted from the column by lowering of salt concentration. Figure 19 shows the pattern of adsorption chromatography of crude rRNA on porous glass (93). RNAs in those fractions in Figure 19 were analyzed by electrophoresis on 8% polyacrylamide gel. The Major RNA in fraction 1 of Figure 19 was tRNA. Fraction 2 contained 5S rRNA and rRNA. The electrophoretic patterns of fractions 4 and 5 showed that those fractions contained high molecular rRNA. tRNA was adsorbed on porous glass in 5M NaCl at pH 4.1 and eluted at 3M NaCl (92). tRNA digested with pancreatic ribonuclease A was passed through the column and $Q_\beta$ RNA was eluted at 2M NaCl (92) as shown in Figure 20. These results suggest that a high molecular weight of RNA is essential for adsorption to porous glass surfaces in 5M NaCl. A half of DNA was adsorbed and the residual was passed through. This system is better for separation of RNA and the recovery was very good.

Siliconized porous glass (1 g, 2 ml) adsorbs 32 mg of RNA in 5M NaCl-10mM Tris-HCl at pH 7.6 (96). Bovine liver tRNA (2.5 mg) was chromatographed on a column

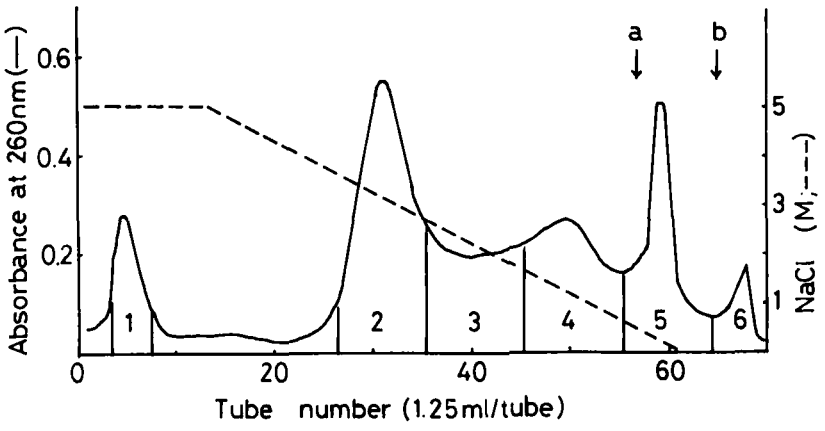


FIGURE 19
Chromatographic pattern of crude rRNA (15 $A_{260}$ units) on porous glass column (0.6 cm x 9 cm). Elution was carried out at 28°C with a linear gradient (total volume 60 ml) from 5M NaCl in 10mM Tris-HCl at pH 6.3 to 10mM Tris-HCl at pH 7.6 at a flow rate of 1.5 ml/$cm^2$/min. At points a and b, 10mM Tris-HCl at pH 7.6 and 1% SDS in the buffer, respectively, were eluted on the column. Reproduced with permission from J. Chromatogr., 262, 441(1983).



(0.5 cm x 3 cm) with a linear gradient (total volume 50 ml) from 5M NaCl to 0M in 10mM Tris-HCl at pH 7.6 as shown in Fig. 21 (96). These tRNAs in each fraction were purified 5-10 fold over those in crude tRNA. Finally, the order of elution of tRNA species from the column was $tRNA^{Pro}$, $tRNA^{Val}$, $tRNA^{Ile}$, $tRNA^{Thr}$, $tRNA^{Ser}$, and $tRNA^{Phe}$. The amino acid acceptor activity was not found in fractions 5 and 6, in which high molecular weight impurities must be present, as described later. Thus, it was found that tRNAs were fractionated on the siliconized porous glass column by decreasing NaCl concentrations in the mobile phase and that adsorption of tRNA on the glass was reversible.

The order of elution of tRNA species from the column was compared with the results on RPC-5 or BD-cellulose

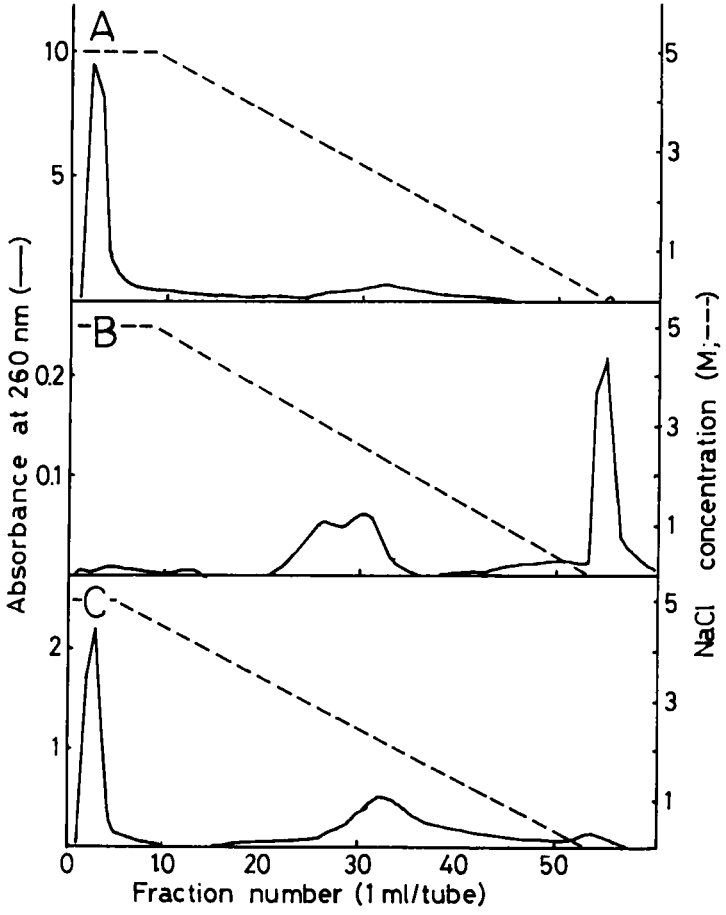


FIGURE 20

Elution patterns from a porous glass column of tRNA digested with pancreatic ribonuclease A, $Q_\beta$ RNA, and calf thymus DNA. The conditions of elution were identical with those in Figure 19. (A) tRNA digested with ribonuclease A (20 $A_{260}$ units), (B) $Q_\beta$ RNA (1.2 $A_{260}$ units), (C) calf thymus DNA (10 $A_{260}$ units); ——, Absorbance at 260 nm; – – –, molarity of NaCl.

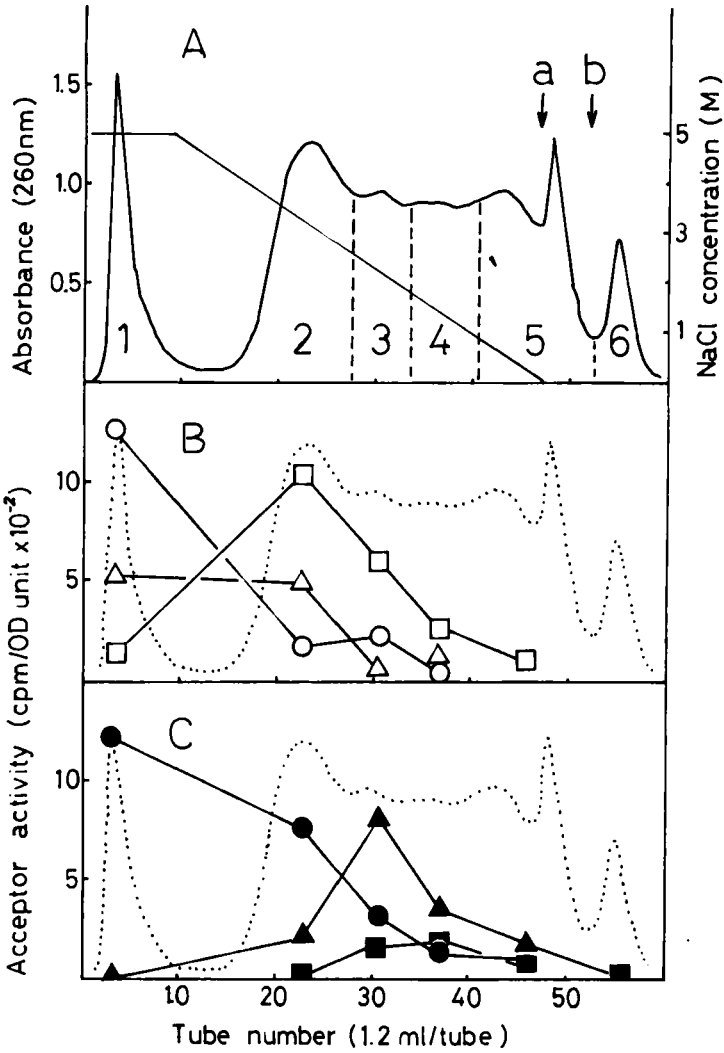


FIGURE 21

Chromatography of tRNA (2.5 mg) on siliconized porous glass. The column (0.5 cm x 3 cm) was eluted with a linear gradient (total volume 50 ml) from 5M NaCl to 0M in 10mM Tris-HCl at pH 7.6. In figure A, absorbance at 260 nm is shown by a solid line and NaCl concentration by a thin line. Arrow a is the point of elution with 10mM Tris-HCl at pH 7.6 and b is the point of elution with 1% SDS in phosphate buffer at pH 7.3. The amino acid-accepting activities of tRNA in fractions of A are shown in B and C and the $A_{260}$ in figure A is shown by broken lines. ○, proline; △, isoleucine; □, threonine; ●, valine; ▲, serine; ■, phenylalanine. Reproduced with permission from J. Biochem., 94, 163 (1983).

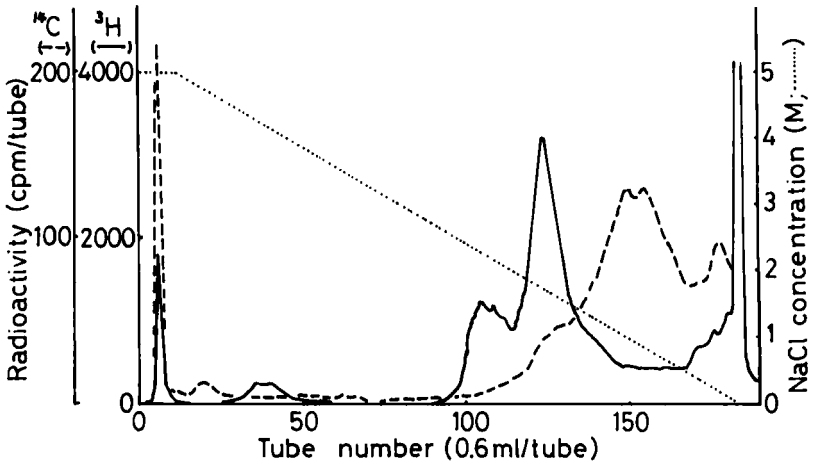


FIGURE 22
Elution patterns of [$^{14}$C]seryl-tRNA and [$^{3}$H]methionyl-tRNA on an siliconized prous glass column (0.3 cm x 30 cm). The soild line indicates [$^{3}$H]methionyl-tRNA and the broken line indicates [$^{14}$C]seryl-tRNA. Elution was carried out with a linear gradient (total volume 100 ml) of 5-0M NaCl in 10mM sodium acetate at pH 4.6. Reproduced with permission from J. Biochem., 94, 163 (1983).



(98). The order of elution of yeast tRNA species from BD-cellulose was tRNA$^{Ile}$, tRNA$^{Val}$, tRNA$^{Pro}$, tRNA$^{Thr}$, tRNA$^{Ser}$, and tRNA$^{Phe}$. On both the siliconized glass and BD-cellulose, tRNA$^{Ser}$ and tRNA$^{Phe}$ were eluted later. tRNA$^{Pro}$ showed the greatest difference in behavior, because the tRNA was eluted first from the siliconized glass column. The order of elution of aminoacyl-tRNAs of mouse liver on RPC-5 chromatography (99) was similar to that on BD-cellulose described above. The siliconized porous glass was a better stationary phase (giving a higher flow rate) than BD-cellulose and RPC-5.

Figure 22 shows the results of cochromatography of [$^{14}$C]seryl-tRNA and [$^{3}$H]methionyl-tRNA on the glass by elution with decreasing NaCl concentrations in acetate buffer at pH 4.6. The aminoacyl-tRNA, prepared by the

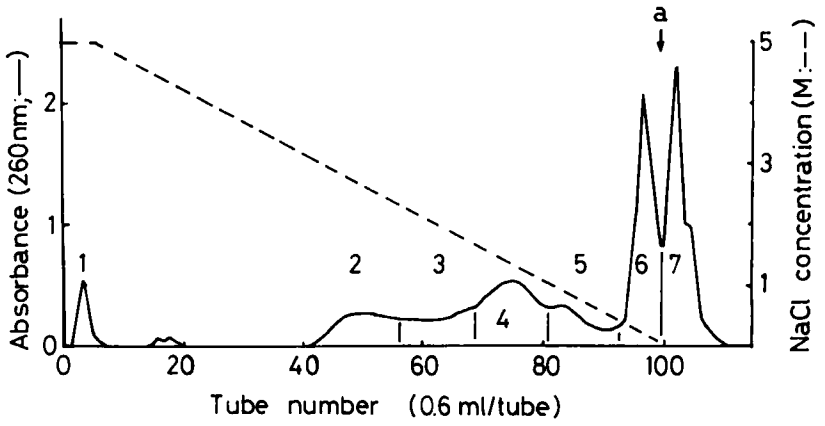


FIGURE 23
Elution profile of E. coli crude rRNA (27 $A_{260}$ units) on an siliconized porous glass column (0.3 cm x 30 cm). Elution was performed at 24°C with a linear gradient (total volume 60 ml) from 5M NaCl to 10mM Tris-HCl at pH 7.6. At point a, 10mM Tris-HCl was applied to the column. Reproduced with permission from J.Biochem., 94, 163 (1983).



usual method, was applied to a column and eluted with a linear gradient from 5M NaCl to 0M in 0.01M sodium acetate buffer at pH 4.6 (flow rate, 2 ml/$cm^2$/min; 13°C; fraction volume, 0.6 ml). As shown in Figure 22, [$^3$H]methionyl-tRNA was eluted at three points, 4.2M, 2.3M, and 1.8M NaCl, and [$^{14}$C]seryl-tRNA was eluted at around 1M NaCl as two peaks. Thus, [$^3$H]methionyl-tRNA was clearly separated from [$^{14}$C]seryl-tRNA, and isoaccepting species of each tRNA were also separated. $tRNA^{Ser}$, not aminoacylated, was eluted with 2.3M NaCl at pH 7.6 (Figure 21), but seryl-tRNA was eluted with 1M NaCl at pH 4.6 at a similar temperature (Figure 22). This difference in NaCl concentration required to elute may depend on the aminoacyl residues on tRNA or the pH value of the solvent. Figure 23 shows the elution profile of crude rRNA on a siliconized porous glass column. Fractions 1

and 2 contained tRNA and fraction 4 contained 5S rRNA (96). The major RNA in fractions 6 and 7 was rRNA. Thus, RNAs of high molecular weight were sepaparted on porous glass or siliconized glass but these support might not be suitable for the fractionation of DNA.

## RPC-5 TYPE CHROMATOGRAPHY ON POROUS GLASS

The mechanism of information exchange between nucleic acids and proteins (tRNA and aminoacyl-tRNA synthetase) is unknown (100). For the purification of aminoacyl-specific tRNA, two chromatographic systems are used: One uses benzoylated DEAE-cellulose (BD-cellulose) in ion exchange-affinity chromatography (98) and the other is RPC-5 chromatography as reversed-phase chromatography (97). The chromatographic patterns of several mouse plasmacytoma tRNA's on RPC-5 columns were compared (99) and organ-specific elution patterns of tRNA on RPC-5 columns were also reported (101). RPC-5 type adsorbent was prepared from siliconized porous glass (102), as follows.

The siliconized glass (10 g) was shaken for 2 hr according to the literature (97) with 20 ml of chloroform containing 0.4 ml of Adogen 464 (a trialkylmethylammonium chloride with the predominant chain length of the alkyl groups being $C_8$-$C_{10}$)(Ashland Chem. Co.). The slurry of siliconized glass in chloroform was dried in a glass tray. The glass treated with Adogen 464 immediately sank in an aqueous medium even though siliconized porous glass floated on the surfaces of water.

The siliconized glass coated with Adogen 464 was suspended in a solution composed of 0.45M sodium chloride, 0.01M magnesium chloride, 0.01M Tris-HCl buffer at pH 7.6

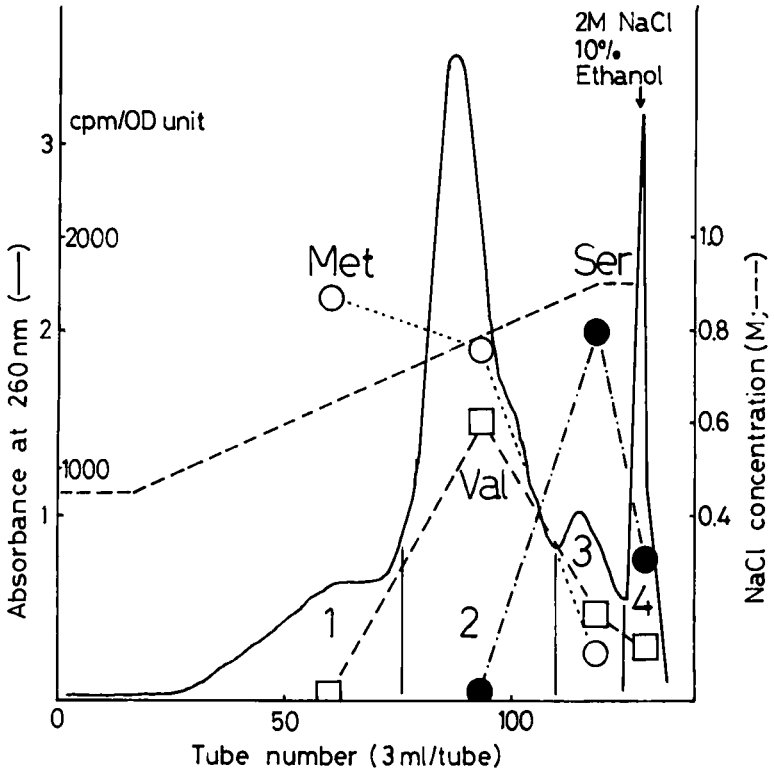


FIGURE 24
Elution profile of crude tRNA (20 mg) on siliconized porous glass coated with Adogen 464. Column, 0.8 cm x 45 cm). Elution was carried out at 37°C with a linear gradient (total volume 300 ml) from 0.45 to 0.9M sodium chloride in 0.01M magnesium chloride, 0.01M Tris-HCl (pH 7.6) and 1mM β-mercaptoethanol at a flow rate of 0.6 ml/cm$^2$/min. Amino acid acceptor activity of tRNA is shown by cpm/absorbance unit as follows; ○, Met; □, Val; ●, Ser. Reproduced with permission from J. Chromatogr., 236, 513 (1982).

and 1mM β-mercaptoethanol. After being washed with the solution, the glass was packed in a column having a water jacket maintained at 37°C. A sample of tRNA was dissolved at a concentration of 10 mg/ml in the above solution, elution was carried out with a linear gradient of sodium chloride at a flow-rate of 0.6 ml/cm$^2$/min.

Figure 24 shows the results of chromatography at 37°C of bovine liver tRNA on a column of the glass. The activity of tRNAs accepting methionine, valine and serine is also shown in Figure 24. Fraction 1 did not contain $tRNA^{Val}$ and $tRNA^{Ser}$, but contained $tRNA^{Met}$. $tRNA^{Val}$ and $tRNA^{Ser}$ were contained in fractions 2 and 3, respectively. Thus, tRNA species were separated on the siliconized glass coated with Adogen 464. The order of elution of tRNA species from the glass column is methionine, valine and serine, which is similar to that on an RPC-5 column (97). However, the concentration of sodium chloride necessary for eluting tRNA on the glass column is higher than the system on an RPC-5 column. The recovery of tRNA was about 90%.



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