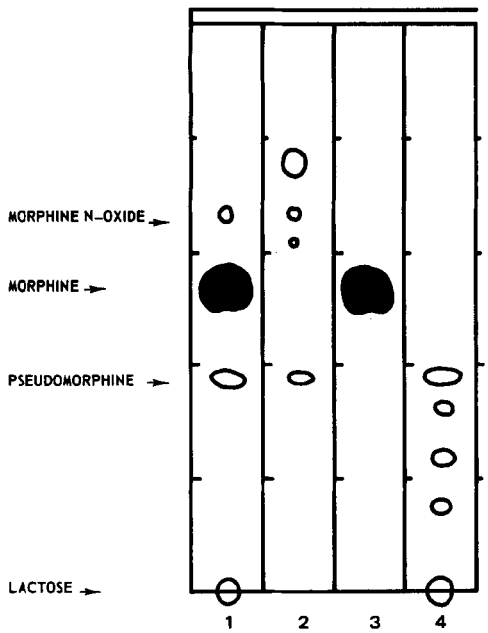

Figure 9—*Chromatogram showing the separation of morphine from decomposition products in morphine tablets. Key: 1, mixture of morphine sulfate, lactose, morphine* N*-oxide, and pseudomorphine; 2, 20 μl of chloroform eluate; 3, 20 μl of chloroform–isobutyl alcohol eluate; and 4, 20 μl of 0.05* N *HCl–methanol eluate.*

trap layer elutes morphine *N*-oxide and other impurities. Morphine, which is partially eluted by the chloroform, is retained by an acidic trap layer (1 *M* phosphate buffer, pH 6.5) and is finally eluted from the column using 15% (v/v) isobutyl alcohol in chloroform. Other tablet ingredients and remaining decomposition products are retained on the column.

On freshly manufactured lots, a direct dilution spectrophotometric assay should be possible. On aged lots, a separation step is necessary (Fig. 7).

The procedure presented was monitored by TLC to check for completeness of separation. In every instance, complete and clean separation of intact morphine was obtained from other substances either present or added (Figs. 8 and 9).

Recovery of morphine from the standard formulations was quantitative (Table II). Morphine was completely and cleanly separated from its decomposition products and lactose (Fig. 9). Seven samples of tablets from two manufacturers were assayed by the column chromatographic procedure (Table I). The results obtained by direct dilution and spectrophotometry (Table I) support the enhancing effect of interfering substances on the morphine assay results as obtained at the maximum at 285 nm. The USP XVIII procedure gave consistently higher assay values than those obtained by the proposed method. The column chromatographic procedure was applied successfully to determine content uniformity of two lots of morphine tablets. Both lots met the USP requirements for content uniformity (Table III).

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Estimation of Adsorption of Drugs and Proteins on Glass Surfaces with Controlled Pore Glass as a Reference

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Abstract □ The amount of drugs adsorbed to glass surfaces was studied, with controlled pore glass as a reference standard, by elution of a solution containing each drug on a pore glass column. The amount of basic drugs, such as epinephrine, physostigmine, and atropine, adsorbed was 1.5–2.0 mg/g of controlled pore glass (97 m^2) in a distilled water medium, but this amount in physiological saline or 0.1 *M* tris(hydroxymethyl)aminomethane hydrochloride (pH 8.6) was negligible. Ascorbic acid, barbital, aspirin, sulfamethoxazole, and acetylcholine were minimally adsorbed to the glass in a water medium or a physiological saline medium. Insulin was adsorbed in a water medium and a glycerin isotonic solution medium at pH 2.6; the amount was 5.5–5.9 mg/g of glass. For clarification of the adsorption mechanism of protein drugs, adsorption of proteins such as bovine serum albumin, chymotrypsin, and lysozyme to glass surfaces was investigated under the various conditions. The maximum amounts of proteins adsorbed on 1 g of controlled pore glass in a distilled water medium were 136, 233, and 84 mg, respectively. The two major forces for adsorption of proteins were ionic amine–silanol bonding and a cooperative cohesive force between proteins and glass. The amount of drugs and proteins to be adsorbed on the inner surface of a glass container and conditions for preventing this adsorption are discussed.

Keyphrases □ Adsorption—various drugs and proteins to glass surfaces studied using controlled pore glass, effect of pH □ Glass surfaces—adsorption of various drugs and proteins studied using controlled pore glass

Adsorption of biological materials on glass surfaces is well known, *e.g.*, the blood clotting reaction (1), macrophage adhesion (2), an antigen-coated column (3), and low adhesion of transformed cells (4). Controlled pore glass was developed for exclusion chromatography (5), and adsorption chromatography of proteins on controlled pore glass was recently reported (6, 7). Applications of porous glass as a carrier for immobilized enzyme were investigated, and the forces involved in the reactions between proteins and glass surfaces were suggested to be ionic amine–silanol bonding and hydrogen bonding (8).

Adsorption of biochemical materials on glass was studied (9) to clarify the adsorption mechanisms. It was reported that about 5 μmoles of cationic biological materials, such as basic amino acids and amino sugars, were adsorbed on 1 g of controlled pore glass, which had a large

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surface area (97 m^2/g) and a high flow rate, and that ionic amine–silanol bonding was one major force of the reactions (9).

Standards for pharmaceutical glass containers, in which drugs are stored or maintained, are provided in the USP (10) and the JP (11). However, the kind and amount of drugs adsorbed on the glass surface of a container have not been defined. The purpose of this investigation was to study the kind and amount of some drugs and proteins adsorbed on glass surfaces using controlled pore glass.

EXPERIMENTAL

Methods—The controlled pore glass[1] was composed of 100-μm particles with a pore diameter of 240 Å and a surface area of 97 m^2/g. After being washed with a chromic acid mixture, water, and 60% nitric acid and then washed thoroughly with distilled water to neutrality, the glass was packed in a 0.65 × 11-cm column. Solutions containing a drug or protein, 0.05–0.8 mg/ml, were applied onto the column at a flow rate of 0.5 ml/cm^2/min, and 1.6-ml fractions were collected.

The amount of sample adsorbed on the glass surfaces was estimated from the loss of the sample in the eluate from the solution applied onto the pore glass column; this value was corrected by subtracting the column volume, which was calculated from the void and inner volumes of the column obtained by elution of 0.1 ml of bromphenol blue or dextran marker[2]. The surface areas of the glass tube column and other glassware were neglected because they were very small.

Adsorption experiments were carried out at room temperature, but the adsorption profiles of proteins and basic biological materials at 30° were identical with those at 4°, as described previously (7).

Materials—The drugs and analytical wavelengths were: insulin[3], 280 nm; atropine sulfate[4], 220 nm; barbital[4], 230 nm; epinephrine[5], 280 nm; physostigmine salicylate[6], 260 nm; sulfamethoxazole[7], 300 nm; aspirin[8], 280 nm; acetylcholine[5], 210 nm; and ascorbic acid[4], 290 nm. These drugs were dissolved in distilled water, physiological saline, or pH 8.6 tris(hydroxymethyl)aminomethane hydrochloride buffer in concentrations of 0.05–0.8 mg/ml and loaded on the pore glass column. Insulin was dissolved in a pH 2.6 glycerin isotonic solution.

Bovine serum albumin[9], chymotrypsin[10], and lysozyme[11] were dissolved in water, 6 *M* guanidine hydrochloride[5], or 8 *M* urea[4], and the solution pH was adjusted with 0.1 *N* NaOH or 0.1 *N* HCl to the particular pH as shown in the figures. The concentration of the protein solutions was determined with an extinction coefficient of 6.7 for $E^{1\%}_{1cm}$ of bovine serum albumin (12), 20.7 for chymotrypsin (13), and 26.4 for lysozyme (14) at 280 nm. Succinylated bovine serum albumin was prepared by the method of Habeeb *et al.* (15). The adsorption of leucine and glycine[12] was studied at pH 2.6, 6.0, and 10.3, and the concentration of these amino acids was determined by the color reaction with ninhydrin.

RESULTS AND DISCUSSION

Drug Adsorption—The control experiments illustrated in Figs. 1 and 2 show the eluting position (tube 2) of the dextran marker and bromphenol blue, by which the void and inner volumes of the column were obtained, respectively. Epinephrine in water was eluted at tube 35 (Fig. 1). Epinephrine was eluted at tube 3 in the tris(hydroxymethyl)aminomethane hydrochloride buffer and saline media and was minimally adsorbed to the glass. Epinephrine also was not adsorbed to the glass in the physiological saline containing bisulfite and chlorobutanol.

The adsorption patterns of atropine and physostigmine are shown in Fig. 1. Both drugs were adsorbed to the glass in a water medium, and the amount in a tris(hydroxymethyl)aminomethane hydrochloride buffer medium was lower than that in a water medium. The amount of atropine and physiostigmine adsorbed to the glass in the saline solution was low and negligible. Measurement of absorbance at 295 nm showed that salicylate ions in physostigmine salicylate molecules were not adsorbed to porous glass in water and eluted at tube 2, even though physostigmine was adsorbed and eluted at tube 10 (Fig. 1). Figure 1 also shows the adsorption patterns of insulin in water at pH 2.6 and a pH 2.6 glycerin isotonic solution. Insulin was well adsorbed in both media.

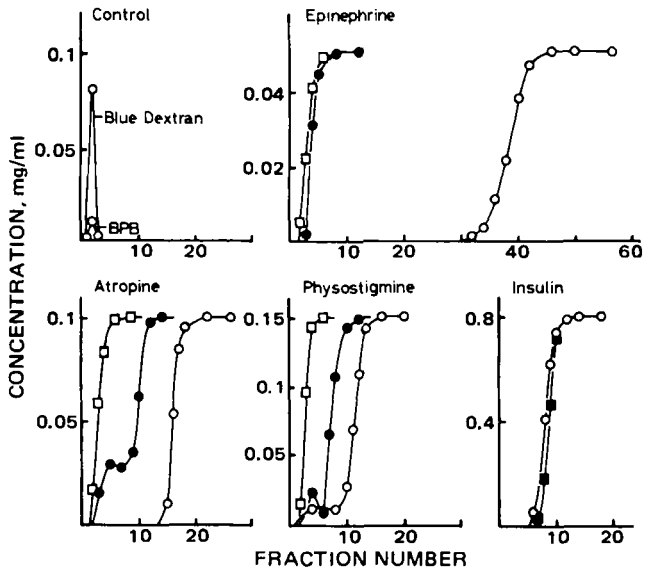

Figure 1—*Adsorption profiles of drugs on controlled pore glass. Key: ○, distilled water; ●, 0.1 M tris(hydroxymethyl)aminomethane hydrochloride, pH 8.6; □, physiological saline; and ■, glycerin isotonic solution. The column size was 0.65 × 11 cm, the fraction volume was 1.6 ml, BPB is bromphenol blue, and blue dextran is a dextran marker of high molecular weight.*

The amounts of drugs adsorbed on 1 g of controlled pore glass are shown in Table I. The adsorption of basic drugs, such as epinephrine, atropine, and physostigmine, to glass surfaces in water was 1.5–2.0 mg (about 3–10 μmoles)/g of glass. The amount of basic drugs adsorbed to the inner surface (50 cm^2) of a glass container (20 ml) in water was about

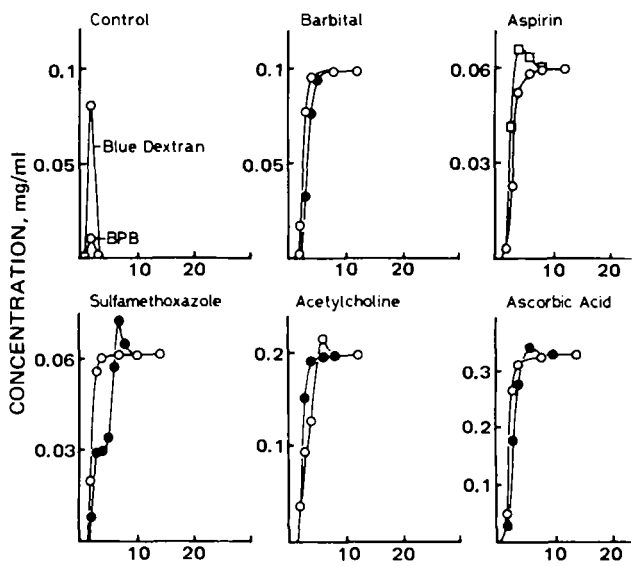

Figure 2—*Elution profiles of drugs on controlled pore glass. Key: ○, distilled water; ●, 0.1 M tris(hydroxymethyl)aminomethane hydrochloride, pH 8.6; and □, physiological saline. The column size was 0.65 × 11 cm, the fraction volume was 1.6 ml, BPB is bromphenol blue, and blue dextran is a dextran marker.*

[1] CPG-10, Electro-Nucleonics, Fairfield, N.J.
[2] Blue Dextran 2000, Pharmacia Fine Chemicals, Uppsala, Sweden.
[3] Fluka AG., Buchs, Switzerland.
[4] Wako Pure Chemicals Ltd., Osaka, Japan.
[5] Katayama Chemical, Osaka, Japan.
[6] E. Merck AG, Darmstadt, Germany.
[7] Shionogi Co. Ltd., Osaka, Japan (see "The Japan Pharmacopoeia," 9th ed., Hirokawa Shoten, Tokyo, Japan, 1976, pp. 370, 371).
[8] Bayer AG, Leberkusen, Germany.
[9] Armour Pharmaceutical Co., Chicago, Ill.
[10] Boehringer GmbH, Mannheim, Germany.
[11] Seikagaku Kogyo Co., Tokyo, Japan.
[12] Ajinomoto Co. Inc., Kawasaki, Japan.

Table I—Amount of Drugs Adsorbed to Glass Surfaces

Drug	Amount, mg/g of Controlled Pore Glass			
	Water	Tris(hydroxy methyl) aminomethane Hydrochloride	Saline	Glycerin Isotonic Solution
Epinephrine	2.00	0.03	0.03	—
Atropine sulfate	1.70	0.70	0.05	—
Physostigmine salicylate	1.54	0.86	0.08	—
Insulin	5.46[a]	—	—	5.95[a]
Barbital	0	0.10	0	—
Aspirin	0	0.03	0	—
Sulfamethoxazole	0	0	0	—
Acetylcholine chloride	0.35	0	0	—
Ascorbic acid	0	0.01	0.03	—

[a] Measured at pH 2.6.

0.1 μg, but the amount in saline was negligible. However, it was also necessary to examine the adsorption of basic drugs that are effective in a microdose. The amount of insulin adsorbed to 1 g of controlled pore glass was 5.5–5.9 mg in water and in the glycerin isotonic solution; 0.3 μg of insulin might be adsorbed on 50 cm^2 of the surface of a glass container.

Elution patterns of nonbasic drugs, such as ascorbic acid, barbital, aspirin, sulfamethoxazole, and acetylcholine, are shown in Fig. 2. The amount of these drugs adsorbed to the glass in water, physiological saline, and 0.1 *M* tris(hydroxymethyl)aminomethane hydrochloride buffer (pH 8.6) was 0–0.3 mg (Table I). It was concluded from these results that basic drugs and proteins were well adsorbed to glass surfaces in water but that neutral or acidic drugs were not adsorbed in water.

Protein Adsorption—To clarify the mechanism of adsorption of insulin, adsorption using bovine serum albumin, chymotrypsin, and lysozyme was studied under various conditions. Adsorption patterns of bovine serum albumin and succinylated bovine serum albumin in distilled water, 8 *M* urea, and 6 *M* guanidine hydrochloride at pH 5.4 are shown in Fig. 3. Serum albumin was not liberated as a milky precipitate in these solutions. The amount of native albumin adsorbed was 136 mg/g of controlled pore glass in water, but the amount of modified albumin adsorbed was 3 mg under the same conditions.

These results showed that one major force of adsorption of proteins on glass surfaces was ionic bonding between amines in the protein molecules and terminal silanol groups on glass surfaces. The amount of bovine serum albumin adsorbed in 8 *M* urea (pH 5.4) was slightly smaller than that in water; therefore, hydrogen bonding seemed to be related to the protein adsorption on the glass. Succinylated albumin was not adsorbed in 8 *M* urea, so modified serum albumin was adsorbed by hydrogen bonding in water.

Meanwhile, the adsorption of native albumin in 6 *M* guanidine hydrochloride (pH 5.4) was lower than that in water, and this phenomenon must be due to inhibition of ionic bonding of proteins to glass by concentrated guanidinium ions. The effect of guanidinium ions on protein adsorption was not so strong that a definite amount of native bovine serum albumin was adsorbed in 6 *M* guanidine hydrochloride. This result is consistent with the previous findings that zwitterions such as amino acids inhibited adsorption but that ammonium ions did not prevent adsorption of proteins (16).

Figure 4 shows adsorption profiles of bovine serum albumin, chymotrypsin, and lysozyme in water at various pH values. Albumin was adsorbed the most at pH 5.4, and the amount adsorbed decreased at above or below this pH. It was concluded from these results that a protein was adsorbed most extensively to glass surfaces at its isoelectric point; this fact was ascertained from adsorption patterns of lysozyme, whose isoelectric point is at pH 10–11 (Fig. 4). Chymotrypsin, having an isoelectric point of pH 8, also was adsorbed the most at neutral pH 8.0 (Fig. 4). Proteins must be adsorbed most extensively to glass at their isoelectric points because of the cooperative aggregative action between glass and proteins, which are most precipitable at their isoelectric points.

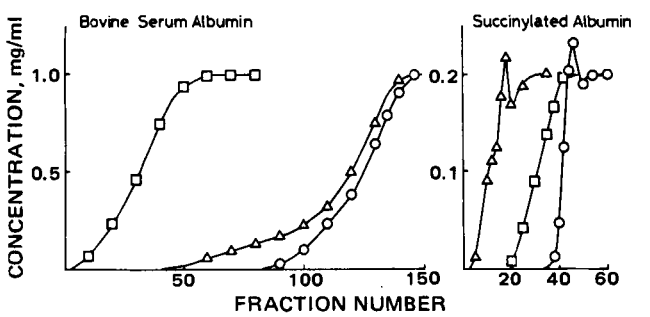

Figure 3—*Adsorption patterns of bovine serum albumin and succinylated albumin in detergent solutions (pH 5.4) on controlled pore glass. Key: ○, distilled water; △, 8 M urea; and □, 6 M guanidine hydrochloride. The fraction volumes were 1.6 ml for bovine serum albumin and 0.52 ml for succinylated albumin.*

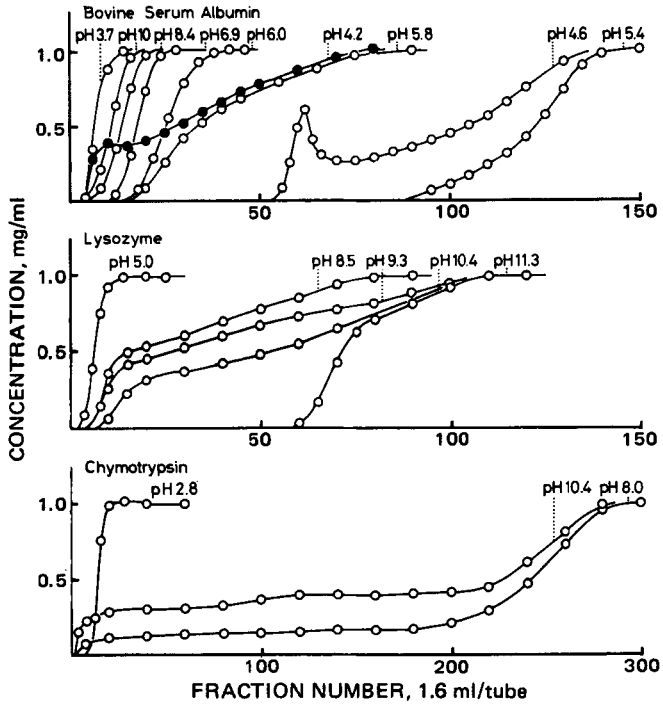

Figure 4—*Adsorption patterns of bovine serum albumin, lysozyme, and chymotrypsin on controlled pore glass at various pH values in distilled water. The column size was 0.65 × 11 cm, and the fraction volume was 1.6 ml.*

The amount of insulin adsorbed at the isoelectric point was not estimated because insulin precipitates at neutral pH. However, the amount adsorbed at the isoelectric point might be more than that at pH 2.6 if insulin were soluble at its isoelectric point. The maximum amounts of proteins adsorbed on 1 g of controlled pore glass were 136 mg (2 μmoles) for bovine serum albumin, 233 mg (9 μmoles) for chymotrypsin, and 84 mg (6 μmoles) for lysozyme. These values might not depend on molecular weights but rather on the amounts of residue for adsorption on the protein surface. These values (2–9 μmoles) of the proteins agreed with the values of 5 μmoles for basic amino acids and amino sugars and of 3–10 μmoles for basic drugs, and these results showed that the glass had adsorption sites of 2–10 μmoles/97 m^2 of surface area. Therefore, one to six molecules were adsorbed on the glass surface having an area of (100 Å)^2.

If the adsorption reactions between proteins and glass surfaces simply depended on ionic amine–silanol bonding, proteins should be adsorbed more at acidic pH, at which proteins would have a positive charge. However, proteins were adsorbed most extensively at their isoelectric points. Therefore, another important factor for adsorption would be the cooperative aggregative force between glass surfaces and proteins. This aggregative force might depend on bondings such as intermolecular forces, hydrogen bonding, and/or hydrophobic bonding, including ionic bonding.

From the values of chymotrypsin (233 mg/g), the amount of proteins adsorbed on the surfaces of a glass container (50 cm^2) and a glass injector was estimated to be more than 12 μg. This 12-μg value must be significant when protein drugs, such as hormones and vaccines, are used in microdoses and accurate doses are required. The purified protein derivative of tuberculin is used at 0.5 μg/ml in phosphate buffer containing sodium chloride, in which proteins are well adsorbed to the glass. The 0.5-μg/ml concentration must be a marginal point not affected by adsorption, and the doses of biologics used at lower concentrations would be ambiguous by adsorption to glass surfaces. The accurate dose of an injection at low

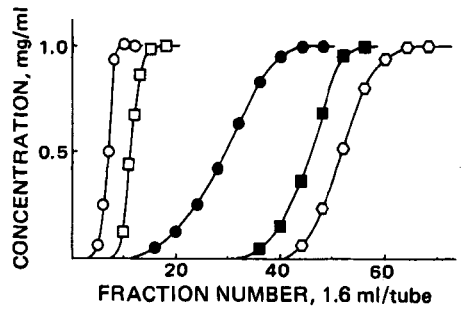

Figure 5—*Adsorption of bovine serum albumin in amino acid solutions. Key:* ○, *0.15* M *glycine, pH 8.0;* ●, *0.15* M *glycine, pH 5.4;* □, *0.15* M *lysine, pH 8.0;* ■, *0.15* M *lysine, pH 5.4; and* ○ *saline, pH 5.4.*

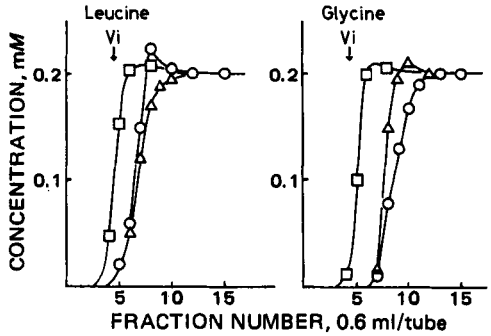

Figure 6—*Adsorption patterns of leucine and glycine on controlled pore glass in water. Key:* ○, *pH 6.0;* △, *pH 2.6;* □, *pH 10.3; and Vi, inner volume of the column. The column size was 0.65 × 11 cm.*

concentrations of biologics must be estimated after evaluating the value of the protein adsorbed to glass containers and injectors.

It was previously shown that the amino acid buffer was the most effective of several buffers for preventing adsorption of albumin on the glass (16). The conditions needed for the solvent to prevent adsorption were examined with bovine albumin, and adsorption patterns in solutions of glycine or lysine are shown in Fig. 5. The amount of albumin adsorbed to the glass in the glycine buffer or the lysine buffer of pH 5.4 was smaller than that in water at pH 5.4 (Fig. 4) and similar to that in saline. The amount in the amino acid buffers of pH 8.0 was smaller than at pH 5.4. The amount of albumin adsorbed to the porous glass in 0.15 *M* glycine of pH 8.0 was the smallest compared to the amounts in lysine and glutamic acid of pH 8.0; therefore, it may be desirable to add glycine to protein drug solutions to prevent protein adsorption. It is also important to select the optimum pH for minimum adsorption of protein drugs in the pH range of retention of drug activity.

It was previously reported that 5 μmoles of basic amino acids was adsorbed on 1 g of controlled pore glass and that the amount of other amino acids was 0.2–0.8 μmole/g of glass (9). In this work, adsorption of leucine and glycine on glass was studied at three pH values. Adsorption patterns of the amino acids in water at pH 2.6, 6.0, and 10.3 are shown in Fig. 6. The amino acids were adsorbed in an acidic solution but not in a basic solution, because the positively charged amino acids in an acidic condition were adsorbed to silanol but the negatively charged amino acids in a basic condition were repulsed by the negatively charged terminal silanol groups on glass surfaces. The amount of leucine and glycine adsorbed at pH 6.0 and 10.3 was 0.2 μmole/g of controlled pore glass. Leucine and glycine were greatly adsorbed to glass at their isoelectric point (pH 6.0). The force of this bonding has not been resolved, but this phenomenon may be correlated with the maximum adsorption of proteins at their isoelectric point and with the prevention of adsorption of proteins.

The amount of samples adsorbed was estimated by the column method, not by a batch operation. The former approach was preferable for the determination of the maximum amount adsorbed, even though the latter approach was preferable for the estimation of the dissociation constant or the reaction rate. The sample solution was eluted from the column with a constant flow rate to normalize the reaction rate.

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