Estimation of Protein and Drug Adsorption onto Silicone-Coated Glass Surfaces

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Abstract □ Porous glass coated with silicone adsorbed proteins such as albumin, globulin, hemoglobin, lysozyme, and peroxidase in pH 7.2 phosphate-buffered saline in the amount of 33–53 mg of protein/g of glass (39.4 m^2/g of glass). These proteins were not adsorbed from distilled water (except peroxidase). The amounts of albumin adsorbed onto coated glass from several buffers at ionic strength >0.05 μ were similar to amounts adsorbed from saline, despite the difference in salts and pH. The enzymes adsorbed onto coated glass were stable for at least 1 month. Drugs such as insulin, atropine sulfate, physostigmine salicylate, and epinephrine were adsorbed onto the coated glass in the amounts of 28, 0.32, 1.53, and 0.01 mg/g of glass, respectively. The adsorption of pharmaceutical biologicals composed of proteins such as hormones and vaccines onto silicone-coated glass containers must be recognized and compensated for, particularly at low concentrations. Protein adsorption onto coated glass might be caused by hydrophobic interaction.

Keyphrases □ Adsorption—proteins, drugs onto silicone-coated glass □ Silicone-coated glass surfaces—adsorption of drugs and proteins □ Proteins—adsorption onto silicone-coated glass surfaces □ Glass containers—adsorption of proteins onto silicone-coated surfaces

Uncoated porous glass was developed for exclusion chromatography (1). Glass with a large surface area is useful for the accurate estimation of drugs and proteins adsorbed onto glass surfaces (2). From the maximum amount of protein adsorbed (233 mg/97 m^2), the amounts of protein drugs adsorbed onto a glass container with a 20-ml capacity were estimated to be >12 μg (2). After adsorption onto uncoated glass surfaces for a long time, protein activity decreased (3).

Glass surfaces are anionic, and 1 g of glass (97 m^2) adsorbs 5 μmoles of cationic drugs, such as atropine and physostigmine, and other biological components (4). Insulin also was adsorbed onto glass surfaces from a pH 2.6 isotonic glycerin solution (2). Serum globulin was adsorbed to a greater extent than albumin; antibodies (IgG and IgM) are also well adsorbed onto glass from physiological saline (5).

Pharmaceutical glass containers generally are coated with silicone to prevent drug adsorption and inactivation during storage. Surfaces coated with silicone can repulse a water layer and thus decrease adsorption. However, silicone-coated glass has been shown to adsorb the hormone secretin, although albumin blocks secretin adsorption (6). Adsorption of albumin and cells such as platelets and leucocytes onto silicone-coated glass also was reported (7–9). The USP (10) and the JP (11) do not provide standards for silicone coating, and the properties of the glass surfaces coated with silicone are not defined. The purpose of this investigation was to estimate drug and protein adsorption onto silicone-coated glass surfaces.

EXPERIMENTAL

Methods—Adsorption of proteins and other substances was studied using silicone-coated porous glass packed in columns. Silicone-coated porous glass floats on water because of the repulsion between water and silicone, making an accurate estimation of adsorption impossible. Therefore, silicone-coated glass was precipitated as follows.

The controlled porous glass[1], composed of 100-μm particles with a pore diameter of 240 Å, was suspended in a chromic acid mixture. The glass then was packed in a column and washed thoroughly with distilled water to neutrality. The beads were dried at 180° in an evaporating dish, and 1 g of the dried beads was added to 3 ml of carbon tetrachloride containing 200 mg of silicone[2] (dimethylpolysiloxane). After evaporation of carbon tetrachloride, the glass was heated at 300° for 10 min to enhance a tight coating with silicone. The surface area of the silicone-coated glass measured 39.4 m^2/g of glass using equipment[3] with nitrogen gas.

The porous glass coated with silicone was precipitated in 0.05 *M* phosphate (pH 7.3) by using a detergent, 0.7% sodium lauryl sulfate, and the precipitate was packed in columns (12). Precipitation was caused by the loss of repulsion between water and silicone, because the glass surfaces were coated with sulfate residues of lauryl sulfate, which were bound to silicone residues by hydrophobic bonding. After washing with ~100 column volumes of hot water, lauryl sulfate residues on the glass were completely removed. The complete removal was confirmed by passing a 0.2% $BaCl_2$ solution through the column (13). Frontal analysis was used to estimate the amount of sample adsorbed onto silicone-coated glass (14). Sample solutions were loaded at a flow rate of 1.5 ml/min/cm^2 at room temperature. The column size was 0.6 × 4.0 cm.

Materials—The proteins used were bovine serum albumin[4], egg lysozyme[5], horseradish peroxidase[5], bovine hemoglobin[5], and bovine serum globulin prepared from bovine serum by fractional precipitation with ammonium sulfate. These proteins were dissolved at a concentration of 1 mg/ml in distilled water or pH 7.2 phosphate-buffered saline composed of 0.137 *M* NaCl, 2.7 m*M* KCl, and 9.5 m*M* phosphate. Other active enzymes were alkaline phosphatase[6] from *Escherichia coli* and malate dehydrogenase[7].

The drugs used were insulin[8], atropine sulfate[9], epinephrine[10], and physostigmine salicylate[11]. Insulin was dissolved in pH 2.6 isotonic glycerin solution, and epinephrine was dissolved in saline containing 0.01 *N* HCl. Atropine and physostigmine were dissolved in pH 7.2 phosphate-buffered saline. Amino acids[12], glucose, and nucleic acid components[5] were commercial reagents. The concentrations of proteins and nucleic acid components were determined by absorbance measurements at 280 and 260 nm, respectively. The amino acid concentrations were determined by the color reaction with ninhydrin.

RESULTS AND DISCUSSION

Protein Adsorption—Figure 1 shows the patterns of protein adsorption onto silicone-coated glass columns (0.6 × 4 cm, 0.5 g). In phosphate-buffered saline, proteins were well adsorbed onto the glass; the amounts of albumin, globulin, lysozyme, hemoglobin, and peroxidase adsorbed onto 1 g (39.4 m^2) of the glass were 42 mg (0.64 μmole), 53 mg (0.3 μmole), 38 mg (2.7 μmoles), 47 mg (0.73 μmole), and 33 mg (0.83 μmole), respectively. From these values, the amount of proteins adsorbed onto silicone-coated surfaces of a glass container (50 cm^2 of surface area, 20-ml capacity) and a glass injector was estimated to be >7 μg. The 7-μg/20-ml concentration must have been a minimum point not significantly affected by adsorption onto silicone-coated glass surfaces.

[1] CPG-10, Electro-Nucleonics, Fairfield, N.J.
[2] KF 96, Shin-Etsu Chemicals, Tokyo, Japan.
[3] Orr surface-area pore-volume analyzer model 2100D, Micromeritics Instrument Corp., Norcross, Ga.
[4] Miles Laboratories, Elkhart, Ind.
[5] Sigma Chemical Co., St. Louis, Mo.
[6] P-L Biochemical Inc., Milwaukee, Wis.
[7] Boehringer GmbH, Mannheim, West Germany.
[8] Fluka AG, Buchs, Switzerland.
[9] Wako Pure Chemicals Ltd., Osaka, Japan.
[10] Katayama Chemicals, Osaka, Japan.
[11] E. Merck AG, Darmstadt, West Germany.
[12] Ajinomoto Co. Inc., Kawasaki, Japan.

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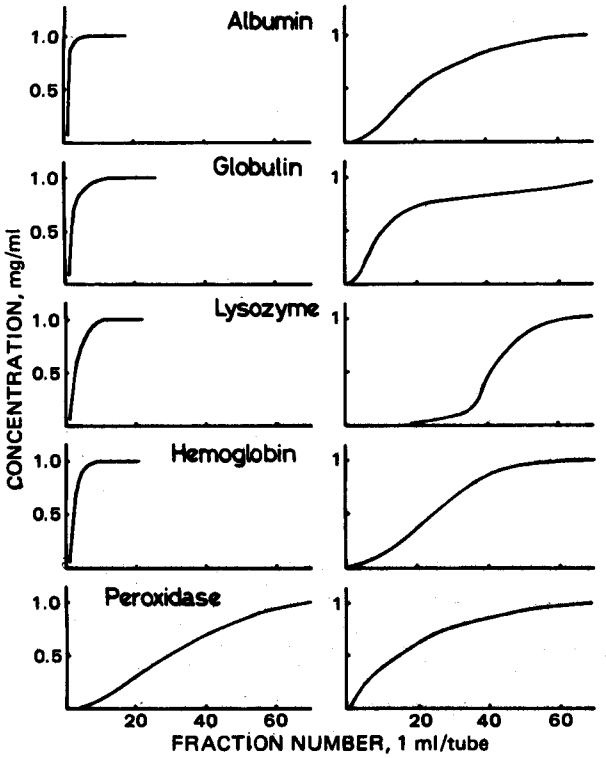

Figure 1—*Adsorption patterns of protein (1 mg/ml) from distilled-water medium (left) and phosphate-buffered saline (PBS) (right) on the silicone-coated porous glass column (0.6 × 4 cm, 0.5 g). Proteins are the same in both sides of the figure.*

One example of a protein solution being used at a low concentration is the purified protein derivative of tuberculin, which is used at 0.5 μg/ml in phosphate-buffered saline. Therefore, the adsorption of pharmaceutical biologicals composed of proteins such as hormones and vaccines onto silicone-coated glass must be recognized and compensated for, particularly at low concentrations and when an accurate dose of an injection is required.

The amounts adsorbed onto silicone-coated glass (83–133 mg/100 m^2 of glass) were similar to those (100–200 mg/100 m^2) of protein adsorbed onto noncoated glass in a distilled water medium. The value (133 mg/100 m^2) of bovine serum globulin was slightly higher than that (83 mg/100 m^2) for noncoated glass (5). Thus, the antibody in antiserum also is well adsorbed onto silicone-coated glass surfaces, and this adsorption must be seriously considered upon storage of purified antiserum in pharmaceutical glass containers coated with silicone.

In distilled water, the amounts of albumin, globulin, lysozyme, and hemoglobin adsorbed were 1.2, 2.7, 1.7, and 2.4 mg/g of glass, respectively. These values were lower than those in phosphate-buffered saline (Fig. 1). The discrepancy in protein affinities for the coated glass in phosphate-buffered saline and in distilled water must have been due to the ionic environment in the solution. The peroxidase from horseradish adsorbed well onto silicone-coated glass in water (62 mg/g of glass). This adsorption of peroxidase onto coated glass contrasts to that onto noncoated glass; peroxidase adsorption in distilled water onto coated glass was the strongest, but on noncoated glass, it was the weakest among the proteins examined.

This phenomenon might be explained by the difference in bonding between proteins and glass. On noncoated glass, proteins are adsorbed mainly by amine–silanol bonding and a cooperative aggregative force between silica and proteins. On coated glass, proteins must be adsorbed mainly by hydrophobic bonding between aliphatic residues on proteins and silicone residues on coated glass. This hypothesis is supported partly by the observation that some proteins adsorbed onto noncoated porous glass were eluted with several different buffers, while proteins adsorbed onto the coated glass were not unless detergents such as sodium lauryl sulfate were added to the elution buffers.

The difference in the strength of adsorption onto silicone-coated glass was examined next by using protein mixtures in phosphate-buffered saline. In the top section of Fig. 2, the absorbance at 403 nm shows the elution of peroxidase; the absorbance at 280 nm was due to total proteins (peroxidase plus albumin). The ratio of A_{280} to A_{403} was 0.6 at the 20th

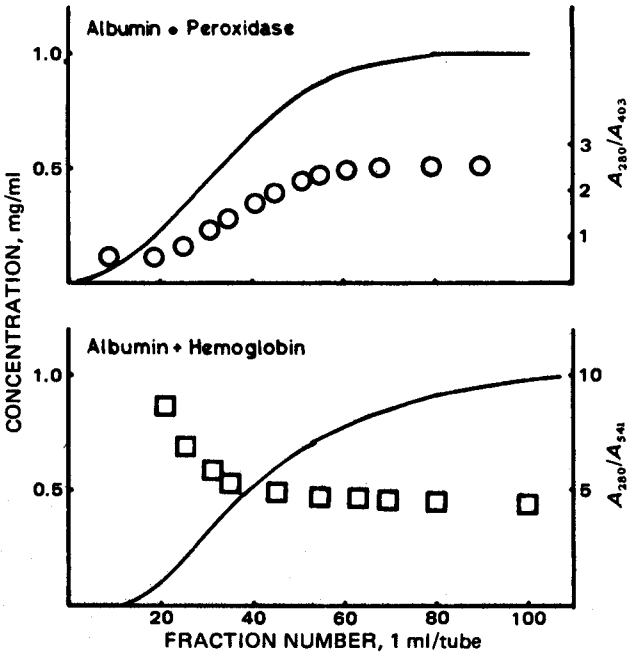

Figure 2—*Adsorption patterns of mixtures on silicone-coated glass columns in pH 7.2 phosphate-buffered saline. The upper profile is for a mixture of bovine serum albumin (0.5 mg/ml) and horseradish peroxidase (0.5 mg/ml); the lower profile is for a mixture of albumin (0.5 mg/ml) and hemoglobin (0.5 mg/ml). Key: ○, ratio of A_{280} to A_{403}; and □, ratio of A_{280} to A_{541}.*

tube and increased to 2.3 at the 60th tube, which is identical to that of the original mixture. This result indicates that peroxidase was adsorbed more weakly onto coated glass surfaces than was albumin.

The absorbance at 541 nm shown at the bottom of Fig. 2 represents hemoglobin. The ratio (8.6) of A_{280} to A_{541} at the 21st tube indicates that hemoglobin adsorption was stronger than that of albumin. Thus, various proteins have different affinities for silicone-coated glass surfaces, and the affinities of three proteins onto the coated glass are in the order of hemoglobin > albumin > peroxidase. This order parallels the aliphatic amino acid content (44% in hemoglobin, 36% in albumin, and 31% in peroxidase) and is independent of molecular weight.

These results suggest that the protein adsorption onto silicone-coated glass was caused at least partially by hydrophobic interaction. These results also suggest that prevention of adsorption of hormones or vaccines by the addition of nonactive proteins such as albumin should depend on the ratio of those drugs to the adduct and on the difference in the affinities of the proteins to the coated glass.

Adsorption Properties of Proteins—Figure 3 shows albumin adsorption patterns (isoelectric point 4.7) in 0.1 *M* citrate buffer at pH 3.0

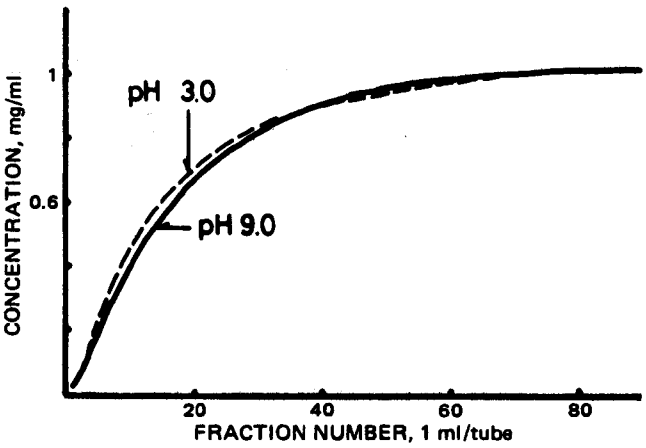

Figure 3—*Adsorption patterns of bovine serum albumin on the coated glass column at pH 3.0 and 9.0 with buffers of 0.1 M sodium citrate and 0.5 M tris(hydroxymethyl)aminomethane hydrochloride, respectively.*

Table I—Amounts of Bovine Serum Albumin Adsorbed onto Silicone-Coated Porous Glass in Buffers

Buffer	Ionic Strength, μ	pH	Amount, mg/g (39.4 m^2 of the glass)
Distilled water	0	—	1.2
Phosphate-buffered saline	0.16	7.2	42
0.1 *M* Citrate	0.20	3.0	32
0.5 *M* Tris(hydroxymethyl)-aminomethane hydrochloride	0.05	9.0	31
0.01 *M* Phosphate	0.026	7.3	1.0
0.5 *M* Phosphate	1.3	7.3	32
0.01 *M* Phosphate + 0.05 *M* NaCl	0.076	7.3	20
0.01 *M* Phosphate + 2 *M* NaCl	2.026	7.3	43
0.1 *M* Tris(hydroxymethyl)-aminomethane hydrochloride	0.09	7.6	41
0.1 *M* Acetate	0.1	7.0	19
0.5 *M* Acetate	0.45	5.6	36
0.2 *M* Glycine	0.01	8.0	2.4
0.5 *M* Glycine	0.1	9.0	28
20% Sucrose	—	—	10

and in 0.5 *M* tris(hydroxymethyl)aminomethane hydrochloride at pH 9.0. The albumin adsorption pattern at pH 7.2 is shown in Fig. 1. At these three pH points, the patterns were similar, indicating that pH did not affect protein adsorption onto silicone-coated glass. Thus, the adsorption is not caused by ionic bonding, even though protein adsorption onto noncoated glass is caused partially by ionic bonding (2).

Table I shows the amounts of bovine serum albumin adsorbed onto silicone-coated glass in various buffers. Albumin was well adsorbed (from 20 to 43 mg/g) onto the glass in phosphate, acetate, glycine, and tris(hydroxymethyl)aminomethane hydrochloride buffers at ionic strengths higher than 0.05 μ. However, only 1–2.4 mg of albumin/g was adsorbed from buffers at ionic strengths of <0.026 μ. Thus, ionic strength is an important factor for protein adsorption onto coated glass; the marginal ionic strength for albumin was ~0.02–0.05 μ. Meanwhile, a small amount of albumin was adsorbed at high concentrations (20%) of nonionic substances such as sucrose. Glycine buffer prevented protein adsorption onto noncoated glass (15) but did not interfere with adsorption onto silicone-coated glass. These results also demonstrate that protein adsorption onto coated and noncoated glass is different.

The amounts of amino acids and other biological components adsorbed onto coated glass are shown in Table II. The values were low, 0.03–0.7 μmole/g (6–60 μg/g). The values for lysine, nucleic acid components, and glucose were small (0.03–0.08 μmole/g, the minimum values to be measured accurately under these conditions). Some hydrophobic amino acids such as isoleucine, methionine, and phenylalanine were adsorbed in the amounts of 0.3–0.7 μmole/g (39.4 m^2); these values were similar to those of proteins (0.3–2.7 μmoles/g) in Fig. 1. Some amino acids were adsorbed in distilled water, even though those proteins in Fig. 1 (except peroxidase) were not adsorbed in water.

Drug Adsorption—The adsorption patterns of some drugs on silicone-coated glass are shown in Fig. 4. Insulin adsorbed well (71 mg/100 m^2) onto the glass in pH 2.6 isotonic glycerin solution; this value was similar to the values in Fig. 1 but was higher than the value (6.14 mg/100 m^2) on noncoated glass (2). In phosphate-buffered saline, the values for atropine and physostigmine were 0.81 and 3.88 mg/100 m^2, respectively, about five times higher than the amounts on noncoated glass in saline.

Table II—Amounts of Biological Components Adsorbed onto Silicone-Coated Glass in pH 7.2 Phosphate-Buffered Saline

Sample	Amount on Silicone-Coated Glass, μmole/g	μg/g
Lysine	0.04	7
Aspartic acid	0.39	52
Isoleucine	0.38 (0.16)[a]	50
Methionine	0.24	36
Serine	0.76	80
Asparagine	0.36	48
Tryptophan	0.08 (0.02)[a]	16
Tyrosine	0.16 (0.26)[a]	29
Phenylalanine	0.36 (0.18)[a]	59
Adenosine	0.18	47
Cytidine	0.04	9.6
Guanosine	0.07	20
Uridine	0.06	15
5′-Adenylic acid	0.08	29
Crude tRNA	0.05[b]	15
Glucose	0.03	5.4

[a] Values in a distilled water medium. [b] Value of phosphate.

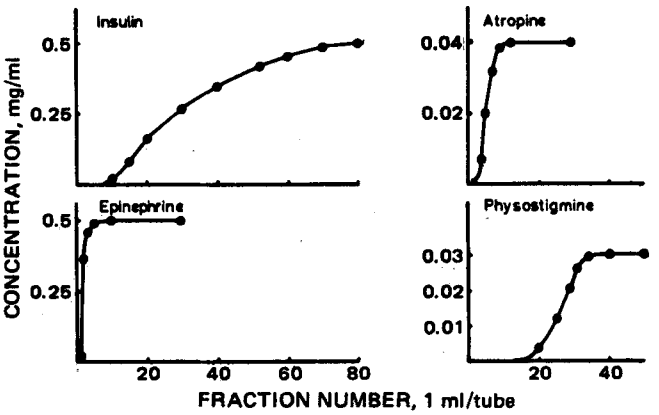

Figure 4—*Adsorption patterns of drugs on columns of the silicone-coated porous glass. The concentrations of insulin and epinephrine were measured by absorbance at 280 nm. Atropine and physostigmine concentrations were measured at 220 and 260 nm, respectively. The column size was 0.6 × 4 cm. Insulin was dissolved in pH 2.6 isotonic glycerin solution, epinephrine was dissolved in saline containing 0.01* N *HCl, and atropine sulfate and physostigmine salicylate were dissolved in pH 7.2 phosphate-buffered saline.*

Very little epinephrine was adsorbed (<0.02 mg/100 m^2) onto silicone-coated glass. The amount of insulin adsorbed onto the surface of a coated glass container (50 cm^2, 20 ml) was 0.085 U. This value was relatively low compared to the usual dosage, although an accurate dosage should be influenced by this adsorption. The values for atropine and physostigmine adsorbed onto the surface of a coated glass container were very low (60–200 ng).

Stability of Adsorbed Proteins—The stability of enzymes adsorbed onto silicone-coated glass was studied using alkaline phosphatase, malate dehydrogenase, and horseradish peroxidase. After these enzymes were adsorbed onto the coated glass column, the solutions containing substrates, such as *p*-nitrophenylphosphate for phosphatase, β-nicotinamide-adenine dinucleotide in the reduced form for dehydrogenase, and guaiacol hydrogen peroxide for peroxidase, were loaded on the columns and the absorbance of the eluates was measured.

The alkaline phosphatase activities after adsorption for 1 week and 1 month were 98 and 96%, respectively, of the value obtained on the 1st day. The malate dehydrogenase activities after adsorption for 1 week and 1 month were 97 and 89%, respectively, under the same conditions. Peroxidase retained full activity after being adsorbed onto the column for 1 month. The activities of phosphatase in free and in bound forms were

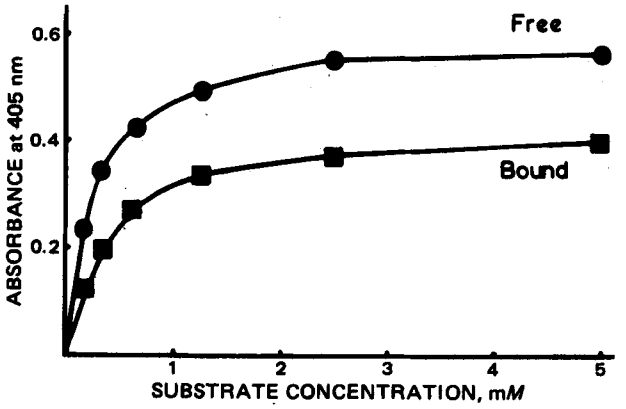

Figure 5—*Substrate concentration curve of alkaline phosphatase immobilized on the silicone-coated glass and in free solution. The reaction mixtures of 3 ml in 1* M *tris(hydroxymethyl)aminomethane hydrochloride (pH 8.6) contained substrate and 25 μg of free or bound enzyme on 1 ml of coated glass. The reaction was carried out at room temperature; the absorbance at 405 nm was measured after incubation for 30 sec in both cases. The substrate was* p*-nitrophenyl phosphate. Key: ●, free solution; and ■, enzyme immobilized on the glass.*

compared as a function of substrate concentration (Fig. 5). The activity of bound enzyme was determined by mixing with the substrate solution for 30 sec, using a mechanical mixer, and measuring the absorbance of the supernate after standing for 10 sec. The maximum velocity of the immobilized enzyme was slightly lower than that of the free enzyme, but the K_m values were similar. These properties did not vary on adsorption for 1 week. Hence, the surfaces of silicone-coated glass may be useful as a support for the enzymes to be immobilized, but the surfaces of noncoated glass are not appropriate for this purpose (3).

With the noncoated glass in the column, the top portion of the glass turned milky white after albumin solution was applied whereas no color change was observed when coated glass was used. Albumin might have precipitated on the noncoated glass but was well adsorbed onto the coated glass. The difference in the adsorption forces between amine–silanol ionic bonding and hydrophobic interaction might have caused this difference.

Adsorption on Synthetic Polymers—Synthetic polymers such as polyethylene and polyethylmethacrylate are useful for pharmaceutical containers. However, polyethylene was shown to adsorb 155 mg of human serum albumin/100 m^2 of polyethylene surface (16). Hemoglobin also is adsorbed onto polyethylene (17). Polyethylmethacrylate and polyhydroxyethylmethacrylate adsorb plasma proteins such as hemoglobin, albumin, fibrinogen, and γ-globulin (18). Polystyrene adsorbs human serum albumin, ribonuclease, and muscle proteins (19, 20). The reported amounts of proteins adsorbed onto synthetic polymers are comparable to those adsorbed onto coated and noncoated glass, although the mechanisms of protein adsorption onto synthetic polymers and glass must be different.

Pharmaceutical preparations composed of proteins such as hormones and vaccines adsorb onto the surfaces of their containers. This adsorption is particularly significant at low protein concentrations since at least 7 μg of protein is adsorbed by the surface of a 20-ml glass container. Thus, it is important to compensate for this adsorption when dispensing low doses of such substances.

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Synthesis of Tetracycline Ring A Analogs

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Abstract □ Studies directed at the synthesis of tetracycline ring A analogs are described. 4-Carbethoxycyclohexane-1,3-dione was converted to the ethyl urethan dispiro[1,3-dioxolane-2,2′-cyclohexane-4′,2″(1,3)-dioxolane]-1′-carbamic acid ethyl ester *via* the dispiro[1,3-dioxolane-2,2′-cyclohexane-4′,2″(1,3)-dioxolane]-1′-carboxylic acid hydrazide. An improved synthesis of another cyclohexenone from methyl vinyl ketone and ethyl nitroacetate is reported. Reaction of *N*-(3-hydroxy-1-oxo-2-cyclohexen-4-yl)benzamide with α-chloroacetyl isocyanate afforded a 4(5*H*)-oxazolone derivative, as did the identical reaction on 5,5-dimethyl-1,3-cyclohexanedione (dimedone). This reaction provides a novel approach to these oxazolones with potential therapeutic importance. Other ring A analogs were synthesized also.

Keyphrases □ Tetracycline—synthesis of ring A analogs □ 4(5*H*)-Oxazolones—tetracycline ring A analogs, synthesis □ Analogs—tetracycline ring A analogs, synthesis

Following a study of the mechanism of the polarographic reduction of tetracycline compounds (1), the need arose for simpler analogs encompassing some or all structural features of ring A of the tetracyclines. A literature search, followed by some preliminary attempts at duplicating one synthetic approach, revealed that a ready access to these compounds had not been published.

Smissman *et al.* (2) attempted, without success, to prepare ring A analogs by cyclizing appropriate alicyclic molecules. Muxfeldt *et al.* (3) prepared several ring A analogs by the condensation of unsaturated oxazolones with active methylene compounds. Compound I was obtained, which could be hydrolyzed and decarboxylated to give II. Alternatively, treatment of I with Meerwein's reagent afforded III (Scheme I).

A different approach was reported (4, 5) for preparing ring A analogs, involving catalytic hydrogenation of substituted resorcinols to yield the corresponding 1,3-cyclohexanedione derivatives. All efforts at duplicating this synthesis were unsuccessful. Several reported approaches

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