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The limiting role of mucus in drug absorption: drug permeation through mucus solution

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Abstract

A purified model in vitro mucus system containing primarily the large, 400 kDa glycoprotein fraction of mucus has been developed for use in drug permeability and drug binding studies. The effect of protein solutions, either bovine serum albumin (BSA) or purified porcine gastric mucus, on the permeability behavior of five drugs was studied. The drugs chosen were isoniazid, pentamidine, rifampicin, *p*-aminosalicylic acid, and pyrazinamide, all of which can be potentially delivered as pulmonary aerosols. BSA was included in the permeability studies for comparison with previously obtained data regarding their binding behaviors to mucin relative to BSA. A custom membrane holder with a 3 mm chamber for mucin or other solutions was used in a Side-Bi-Side® diffusion apparatus to measure drug permeation through the solutions. Apparent permeability coefficients were calculated for each barrier in the series barrier system, with a protein solution being one of the barriers. The protein solutions significantly reduced the permeability of the drugs studied compared with their permeability through blank buffer solution. Both the lag time and the steady-state flux of the compounds were altered in the presence of protein indicating that there is more than protein binding affecting permeability. Such reductions in permeability coefficients need to be considered for all compounds that must traverse any mucosal surface prior to absorption or action.

Keywords: Mucus; Mucin; Drug transport; Drug permeation; Diffusion; Aqueous diffusion layer; Aminosalicylic acid; Isoniazid; Pyrazinamide; Pentamidine; Rifampicin

1. Introduction

Mucus is a viscous endogenous solution serving primarily a cytoprotective and lubricative role for the underlying mucosal tissues. Mucus is composed chiefly of a high molecular weight glycoprotein, mucin, which is the rheologically active fraction responsible for the viscous and gel-forming nature of mucus (Allen and Snary, 1972; Meyer and Silberberg, 1978). Non-mucin components of mucus include secretory IgA, lysozyme, lactoferrin, lipids, polysaccharides, and ionic species. Some of the non-mucin components are responsible for the bacteriostatic action of mucus (Allen and Snary, 1972).

There have been previous reports of the interactions of mucus with various drugs (Braybrooks et

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al., 1974; Kearney and Marriott, 1986, 1987; Matthes et al., 1992; Karlsson et al., 1993), with enzymes (Shora et al., 1975; Hao et al., 1977) and with inorganic cations (Bella and Kim, 1974; Forstner and Forstner, 1975, 1977). There are also reports indicating that the rate-limiting step to the absorption of lipophilic solutes is their transfer across the aqueous diffusion barrier adjacent to the intestinal wall (Komiya et al., 1980; Poelma et al., 1990). A part of this aqueous diffusion resistance is the mucus layer, but it is difficult to determine the exact contribution of mucus to the overall aqueous barrier. Studies have indicated that the presence of mucus retards the transport of many compounds. Braybrooks et al. (1974) observed an $\sim 50\%$ decrease in the apparent permeability coefficients for tetracycline in the presence of mucus. Kearney and Marriott (1986) found an increase in lag-time as well as a decrease in the tetracycline transport rates using everted gut experiments in the presence of mucus. Matthes et al. (1992) found that the disappearance rate of compounds of various polarities through a diffusional chamber was reduced when buffer solutions in a drug-buffer mixture were replaced with mucus solution. Karlsson et al. (1993) found, in a study of testosterone permeation through a mucus-producing human goblet cell line (HT29-H), that the permeability coefficients increased by $\sim 50\%$ when the mucus layers were removed. Therefore, drug interactions with mucin may limit the bioavailability of drugs being delivered via any mucosal surface by retarding their rate of membrane transport.

To determine the effect of mucin on transport, five drugs, each having the potential to be delivered in aerosolized formulations to the lungs, were selected for study. The drugs, isoniazid, rifampicin, *p*-aminosalicylic acid, pyrazinamide, and pentamidine, are structurally diverse and have a broad range of protein binding characteristics (Bhat et al., 1995). The selection of model compounds was on the basis of their potential therapeutic application for pulmonary delivery rather than on their physicochemical properties. The compounds, however, cover a range of aqueous solubilities, molecular sizes, and ionization states at the experimental pH of 7.4.

Permeation rates were obtained using a diffusion cell with a custom membrane holder. This holder had a 3 mm chamber for protein or buffer solution which was held in place by two ultrafiltration membranes. This system has the advantage over other test methods (e.g. tube diffusion, cell culture systems) in that the mucus composition can be systematically changed by adding substances known to alter its characteristics thus simulating various disease states or physiological conditions. Such systems can then be used to estimate changes in drug permeabilities in these conditions.

2. Experimental

2.1. Materials and methods

Sodium *p*-aminosalicylate (PAS), isonicotinic acid hydrazide (INH), pyrazinamide, and rifampicin were obtained from Sigma Chemical Co. (St. Louis, MO) while pentamidine was obtained from the Walter Reed Army Institute of Research. The proteins, bovine serum albumin, (BSA; Fraction V) and pig gastric mucin (Type II, lyophilized) were also obtained from Sigma Chemical Co. (St. Louis, MO). BSA was used without further purification while the gastric mucus was purified as described below. Distilled deionized water was used in the preparation of all drug and buffer solutions. The buffer system used in all the binding studies was Sørenson's phosphate buffer, 0.067 M, pH 7.4. The buffer was prepared from monobasic sodium phosphate and dibasic sodium phosphate, heptahydrate, (EM Science, Gibbstown, NJ).

2.2. Reconstitution and purification of mucin

Mucus solutions were prepared by a modification of the reconstitution procedure of List et al. (1978). These were purified, the molecular weight distributions of proteins measured and the total solids content determined by methods reported elsewhere (Bhat et al., 1995).

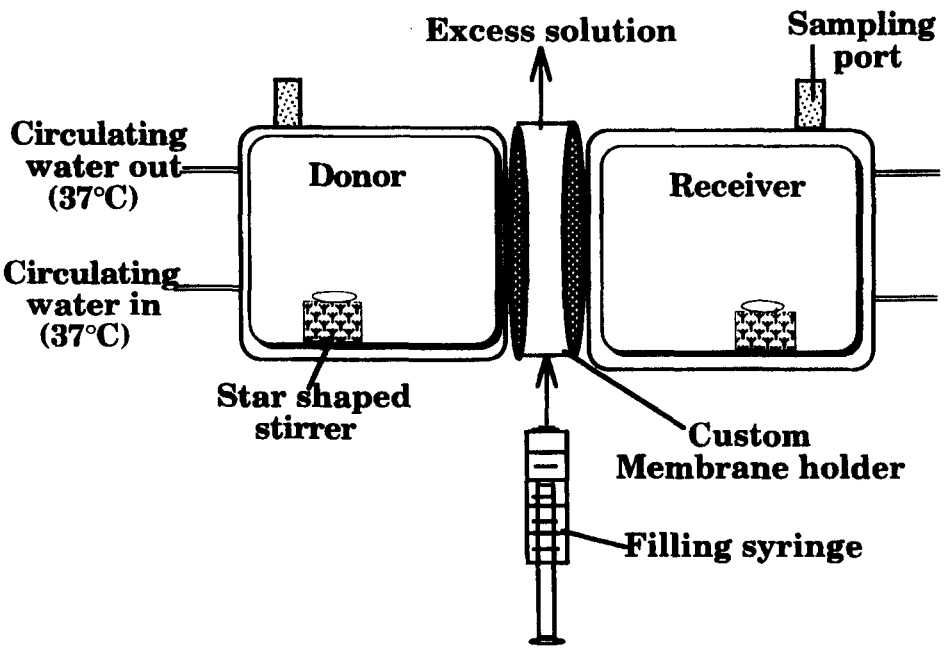

Fig. 1. Schematic of the diffusion chamber system used for the drug transport studies through a solution.

2.3. Permeation studies

2.3.1. Membrane permeability studies

Drug permeation studies were conducted using a Side-By-Side® diffusion cell with a custom membrane holder (Crown Bioscientific, Somerville, NJ) as shown in Fig. 1. All drug permeation experiments were conducted at 37°C with constant stirring using magnetic stir bars (Cell Spinbar, Bel-Art Products, Pequannock, NJ).

To determine if the system was under aqueous layer or membrane control, two sets of initial diffusion experiments were conducted. First, the stirring speed in the diffusion cell was varied between 60 and 200 rpm with overhead stirrers (Hurst® Model CA, Princeton, IN) on both donor and receiver sides. If the apparent permeability coefficient decreased with increased stirring speeds, there was a significant contribution from the aqueous boundary layers. In the second set of experiments, drug permeability studies through two Amicon® PM-10 (MWCO 10000) membranes were conducted in these diffusion cells with conventional Snap Tight® membrane holders (Crown Bioscientific, Somerville, NJ) and with magnetic stirrer agitation. The two membranes were mounted in the Snap Tight® membrane holder instead of one thereby doubling the membrane diffusional distance. If the apparent permeability coefficient decreased in proportion to the increasing membrane thickness, the system was primarily under membrane control. Permeability studies through a single membrane were also conducted for each drug to obtain the permeability coefficient through this barrier.

The extent of drug-binding to the Amicon PM-10 membranes used in these permeability studies has been reported (Bhat et al., 1995). It was found that only rifampicin showed any significant binding to these membranes which are composite in nature, with a polymeric membrane overlying a spongy cellulosic support. To limit the influence of drug adsorption on the permeability of rifampicin, the membranes were presaturated with drug solution for approximately 12 h at 37°C. The membranes were thoroughly washed with isotonic buffer solution until excess drug was removed. These pre-saturated membranes were then used

for rifampicin permeation studies. The change in the mucin concentration in solution due to adsorption or non-specific protein binding to the Amicon® membranes was determined by the Alcian blue binding technique (Hall et al., 1980) and was found to be about 3.5% of the original mucus concentration (Bhat et al., 1995). This negligible binding should not significantly affect transport across the Amicon membranes.

Drug donor solutions (0.5–15 mg/ml) in isotonic pH 7.4 phosphate buffer, were filled into the donor compartment (8 ml). The receiver compartment was filled with 8 ml of blank isotonic pH 7.4 phosphate buffer. A receiver compartment sample was taken after approximately 2–3 min to check for any leaks in the apparatus. After ensuring that there were no leaks, samples were taken at appropriate intervals by removing the entire receiver volume and replacing it with fresh buffer so that sink conditions were maintained. The drug donor concentrations were typically maintained at ~90–95% of the initial concentrations thereby assuring sink conditions throughout the experiment. The amount of diffusant in each receiver aliquot was determined by measuring the absorbance with a UV-visible diode array spectrophotometer (Model 8450A, Hewlett-Packard Company, Palo Alto, CA) at the appropriate wavelength maximum for each drug.

2.3.2. Solution permeability studies

Drug-permeation studies through isotonic Sørenson's phosphate buffer solution and the protein solutions were conducted in addition to the membrane permeability studies using the custom membrane holder. Drug permeability through buffer was measured to determine the diffusivities of these drugs through an aqueous environment without effects from protein. For these studies, the custom stainless steel membrane holder (Performance Systematix, Grand Rapids, MI) which contained buffer or protein solution, retained by two Amicon PM-10 membranes was placed between the diffusion compartments. The custom membrane holder was designed to hold a fixed solution volume (~ 0.8–1.0 ml) in an ~ 3.0 mm thick chamber. The protein solutions were injected into the chamber with a syringe (25 G needle) through a port on the side of the membrane holder. Any excess solution in the chamber after filling caused the solution to rise through a second port at the top of the holder.

3. Results and discussion

3.1. Membrane permeability studies

The steady-state permeation results were analyzed using Fick's first law of diffusion:

$$J = \frac{1}{A} \cdot \frac{dQ}{dt} = \frac{DK(C_d - C_r)}{h} \quad (1)$$

where J is the steady-state flux, Q is the cumulative amount of drug in the receiver cell, dQ/dt is the drug permeation rate, A is the membrane area, D is the diffusion coefficient, K is the partition coefficient, C_d is the donor drug concentration, C_r is the receptor drug concentration and h is the membrane thickness.

DK/h is the permeability coefficient (P). Assuming sink conditions ($C_r = 0$) and rearranging Eq. 1 gives the total permeability coefficient (P_T) as:

$$P_T = \frac{DK}{h} = \frac{\text{Slope}_{ss}}{AC_d} \quad (2)$$

where Slope_{ss} is the steady-state slope from a plot of cumulative amount vs. time.

For permeation through a series of barriers, the permeation rate is inversely proportional to the sum of the diffusional resistances of the barriers. In the case of permeation through only one Amicon® PM-10 membrane, the barriers are the membrane and the aqueous diffusion layers on the donor and the receiver sides of the membrane. The permeation rate for this system is given by:

$$\frac{dQ}{dt} = \frac{C_d A}{\frac{2}{P_{aq}} + \frac{1}{P_m}} \quad (3)$$

where P_{aq} is the aqueous boundary layer permeability coefficient and P_m is the membrane permeability coefficient.

Two different permeation studies were performed to determine if this system was under

aqueous or membrane control, as described earlier. The results obtained are shown in Table 1. Doubling the membrane thickness decreased the apparent permeability coefficients of *p*-aminosalicylic acid, pyrazinamide and isoniazid by nearly one-half indicating that diffusion for the drugs in this system was under membrane control. This was confirmed by the permeability results obtained from experiments using different stirring speeds in the two halves of the diffusion cell. The permeation results showed that between 60 and 200 rpm, no significant change in pyrazinamide permeability coefficient was observed, i.e. $P_{app} = 1.4 \times 10^{-5}$ cm/s (60 rpm), $P_{app} = 1.57 \times 10^{-5}$ cm/s (120 rpm), and $P_{app} = 1.67 \times 10^{-5}$ cm/s (200 rpm). After establishing that drug diffusion was under membrane-control for the three compounds listed, the experiments were not duplicated for rifampicin and pentamidine and it was assumed that these drugs were also diffusing under membrane control. Magnetic stir bars were used in subsequent permeation experiments at speeds equivalent to 60–200 rpm overhead stirring (Tables 2 and 3).

The results obtained for drug permeabilities through PM-10 membranes are shown in Table 3. The permeability coefficients were calculated using Eq. 2. It can be seen that the apparent permeability coefficients through PM-10 membranes for all the compounds were similar except for rifampicin. This may due to the higher molecular weight of the rifampicin molecule which would be expected to have a lower membrane permeability.

Table 1
Effect of membrane thickness on permeability coefficients

Drug[a]	One PM-10 membrane ($\times 10^5$ cm/s)	Two PM-10 membranes ($\times 10^5$ cm/s)
Isoniazid	1.32 (0.12)	0.71 (0.02)[b]
PAS	1.60 (0.12)	0.61 (0.03)
Pyrazinamide	1.71 (0.31)	0.61 (0.03)[b]

[a]$n = 3$, Average ± (S.D.). [b]$n = 2$.

Table 2
Comparison of experimental and theoretical buffer diffusion coefficients

Drug	Theoretical D_{aq} ($\times 10^6$ cm^2/s)[a]	Experimental P_B ($\times 10^6$ cm^2/s)	Experimental D_B ($\times 10^6$ cm^2/s)
INH	10.0	23.8	7.14
PAS	9.7	16.9	5.07
Pentamidine	7.42	18.9	5.67
Pyrazinamide	10.4	18.0	6.00
Rifampicin	5.53	25.4	7.62

[a]Calculated using Eq. 6.

3.2. Solution permeability studies

There are potentially five barriers in series for the diffusion through the buffer, BSA, or mucus solution in the custom membrane holder system. If the aqueous diffusion layers are not included, then this system becomes a three barrier system (i.e. two membranes and the solution chamber). The equation for these three physical barriers is similar to Eq. 3 with P_{soln} in place of P_m and P_m in place of P_{aq} and is given by:

$$\frac{dQ}{dt} = \frac{C_d A}{\frac{1}{P_{soln}} + \frac{2}{P_m}} \tag{4}$$

where P_{soln} is the permeability coefficient of the solution between the membranes and P_m is the permeability coefficient of the Amicon PM-10 membrane. Rearranging Eq. 4 and substituting the steady-state slope (S_{ss}) from the permeation plot for dQ/dt gives:

$$P_{soln} = \frac{-P_m}{2 - (XP_m)} \tag{5}$$

where

$$X = \frac{C_d A}{\text{Slope}_{ss}}.$$

Using the experimental steady-state fluxes from buffer or protein solutions and the apparent membrane permeability coefficients (P_m), solution permeability coefficients (P_{soln}) were obtained using Eq. 5. The apparent buffer permeabilities and the apparent protein solution permeabilities for each

Table 3
Permeability coefficients and lag-time changes of various drugs for a PM-10 membrane (P_m), buffer (P_B), mucus (P_{Mc}) and BSA (P_{BSA})

Drug[a]	P_m ($\times 10^6$ cm/s)	P_B ($\times 10^6$ cm/s)	P_{Mc} ($\times 10^6$ cm/s)	Δt_L[c] (h)	P_{BSA} ($\times 10^6$ cm/s)
INH	13.2 (1.2)[b]	23.8 (10.8)[b]	5.68 (0.62)[b]	0.76	6.40 (1.80)[b]
PAS	16.0 (2.0)	16.9 (3.6)	6.03 (0.24)	0.96	3.56 (0.13)
Pentamidine	9.3 (0.2)	18.9 (0.8)	4.64 (0.94)	2.83	4.2 (0.73)
Pyrazinamide	17.1 (3.1)	18.0 (2.8)	7.93 (0.15)	2.19	6.46 (0.15)
Rifampicin	4.8 (0.7)	25.4 (4.2)	2.31 (0.70)	2.21	1.1 (0.13)

[a] $n = 3$. [b](S.D.). [c]Lag-time difference between buffer and mucus solutions.

drug are given in Table 3. The buffer permeability studies were conducted to estimate the drug diffusivities through an aqueous environment and to provide a basis for comparing the drug permeabilities through the protein solutions. In addition to buffer, BSA was used as a reference protein solution since mucin-binding of these compounds was compared with BSA binding in another study (Bhat et al., 1995). The concentration of the protein solutions used were 4% BSA solution, which is the albumin concentration in plasma, and 2.85% mucus solution, this being the concentration of mucus obtained by the reconstitution, purification and equilibration procedure. On a molar basis, the BSA solution was approximately 0.6 mM while mucin was approximately 0.07 mM, thus BSA was ~ 8.4 times more concentrated than the mucin solution.

Using mannitol as a reference solute (MW: 182 g/mol, $D_{aq} = 9.14 \times 10^{-6}$ cm^2/s, Karlsson et al., 1993), theoretical aqueous diffusion coefficients for these drugs were calculated using the following equation:

$$D \times \mathrm{MW}^{1/3} = \text{Constant} \tag{6}$$

Theoretical aqueous diffusion coefficients and experimentally obtained buffer diffusion coefficients are shown in Table 2. Since the apparent solution permeability coefficients were defined as $P = DK/h$, the apparent buffer diffusion coefficients were obtained from the product of the permeability coefficient and the solution thickness in the membrane holder (0.3 cm) with $K = 1$. It can be seen that the experimental buffer diffusion coefficients are similar to the calculated values.

The buffer diffusion coefficients reported in Table 2 are all of the same order of magnitude. Even though rifampicin has the highest molecular weight, its apparent buffer permeability coefficient is also the highest of the compounds studied. This may be because the range of molecular weights of the compounds spanned in this study is rather narrow (123 g/mol–823 g/mol). Hence, the theoretical range of D_{aq}, $5.53–10.4 \times 10^6$ cm^2/s, and the experimental range of D_B of $5.07–7.62 \times 10^6$ cm^2/s, are well within the experimental error possible with this technique. Therefore, there appear to be no significant differences in the observed buffer permeabilities among the compounds studied.

To evaluate the contribution of each barrier to the overall diffusional resistance, individual barrier resistances were calculated. For this series barrier system, the apparent total resistance (R_{total}) is the sum of the reciprocals of the apparent individual barrier permeabilities ($P_{barrier}$).

$$R_{total} = \sum_{i=1}^{n} R_{barrier,i} = \sum_{i=1}^{n} \frac{1}{P_{barrier,i}} \tag{7}$$

For drug-permeation through the custom membrane holder consisting of a solution between two Amicon PM membranes, Eq. 7 can be written as:

$$R_{total} = \sum_{i=1}^{3} \frac{1}{P_{barrier,i}} = \frac{2}{P_{membrane}} + \frac{1}{P_{soln}} \tag{8}$$

Thus, knowing the apparent permeability coefficient of the compound through the membrane and the steady-state flux through the total system, it was possible to calculate the individual apparent permeability coefficients through each of the barriers. Using Eq. 8, the apparent resistances

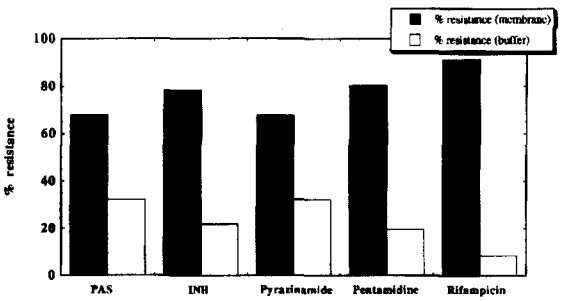

Fig. 2. Comparison of diffusional resistances of Amicon PM-10 membranes and buffer solution.

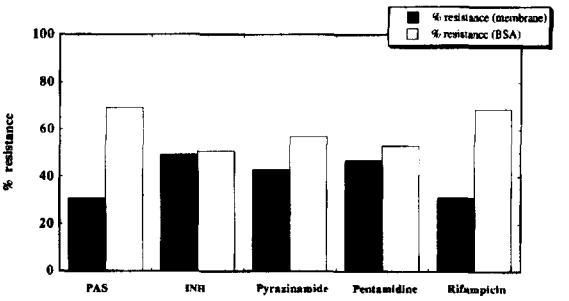

Fig. 4. Comparison of diffusional resistances of Amicon PM-10 membranes and 4% BSA solution.

($R_{barrier}$) of each of the barriers were calculated from individual permeability coefficients.

Figs. 2–4 show histogram plots comparing the relative percentage resistances of the barriers in the series. Fig. 2 compares the diffusional resistances through the custom membrane holder with Sørenson's phosphate buffer. In this case, the membranes account for most of the transport resistance (67.8–92.0%) thus making the solution resistances small (8.2–32%). For the cases when a protein solution was placed within the chamber, the fraction of the membrane resistance decreased to 43.0–50.0% of the total resistance and the apparent solution resistances increased proportionately. Thus, the mucus resistance value was 50.0–57.0% (Fig. 3) while BSA resistance value was 50.8–69.2%. The resistance of the protein solution is due to a combination of factors such as protein binding, viscosity, physical obstruction and/or effects due to bound water.

The apparent permeability coefficients (P_{soln}) obtained for the various drugs through the two

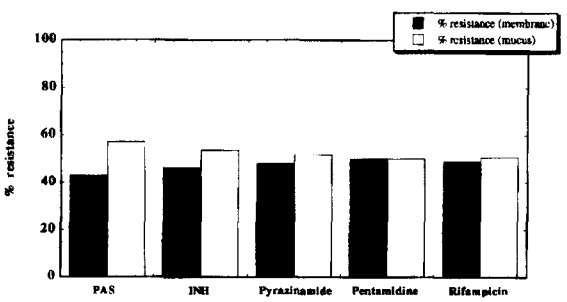

Fig. 3. Comparison of diffusional resistances of Amicon PM-10 membranes and 2.85% mucus solution.

protein solutions were about one order of magnitude less than those obtained through the buffer solution. If the mucus glycoproteins were acting merely as inert fillers, then on saturation of the mucin binding sites, the drugs should have exhibited steady-state fluxes similar to those for the buffer solution. The reduction in apparent permeabilities could be due to interaction with protein, physical obstruction of diffusion by the highly intertwined mucus glycoproteins, and/or binding of water to the glycoprotein matrix which reduces the amount of "free water" available for diffusion. Mucus is a viscoelastic gel (Silberberg, 1988) and it has been suggested that such a system is a macromolecular network of cross-linked glycoproteins which gives rise to macroscopic aggregates (Gu et al., 1988). Whether permanently cross-linked or not, the molecular units of the mucus glycoproteins overlap and interpenetrate to form a linked macroscopic network that could function as a barrier to diffusion (Forstner and Forstner, 1975).

The sialic acid residues on mucin glycoproteins have a pK_a of 2.6 (Johnson and Rainsford, 1972) which results in mucin being negatively charged at pH 7.4. The repulsion between the anionic sialic acid residues and the sulfate residues places the mucin glycoproteins in a stretched conformation similar to other charged polymers in solution. It is this expanded nature of the mucin network which facilitates interpenetration of the glycoprotein molecules giving mucin a meshed structure and significantly increases the system's tortuosity. This increased tortuosity could also be responsible for the reduction in these apparent permeability coefficients.

Mucus glycoproteins also contain a significant fraction of associated carbohydrate residues — 160–200 chains per molecule. The carbohydrate composition is polydisperse with respect to oligosaccharide composition, sequence, and chain length. (Rosbottom, 1968; Carlson, 1968; Spiro, 1970; Oates et al., 1974). These carbohydrate chains form a dense, hydrophilic layer surrounding the protein backbone. The high carbohydrate content ensures that the equivalent hydrodynamic volumes of all mucins are quite high (Allen et al., 1974; Forstner, 1978) and considerable amounts of water tend to be immobilized within the fibrillar network (Creeth et al., 1977). This means that even though the bulk of the mucus solution is aqueous in nature, not all of the water may be available for free diffusion of solutes. Carlstedt et al. (1983, Carlstedt et al., 1985) have shown by analytical ultracentrifugation, buoyant density, and electron microscopy studies that mucin is a highly flexible linear polymer with the subunits joined end-to-end. To fit the spheroidal domain predicted by hydrodynamic data, they suggested that the linear mucin threads must be sufficiently flexible to approach the behavior of a random coil. Such a model suggests that the mucin chains must assume a random configuration with a large hydrodynamic radius. Such a configuration would have large amounts of water not only associated in shells of hydration but also entrapped within the random coil structure, thus decreasing the amount of free water available for diffusion.

Similar reductions in apparent permeability coefficients to those observed in these studies have been reported by previous investigators. As stated earlier, Kearney and Marriott (1986) found increased lag-times as well as decreased transport rates for tetracycline in everted gut experiments. Braybrooks et al. (1974) observed an $\sim 50\%$ decrease in the apparent permeability coefficients for tetracycline in the presence of mucus using a combination of perfusion, everted gut studies, diffusion cell techniques and a Sartorius absorption apparatus. Karlsson et al. (1993) found, in a study of testosterone permeation through a mucus-producing human goblet cell line (HT29-H), that the permeability coefficients increased by $\sim 50\%$ when the mucus layers were removed.

If all the factors which affect transport remain constant, for chemically dissimilar compounds it would be expected that the resistance due to mucus would depend on the interactions of the drug with mucus glycoproteins. Resistances of 50.0–57.0%, seen for the various drugs, in mucin indicates that it equivalently inhibited the diffusion of all drugs. Thus, the diffusional reduction by mucin is non-specific in nature and similar mechanisms are likely involved in the slowing of the diffusion of all compounds.

Permeabilities through BSA solutions also indicated a significant decrease in the apparent permeability coefficients through this macromolecular solution. Protein binding, viscosity of BSA solution, and/or the "bound water" could also be affecting the permeabilites.

4. Conclusions

A model in vitro model mucus system has been developed for use in in vitro binding and permeability studies, and a new permeability system has been developed for performing permeation studies through a test solution containing macromolecules. Results from permeation experiments showed that more than protein binding between the diffusing compounds and the mucus solution slows diffusion. Increased lag-times were associated with decreased steady-state fluxes for the solutes when buffer solutions were replaced by mucus or BSA solutions. Besides binding to mucus glycoproteins, there could be an obstructive effect by mucin, increased viscosity of the solution, and/or a decrease in the fraction of "free" water, all of which could contribute to a reduction in the apparent permeability coefficients. Mucus diffusional resistances were 50.0–57.0% of the total observed resistance and those for BSA were 50.8–69.2%. These results indicate adequate sensitivity of the diffusional system for the experimental determination of such permeability parameters since the resistances were over half the total observed resistances.

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