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*Research Paper*

# Interplay of Secondary Structure and Charge on the Diffusion of a Polypeptide through Negatively Charged Aqueous Pores

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***Purpose.*** This study was conducted to investigate the interplay of secondary structure and charge of a polypeptide on its permeability through negatively charged pores of synthetic porous membranes and Caco-2 cell monolayers.

***Materials and Methods.*** Poly(D-glutamic acid) [Poly(D-Glu)] was used as a model polypeptide. Transport studies were conducted at 37°C through both track-etched polycarbonate membranes (using side-by-side diffusion cells) and Caco-2 cell monolayers. Apparent permeability coefficients and diffusion coefficients were calculated.

***Results.*** When diffusion was unhindered, poly(D-Glu) appeared to be transported at the same rate regardless of whether it existed in the random coil or the α-helix secondary structure. When moderately hindered diffusion was evaluated, poly(D-Glu) with partial α-helix secondary structure, exhibited significantly greater transport than when the polypeptide predominantly existed as the highly negatively charged random coil. This trend was reversed when the diffusion was severely hindered by the tight junctions of the Caco-2 cell monolayers.

***Conclusions.*** Neither charge, nor secondary structure, played a significant role in the unhindered diffusion of poly(D-Glu). When the molecules were moderately hindered, polypeptide/membrane charge interactions significantly influenced the rate of aqueous diffusion. As the overall molecular dimensions of the polypeptide approached the pore size, the inherent molecular flexibility of the random coil secondary structure overcame the effect of charge repulsion.

**KEY WORDS:** charge; diffusion; paracellular transport; polypeptide; secondary structure.

## INTRODUCTION

Recent advances in the area of biotechnology and the understanding of physiological processes have led to the identification and economical production of a large number of biologically active peptides and proteins in large quantities and with high purity. These compounds generally show high potency, high specificity, and low generalized toxicity, rendering them attractive therapeutic agents. Unfortunately, a major impediment associated with the administration of therapeutic peptides and proteins is that most need to be given by injection as a result of both their poor membrane permeability and their extensive enzymatic degradation. Consequently, there is an urgent need for improved delivery of this class of therapeutic compounds.

Due to their hydrophilic nature, therapeutic polypeptides and proteins are generally unable to permeate the lipid bilayer of cell membranes. Thus, their transport across biological membranes normally occurs by the paracellular route. Even though the paracellular route is usually considered to be an absorption pathway for small hydrophilic molecules, there have continuously been reports that this route can also accommodate hydrophilic macromolecules, including peptides and proteins. This may appear to be counterintuitive, based on knowledge that has arisen from the pharmaceutical scientific community directed at the absorption of traditional low-molecular-weight drug compounds. However, it has been shown that large-molecular-weight polypeptides and proteins are absorbed via the paracellular pathway, albeit in small quantities (1,2).

There have been numerous attempts to improve membrane permeability of peptide and protein drugs by co-formulating them with permeation enhancers, such as surfactants, polymers, etc., in order to overcome significant diffusion barriers through various mechanisms. Unfortunately, success has not only been limited, but also appears to be compound-specific. Additionally, such a strategy involving modification of either transcellular or paracellular permeability usually leads to potential safety issues associated with both the reduced barrier function of the absorptive epithelium and other non-specific effects of these pharmaceutical additives. For this reason, the approach taken in the present study involved modifying the permeant rather than the absorptive epithelium.

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It is usually assumed that the primary resistance to the paracellular flux of drug molecules results from the presence of the tight junctions. Tight junctional strands can be viewed as relatively impermeable structures, in which "channels" or "pores" reside (3). The interior of these channels appears to be highly hydrated, and contains fixed negative charges (4). Therefore, the tight junctions can also be viewed as negatively charged, aqueous-filled pores. Since the molecular size of therapeutic peptides and proteins typically either approaches or exceeds the size of the aqueous channels, these macromolecules are likely to experience frictional resistance and hindrance by the pore wall while diffusing through the aqueous-filled pores that comprise the paracellular pathway.

In addition to size limitations, movement of solutes through the tight junctions is also restricted based on their charge. For small molecules, it has been demonstrated that positively charged cations of weak bases permeate the aqueous pores of the paracellular pathway at a faster rate than their neutral images, whereas, the negatively charged anions of weak acids permeate at a much slower rate (5,6).

Although selectivity to paracellular transport is based predominantly on a permeant's size and charge (7), several studies have suggested that other molecular properties of solutes may influence their paracellular permeability. These additional properties include molecular geometry (8), flexibility (9), and conformation (10,11). These properties can be interrelated and, therefore, may act either alone, or collectively, to determine the overall rate and extent of paracellular transport.

In solution, polypeptides exhibit different types of secondary structure (such as random coil, α-helix, and β-sheet) based, in part, on their amino acid sequence and the solution microenvironment. The potential to modulate both the rate and extent to which a polypeptide undergoes paracellular transport may be possible by intentional selection of the secondary structure that possesses optimal molecular geometry. The influence of various structural features (especially molecular size and ionic charge) on paracellular permeability has been investigated using small peptides, in which formation of various types of secondary structure (other than β-turns) is not possible (12,13). Therefore, the effect of secondary structure on paracellular transport has not been systematically evaluated.

Traditionally, a series of molecules are used to assess the effects of size/charge on transport. However, the approach of using a series of molecules does not work well when investigating the effects of overall molecular dimensions/geometry (conferred by secondary structure) of such a complex molecule as a polypeptide on its paracellular transport. This is due to the confounding variables that might have resulted from widely varying physicochemical properties if two different polypeptides were used; i.e., one model polypeptide to represent a random coil conformation and a second, different polypeptide to represent an α-helix secondary structure. It is worth mentioning that the β-sheet conformer was not investigated in the present study because it appeared to significantly delay the aqueous diffusion of a polypeptide (14).

Poly(D-glutamic acid), a synthetic homopolypeptide, was chosen as a model polypeptide in the present work because of the ease associated with converting the random coil secondary structure to the α-helix secondary structure and because of the polypeptide's negligible permanent tertiary structure. By using the same polypeptide, which we intentionally forced to adopt either of the two different secondary structures by a simple stimulus, it was possible to have two conformations of the same chemical composition, identical MW, and the same net overall charge (both negative in the case of poly(D-glutamic acid), albeit, the random coil has a greater negative charge density than the α-helix conformation). Thus, as many variables as possible were held constant so that the effects of charge and secondary structure of a polypeptide on its diffusive transport through negatively charged, aqueous-filled pores could be evaluated.

The purpose of the present study was to investigate the interplay of molecular geometry (conferred by secondary structure) and charge of a polypeptide on its permeability through negatively charged pores. Various diffusion barriers with decreasing pore sizes were used to assess the relative contributions of charge and molecular flexibility of poly(D-glutamic acid) on its diffusion through negatively charged pores under various degrees of hindrance (i.e., unhindered, moderately hindered, and severely hindered situations).

## MATERIALS AND METHODS

### Materials

Poly(D-glutamic acid) sodium from the 4–15 kDa molecular weight range (with an average molecular weight of 13.0 kDa, a degree of polymerization of 86, and a polydispersity index of 1.1, as determined by the supplier), potassium phosphate (monobasic, anhydrous), hydrochloric acid, and sodium hydroxide were purchased from Sigma Chemical Company (St. Louis, MO). Whatman® Nuclepore® track-etched membranes (polyester or polycarbonate; 13-mm membrane diameter; 0.015-, 0.4-, or 1-μm pore diameters) were obtained from Fisher Scientific (Hanover Park, IL). Micro BCA™ reagents were purchased from Pierce (Rockford, IL). All materials were used as received.

Human colon adenocarcinoma Caco-2 cells were obtained from the American Type Culture Collection (ATCC) Cell Repository (Rockville, MD). Cell culture reagents were from Invitrogen (Grand Island, NY). Costar® Transwell® inserts (polyester, 12-mm diameter, 3.0-μm pore diameter) for 12-well microplates were obtained from Fisher Scientific (Hanover Park, IL).

### Methods

All experiments were performed in triplicate or quadruplicate, unless otherwise stated, and the results are expressed as the mean value ± standard deviation. Statistical significance of the differences between group means was determined by a two-tailed Student's *t*-test, and the differences deemed significant at $P < 0.05$.

In this study, poly(D-glutamic acid) with a polydispersity index of 1.1 was used as received. Ideally, a monodisperse polypeptide would be preferred. Unfortunately, it is rather difficult to achieve, due to the nature of the polymerization method used to synthesize this homopolypeptide. In addition, although the model polypeptide was not perfectly monodis-

perse, the same polypeptide, with the same molecular weight distribution and the same chemistry, was used in all experiments. More importantly, the polydispersity index, which is the weight-average molecular weight ($M_w$) divided by the number-average molecular weight ($M_n$), of 1.1 for the poly(D-glutamic acid) used in this study is considered by many to represent a fairly narrow molecular weight distribution that does not require further fractionation (15,16).

### *Induction of Secondary Structural Changes in Poly(D-glutamic acid)*

At neutral pH, poly(D-glutamic acid) exists as a highly negatively charged random coil conformer. The α-helix conformer can be induced by decreasing the solution pH to below 5.0. A sufficient amount of poly(D-glutamic acid) sodium was first dissolved in phosphate buffer (10 mM) to achieve a concentration of 1 mg/ml, unless otherwise noted. The percentage of the α-helix conformer formed as a function of pH was examined by successively decreasing the pH of five identical aliquots of the poly(D-glutamic acid) solution to pH values of 7.4, 6.0, 5.0, 4.5, and 4.0. This was accomplished by adding a small volume of either 1 N HCl or 1 M NaOH.

To verify formation of the appropriate secondary structure, circular dichroism (CD) studies were conducted as described previously (17) using a Jasco J-720 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan). The percentages of each secondary structure (i.e., random coil, α-helix, and β-sheet) present in the solutions were determined using the JFIT program developed by Dr. Bernhard Rupp, Lawrence Livermore National Laboratory, Livermore, CA (18). The software deconvolutes the recorded CD spectra into three individual contributions from the random coil, α-helix, and β-sheet secondary structural elements by fitting the CD spectra in the region of 190–250 nm to the standard curves obtained from different conformations of poly(L-lysine).

### *Determination of Aqueous Diffusion Coefficients ($D_{aq}$) and Hydrodynamic Radii ($r_H$)*

In order to estimate the hydrodynamic radius of poly(D-glutamic acid), its aqueous diffusion coefficient was first determined from diffusion experiments across track-etched polyester membranes having a pore diameter of 1 μm, as described previously (14). Briefly, diffusion studies were conducted at 37 ± 1°C for 3 h, using side-by-side diffusion cells (Crown Glass Company, Inc., Somerville, NJ) and a donor solution of poly(D-glutamic acid) (1 mg/ml) with a pH of either 7.4 or 4.5, while phosphate buffer (10 mM) with the same pH was simultaneously added to the receptor chamber. Samples (200 μl) were periodically withdrawn at predetermined time points (0, 5, 10, 15, 30, 45, 60, 90, 120, 150, and 180 min) from the receiver compartment with buffer replacement, and subsequently analyzed for the polypeptide in duplicate using the Micro BCA™ protein assay method modified for 96-well microtiter plates, as described previously (14). At the end of each experiment, CD measurements were performed with an aliquot of the donor and receptor solutions in order to verify that the desired percentages of each conformer were maintained throughout the experiments.

For each transport profile, the apparent permeability coefficient ($P_{app}$ in cm/sec) and the aqueous diffusion coefficient ($D_{aq}$ in $cm^2$/sec) were calculated as described previously (14). The electrochemical energy function term and the Renkin molecular sieving function were not included in the calculation of $D_{aq}$, since the membrane pores were uncharged and also much larger than the radius of the molecules, thus allowing free diffusion of the solute molecules across the membrane. The rearranged Stokes–Einstein equation was then used to estimate the hydrodynamic radius ($r_H$ in cm) from the calculated $D_{aq}$, along with the viscosity of phosphate buffer (10 mM) at 37°C.

### *Diffusion through Negatively Charged Aqueous Pores of Synthetic Polycarbonate Membranes*

Diffusion studies analogous to the previous section were conducted at 37 ± 1°C, except that track-etched polycarbonate membranes with a pore diameter of either 0.4 or 0.015 μm were used as the diffusion barrier. Transport studies across the 0.4-μm polycarbonate membranes were carried out for 3 h, and the sampling time points were 0, 5, 10, 15, 30, 45, 60, 90, 120, 150, and 180 min. In contrast, the experiments with the 0.015-μm polycarbonate membranes were continued for 24 h due to their low membrane porosity (Table I). Thus, samples were obtained at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, and 24 h. All samples were analyzed for poly(D-glutamic acid) in duplicate using the Micro BCA™ protein assay method modified for 96-well microtiter plates, as described previously (14). The apparent permeability and diffusion coefficients were calculated from each transport profile.

### *Transport Studies across Caco-2 Cell Monolayers*

Caco-2 cells were grown as monolayers in T-75 tissue culture flasks with a flat surface of 75 $cm^2$. The cells were maintained and subcultured weekly in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% Modified Eagle's Medium (MEM) non-essential amino acids (100X solution), 166.8 Units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. For transport studies, Caco-2 cells were plated at a density of 100,000 cells/well onto 12-mm-diameter Transwell® inserts (previously coated with rat tail collagen) in 12-well microplates, and also maintained in the same culture medium as described above. All the cells were incubated at 37°C in a highly humidified atmosphere of 95% air/5% $CO_2$. The culture medium was changed every other day for the first

**Table I.** Porosity and Thickness of the Track-etched Polycarbonate Membranes Used in This Study

| Membrane | Pore Radius (Å) | Pore Density (pores/$cm^2$) | Porosity | Thickness (μm) | $\varepsilon/l$ ($cm^{-1}$) |
|---|---|---|---|---|---|
| 0.4-μm Polycarbonate | 2,000 | $1\times10^8$ | 0.1257 | 10 | 125.66 |
| 0.015-μm Polycarbonate | 75 | $6\times10^8$ | 0.0011 | 6 | 1.77 |

7 days and daily afterwards. Cell monolayers were used between 21 and 28 days post-seeding at passages 20–40, and only those displaying a transepithelial electrical resistance (TEER) value between 170 and 250 Ω $cm^2$ were used for transport studies.

All transport studies with Caco-2 cell monolayers were carried out at 37 ± 1°C for 3 h in 12-well microplates with the cells grown on Transwell® inserts. Dulbecco's phosphate buffered saline (DPBS) was used as the transport medium. Prior to the experiments, confluent Caco-2 cell monolayers were rinsed free of culture medium 2–3 times using DPBS (pH 7.4, prewarmed at 37°C). For detection purposes in these Caco-2 cell monolayer diffusion experiments, the N-terminus of poly(D-glutamic acid) sodium was labeled with fluorescein isothiocyanate (FITC) using a FluoroTag™ FITC Conjugation Kit from Sigma Chemical Company (St. Louis, MO), as per the manufacturer's protocol.

Solutions of FITC-labeled poly(D-glutamic acid) (200 μg/ml) with a pH of either 7.4 or 4.5 were placed in the donor (apical) chamber (0.5 ml/well), while DPBS (1.5 ml/well) with the same pH as the donor solution was added to the receptor (basolateral) side. At predetermined time points (0, 15, 30, 45, 60, 80, 100, 120, 140, and 180 min), samples (200 μl) were periodically obtained from the receiver compartment with buffer replacement. The samples were transferred to 96-well microtiter plates, and analyzed for FITC-labeled poly(D-glutamic acid) in duplicate using a SpectraFluor Plus microplate reader (Tecan U.S. Inc., Durham, NC) at the excitation and emission wavelengths of 485 and 535 nm, respectively. The apparent permeability and diffusion coefficients were then determined.

Another set of experiments were conducted to confirm the pathway by which poly(D-glutamic acid) traversed the Caco-2 cell monolayers at both pH 7.4 and 4.5. A selective calcium chelator, EGTA, was used to bind free extracellular $Ca^{2+}$ in order to reversibly open up the tight junctions. Theoretically, this should lead to a significant increase in paracellular transport, whereas, permeation by the transcellular route should remain unaffected. All EGTA experiments were performed under the same conditions as the transport studies previously described above, except that prior to the experiments, the Caco-2 cell monolayers were pretreated at 37°C for 45 min with prewarmed DPBS (pH 7.4) containing 2.5 mM EGTA in both the apical and basolateral compartments. At the end of the incubation period, the treated cell monolayers were then rinsed 2–3 times with prewarmed DPBS (pH 7.4) without EGTA. Transport studies of poly(D-glutamic acid) were subsequently conducted for 3 h. Integrity of the monolayers was confirmed at the end of all experiments by TEER measurements and determinations of [$^{14}$C]mannitol transport.

## RESULTS AND DISCUSSION

### Induction of Secondary Structural Changes in Poly(*D*-glutamic acid)

pH was used in the present study as a stimulus to induce and maintain the desired secondary structure in poly(D-glutamic acid). Ideally, a model polypeptide in which secondary structural changes could be induced with a simple stimulus, and the desired secondary structure maintained after the stimulus is removed, would be preferred. Unfortunately, no polypeptide with this property could be identified after an extensive search.

The effect of pH on the percentages of each conformer present in a poly(D-glutamic acid) solution is shown in Fig. 1. As the pH was decreased to below 6.0, the carboxylate side chains underwent charge neutralization, allowing the α-helix structure to be formed by intramolecular hydrogen bonding. The percentage of the induced α-helix conformer started to level off at pH 4.5. A further decrease in the solution pH to 4.0 did not result in a significant increase in the percent of the α-helix conformer that was formed. Additionally, as the solution pH approached 3.0, precipitation of the polypeptide was observed. This is why the less acidic pH of 4.5 was selected for induction and preservation of the α-helix conformer in all of our experiments.

The two lines in Fig. 1 intersected at a pH of approximately 4.6–4.7, representing a 50:50 ratio of the two conformers, which was in relatively good agreement with the reported p$K_a$ of 4.3–4.6 for the carboxylic acid side chain of glutamic acid residues in proteins. This result supports the premise that charge neutralization is the mechanism by which the α-helix secondary structure of poly(D-glutamic acid) is induced. It should be noted that the extent of α-helix formation in poly(D-glutamic acid) was comparable to that in poly(L-glutamic acid) from the same molecular weight range (19). Therefore, poly(D-glutamic acid) was used in all experiments to prevent enzymatic degradation in subsequent studies involving biological membranes.

### Determination of Aqueous Diffusion Coefficients ($D_{aq}$) and Hydrodynamic Radii ($r_H$)

The effect of pH on the percentages of each conformer in a poly(D-glutamic acid) solution described in the previous section was investigated in order to identify an optimum pH for inducing the α-helix structure. Therefore, the experiments

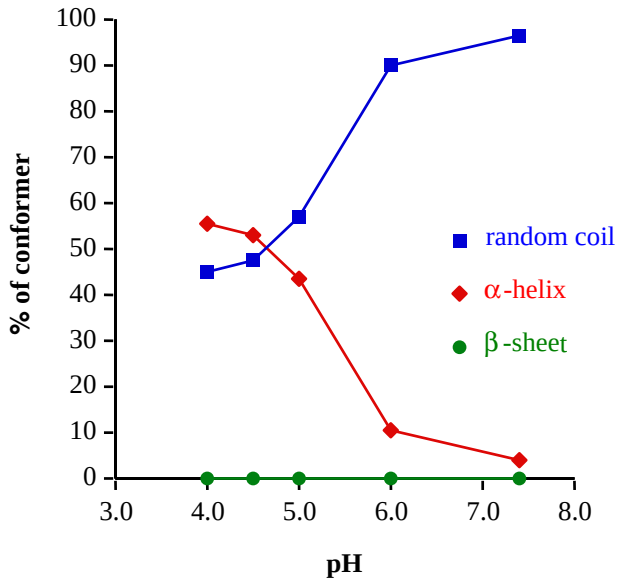


**Fig. 1.** Effect of pH on the percentages of each conformer in a poly(D-glutamic acid) solution at 25°C.

**Table II.** Characteristics of Poly(D-glutamic acid) Used in This Study Determined at 37°C

| pH | Random Coil (%) | α-Helix (%) | $P_{app} \times 10^4$ (cm/sec)[a] | $D_{aq} \times 10^6$ (cm$^2$/sec)[a] | $r_H$ (Å)[b] |
|---|---|---|---|---|---|
| 7.4 | 96.5 ± 0.1[c] | 3.5 ± 0.1 | 2.0 ± 0.3 | 2.8 ± 0.4 | 10.8 ± 1.4 |
| 4.5 | 47.4 ± 1.0 | 52.6 ± 1.0[c] | 2.5 ± 0.6 | 3.5 ± 0.8 | 9.0 ± 2.2 |

[a] Determined from the diffusion across track-etched polyester membranes with a nominal pore diameter of 1 μm.
[b] Hydrodynamic radius estimated from the $D_{aq}$ using the Stokes–Einstein equation.
[c] Represents the percentage of the predominant conformer in the solution.

were conducted at room temperature, which is normally used for CD measurements. Although our previous studies have shown that increasing the temperature to 37°C does not affect the extent of the pH-induced α-helix conformer in this type of synthetic polypeptide (17), the percentages of each conformer present at pH 7.4 and 4.5 in the solutions used for transport studies were also determined at 37°C, and the results are shown in Table II.

As reported previously, the hydrodynamic radius of a molecule can be determined from its diffusivity through membranes with large pores (14). When the average pore radius of these membranes is much larger than the radius of the solutes, their diffusion through the pores is not hindered, and the resulting diffusion coefficient approaches the value in the absence of constraints introduced by the pores ($D_\infty$). In this study, diffusion experiments across track-etched polyester membranes with a pore diameter of 1 μm were conducted in order to estimate the $D_{aq}$ of poly(D-glutamic acid) at pH 7.4 and 4.5.

Table II shows the calculated transport parameters associated with poly(D-glutamic acid) diffusion through 1-μm diameter pores of a polyester membrane at two pH values, in which different percentages of the random coil and α-helix conformers were present. There was no significant difference ($P > 0.05$) in the mean values of either the $P_{app}$ or the $D_{aq}$ between pH 7.4 and 4.5. This may be because the change in shape, upon formation of the α-helix, was not drastic enough to alter the aqueous diffusion of the polypeptide in bulk solution.

The hydrodynamic radius of poly(D-glutamic acid) at each pH was subsequently estimated. As shown in Table II, conversion of 53% of the molecules to the α-helix conformer by decreasing the solution pH to 4.5 resulted in a slightly smaller hydrodynamic radius, but it was not significantly different ($P > 0.05$) from the hydrodynamic radius calculated for the random coil conformer at pH 7.4. These results suggest that the overall molecular dimensions of these two conformers are comparable.

### Diffusion through Negatively Charged Aqueous Pores of Synthetic Polycarbonate Membranes

Another set of diffusion experiments were conducted using synthetic polycarbonate membranes with two different pore sizes in order to examine the effects of different degrees of hindrance (i.e., no hindrance and moderate hindrance) on the passive diffusion of a macromolecule through a negatively charged, aqueous-filled pore. The molecular size restriction (or degree of hindrance) imposed by the pores was estimated for each membrane by calculating the Renkin molecular sieving function ($F(\frac{r}{R})$), using the hydrodynamic radii of poly(D-glutamic acid) determined at the corresponding pH (Table II), as described previously (14). The mean values of the Renkin molecular sieving function at pH 7.4 and 4.5 were not significantly different ($P > 0.05$) from one another for any of the diffusion barriers evaluated (Table III).

In unhindered diffusion (i.e., the Renkin molecular sieving function was close to 1.0), poly(D-glutamic acid), whether in the random coil or α-helix secondary structure, was transported across a 0.4-μm polycarbonate membrane at approximately the same rate and to the same extent (Fig. 2). The apparent diffusion coefficients, calculated from the fluxes during the first 30 min (linear portion of the profiles), were not significantly different, i.e., 1.99 (± 0.15) $\times 10^{-6}$ cm$^2$/sec at pH 7.4, compared to 2.16 (± 0.17) $\times 10^{-6}$ cm$^2$/sec at pH 4.5. These results indicate that charge interaction did not appear to play a significant role in unhindered diffusion, since these large, negatively charged pores did not distinguish between the highly negatively charged random coil conformer and the α-helix structure with partial negative charges. This inference appears reasonable considering the Coulomb's Law $\left(F = kq_1q_2/d^2\right)$, which states that the charge interaction ($F$) is inversely proportional to the square of the distance ($d$) between two charges ($q_1$ and $q_2$). Since the pores were so large in this case, the interaction between the charges associated with the pore wall and the permeant was likely to be minimal.

**Table III.** Estimated Renkin Molecular Sieving Function of Poly(D-glutamic acid)

| | $\left(\frac{r}{R}\right)$ | | $F\left(\frac{r}{R}\right)$ | |
|---|---|---|---|---|
| Membrane | pH 7.4 (96% Random Coil) | pH 4.5 (53% α-Helix) | pH 7.4 (96% Random Coil) | pH 4.5 (53% α-Helix) |
| 0.4-μm Polycarbonate ($R$ = 2,000 Å) | 0.005 ± 0.001 | 0.005 ± 0.001 | 0.974 ± 0.003 | 0.978 ± 0.005 |
| 0.015-μm Polycarbonate ($R$ = 75 Å) | 0.144 ± 0.019 | 0.120 ± 0.029 | 0.480 ± 0.047 | 0.547 ± 0.084 |
| Caco-2 Cell Monolayers ($R \cong$ 12 Å)[a] | 0.902 ± 0.117 | 0.753 ± 0.184 | 0.002 ± 0.003 | 0.014 ± 0.016 |

[a] Literature value of the pore radius in Caco-2 cell monolayers (5).

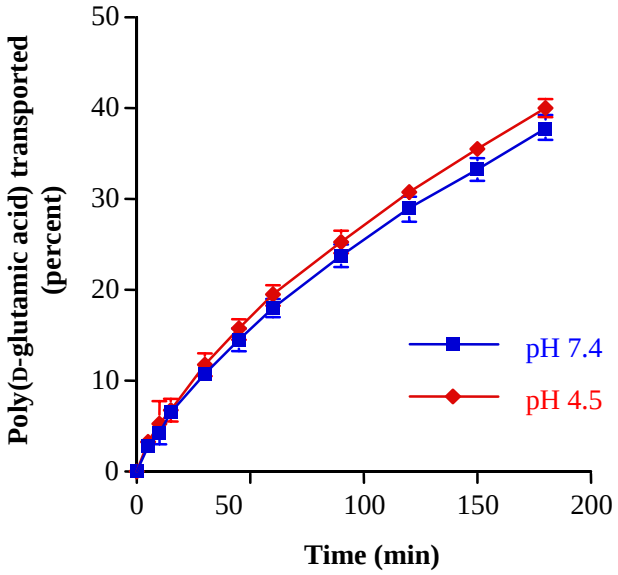


**Fig. 2.** Percentages of poly(D-glutamic acid) transported across a track-etched polycarbonate membrane with an average pore diameter of 0.4 μm at 37°C.

When the present study evaluated moderately hindered diffusion of poly(D-glutamic acid) across a 0.015-μm polycarbonate membrane, the Renkin molecular sieving function was calculated to be approximately 0.48 and 0.55 at pH 7.4 and 4.5, respectively (Table III). It is evident from Fig. 3 that poly(D-glutamic acid) at pH 4.5 was transported across a 0.015-μm polycarbonate membrane at a faster rate and to a greater extent than at pH 7.4. The calculated apparent diffusion coefficients through the negatively charged pores at both pH values were significantly different ($P < 0.05$), i.e., 1.10 ($\pm 0.04$)$\times 10^{-6}$ cm$^2$/sec at pH 7.4, compared to 2.38 ($\pm 0.14$)$\times 10^{-6}$ cm$^2$/sec at pH 4.5. The observed trend corroborates the previously reported charge selectivity of the negatively charged paracellular pathway, in which organic anions permeated the pores more slowly than their neutral images, while protonated amines permeated at a much faster rate (5). These data seem to suggest that the enhanced transport of poly(D-glutamic acid) at pH 4.5, in which a smaller number of the side chain groups exist as carboxylate anions, occurred because the α-helix structure was subjected to less repulsion by the fixed negative charges associated with the pore wall, compared to the highly negatively charged random coil conformer predominantly present at pH 7.4. It is possible that, in moderately hindered diffusion, the charges associated with the permeant and the pore wall were in closer proximity, leading to stronger charge interactions, and thus, played a more important role compared to when the diffusion was unhindered.

### Transport Studies across Caco-2 Cell Monolayers

The paracellular diffusion of polypeptides across biological membranes represents the case of severely hindered diffusion. Caco-2 cell monolayers have tight junctions that are well characterized and thus provide an ideal model for paracellular transport under hindered conditions. Adson *et al.* (5) have estimated the effective pore radius in Caco-2 cell monolayers to be 12.0 ± 1.9 Å. With a hydrodynamic radius of approximately 10 Å for poly(D-glutamic acid) at both pH 7.4 and 4.5 and a pore radius of 12 Å, the Renkin molecular sieving function approached zero (Table III), indicating that the diffusion of this polypeptide across Caco-2 cell monolayers was likely to be severely hindered at both pH values.

Although most transport studies using Caco-2 cells have been conducted at neutral or mildly acidic pH, these cells have been shown to be able to tolerate a more acidic pH, which, in our case, was necessary to induce the α-helix conformer of poly(D-glutamic acid). For example, Hilgers *et al.* (20) have reported that pH 4.5 did not damage either the morphology or the barrier properties of Caco-2 cell monolayers. In addition, a previous study conducted in our laboratory using both diazepam (transcellular marker) and mannitol (paracellular marker) demonstrated that neither pH 4.7, nor the presence of poly(D-glutamic acid) in the donor solution, affected the integrity of Caco-2 cell monolayers during the course of a typical 3-h transport study (21). In the present study, the TEER values before and after the transport experiments of poly(D-glutamic acid) at pH 4.5 were not significantly different from each other (i.e., 189.7 ± 13.8 Ω cm$^2$ and 185.6 ± 11.1 Ω cm$^2$, respectively), which confirms that the integrity of Caco-2 cell monolayers was not affected after having been exposed to pH 4.5 for 3 h.

It should be noted that since poly(D-glutamic acid) lacks a chromophore that would allow effective detection, sample analysis in the previous experiments involving synthetic membranes was made possible by using the Micro BCA assay. Unfortunately, this assay could not be used in the Caco-2 cell monolayer experiments due to the interference by other proteins released from Caco-2 cells. Therefore, a new method of analysis was required. After an extensive search, labeling the polypeptide with FITC appeared to be the most suitable alternative in this case, not only because the

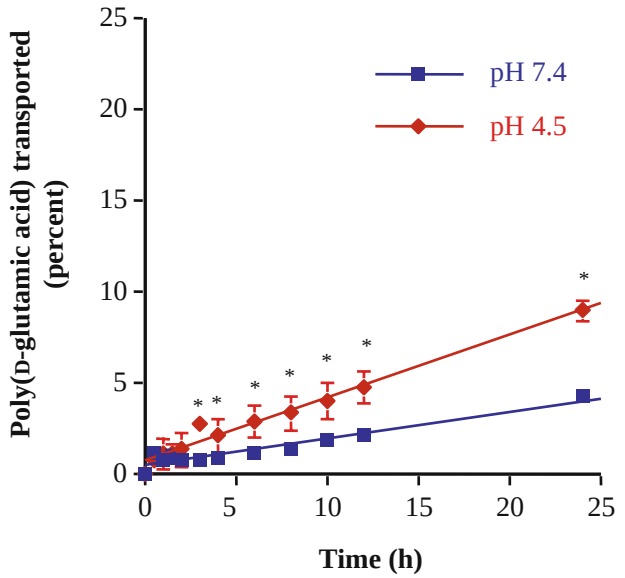


**Fig. 3.** Percentages of poly(D-glutamic acid) transported across a track-etched polycarbonate membrane with an average pore diameter of 0.015 μm at 37°C. *Asterisks* indicate a significant difference ($P < 0.05$) compared to pH 7.4.

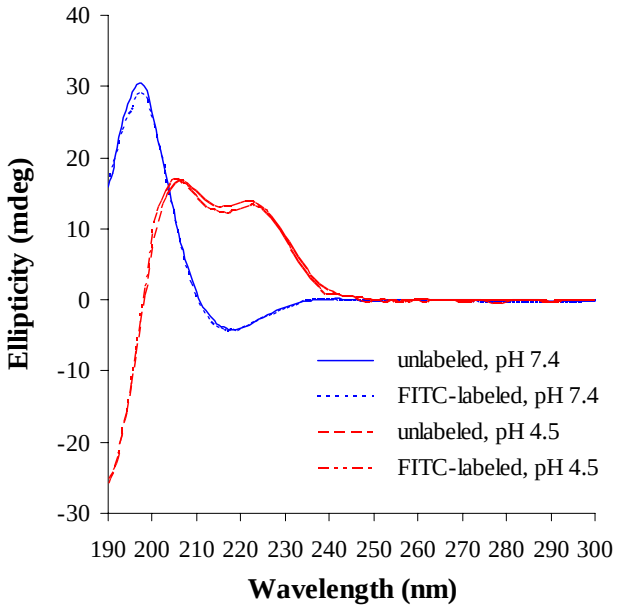


**Fig. 4.** CD spectra of unlabeled and FITC-labeled poly(D-glutamic acid).

conjugation is stable, but also because it occurs through the reaction between FITC and only the terminal amino group of poly(D-glutamic acid). Thus, the presence of FITC should not interfere with α-helix induction in this polypeptide, as subsequently confirmed by CD measurements (Fig. 4). Since FITC-labeled poly(D-glutamic acid) is not commercially available, the FITC labeling was performed in our laboratory.

It has been shown that even small molecules bearing numerous negative charges, such as ferrocyanide anion $\left[Fe(CN)_6^{4-}\right]$, are completely excluded from passage through the paracellular pathway, presumably due to repulsion by like charges and van der Waal's forces, which causes "closure" of this route of drug transport (22). Based on overall charge density considerations and potential charge repulsion, the highly negatively charged random coil conformer of poly(D-glutamic acid) was expected to experience stronger repulsion. Hence, we originally hypothesized that the highly negatively charged random coil conformer of poly(D-glutamic acid) would permeate through the negatively charged tight junctions to a smaller extent than the α-helix conformer with partial negative charges, similar to the study with track-etched polycarbonate membranes having a pore diameter of 0.015 μm.

Figure 5 shows the transport profiles of poly(D-glutamic acid) across a Caco-2 cell monolayer at 37°C. Interestingly, when severely hindered by the negatively charged tight junctions, poly(D-glutamic acid) at pH 7.4 permeated the monolayers to a significantly ($P < 0.05$) greater extent than at pH 4.5, which is completely opposite to the trend observed in the moderately hindered diffusion case.

The reverse trend observed in this experiment was unexpected based on charge density considerations. Nevertheless, it has been reported that the effect of electric field plays a larger role when the permeating solutes are small relative to the pores, whereas, for large molecules, sieving by the pores based on molecular size becomes an increasingly important and discriminating factor, and overcomes the influence of the electric field (5). Pauletti *et al*. have observed that charge selectivity for the permeation of solutes through the paracellular pathway is only effective for small peptides, and the permeability of cell monolayers becomes less sensitive to the net charge of the permeants as the size of the peptides increases (23). It was concluded by these authors that, with increasing molecular radius, molecular size restriction by the pores becomes more dominant and, finally, eliminates the influence of the electric field at the junctional complex. For poly(D-glutamic acid), it is possible that size restriction was more dominant than the effect of overall charge. However, because the hydrodynamic radius (Table II) and the Renkin molecular sieving function (Table III) calculated for poly(D-glutamic acid) at pH 7.4 were not significantly different from those at pH 4.5, a difference in molecular size restriction was not likely to have been responsible for the significant difference we observed in their paracellular transport (Fig. 5).

Since the discrepancy in the paracellular transport of poly(D-glutamic acid) between pH 7.4 and 4.5 could not be attributed to the differences in either size or charge of the random coil and α-helix conformers, additional properties of these two conformers were considered. One major change known to occur when a polypeptide chain adopts the α-helix conformation is the significantly increased rigidity of the molecule. We suggest that the flexibility of the random coil conformer, which allows for changes in the overall molecular geometry, might have relieved the potential steric hindrance of the polypeptide as it was entering the pores, as well as the diffusional resistance inside the pores, and hence, assisted its diffusion through the tight junctions. These same effects may not have played a role in the paracellular transport of the rigid α-helix conformer.

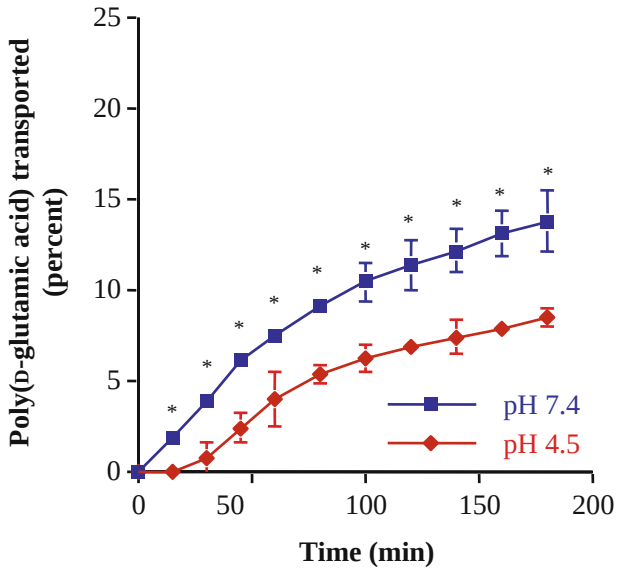


**Fig. 5.** Percentages of FITC-labeled poly(D-glutamic acid) transported across a Caco-2 cell monolayer at 37°C. *Asterisks* indicate a significant difference ($P < 0.05$) compared to pH 4.5. The TEER values before and after the transport experiments of poly(D-glutamic acid) at pH 4.5 were not significantly different from each other (i.e., $189.7 \pm 13.8$ and $185.6 \pm 11.1$ Ω cm$^2$, respectively), which confirms that the integrity of Caco-2 cell monolayers was not affected after having been exposed to pH 4.5 for 3 h.

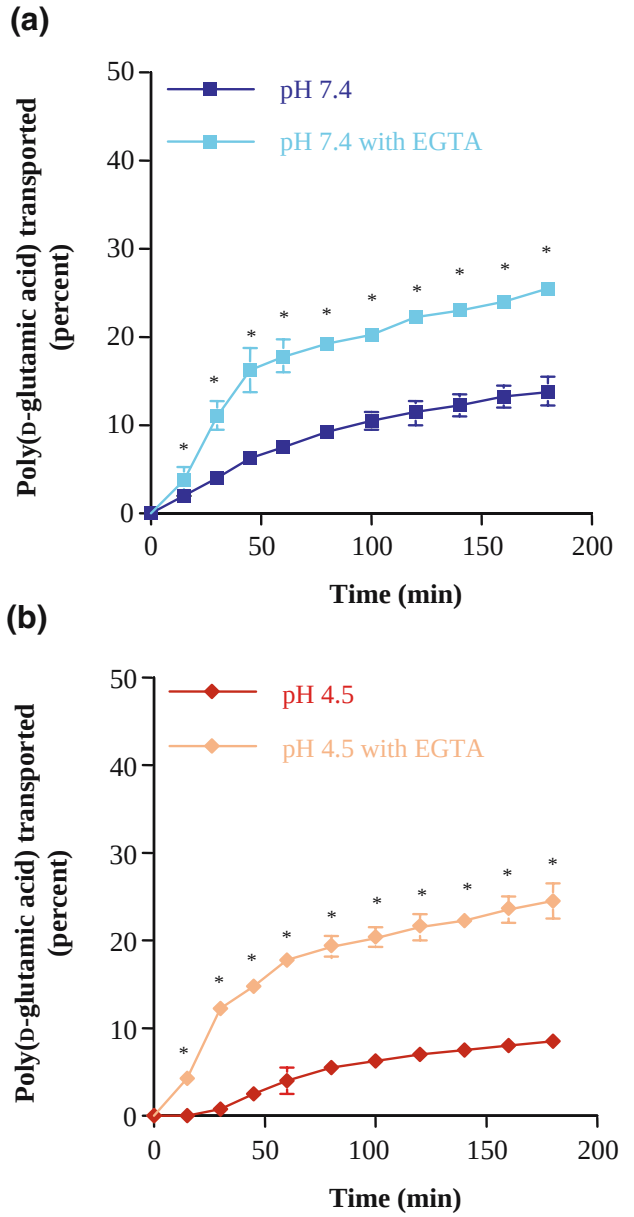


**Fig. 6.** Effect of EGTA on the percentages of FITC-labeled poly(D-glutamic acid) transported across a Caco-2 cell monolayer at pH 7.4 (a) and pH 4.5 (b) at 37°C. *Asterisks* indicate a significant difference ($P < 0.05$) compared to in the absence of EGTA.

Molecular flexibility has been previously demonstrated to enhance the diffusion of macromolecules through small pores. One example is the greater diffusivity of dextran (with a nearly linear structure) in small pores, compared to highly cross-linked ficoll at any given Stokes–Einstein radius (24). Additionally, Pluen *et al.* (25) have shown that flexible macromolecules exhibited greater mobility in the porous matrix of agarose gels than that of rigid spherical particles with comparable size. The authors suggested that, in spite of the same hydrodynamic radius in solution, rigid macromolecules quickly became and remained trapped in the pores.

Similar findings have been reported by Baker and Strathmann (26). These authors conducted ultrafiltration of macromolecular solutions, and observed that a protein was always retained by the ultrafiltration membrane to a greater extent than that of a polymer (such as dextran or poly(ethylene glycol)) with the same molecular weight. This unexpected behavior was ascribed to flow-induced deformation of flexible polymers, which allows the polymer chains to alter their conformation and pass through even very small pores. In ultrafiltration, when transmembrane pressure is applied and solvent flows through the pores, shear is exerted on the polymer molecule by frictional forces, and causes the polymer chain to uncoil. The smallest cross-sectional diameter of the uncoiled polymer chain is now smaller than the pore size, thus allowing the polymer to enter the pore more easily. A similar effect of flow-induced deformation on the retention of polystyrene chains by ultrafiltration membranes has recently been reported by Beerlage (27).

Since no transmembrane pressure was applied in the transport studies across the Caco-2 cell monolayers, the transport mechanism through the monolayers was exclusively passive diffusion; i.e., flow-induced deformation did not take place. Nevertheless, deformation of the polypeptide chain may have occurred, albeit to a lesser extent, with the more flexible random coil conformer, whereas, the rigidity of the α-helix conformer reduced the potential for molecular deformation. This may be a reason why the paracellular transport of poly(D-glutamic acid) was enhanced for the flexible random coil conformer compared to the more rigid α-helix structure in our Caco-2 transport studies. The findings in the present study can serve as a prelude for additional investigations with clinically useful polypeptide drugs in order to see whether one specific secondary structure of a therapeutic polypeptide capable of residing in different secondary structures is always preferentially absorbed by the paracellular pathway.

The higher percentages of poly(D-glutamic acid) transported across the Caco-2 cell monolayers (Fig. 5) compared to the percentages of the polypeptide transported across the 0.015-μm polycarbonate membranes (Fig. 3) may be explained by the fact that the percentages of a paracellular permeant transported depend not only on the aqueous diffusion coefficient of the molecule, but also on the porosity ($\varepsilon$) and the thickness ($l$) of the membrane, as well as the surface area available for diffusion. The porosity of a membrane is a function of both the pore size and the number of pores per unit area. As a result, it is not necessarily true that a membrane with a larger pore size will have a higher porosity. For example, the 1-μm membranes used in the present study (to estimate the aqueous diffusion coefficients and the hydrodynamic radii) had a pore radius of 5,000 Å, and the porosity was calculated to be 0.079. On the other hand, the 0.4-μm membranes had a 2.5-fold smaller pore radius, i.e., 2,000 Å, and yet still had a higher porosity of 0.126 due to a larger number of pores per unit area. As a result, the percentages of poly(D-glutamic acid) transported across the 0.4-μm membranes at both pH 7.4 and 4.5 approached 40% after 180 min (Fig. 2), compared to about 30% for the 1-μm membranes (data not shown).

In the case of 0.015-μm membranes with a pore radius of 75 Å, the porosity was calculated to be very low, i.e., 0.0011. Unfortunately, the porosity of the Caco-2 monolayers could not be directly calculated, since the number of pores was unknown, even though the pore radius has been estimated to be approximately 12 Å (5). However, the $\varepsilon/l$ has been

estimated to be 1.22 $cm^{-1}$ (5), which is not much different from 1.77 $cm^{-1}$ calculated for the 0.015-μm membranes. Additionally, the surface area available for diffusion in the Caco-2 experiments was 1.13 $cm^2$, almost two times larger than in the experiments which utilized 0.015-μm membranes (0.64 $cm^2$). Thus, we did not attempt to compare the percentages transported across different membranes, but instead, focused on the difference in the transport at pH 7.4 and 4.5 across each membrane.

Lastly, the mechanism of transport of poly(D-glutamic acid) across the Caco-2 cell monolayers was confirmed by determining the permeability of this polypeptide at two different pH values in the presence and absence of EGTA, a chelating agent that temporarily disrupts the tight junctions. As shown in Fig. 6, EGTA treatment significantly ($P < 0.05$) increased the transport of poly(D-glutamic acid) at pH 7.4, thus supporting the premise that this polypeptide was transported by the paracellular pathway. Similarly, EGTA treatment also significantly ($P < 0.05$) increased the transport of poly(D-glutamic acid) at pH 4.5; again indicating that the polypeptide was transported by the paracellular pathway at this pH as well. Interestingly, the rate and extent of poly(D-glutamic acid) transport across the Caco-2 cell monolayers treated with EGTA were approximately the same at both pH 7.4 and 4.5, which suggests the possibility that secondary structure-dependent paracellular transport was lost when the tight junctions were deliberately expanded with EGTA.

## CONCLUSIONS

When the pores were very large compared to the molecular size of the two types of secondary structure of poly(D-glutamic acid) evaluated in this study, neither the net charge, nor the predominant secondary structure, played a significant role in its unhindered diffusion through negatively charged, aqueous-filled pores. On the other hand, when the molecules were moderately hindered by the pores, charge repulsion between the like charges associated with the pore wall and the permeant significantly influenced the aqueous diffusion of this negatively charged polypeptide through the pores. In this case, selectivity, with regard to diffusion across microporous membranes having negatively charged pores, was exhibited towards the α-helix conformer possessing less negative charges. As the overall molecular dimensions of the polypeptide approached the size of the aqueous pores in the Caco-2 cell monolayers, the diffusion was severely hindered by the pores. In the severely hindered diffusion situation, the flexibility of the highly negatively charged random coil conformer of poly(D-glutamic acid) appeared to overcome the repulsion by negative charges associated with the tight junctions, and enhanced the paracellular transport of this secondary structure. These results suggest that, in addition to molecular size restriction and charge interactions, secondary structure of a polypeptide can be a determining factor governing its paracellular transport, especially when the molecular dimensions approach the size of the pores. This finding could potentially be used to identify the optimal secondary structure of a therapeutic polypeptide to enhance its membrane permeation, which would represent a significant advancement in the delivery of this class of drug compounds.

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