A study of macromolecular diffusion through native porcine mucus

M. A. Desai, M. Mutlu[a] and P. Vadgama

Department of Medicine (Clinical Biochemistry), University of Manchester, Hope Hospital, Salford M6 8HD (England), and [a] Department of Chemical Engineering, Hacettepe University, Ankara (Turkey)
Received 12 April 1991; accepted 20 June 1991

Abstract. A diffusion chamber technique based on time-lag analysis for the estimation of effective diffusion coefficients of radiolabelled macromolecules of varying molecular weights through native mucus gel is reported. For all solutes studied, a reduction in effective diffusion coefficients was observed with a retardation of solute flux in both aqueous and mucus layers. Over the molecular weight range of solutes investigated (126–186000 Daltons), a consistent effect of molecular weight was evident with regard to the retarding effect of mucus. No apparent or absolute molecular weight cut-off for macromolecular transfer was exhibited. However, at high molecular weights (> 30000 Daltons) the retardation was greatly enhanced. The results confirm that mucus can be regarded as a gel with finite pores, but that it does not constitute an absolute barrier to even high molecular weight solutes.
Key words. Diffusion coefficients; macromolecular diffusion; mucus; time-lag.

Mucus forms a continuous viscoelastic and water-insoluble gel layer that covers many epithelial surfaces, most notably those of the gastrointestinal tract, the upper respiratory tract and parts of the genitourinary tract. The precise thickness of the gel has been a matter of controversy depending on the technique used[1,2]. However, the thickness of the gel has been estimated to be between 100–600 μm in the gut[3,4] and its key gel forming constituent has been considered to be a 2×10^6 Dalton glycoprotein subunit[5]. Apart from such high molecular weight glycoproteins, mucus also contains a wide range of substances such as DNA, inorganic material and plasma proteins due to cell breakage. Human gastric mucus has also been shown to contain polysaccharides and lipids[6,7].

The glycoprotein is thought to be responsible for both the physical and structural properties of mucus which includes the protection of the delicate underlying epithelial cell surface[8]. Macromolecules such as secretory IgA, lysozyme and lactoferrin thought to be specifically added to mucus secretions, may also contribute to the protective function of mucus[9].

While rheological properties are important for the action of mucus as lubricant, mixing barrier and particle trap, and have been the focus of much recent attention[10–12], less information is available about mucus as a diffusion barrier. Some controversy also exists as to whether mucus acts as a diffusion barrier by stabilizing a surface water layer[13,14], or could present a more selective and potent barrier to the transfer of both low and high molecular weight nutrients and drugs within the small intestine[15].

Peppas et al.[16] have developed a theory for solute diffusion in intestinal mucus; this takes into account the concentration of the constituent glycoprotein, size of the diffusing species and density of the macromolecular cross-links. There are indications that the diffusion of certain species such as the ergot alkaloids[17], aminoglycosides[18], fatty acids[19] and some antibodies[20,21] is significantly retarded.

Here, we report diffusion coefficients obtained by lag-time analyses for a series of ^{125}I-labeled macromolecules of varying molecular weights through a native mucus gel in an attempt to examine the precise retarding effect of mucus on access of larger species to the intestinal epithelium.

Theory

The diffusion coefficients of macromolecules were determined using lag-time analysis[22]. When a membrane barrier is held between two well mixed chambers of concentration C_1 and C_2 in the component whose diffusion coefficient is to be measured, and assuming that the film mass transfer resistance between the bulk fluids in the two chambers and the membrane is negligible, the transient diffusion process inside one volume element within the membrane is governed by the partial differential equation

$$\frac{\delta C}{\delta t} = D \frac{\delta^2 C}{\delta X^2} \tag{1}$$

where C is the concentration in a volume element of membrane, D is diffusion coefficient, t is time, and X is vertical distance from the membrane surface using the boundary conditions:

$$C = C_1 \quad \text{at} \quad X = 0$$

$$C = C_2 \quad \text{at} \quad X = 1 \tag{2}$$

At the start of the experiment, the membrane is assumed to be free of the diffusing component and therefore the initial condition to equation (1) is

$$C = 0 \quad 0 < x < 1 \quad \text{at} \quad t = 0 \tag{3}$$

The solution to equation (1) at the boundary conditions described in equation (2) under initial condition outlined

in equation (3) has been proposed by Carslaw[23]. This simplified form of the expression relating to the total solute transferred with time can be summarised as

$$Q_{ts} = \frac{A\,D\,C_1}{1}\left(t_s - \frac{F^2}{6\,D}\right) \quad (4)$$

where Q_{ts} is the total amount of solute transferred through membrane of area A at time t and D is the effective diffusion coefficient. A graph of Q vs t approaches a straight line which intercepts the time axis at $t = F^2/6D$. The intercept of the linear part of the curve is referred to as the lag-time. Diffusion coefficients are calculated from the intercept time and the membrane thickness, F.

Materials and methods

Reagents: Lysozyme (muramidase EC 3.2.1.17), rennett, bovine serum albumin (BSA) and glucose oxidase (GOD) were purchased from Sigma (Poole, U.K.). Rennett is a mixture of closely related proteins with a narrow molecular weight range. Radio-active ^{125}I was purchased from Amersham (U.K.). Track-etched porous polycarbonate membranes (0.1-μm pore size) were obtained from Poretics (Livermore, CA). The remaining chemicals including the buffer components were purchased from BDH Chemicals (Poole, U.K.). An isotonic phosphate buffer was used comprising 2.44 g of NaH_2PO_4, 7.5 g Na_2HPO_4, 3 g NaCl and 0.6 g EDTA in 1 l, pH 7.4.

Solutes investigated in this study were selected on the basis that they were cheap and provided a wide molecular weight range (~200–200000 Daltons). These macromolecules were readily radiolabelled with ^{125}I and demonstrated reasonable stability on and after labelling. The proteins were iodinated using a modified method originally described by Hunter and Greenwood[24]. A 10 mg amount of protein was dissolved in 1 ml of 0.5 M sodium phosphate, pH 7.0. A 20-μl aliquot of this solution was added to a plastic vial to which 10 μl of 0.05 M sodium phosphate buffer was also added. To this 100–200 μCi of radioactive NaI was introduced using a Hamilton syringe. Chloramine T (10 μl; 2 mg/ml) was added and with the use of a stop clock, sodium metabisulphite (10 μl; 2 mg/ml) and potassium iodide (300 μl; 1 mg/ml) were each added at 25-s intervals. The sample was then gel-filtered using a 1.0 × 50 cm Sephadex column. Fractions (1 ml) which made up the first 'peak' of radioactivity were collected, pooled and stored at −20 °C until required for experimental purposes.

Porcine gastric mucus was obtained from an abattoir using animals immediately after slaughter. The stomach was split open, the luminal surface washed with water and the gel collected by scraping the intact mucosal surface with a glass slide. The mucus was frozen at −20 °C until required for diffusion studies. Validation experiments on acid diffusion through fresh and frozen porcine gastric mucus[25] indicates that permeability remains unaffected by storage; freezing and thawing of mucus has also been demonstrated to have no apparent effect on permeability[19].

Diffusion experiments: Experiments were carried out at room temperature (22 ± 2 °C) using a Perspex diffusion chamber consisting of two half-cells. Each half-cell contained a round chamber of 170 ml volume, held together with screw connections (fig. 1). Two polycarbonate membranes (50 mm diameter; 0.1-μm pore size) were placed on either side of a 200-μm nylon netting spacer, and the laminate clamped between two stainless steel discs. The chambers were secured with O-rings in position to provide a water-tight connection. The nylon spacer maintained a finite unstirred layer for diffusion. For mucus studies, thawed porcine mucus was layered on the nylon spacer before replacing the upper polycarbonate membrane. Both chambers were filled with isotonic buffer and allowed to equilibrate for 1 h. The chambers were then emptied, and simultaneously filled with buffer in chamber C_1 and a 1-mg/ml solution of the solute in

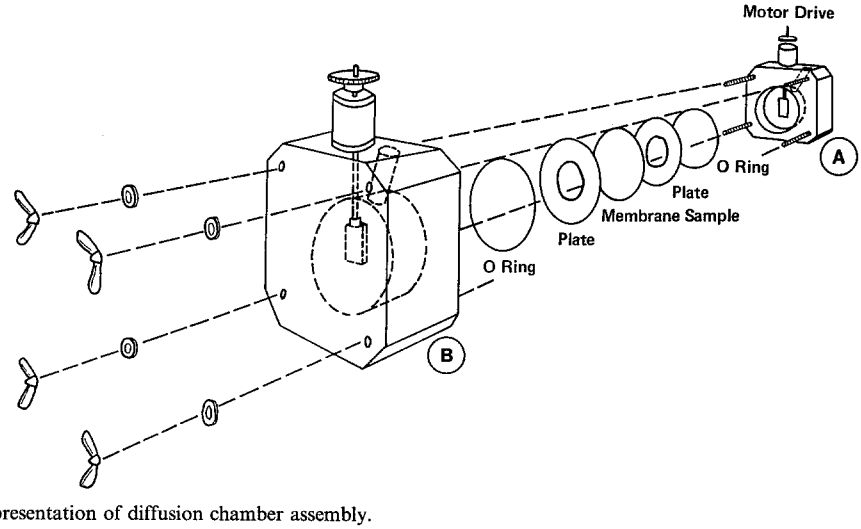

Figure 1. Schematic representation of diffusion chamber assembly.

identical buffer together with 500 µl of the ^{125}I-labelled solute under investigation in chamber C_2 and rapid stirring instituted. A single peristaltic pump was used to draw solution fractions from chamber C_1 and simultaneously replace buffer in chamber C_2 at equivalent flow rates to maintain constant volumes in both chambers. Initial and subsequent fixed time 10-s fractions were collected by a pre-set fraction collector (LKB, Bromma) and the samples were counted for radioactivity using an LKB-Wallac 1260 Multigamma scintillation counter.

Results

The plots of Q (amount of solute transferred) versus time for two selected solutes, lysozyme and glucose oxidase, is shown in figure 2 (a and b, respectively) in the presence and absence of mucus. The plots show that the relationship of Q versus time approaches a straight line with a non-zero intercept on the time axis. This is consistent with theory, allowing application of equation (4), under the operating conditions and duration of the experiment, to calculate effective diffusion coefficients. Linear regression analyses of linear parts of the curves gave regression coefficients of > 0.99 for both aqueous and mucus layers. The effect of mucus is to substantially reduce Q over a given time. The table shows the effects of increasing molecular weight on effective diffusion coefficients through mucus.

For all solutes studied, a reduction in effective diffusion coefficient is observed with molecular weight with considerable retardation of solute flux in both aqueous and mucus layers (fig. 3). Over the molecular weight range of solutes investigated (126–186000 Daltons), a consistent increase in the retarding effect of mucus is evident with increase in molecular weight of the solutes. The results also show that there is no apparent or absolute molecular weight cut-off (at which mass transfer is abolished), however, at high molecular weights (> 30000 Daltons) solute transfer is considerably retarded as shown by the plot of D_A/D_M versus log of molecular weight (fig. 4).

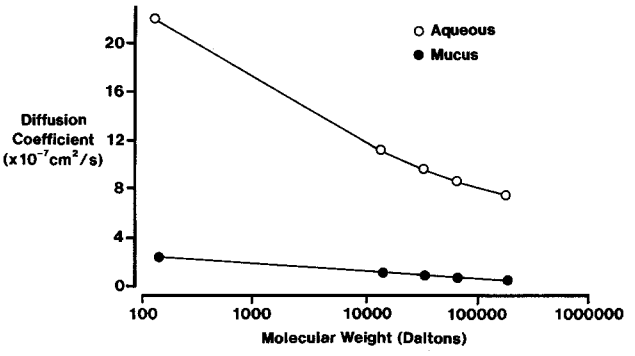

Figure 3. Plots of diffusion coefficients of solutes in aqueous and mucus layers versus log molecular weight.

Discussion

We have described here a diffusion chamber technique based on lag-time analysis for the estimation of effective diffusion coefficients of ^{125}I-labelled solutes having a broad range of molecular weights (126–186000 Daltons). The main advantage of time-lag analysis is the short experimental time required for each experiment, of the order of minutes as compared with several hours for other diffusion measurement techniques such as those involving steady state analyses[26]. The technique is particularly convenient for estimating effective diffusion co-

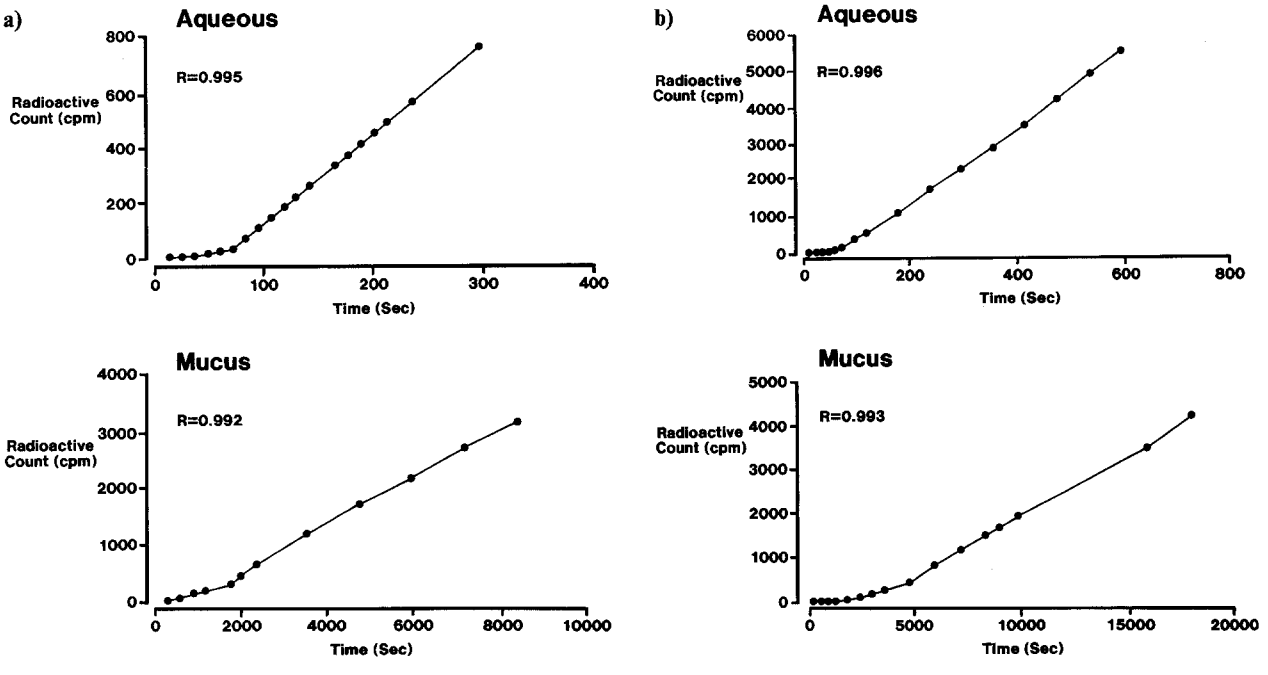

Figure 2. Plots of cpm (amount of ^{125}I-labelled solute transferred) versus time for lysozyme (*a*), and glucose oxidase (*b*) in aqueous and mucus.

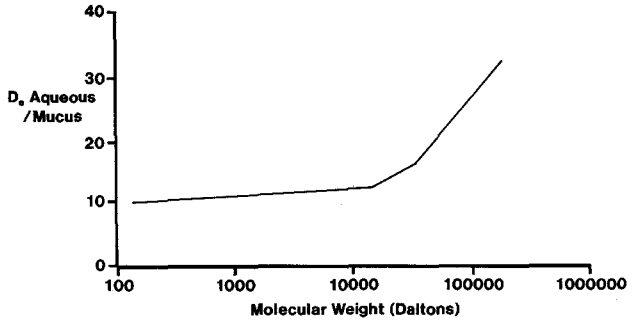

Figure 4. A plot of log molecular weight versus ratio of $D_{e,Aqueous}/D_{e,Mucus}$.

efficients through a medium which offers high resistance to solute transfer (such as mucus layer in this study) thus extending the usually low lag-time at the start of diffusion experiment. Therefore the reliability and reproducibility of effective diffusion coefficients of solutes in such a medium is greatly enhanced as compared to the values obtained from low resistance aqueous layers. Indeed the mean of five experiments for the aqueous layer were used, compared with two experiments for the mucus layer.

It is important to point out that time-lag analysis becomes effective only when sensitive detection techniques are employed (such as radioactivity measurements). The sensitive detection of changes in concentration in the first 2–3 min of the diffusion experiment is critical (fig. 2), such short-time changes can only be followed accurately and conveniently by the use of radio-labelled solutes. Less sensitive detection techniques such as spectrophotometry and conductimetry are therefore not suited to time-lag approach for the estimation of effective diffusion coefficients.

It is important to point out that the D_e values for the solutes reported here are effective diffusion coefficients obtained strictly under the experimental conditions described here and assumptions made in the calculation of D_e. They may therefore show a deviation from literature values normally obtained under ideal diffusion conditions. Indeed the diffusion coefficients of solutes in the aqueous layer are between 6–60% higher (table) than those reported in the literature. However, the main emphasis of the study is to compare different solutes under identical experimental conditions.

The high estimates of D_e values obtained in this study are probably due to the fact that very rapid stirring (1400 rpm) of solutions was instituted to ensure that the barrier to diffusion presented by the stagnant boundary layer was minimized, so that D_e values reflected only the barrier presented by the membrane/gl/water barriers. As a consequence, the model here assumes that the barrier to diffusion presented by such a stagnant layer is negligible. However, under such rapid stirring conditions, some turbulence in the non-stirring aqueous diffusion compartment in the control experiments was unavoidable, and

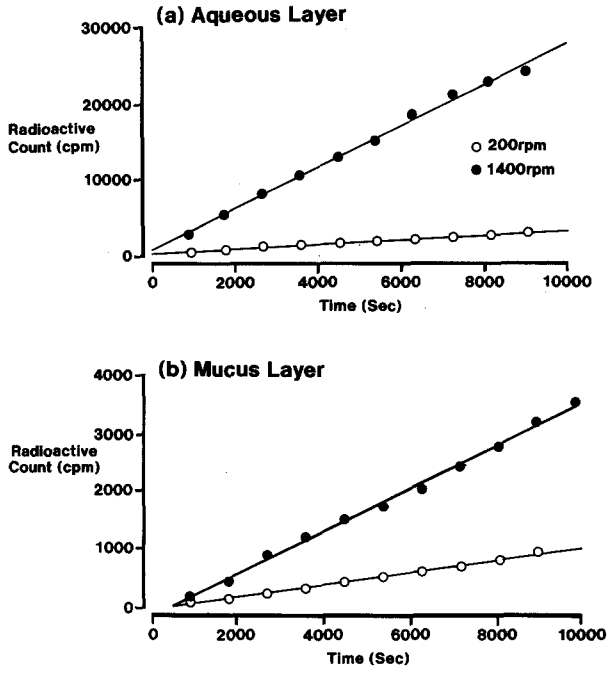

Figure 5. Plots of amount of ^{125}I-lysozyme transferred (cpm) versus time through aqueous (*a*), and mucus (*b*) layers.

may have given rise to higher D_e values as a result of convection.

Figure 5 which depicts plots of cpm (amount of ^{125}I-lysozyme transferred in a steady state experiment) versus time in aqueous layers at 200 and 1400 rpm stirring speeds, clearly shows that increasing stirring speeds resulted in an increase in the transfer of solute across the membranes possibly due to depletion of the boundary layer and the resultant convection in the diffusion layer. Lucas[27] has also reported diffusion coefficients of sodium and hydrogen ions to be of one order of magnitude higher than free solution values when similar stirred compartmental systems were used. The use of smaller pore polycarbonate membranes could have avoided this problem, but then the diffusion barrier presented by the membranes themselves would have been significant, particularly for macromolecular diffusion.

Gastrointestinal mucus forms a tightly bound gel thought to restrict the diffusion of protein molecules[28]. The table clearly demonstrates that the retarding effect of

Effective diffusion coefficients of ^{125}I-labelled solutes through aqueous and porcine mucus layers

Solute	Mol. wt	Diffusion coefficient, D_e ($*10^7$ cm^2/s)			
		Aqueous*	Lit. value	Mucus	D_A/D_M
^{125}I-Na	148	20	-	2.2	9.1
^{125}I-Lysozyme	14,400	11	10.4	0.87	12.4
^{125}I-Rennett	~35,000	9.4	7.8[1]	0.56	16.8
^{125}I-BSA	68,000	8.5	6.1	0.36	23.6
^{125}I-GOD	186,000	7.3	4.6[2]	0.22	33.2

*mean of 5 experiments +0.5; mean of 2 experiments +0.05; [1]D_e for B-lactoglobulin (37,100 Daltons)[33]; [2]D_e for aldolase (149,000 Daltons)[33].

mucus to solute flux increases with increase in molecular weight from 10-fold for $Na^{125}I$ (126 Daltons) to over 30-fold for glucose oxidase (186000 Daltons) when compared to flux through an unstirred water layer of similar thickness. Diffusion of low molecular weight solutes (< 1000 Daltons) through mucus have been previously demonstrated to be retarded approximately 5-fold when compared with diffusion through an unstirred water layer of similar thickness[25,29].

It is notable from the table that solutes as large as BSA (68000 Daltons) and glucose oxidase (186000 Daltons), although significantly retarded, can still diffuse through the mucus polymeric matrix and that defined diffusion coefficients can be measured as for small solutes (fig. 2). A network of channels due to the ordered arrangement of glycoprotein molecules in mucus has been proposed[30], which is apparently sufficient to permit macromolecular transfer. Indeed even multilayer liposomes (~ 200 nm) introduced into the lumen of the small intestine have been shown to be able to penetrate the mucus layer[31]. It may be possible therefore, that the existence of such channels in porcine gastric mucus can account for the finite diffusion of these macromolecules.

It is important to point out that lysozyme did not seem to have any proteolytic effect on mucus under the conditions of this study as confirmed by the plot of Q versus time (fig. 2), which dies not show any deviation from linearity that would have otherwise occurred in the presence of any decomposition of mucus.

The highly retarded diffusion of large solutes however, emphasizes the role of mucus as a barrier to luminal nutrient peptide or saccharide molecules which are required to diffuse to their hydrolase or transport sites on the epithelial membrane. The intestinal mucus coat has been shown by some to be an important diffusion barrier for such nutrients and other oligomers that require to be digested, transported, or bound to receptor sites on the outer intestinal membrane[15]. The high diffusional resistance demonstrated for mucus here is also of relevance to the absorption of therapeutic and other exogenous compounds to which the small intestine is exposed.

It is likely that the variation of effective diffusion coefficients with molecular weight reported here may not solely represent increases in molecular weight, but, will also involve molecular charge and shape. Thus, solutes bearing nitrogen groups have been shown to be selectively retarded, a likely consequence of ionic interaction[18]. Mucus gel has also been shown to retard diffusion of ionic species such as K^+ ions[12]. This may be explained by the fact that mucus glycoprotein has a net surplus of negative charges due to the presence of charged amino, carboxyl, sulphate and neuraminic acid groups providing a hugely electrified surface. Diffusion retardation of negatively charged species will also occur by Donnan exclusion[32]. The reduction of net charge by treatment with a species such as N-acetyl neuraminidase[12] or N-acetyl cysteine, sodium taurodeoxycholate and acetyl salicylic acid[29] has been reported to decrease the effectiveness of the mucus as an ionic diffusion barrier.

Our results confirm that mucus is acting as more than a high viscosity aqueous phase. However, it is not clear from this general study what the precise relationship of molecular weight may be to mucus resistance since the solutes examined varied somewhat in charge, shape and class of compound. Future work will determine diffusional resistance using model diffusants of consistent shape and charge.

1 Kerss, S., Allen, A., and Garner, A., Clin. Sci. *63* (1982) 187.
2 Bickel, M., and Kauffman, G. L., Gastroenterology *80* (1981) 770.
3 Munster, D. J., Roberton, A. M., and Bagshaw, P. F., N. Z. med. J. *102* (1989) 607.
4 Van Hoogdalem, E. J., De Boeboer, A. G., and Briemer, D. D., Pharmac. Ther. *44* (1989) 407.
5 Carlstedt, I., and Sheehan, J. K., Biochem. Soc. Trans. *12* (1984) 615.
6 Slomiany, A., Slomiany, B. L., Witas, H., Aono, M., and Newman, L. J., Biochem. biophys. Res. Commun. *113* (1983) 286.
7 Clamp, J. R., Cooper, B., Creeth, J. M., Ene, D., Barrett, J., and Gough, M., Biochem. J. *215* (1983) 421.
8 Neutra, M. R., and Forstner, J. F. in: Physiology of the Gastrointestinal Tract, 2nd edn, p. 975. Ed. L. R. Johnson. Raven Press, New York 1987.
9 Clamp, J. R., and Creeth, J. M., in: Mucus and Mucosa, p. 121. Ciba Foundation Symposium 109. Pitman, London 1984.
10 Crowther, R. S., Marriott, C., and James, S. L., Biorheology *21* (1984) 253.
11 Allen, A., in: Physiology of the Gastrointestinal Tract, vol. 1, p. 617. Ed. L. R. Johnson. Raven Press, New York 1981.
12 Lee, S. P., and Nicholls, J. F., Biorheology *24* (1987) 565.
13 Morris, G. P., Clin. Biol. *9* (1985) 106.
14 De Simone, J. A., Science *220* (1982) 221.
15 Smithson, K. W., Millar, D. B., Jacobs, L. R., and Gray, G., Science *214* (1981) 1241.
16 Peppas, N. A., Hansen, P. J., and Buri, P. A., Int. J. Pharm. *20* (1984) 107.
17 Nimmerfall, F. N., and Rosenthaler, J., Biochem. biophys. Res. Comm. *94* (1980) 960.
18 Niibuchi, J. J., Aramaki, Y., and Tsuchiya, S., Int. J. Pharmaceutics *30* (1986) 181.
19 Smith, G. W., Wiggins, P. M., Lee, S. P. and Tasman-Jones, C., Clin. Sci. *70* (1986) 271.
20 Cheema, M. S., Rassing, J. E., and Marriott, C., J. Pharm. Pharmac. Suppl. *38* (1986) 53.
21 Kearney, P., and Marriott, C., Int. J. Pharmaceut. *38* (1987) 211.
22 Hanoun, B. J. M., and Stephanopoulos, G., Biotech. Bioeng. *28* (1986) 829.
23 Carlslaw, H. S., in: Fourier's Series and Integrals, p. 263. McMillan, New York 1906.
24 Hunter, W. M., and Greenwood, F. C., Nature *194* (1962) 495.
25 Nicholas, C. V., Desai, M., Vadgama, P., McDonnell, M. B., and Lucas, S., Analyst *116* (1991) 463.
26 Desai, M., and Vadgama, P., Analyst (1991) in press.
27 Lucas, M. L., Digest, Dis. Sci. *29* (1984) 336.
28 Allen, A., Br. med. Bull. *34* (1978) 28.
29 Turner, N. C., Martin, G. P., and Marriott, C., J. Pharm. Pharmac. *37* (1985) 776.
30 Lazarev, P. I., Dokl. Akad. Nauk. SSr. *286* 81986) 761.
31 Brodskii, R. A., Gal'perin, Yu. M., Lazarev, P. I., Nadkochii, V., and Popov, G. A., Dokl. Akad. Nauk. SSR *273* (1983) 464.
32 Bokris, J. O'M., and Reddy, A. K. N., in: Modern Electrochemistry, vol. 2, p. 623. Eds J. O'M. Bokris and A. K. N. Reddy. Plenum Press, New York 1973.
33 Lehninger, A. L., in: Biochemistry, 2nd edn, p. 176. Worth Publishers, New York 1975.

0014-4754/92/010022-05$1.50 + 0.20/0