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The effects of mucus glycoproteins on the bioavailability of tetracycline. II. Binding

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Summary

Equilibrium dialysis was used to study the binding of tetracycline to the isolated glycoprotein of gastrointestinal mucus. At neutral pH, the binding characteristics were consistent with a non-specific interaction such as might arise through a hydrophobic process. At acidic pH, a more specific binding was indicated involving one class of binding site. The overall level of binding was markedly pH-dependent with a maximum occurring around pH 3. The shape of this pH/binding profile could be accurately described in terms of both hydrophobic and electrostatic processes. Ca^{2+} and Mg^{2+} ions increased binding at neutral pH only. Fluorimetric analysis suggests that this enhanced binding is unlikely to occur through the formation of a metal ion bridge and hence is probably due to the higher lipophillicity of the tetracycline–calcium chelate.

Introduction

The binding of drugs at sites or receptors other than the target region has long been accepted as a possible cause of low bioavailability. Such problems are usually envisaged once the drug has entered the circulation. In addition, there are the possible binding interactions that may occur between orally administered drugs and the contents of the gastrointestinal tract which may be either exogenous or endogenous in origin. Exogenous substances may be food or co-administered formulations and it is with this latter class that most of the possible interactions have been anticipated. However, the possible interactions with endogenous substances have received much less attention. The mucus secretion of the epithelium is by far the most copious endogenous material within the gastrointestinal tract.

Certain studies have illustrated idiosyncratic drug interactions (Levine et al., 1955; Franz et al., 1980; Rimele et al., 1982) with the major component of this secretion, namely, mucus glycoprotein, and in some instances this has resulted in extremely low availability. Similarly, these glycoproteins or 'mucins', have been shown to bind the antibiotic tetracycline (Saggers and Lawson, 1966) and the important mechanical properties of mucus gels are significantly altered in the presence of this drug (Marriott and Kellaway, 1975; Davis and Deverall, 1977). Both tetracycline and mucus glycoproteins associate with certain divalent cations (Albert and Rees, 1956; Forstner and Forstner, 1975) and this has been suggested as a possible mode for their mutual interaction. However, no

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specific mechanisms for the mucin–tetracycline interaction have been shown and the extent of the contribution of this binding process to the low absorption levels measured is uncertain.

In this work we have used equilibrium dialysis to study the binding of tetracycline to mucus glycoproteins and attempted to determine the nature and significance of the interaction by investigating both the concentration and pH dependence of the process.

Materials and Methods

Mucus was obtained from 3 sources, a commercially available powdered form (Hog gastric mucin, Sigma Chemical Co., Poole) and the native material obtained directly from pig stomachs and rat intestine. The mucin powder (PPM) was purified and prepared as described previously (Kearney and Marriott, 1986). Native gastric mucin (NGM) was obtained from the stomach scrapings of freshly slaughtered pigs. This material was homogenised in 0.1 M phosphate buffer, pH 7.4, containing 0.22 M potassium thiocyanate, centrifuged (30 min, $27{,}000 \times g$) and fractionated on column of Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The high molecular weight mucin fraction which was eluted in the void volume was retained. Rat intestinal mucin (RIM) was isolated from the epithelial surface of excised, everted, jejuno-ileal sections after incubation for 2 h in physiological buffer. This material was homogenised and washed on an ultrafiltration membrane (PM 30, Amicon Corp., Working, Surrey).

The sialic acid content of the mucin was determined by the method of Warren (1959).

Materials used in the preparation of the various buffer systems were reagent grade and supplied by BDH Chemicals, Poole, Dorset.

Tetracycline (TC) hydrochloride and Tris base (Sigma 7-9) were supplied by the Sigma Chemical Co., Poole, Dorset.

[7-^{3}H(N)]tetracycline was obtained from New England Nuclear, Boston, MA and had a spec. act. of 37 GBq/mmol.

Equilibrium dialysis

Each dialysis cell consisted of two circular perspex half cells which were assembled with a hydrated Visking membrane between them. The resulting sample compartments had a capacity of 1 ml and were accessed via ports in each of the half cells. A total of 6 such cells could be clamped together in a steel frame enabling simultaneous equilibration of different samples and duplicate determinations. Filling and sampling was aided by the use of hypodermic syringes and needles.

Solutions of glycoprotein and drug were prepared having a final activity of 200.000 dpm/ml as determined by liquid scintillation counting.

The glycoprotein solutions were placed in one compartment of the dialysis cell while the matching compartment contained drug in buffer only. After sealing the sampling ports with nylon screws, the cells were left to equilibrate for a period of 20 h at 4°C in the dark. Such a procedure reduced the thermal and photolytic breakdown of the drug. At the end of the equilibration period, 0.2 ml aliquots were taken, in duplicate, from each compartment of each cell and assayed for activity. The difference in activity between the two compartments in a given cell was a direct measure of the amount of drug bound to the macromolecule.

Fluorescence spectra were recorded in Tris buffer, pH 7.4, using an excitation wavelength of 390 nm (Aminco Bowman Instrument Co., Silver Spring, U.S.A.).

Results and Discussion

The standard equation describing ligand binding phenomena is (Thompson and Klotz, 1971):

$$r = n \cdot k \cdot c/(1 + k \cdot c) \qquad (1)$$

where r = moles of drug (ligand) bound per mole of glycoprotein; c = concentration of unbound drug; k = association constant; and n = number of binding sites on the macromolecule.

Alternatively, the data may be plotted in Scatchard form (Scatchard, 1949):

$$r/c = k \cdot n - k \cdot r \qquad (2)$$

For a single class of binding site such a plot

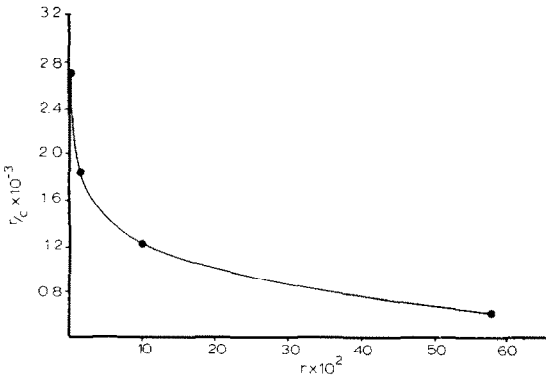

Fig. 1. Scatchard plot of tetracycline binding in PPM (15 mg/ml) in 0.05 M Tris-HCl, pH 7.4.

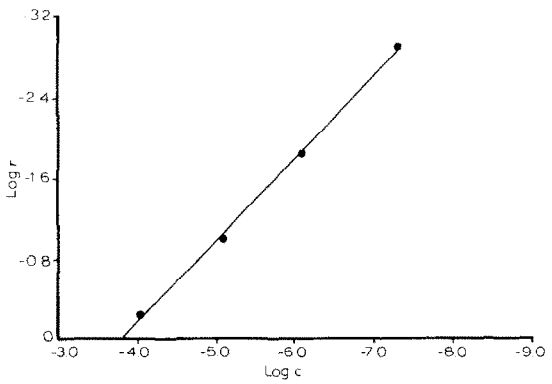

Fig. 2. Freundlich plot of data from Fig. 1.

yields a straight line with intercept on the ordinate of n and slope $-k$. More than one site results in a hyperbolic curve and such a curve is obtained with the TC/glycoprotein data (Fig. 1).

Similar Scatchard plots have been described previously for a glycoprotein/TC system (Brown, 1980) and the asymptotes explained as arising from two types of sites; namely, a high capacity low affinity site and a low capacity high affinity site. With these plots, an approximate n value for each of the two sites may be determined by the extrapolation of the asymptotes of the curve. With the present data, this gives n values of 1 and 0.043, respectively.

The Scatchard equation and the analogous enzyme kinetics equations are derived from the Langmuir adsorption isotherm:

$$x/m = k' \cdot k \cdot c/(1 + k \cdot c) \quad (3)$$

where x/m = amount of adsorbate (drug) bound per gram of adsorbant (protein); c = concentration of free adsorbate; and k and k' are constants.

This equation is derived assuming equal or uniform affinity of the binding sites and a non-cooperativity between them.

A related isotherm is the Freundlich isotherm:

$$x/m = k \cdot c^{1/z} \quad (4)$$

where z is a constant. This is an empirical relation and applies when the affinity of sites decreases proportionately with the log fraction of the sites occupied (Moore, 1972). As $r \propto x/m$, the data may be analysed according to the Freundlich equation by plotting log r versus log c. Fig. 2 shows such a plot which results in a straight line fit. This suggests that the data are also compatible with the presence of sites possessing a wide range of binding affinities as opposed to just two. Binding characteristics of this type are typical of non-specific processes such as hydrophobic interactions.

Binding and pH

As a greater fraction of drug is bound at low concentrations, determinations at low total drug concentrations will be most sensitive, hence pH effects were studied using the stock-labelled drug alone which gave a final total drug concentration of 72.5 nM.

The percentage of drug bound was determined as a function of pH and this is shown in Fig. 3. At a pH of 1, approximately 15% of the drug is bound to the glycoprotein and this rises to a maximum of 60% at pH 3. Through still higher pH values the level of binding gradually decreases to around 15% once again. An anomaly which became evident during these determinations was the consistently low binding values measured when citrate was present as a buffer component, and these values are shown as a separate plot in Fig. 3. The citrate ion has been shown to promote the conversion of TC to 4-epi-TC (Remmers et al., 1963) which may have a lower affinity for the glycoprotein. However, UV analysis showed no

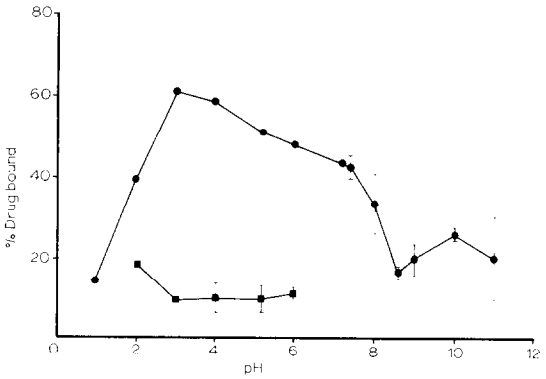

Fig. 3. Plot showing the pH dependence of tetracycline binding to PPM. ●, glycine, acetate and Tris buffer systems; ■, citrate buffer systems. ±S.D. for $n = 4$.

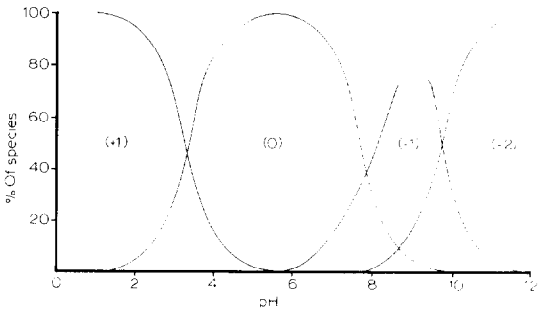

Fig. 4. Plot of the percentage of charged species versus pH for tetracycline calculated from the 3 main pK_a values.

detectable formation of this isomer in any of the buffer systems.

One proposed mechanism for the binding of TC to mucins is via chelation with Ca^{2+} ions already bound to the glycoprotein, and it has been suggested that chelating agents, such as citrate, may reduce the binding of TC to mucins by competing with the antibiotic for the bound Ca^{2+} (Saggers and Lawson, 1966). However, TC–Ca^{2+} complexes do not form below pH 6 (Day et al., 1978) hence this cannot explain the reduced binding at the lower pH values. Relatively large standard deviations were evident in the binding levels at high pH values and this is probably due to the formation of degradation products, such as iso-TC, which are formed in alkaline media.

Parameters which alter as a function of pH are invariably associated with charge effects and Figs. 4 and 5 show the pH–charge profile for TC and the gastric mucin, respectively. The profile for the PPM is simply described as the predominant ionizable groups within the glycoprotein are those of *N*-acetylneuraminic acid and esterified sulphate which both have a pK_a of 2.6 (Eylar et al., 1962). Consequently, around pH 3, the net charge on the glycoprotein changes from neutral to negative. The charge–pH profile for TC is more complicated. At low pH, all ionizable groups are protonated and the net charge of +1 arises from the basic dimethyl-amino group (pK_a 9.7). A change to a net charge of 0 occurs above pH 3 due to the ionization of the acid group of the tricarbonyl methane system (pK_a 3.3) at which point the molecule is zwitterionic. Above pH 7, the net charge changes to −1 as the acidic β-diketone system (pK_a 7.7) ionizes.

Taking the two pH/charge profiles, a scheme of binding can be postulated which is consistent with the observed pH/binding profile. Between pH 2 and 3, the drug and PPM have opposite charges and hence would be able to interact electrostatically. Above pH 3, the drug becomes effectively neutral and although electrostatic binding is reduced, a hydrophobic interaction is now possible. The fall in the binding level above pH 7 is coincident with the reduction in the amount of the neutral species. Any hydrophobic interaction may, at first, seem unlikely considering that the glycoprotein is a highly soluble, extensively hydrated macromolecule. However, part of the structure contains a globular protein region (Pearson et al., 1981) and also, gastric mucus glycoproteins have been shown to be extensively esterified with long-

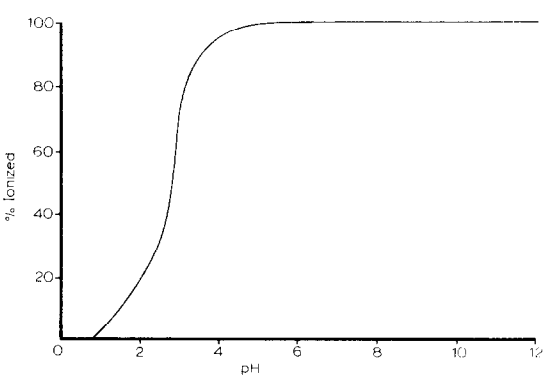

Fig. 5. Plot of relative charge on PPM as a function of pH.

chain fatty acids (Slomiany et al., 1983). With such lipophillic regions in the glycoprotein, a hydrophobic interaction with the drug is a realistic possibility.

With this scheme and using the respective pK_a values, an estimate for the relative amount of drug bound to the glycoprotein can be derived theoretically. If we assume that the binding occurs at conditions well below saturation, then for an electrostatic interaction:

$$\% \text{ bound} \propto [TC(+1)] \cdot [PPM(-)]$$

where TC(+1) refers to tetracycline molecules with net charge +1 and PPM(−) to the ionized acid groups on the glycoprotein. Similarly, for a hydrophobic interaction:

$$\% \text{ bound} \propto [TC(0)] \cdot [PPM]$$

A theoretical plot of the normalised amount of drug bound versus pH for each of the two mechanisms is shown in Fig. 6. Fig. 7 shows the summation of these two plots and it can be seen that there is a favourable comparison with the experimental data (Fig. 3).

With regard to the binding process at low pH, if the sialic acid residues only are involved, then the binding characteristics would be expected to be different to those at neutral pH where a non-specific hydrophobic interaction is suggested. Fig. 8 shows a Scatchard plot of the binding data for a solution of NGM at pH 3.0 and indeed a straight line is observed which indicates a single class of binding site and extrapolation gives the number of binding sites as 50 per glycoprotein molecule. This represents only half the number of sialic acid residues determined (49.3 nmol/mg glycoprotein, 99 residues/mol) and suggests that some of the acid groups may be inaccessible to the drug.

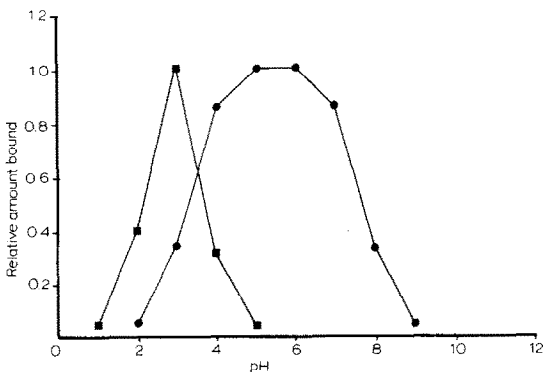

Fig. 6. Theoretical plot of the binding of tetracycline to PPM as a function of pH. ■, electrostatic interaction; ●, hydrophobic interaction.

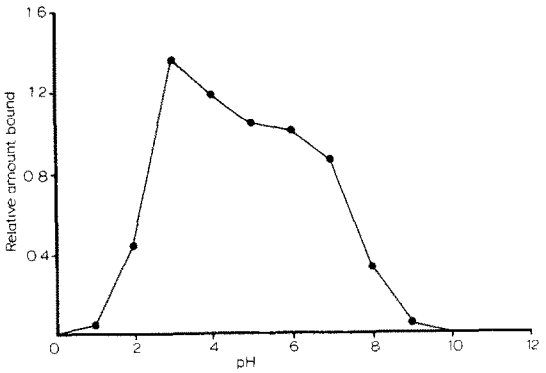

Fig. 7. Theoretical plot of the total amount of tetracycline bound to PPM as a function of pH.

Divalent cations

Table 1 shows the percentage of drug bound to dispersions (1.7 mg/ml) of glycoprotein from each of the three sources at a pH of 7.4, and it is evident that each type shows similar levels of binding. Table 2 shows the effect on binding of two divalent cations at various pH values for a solution of NGM only (4 mg/ml).

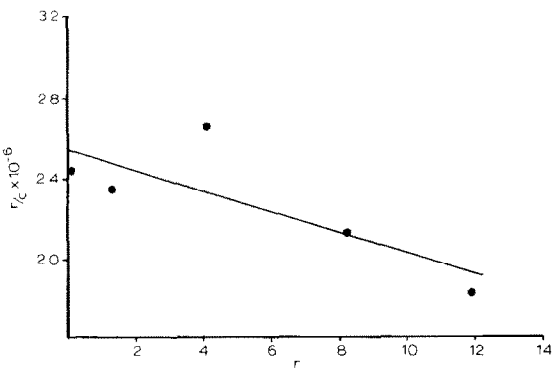

Fig. 8. Scatchard plot of tetracycline binding in NGM (10 mg/ml) in 0.05 M Tris-HCl, pH 7.4.

TABLE 1

Levels of tetracycline bound in dispersions (1.7 mg/ml) of the different gastrointestinal glycoproteins

	Type of mucus glycoprotein		
	PPM	NGM	RIM
Percent bound	29.9 ± 1.6	25.2 ± 1.0	24.7 ± 0.4

Mean ± S.D., $n = 3$.

The increased binding at pH 7.4 in the presence of the cations, must be attributed to a joint drug/ion/glycoprotein interaction for at the low pH values, where there is no formation of a TC–Ca^{2+} chelate, no elevation in the binding is observed. An ionic bridge between the drug and PPM is a possibility and this would be detected by fluorescence measurement. Ca^{2+} bridges do occur between TC and both DNA and serum albumin and this results in an enhanced fluorescence of the antibiotic chelate (Kohn, 1961). Fig. 9 shows the fluorescence spectra of TC in various media. Tetracycline itself gives only a weak signal but becomes strongly fluorescent when chelated with Ca^{2+}. It is clear that the presence of PPM does not produce a significant perturbation of the fluorescence peak, hence an ionic bridge seems unlikely. However, Ca^{2+} does bind specifically to mucus glycoproteins (Forstner and Forstner, 1975) in a pH-dependent manner and as with the formation of the TC chelate, significant binding levels are measured only above pH 6.

As a hydrophobic interaction is suspected at neutral pH, the increased binding in the presence of the cation may be due to the fact that the chelate is more hydrophobic than the free drug.

TABLE 2

The effect of Ca^{2+} and Mg^{2+} on the percentage of tetracycline bound in a dispersion of NGM (4 mg/ml)

	pH:		
	3.0	5.2	7.4
Control	64.3 ± 0.3	45.1 ± 0.7	30.8 ± 0.1
0.5 mM $CaCl_2$	64.2 ± 2.1	44.3 ± 0.4	39.6 ± 0.3
0.5 mM $MgCl_2$	–	–	38.5 ± 0.1

Mean ± S.D., n = 3.

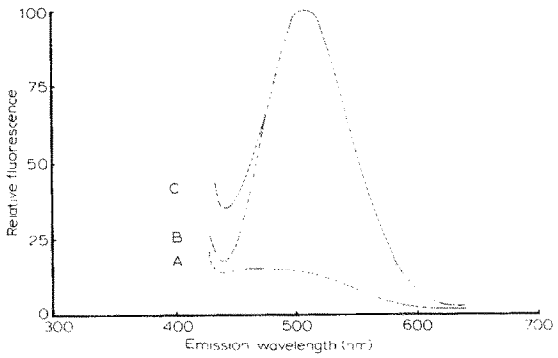

Fig. 9. Fluorescence spectra of tetracycline in 0.05 M Tris-HCl, pH 7.4. Excitation wavelength = 390 nm. A = 2 μM tetracycline; B = 2 μm tetracycline + 10 mM Ca^{2+}; C = 2 μM tetracycline + 10 mM Ca^{2+} + NGM (1 mg/ml).

Indeed, in such experiments with calcium, drug lost to the hydrophobic surfaces of the Perspex cells was significantly higher than in other experiments.

In conclusion, the interaction of TC with mucus glycoproteins exhibited a marked pH dependence. The binding profile can be explained in terms of simultaneous electrostatic and hydrophobic processes. At neutral pH, the binding characteristics appear to be of a non-specific nature as may occur, for example, with a hydrophobic interaction and at the highest concentration studied (1 mM), such an interaction resulted in 15% of total drug bound. With low drug concentrations, the presence of divalent cations increases TC binding by some 30% and this is probably due to the fact that TC/Ca^{2+} chelates are more hydrophobic than the free drug. The binding characteristics at low pH were typical of a more specific interaction which is consistent with a proposed electrostatic interaction between the positively charged TC and the sialic acid residues. At pH levels where such a mechanism could operate, with a drug concentration of 1 mM, as much as 40% of the drug is bound. Ca^{2+} had no effect on the binding levels in this pH range.

Clinically, the relatively high doses of TC used would prevent binding being as significant a problem as that observed with quaternary ammonium compounds (Levine et al., 1955). The net clearance of the drug from the tract would, however, be

somewhat retarded and could account, in part, for the variable oral availability.

Although the increased binding of TC to mucin in the presence of divalent cations may contribute towards the incompatibility of this ion with the drug, the much lower solubility of the chelate compared to the free drug would remain the major disadvantage of such an interaction.

With regard to the reported thickening of mucus gels by TC (Marriott and Kellaway, 1975), it cannot be assumed that this will increase the diffusive resistance to permeating drug molecules. Mucus consists mainly of 'free' water which results in a low 'effective' viscosity at the molecular level (Kearney and Marriott, 1986). Any binding-induced changes in gel structure and resulting increase in gel rigidity will not necessarily alter the viscous properties of the solvent microenvironment.

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