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Counter-ion binding to mucus glycoproteins

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The affinity of pig gastric mucus glycoprotein for counter ions of various valencies was examined. Ions of higher valency were bound with increasing avidity, but the degree of binding was apparently not influenced by the ionic radius. The affinity of the glycoprotein for the ions studied may be ranked: $Fe^{3+} > Al^{3+} > Ca^{2+} > Cs^{+} \simeq Na^{+}$. The binding of calcium was inhibited by high ionic strength and was sensitive to the pH of the environment. Enzymatic removal of sialic acid slightly reduced the calcium binding capacity.

The basic structural unit of mucus glycoproteins is a protein core rich in serine and threonine residues to which oligosaccharide side chains are attached via *O*-glycosidic bonds (Reid & Clamp 1978). Additionally, segments of the protein core are devoid of sugars, and disulphide bonds located in these areas have been shown to be important for maintaining the integrity of the mucus macromolecule (Scawen & Allen 1975; Meyer & Silberberg 1978; Mantle et al 1981; Pearson et al 1981). Of the five sugars that are found in mucins, two, galactose and *N*-acetylglucosamine, may be sulphated and thereby acquire a negative charge and one, sialic acid, is intrinsically negatively charged. The pK_a values of sialic acid and the sulphate groups are such that under most physiological conditions they will be highly dissociated (Forstner & Forstner 1975; Larsson et al 1981) and the glycoprotein should behave as a negatively charged polyelectrolyte. Polyelectrolytes characteristically interact with ions of opposite charge (counter ions) and such an interaction usually affects the physical properties of the macro-ion. Interacting counter ions have lower activity coefficients than do the counter ions of the bulk solution and thus the interaction between polyelectrolytes and counter ions may be viewed as the latter binding to the former.

In-vivo mucus glycoproteins form aqueous gels which overlie and protect the delicate epithelia. These gels typically contain about 1% by weight of salts and other dialysable material, and although the detailed electrolyte analysis varies with origin, it is usually similar to that of serum or bile (Creeth 1978). In addition to the electrolytes which are naturally present, mucus may be confronted with a variety of exogenous ions being employed for therapeutic purposes. Examples include dietary supplements (K^{+}, Ca^{2+}, Fe^{2+}), antacids (Mg^{2+}, Al^{3+}) and intra-uterine devices (Cu^{2+}). Any interaction between these ions and the glycoprotein may impair the ability of the mucus to fulfil its protective function by disrupting the subtle forces which stabilize the gel. In addition, the binding of ions to the glycoprotein may result in reduced absorption of ions which are intended to exert a systemic effect and consequently lead to lower bioavailability.

The purpose of this paper is to report on the binding of a range of ions (Cs^{+}, Na^{+}, Ca^{2+}, Al^{3+}, Fe^{3+}) to purified mucus glycoproteins and to attempt to identify which components of the mucin macromolecule are important for any such binding.

MATERIALS AND METHODS

Freeze dried porcine gastric mucus, type VI neuraminidase (E.C. 3.2.1.18) and Tris 7–9 base were obtained from the Sigma Chemical Co. ^{22}Na, ^{134}Cs, ^{45}Ca and ^{59}Fe were supplied by Amersham International. Toluene scintillant was obtained from the Packard Instrument Co. and dibutylphosphate from Fluka. Sepharose 4B was obtained from Pharmacia Fine Chemicals. All other chemicals were supplied by BDH and were of Analar quality where possible. All the counter ions were used as the chloride because this anion is relatively inert with respect to the solution structure of macro-molecules (von Hippel & Schleich 1969).

Glycoprotein preparation

The glycoproteins were isolated by a procedure based upon that developed by Brown et al (1981). 20 g of freeze dried porcine gastric mucus (desig-

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nated type I, Sigma Chemical Co., Poole, U.K.) were dispersed in 1 l of 0·1 M phosphate buffer, pH 7·4, containing 0·22 M potassium thiocyanate and 0·02% w/v sodium azide (KCNS buffer). Any insoluble material was removed by centrifugation at 23 000 *g* for 1 h at 4 °C. The supernatant was filtered through washed glass wool and the filtrate stored at 4 °C. Aliquots of filtrate, suitably diluted with KCNS buffer, were applied to a column of Sepharose 4B (30 × 9·5 cm) and the glycoprotein fraction separated from lower molecular weight proteins and glycopeptides by elution with KCNS buffer. The glycoprotein peak eluted at the void volume. It has been shown that the biochemical analysis of the material contained in this peak is generally typical of a mucus glycoprotein and that contamination with non-covalently bound protein and DNA is low (Table 1). The glycoprotein fraction was exhaustively dialysed against distilled water and concentrated by ultrafiltration (Model 402 cell and PM30 membrane, Amicon Corp.) to approximately 2% w/w.

Table 1. Biochemical analysis of purified glycoprotein (type I).

Sugar	Concn n mol mg^{-1}	Amino acid	% Total protein
Fucose	451	Asp	2·4
Mannose	89	Thr	21·3
Galactose	1073	Ser	15·1
N-Acetylgalactosamine	1063	Glu	4·8
N-Acetylglucosamine	1344	Gly	3·8
N-Acetylneuraminic Acid	64	Ala	8·1
After Neuraminidase	26	Cys	—
		Val	5·4
		Met	—
		Ileu	1·1
Sulphate (μg mg^{-1})	5·0	Leu	3·03
DNA (μg mg^{-1})	5·0	His	3·6
		Lys	1·0
		Arg	2·4
		Pro	27·7

Non-purulent sputum samples (type II glycoprotein) obtained from patients suffering from non-tubercular chest diseases were pooled and washed in an equal volume of 1·8% w/v NaCl containing 0·04% w/v sodium azide by intermittent shaking over several hours at 4 °C. The mixture was centrifuged for 1 h at 12 000 *g* at 4 °C and the supernatant discarded. This washing procedure was repeated at least twice. An equal volume of double strength KCNS buffer was added to the washed gel and the mixture homogenized for 30 s (Silverson Machines, Chesham, U.K.). The homogenate was stored for at least 24 h at 4 °C and then centrifuged at 23 000 *g* for 2 h at 4 °C. The supernatant was treated as described for type I glycoprotein.

When desired, sialic acid was removed from type I glycoprotein by incubation with neuraminidase as described by Forstner et al (1973). Sixty percent of the initial sialic acid content was removed by this procedure, whereas in control incubations, without neuraminidase, 19% was lost.

Cation binding

The degree of binding was determined by equilibrium dialysis using a stack of 6 Perspex cells, each with a capacity of 2 ml. Each cell was divided by a semipermeable membrane; at acid pH values the membrane used was cellulose with a nominal molecular weight cut-off of 12 000 daltons, while at neutral pH a polycarbonate membrane with a pore diameter of 0·015 μm was used (Nucleopore Corp.). For each ion studied preliminary experiments demonstrated that binding to the apparatus and membranes was negligible (<1%). Experiments were also performed to determine how rapidly equilibrium was attained, and in all cases a period of 18 h at room temperature (20 °C) was sufficient. In addition it was ascertained that the binding characteristics of calcium were independent of the glycoprotein concentration in the range 0·5–2·0% w/w, and accordingly a 1% solution was used unless otherwise stated.

In a typical experiment 0·8 ml of a 1% w/w solution of type I glycoprotein in buffer was injected into one half of a cell and 0·8 ml of buffer into the complementary half. The solutions were equimolar with respect to the cation under investigation. The range of cation concentrations studied varied according to the ionic valency. The buffer concentration was 0·01 M in all cases. Caesium, sodium and calcium chlorides were studied in Tris/HCl, pH 7·4, while to prevent precipitation of the respective hydroxides iron (Fe III) and aluminium chlorides were studied in glycine/HCl, pH 3·0. For comparative purposes, calcium was also studied at this pH. Iron was also studied in glycine/HCl, pH 2·0. In the cases of sodium, caesium, calcium and iron a trace amount of the appropriate radionuclide was included on the buffer side of the membrane, such that the specific radioactivity was approximately 50 000 *d* min^{-1} μmol^{-1}. The stack of cells was agitated until equilibrium was attained. Duplicate 200 μl aliquots of both glycoprotein and buffer solutions were removed and, where radionuclides were present, mixed with a scintillation medium consisting of 4 ml of toluene and 1 ml of dibutyl-

phosphate. The samples were counted (Beckman LS3 133P) and the resulting counts corrected for quenching. Aluminium was assayed in duplicate by the fluorometric method of Willard et al (1965) using Solochrome Dark Blue (C. I. 15705) as the fluorescent species. It was determined that the presence of glycoprotein did not interfere with the assay.

The binding of calcium to mucus glycoproteins was studied in greater detail. The effect of ionic strength on the amount of calcium bound was determined by including NaCl at concentrations of 50, 100 and 200 mM on both sides of the membrane. The buffer was 0·01 M Tris/HCl, pH 7·4, and the initial calcium concentration was 0·5 mM. The same concentration was used in a study designed to assess the influence of pH on the extent of calcium binding. In this experiment the pH was varied between 4·1 and 8·5, using 0·01 M succinate/NaOH buffer for pH values below 7 and 0·01 M Tris/HCl for those above 7. The use of these buffers results in a negligible change in ionic strength (Forstner & Forstner 1975). In an attempt to determine which groups were important for counter ion binding, the amount of calcium bound to type I glycoprotein after treatment with neuraminidase was measured over the calcium concentration range 0·05–1·0 mM. 0·5% w/w solutions of de-sialylated glycoprotein in 0·01 M Tris/HCl buffer, pH 7·4, were prepared and the amount of calcium bound at each concentration determined as described above.

The amount of calcium bound to a 0·5% w/w solution of type II glycoprotein in 0·01 M Tris/HCl, pH 7·4 was measured over the cation concentration range 0·05–5·0 mM.

All binding results were corrected for non-specific Donnan effects (West et al 1966).

RESULTS AND DISCUSSION

The results are presented as plots of r/D_f against r, where r is the number of moles of ligand bound per kg of mucin and D_f is the unbound ligand concentration in mol litre^{-1} at equilibrium (Scatchard 1949). Where possible, the graphs were analysed by a curve fitting computer program in order to obtain values for the number of binding sites per kg of mucin (n) and the intrinsic association constants of the sites (k).

The curves produced by sodium and caesium were similar (Fig. 1). Both were non-linear, although only marginally so in the case of sodium. This non-linearity indicated that more than one class of site was involved in binding these ions. One group of sites was of high affinity (k_1) and low capacity (n_1), whereas the other group was of low affinity (k_2) but

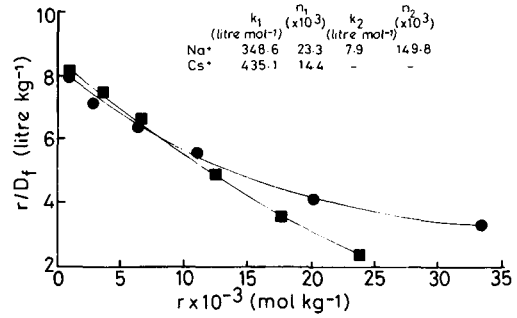

FIG. 1. Scatchard plots for the binding of caesium (●) and sodium (■) in 0·01 M Tris/HCl buffer, pH 7·4, to type I glycoprotein.

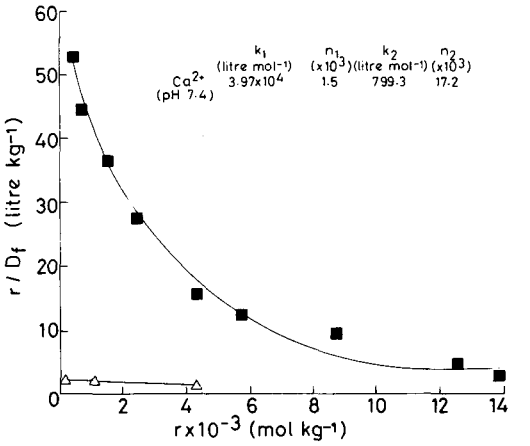

FIG. 2. Scatchard plots for the binding of calcium in 0·01 M Tris/HCl buffer, pH 7·4, (■) and 0·01 M glycine/HCl buffer, pH 3·0, (△) to type I glycoprotein.

high capacity (n_2). Values for n_2 and k_2 could not be obtained for caesium because the low affinity asymptote had a very shallow slope and small variations introduced during the curve fitting procedure produced changes of the order of several magnitudes in the values of n_2 and k_2. The Scatchard plot for calcium at pH 7·4 was successfully resolved into two classes of binding sites (Fig. 2), both of higher affinity than measured for the monovalent ions. At pH 3·0 the affinity of the glycoprotein for calcium was considerably reduced (Fig. 2). The plots for iron and aluminium were non-linear (Fig. 3) and, although they could be fitted to the mathematical model, the association constants of the binding sites were extreme and were sensitive to small experimental errors. Bella & Kim (1973) measured the iron binding capacity at pH 1·8 of glycoproteins obtained from dog gastric and rat intestinal mucus. Values ranging from 300–2100 mmoles iron bound per kg of

glycoprotein were obtained. Although precise extrapolation of the curve for iron binding at pH 2·0 (Fig. 3) is impossible, a reasonable estimate of the iron binding capacity of type I mucin is 200 mmol kg^{-1}. The somewhat higher values presented by Bella & Kim may be a reflection of the generally higher sialic acid and ester sulphate content of the rat intestinal and dog gastric materials (Bella & Kim 1972, 1973), although these workers were not able to show a direct correlation between charge and binding capacity.

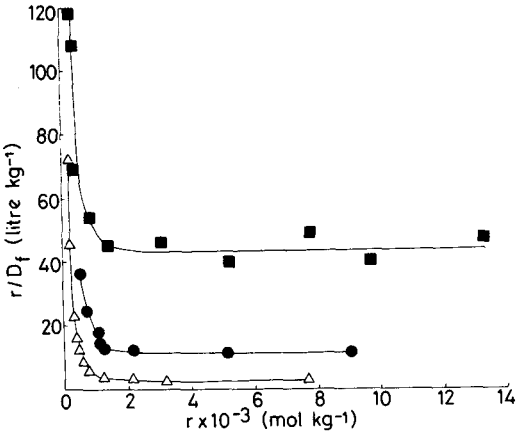

FIG. 3. Scatchard plots for the binding of aluminium (△) and iron (■) in 0·01 M glycine/HCl buffer, pH 3·0, and for iron in pH 2·0, 0·01 M glycine/HCl buffer (●) to type I glycoprotein.

Overall, the affinity of the glycoprotein for the counter ions examined may be ranked: $Fe^{3+} > Al^{3+} > Ca^{2+} > Cs^{+} \simeq Na^{+}$. This is in accord with the theoretical prediction that a polyelectrolyte should interact preferentially with multivalent counter ions (Oosawa 1971). It is similarly predicted that small counter ions should be bound preferentially (Oosawa 1971), and it has been shown for another biological polyanion, DNA, that it is the crystal ionic radius rather than the hydrated radius which is the determining criterion (Ross & Scruggs 1964). In the present system caesium (1·67 Å) and sodium (0·97 Å) were bound with similar affinities, whereas aluminium (0·5 Å) was bound with less affinity than iron (0·64 Å). These deviations from the theoretical ideal may indicate that specific physicochemical interactions were superimposed on the basic polyelectrolyte behaviour. Such interactions are well documented, and it is known for example that calcium binds preferentially to oxyanions (Williams 1971, 1974), whereas copper binds more firmly to sulphur- or nitrogen-containing ligands (Williams 1971).

Increasing the ionic strength by the addition of NaCl reduced the amount of calcium bound in a concentration dependent manner (Table 2). At a sodium concentration of 200 mM r was 11% of the control value. Thus under physiological conditions, the greater affinity of the glycoprotein for calcium may be masked by the higher concentration of monovalent ions present. In a similar manner the binding of trivalent ions may also be reduced, but the higher affinity with which these ions are bound may serve to mitigate this tendency.

Table 2. Effect of increasing NaCl concentration on the number of moles of calcium bound per kg of type I glycoprotein. Solutions were buffered with 0·01 M Tris/HCl, pH 7·4 and the initial calcium concentration was 0·5 mM.

NaCl concn (mM)	$10^3 \times r$ (mol kg^{-1})
0	5·77
50	1·97
100	1·51
200	0·66

Enzymatic removal of sialic acid caused only a slight reduction in the calcium binding capacity of the glycoprotein, when compared with glycoprotein samples which underwent a control incubation (Fig. 4). Surprisingly, both control and enzyme incubated samples bound more calcium than did the unincubated glycoprotein. Such an increase was consistently demonstrated, although the degree of elevation varied. This rise may have been connected with the

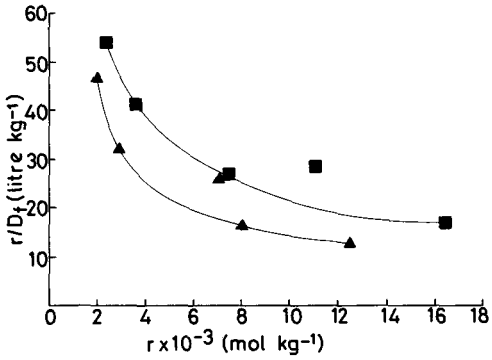

FIG. 4. Scatchard plots for the binding of calcium in 0·01 M Tris/HCl buffer, pH 7·4, to control (■) and neuraminidase treated (▲) type I glycoprotein.

release of a relatively small non-sialylated glycopeptide which occurred during incubation, but this possibility was not pursued. The amount of calcium bound to type I glycoprotein was considerably lower than that bound to type II (Table 3) or to rat intestinal glycoprotein (Forstner & Forstner 1975). In addition these workers found that incubation with neuraminidase caused calcium binding to decrease by almost 90%. The rat intestinal mucin and type II mucin contained substantially more anionic groups than type I but were otherwise biochemically similar (Forstner et al 1973; Crowther 1982). This tends to confirm that, although uncharged mucin sugars can bind calcium ions under certain circumstances (Cook & Bugg 1973, 1975), a high calcium binding capacity is dependent upon a high content of charged groups.

Table 3. Number of moles of calcium bound per kg of types I and II glycoprotein in 0·01 M Tris/HCl buffer, pH 7·4.

Initial calcium concn (mM)	$10^3 \times r$ (mol kg^{-1}) Type I	Type II
0·05	1·50	14·38
0·50	5·77	87·90
5·0	13·82	134·90

Calcium binding to type I glycoprotein was highly pH dependent (Fig. 5). A constant amount of calcium was bound between pH 5–7·4, but at higher pH r rose sharply, whilst at more acid pH values a gradual decline in the value of r was observed. A similar result was reported for rat intestinal glycoprotein, except that with this material the degree of binding increased dramatically as the pH rose above 6·5 rather than 7·4 (Forstner & Forstner 1975). Neither of these results can be accounted for by changing ionization of the charged groups, since at pH 4·0 both sialic acid and the ester sulphate groups will still be approximately 96% dissociated. It may be that at approximately neutral pH both the macromolecules undergo conformational changes which increase the accessibility of the anionic groups, although dilute solution viscosity studies on type I mucin indicated that greater conformational changes occurred in the region of pH 4–6·5 (Crowther 1982), within which calcium binding was relatively constant.

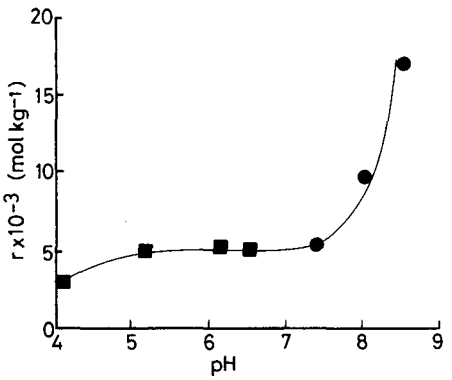

FIG. 5. Influence of pH on the number of moles of calcium bound per kg of type I glycoprotein. Buffers used were 0·01 M succinate/NaOH (■) and 0·01 M Tris/HCl (●).

Although the pH of gastric juice is very low, it has been demonstrated in animals (Williams & Turnberg 1980; Ross et al 1981) and man (Ross et al 1982) that the mucus layer can support a pH gradient, with values approaching neutrality at the epithelial surface when the solution bathing the lumen has a pH of approximately 2. Therefore a significant fraction of the glycoprotein molecules which constitute the gel layer will possess fully dissociated anionic groups and the level of ion binding will be high. Ion binding will be similarly high in the intestine and when the total quantity of mucus present in the gastrointestinal tract is considered the ion binding capacity of the mucus gel layer will be seen to be considerable. This capacity may be altered in diseases such as cystic fibrosis (Clamp & Gough 1979) and ulcerative colitis and Crohn's disease (Clamp et al 1981) in which the biochemical composition of the mucin is abnormal and this may have implications for the systemic absorption of cations.

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