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Studies of the Binding of Copper, Zinc and Calcium to Pectin, Alginate, Carrageenan and Gum Guar in $HCO_3^- - CO_2$ Buffer

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ABSTRACT

Using the equilibrium dialysis technique, the interaction of Cu, Zn and Ca with pectin, alginate, carrageenan and gum guar was studied. The experiments were done either in sodium hydrogencarbonate solution, the physiological $HCO_3^- - CO_2$ buffer of the intestine, or in diluted HCl or NaOH, to keep the ionic strength as low as possible. Great differences in the binding in both media were detected. With increasing pH the amount of metal bound to the isolated acid polysaccharides rose in diluted HCl or NaOH, reaching a maximum binding at pH > 5. In buffer the binding also increased with rising pH (pH 2–5), reaching a maximum at pH 5–6, but declined almost to zero at pH > 6. Because of low affinity no interaction could be detected in buffer between Ca and pectin, Ca, Zn and carrageenan and between Cu, Zn, Ca and gum guar. Based on the pH-dependent binding in the $HCO_3^- - CO_2$ buffer, it is suggested that, under relevant physiological conditions of the intestine (jejunum and ileum), the interaction of Cu^{2+}, Zn^{2+} and Ca^{2+} with the tested isolated polysaccharides is very weak.

INTRODUCTION

High dietary fibre intake has been assumed to correlate with a diminished risk for certain diseases very common in highly industrialized societies (Burkitt & Trowell, 1975; Trowell, 1976; Birch & Parker, 1983). An increase in food rich in dietary fibre, leading to a daily dietary fibre intake in the range of 20–35 g for healthy adults, has been recommended (Pilch, 1987; Deutsche

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Gesellschaft für Ernährung, 1985). Some components of dietary fibre, such as pectins and alginates, act as strong cation exchangers and form stable complexes with bivalent cations. Extensive studies have, therefore, been conducted to see whether an enhanced dietary fibre intake reduces the bioavailability of trace elements or minerals (see the reviews of Kelsay, 1982; Harland & Morris, 1985; Southgate, 1987; as well as Harmuth-Hoene *et al.*, 1982; Oku *et al.*, 1982). For some trace elements such as Pb or Sr a diminished absorption in the presence of pectin and pectate has been reported (Skoryna *et al.*, 1973; Paskins-Hurlburt *et al.*, 1977; Stantschev *et al.*, 1979). The question whether or to what extent pectin and other acid polysaccharides bind essential trace elements or minerals in the intestinal tract, thereby affecing the metal absorption, has still not been finally resolved.

In vitro binding studies demonstrate strong interactions between pectin or pectinaceous fibres and bivalent cations (James *et al.*, 1978; Camire & Clydesdale, 1981; Nair *et al.*, 1987). Feeding studies, using diets rich in pectin, show little or no influence on absorption or balance of Cu^{2+}, Ca^{2+}, Zn^{2+} (Cummings *et al.*, 1975; Kelsay, 1979; Kelsay *et al.*, 1981; Southgate, 1987). It is therefore questionable whether the interactions demonstrated in *in vitro* studies under physiological conditions are sufficiently strong to affect the absorption of essential trace elements or minerals during the gastro-intestinal passage.

More information is needed about the binding capability of dietary fibre under physiological conditions of the intestine, as well as about the influence of various substances present in food, affecting these metal–fibre interactions. Using the equilibrium dialysis technique, the binding of Cu^{2+}, Zn^{2+} and Ca^{2+} to pectin, alginate, carrageenan and gum guar was studied in the physiological HCO_3^-–CO_2 buffer.

MATERIALS AND METHODS

Materials

Pectin

Commercial pectin (apple pectin; Röhm, Darmstadt, FRG; 0·5% w/v) was purified by addition of NaCl/EDTA (resulting concentration 155 mM NaCl/34 mM EDTA) and repeated precipitation in ethanol (resulting solution 64% ethanol/H_2O). It was demineralized by dialysis (Visking dialysis bag, 27/32, Serva, Heidelberg, FRG) against 8 mM EDTA/50 mM NaCl for 3 days and for six more days against H_2O (bidistilled) at +4°C. The dialyzed pectin solution was centrifuged (15 000 × *g*, 15 min) passed through an 0·6 μm filter and kept at −20°C until use. Pectin prepared in this way was

sterile, having the following proportions: Intrinsic viscosity $[\eta] = 3{\cdot}2$ (g/100 ml) and MW 55 600, according to Owens *et al.* (1946); degree of methylation (DM) $52 \pm 2\%$, using the method of Katan & van de Bovenkamp (1981); content of rhamnose $1{\cdot}0 \pm 0{\cdot}2\%$, using the thioglycolic acid method, content of pentoses (arabinose and xylose) $2{\cdot}6 \pm 0{\cdot}3\%$, using the phloroglucinol method (Dische, 1962) and galacturonic acid content 88·4%, determined by a carbazole procedure (Schlemmer, 1986). The final concentration of pectin under the described experimental conditions was 0·8 mg/ml.

Alginate
Commercial Na-alginate was obtained from Henkel & Cie., Düsseldorf, FRG, showing the ratio of mannuronic acid: guluronic acid of ~6·7:1 and a total polysaccharide content of 87% (Harmuth-Hoene & Schelenz, 1980). The alginate solution was demineralized, dialyzed and centrifuged as described for pectin and had a final concentration of 0·8 mg/ml.

Carrageenan
Carrageenan was obtained from Serva (Heidelberg, FRG, kappa type, total polysaccharide content 98%, content of sulphate groups 25% MW ~500 000), prepared as described for alginate with a final concentration of 0·8 mg/ml.

Gum guar
Gum guar was obtained from Serva (Heidelberg, FRG) and prepared as described for alginate. The final concentration of 0·6 mg/ml was selected to obtain a viscosity, which could be handled by the chosen technique.

Methods

Determination of copper, zinc, calcium

Cu, Zn and Ca were determined by atomic absorption spectroscopy (AAS) using an atomic absorption spectrophotometer 2380 Perkin-Elmer (air–acetylene flame; impact bead; slit 0·7 nm; ^{2}H-background compensation; wavelength 324·8 nm; 213·9 nm and 422·8 nm, respectively). All samples and references were adjusted to a final pH 2 by addition of 1N HCl and were measured in duplicate with CV < 3·8%. For Ca^{2+}-determination, lanthanum chloride was added to a final concentration of 0·5%.

Equilibrium dialysis procedure
The dialysis chambers were made of plates of Plexiglas® (Röhm, Darmstadt, FRG) containing hemispherical holes. When two plates were fixed onto each

other hole to hole, cavities of 6 ml were formed. An opened dialysis bag (as used above) was placed as membrane between the plates, thus creating two identical compartments. They were filled and emptied via vertical channels and closed by teflon stoppers, preventing the escape of CO_2. In analogy to the gastrointestinal passage of a meal, pectin, alginate, carrageenan and gum guar were adjusted to pH 1 by HCl and then titrated by $NaHCO_3$ solution (70 mg/ml) to the relevant pH. Alginate was precipitated at pH 1 and carefully redissolved at pH 3 before further use. Two-and-a quarter millilitres of the polymer solutions (pH $\sim$2 to $\sim$8) were filled into compartment 1 of the dialysis chamber and 2·25 ml of 0·1N HCl (pH 1), to which various amounts of $NaHCO_3$ were added to obtain identical pH values as in the polymer solutions, were filled into compartment 2.

To both compartments 100 μl of an aqueous solution of $CuCl_2$, $ZnCl_2$ or $CaCl_2$, varying in molarity, were added. In the binding studies without $NaHCO_3$, the polymers were adjusted to the relevant pH by adding 0·01N HCl or 0·01N NaOH, without initially lowering the pH value to pH 1, in order to keep the ionic strength as low as possible (at pH 2 and pH 7·5, $I < 0{\cdot}009$; in the presence of $NaHCO_3$ at pH 2, $I = 0{\cdot}07$ and at pH 7·5, $I = 0{\cdot}10$).

After reaching binding equilibrium (18 h at 37°C, shaking the dialysis chamber with 100 oscillations/min) the pH value was measured in compartment 2. The dialysis chamber was emptied, first compartment 2, then compartment 1. The contents of both compartments were centrifuged (15 min, 11 000 $\times$ *g*; using closed tubes) and the precipitates separated. Aliquots of the supernatants were acidified (by HCl to pH 2) and the precipitates redissolved by HCl or by EDTA in the case of alginate. All samples were analyzed by AAS (see above) and each experiment was repeated at least twice. The amount of metal bound to the polymers was calculated as the difference between soluble metal contents of the polymers (compartment 1) and of the buffer or HCl/NaOH solutions (compartment 2).

RESULTS AND DISCUSSION

The pH-dependent Cu-pectin binding (0·8 μmol Cu; 1·8 mg pectin, DM 52%) is demonstrated in Fig. 1. To simulate conditions in various sections of the digestive tract the physiological $HCO_3^- - CO_2$ buffer was used and the pH-value was varied between pH 2 and $\sim$8. To detect the influence upon the binding exerted by the buffer, the Cu-pectin binding was also studied in diluted HCl or NaOH.

Obviously the interaction is strongly pH-dependent and more copper is

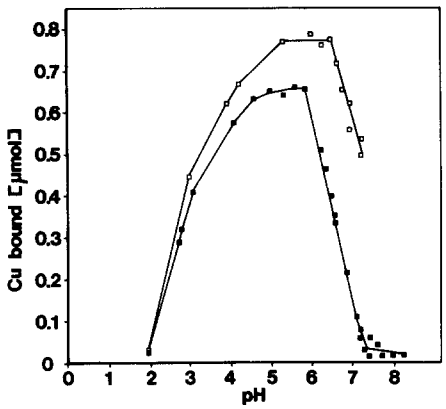

Fig. 1. The pH-dependent binding of 0·8 µmol Cu to 1·8 mg pectin (DM 52%) in HCO_3^-–CO_2, ■——■, and in diluted HCl or NaOH, □——□.

bound in diluted HCl or NaOH than in buffer. Below pH 8 no binding exists. With rising pH (pH 2 to 5) more Cu^{2+} ions are bound, reaching a maximum binding at about pH 5–6. A further increase in pH (pH > 6) decreases the binding. At pH > 7·3 almost no copper–pectin interaction can be determined in buffer, while ~64% of copper is bound in diluted NaOH.

Because of changing concentrations of pectin and Cu^{2+} ions in intestinal contents, the binding was tested at different Cu–pectin ratios. At constant pectin concentration the addition of Cu^{2+} ions was varied without exceeding the stoichiometric relation of one Cu^{2+} ion to two carboxyl groups of pectin (2·3 µmol Cu to 1·8 mg pectin, DM 52%). The results show a higher percentage copper binding when the added amount of copper was decreased (Table 1), indicating that a cooperative binding mechanism (McGhee & von Hippel, 1974) controls the copper–pectin interaction.

At pH 7, however, no changing of the binding occurs when the added amount of copper decreases. This demonstrates that, independent of changing Cu–pectin ratios, copper binds to pectin quite effectively at pH 4–6 but is released again with rising pH. These results confirm the experiments of Nair *et al.*, (1987), who studied the copper–pectin interaction polarographically and found a similar pH-dependent binding in $CuClO_4$ solution, as is demonstrated in Fig. 1.

The amount of bound copper was calculated as the difference of soluble copper of both compartments of the dialysis chamber. Because copper forms hydroxo (Nair *et al.*, 1987) or other low-soluble complexes in the buffer at about neutral pH, the solutions of both compartments were centrifuged and precipitates separated.

When 0·2 µmol or 0·8 µmol are used (Table 1) the copper pectinate is soluble and the amount of other copper precipitates at pH 7 is negligibly small (<4% of total Cu). When higher amounts of copper (>1·5 µmol Cu) are used, copper compounds precipitate in both compartments of the

TABLE 1
The Binding of Cu, Zn, Ca to Pectin[a] and Alginate[a] in HCO_3^-–CO_2 Buffer

Total metal (*μmol*)	*Bound metal in % of total metal at various pH*			
	Pectin		*Alginate*	
	pH 5·0 ± 0·1	*pH 7·0 ± 0·1*	*pH 5·0 ± 0·1*	*pH 7·2 ± 0·1*
Cu				
0·2	86	21	81	5
0·8	80	20	80	4
2·2	61	21	—	—
Zn				
0·2	18	12	18	5
0·8	20	11	21	13
Ca				
0·8	3	2	10	1
3·0	6	0	21	13

[a] 1·8 mg of each polymer.

dialysis chamber at pH >6 (Fig. 2). Under these conditions the decrease in binding is closely related to the reduced copper solubility in buffer (Fig. 2, compartment 2). At pH $>7{\cdot}5$ the solubility of copper compounds in buffer rises again, which, however, does not lead to an enhanced copper–pectin binding. At this pH, copper is bound to soluble complexes, which do not interact with pectin, since free Cu^{2+} ions are probably required for the specific interaction with the carboxyl groups of pectin. Figure 3 shows the

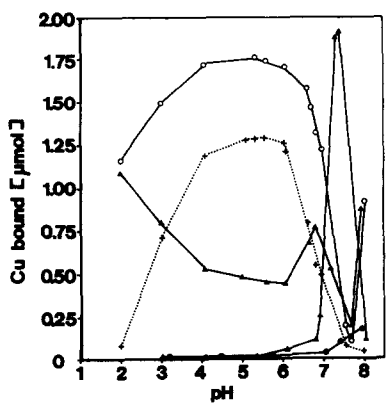

Fig. 2. The distribution of soluble and insoluble Cu in both compartments of the dialysis chamber after addition of 2·2 μmol Cu to 1·8 mg pectin (DM 52%) in HCO_3^-–CO_2 buffer. Bound Cu, + ··· +, is calculated as the difference between soluble Cu of compartment 1 (pectin, ○——○) and compartment 2 (without pectin, △——△); Cu-precipitates in compartment 1, ●——●; Cu-precipitates in compartment 2, ▲——▲.

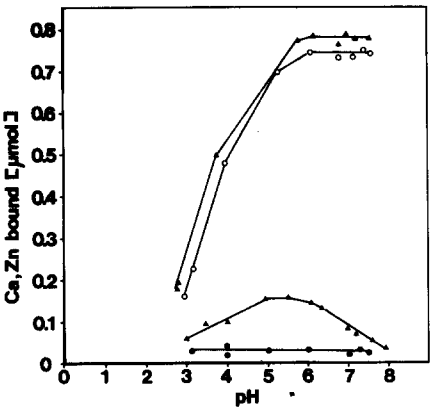

Fig. 3. The pH-dependent binding of 0·8 µmol Zn or Ca to 1·8 mg pectin (DM 52%) in HCO_3^-–CO_2 buffer (Zn ▲——▲; Ca, ●——●) and in diluted HCl or NaOH (Zn, △——△; Ca, ○——○).

binding of zinc and calcium to pectin under conditions equivalent to those shown for the copper–pectin interaction in Fig. 1. As in the case of copper, the binding of zinc and calcium to pectin in HCl/NaOH is much stronger than in HCO_3^-–CO_2 buffer. The binding in HCl/NaOH shows a rise with increasing pH, reaching a maximum level at pH 5–7·5 at which 97% of zinc and 93% of calcium are bound (Fig. 3). This is in accordance with Camire & Clydesdale (1981), who found in aqueous pectin solution, for both metals, a binding of 94%.

In the HCO_3^-–CO_2 buffer, however, the binding is different for both elements. While there is a pH-dependent Zn-binding in buffer, reaching a maximum at pH 5–6, no interaction at all can be detected between calcium and pectin (pH 3–7·5). At optimal pH in buffer (pH 5–6) just 20% of total zinc is bound and $<11\%$ at pH >7. For comparison, the copper–pectin interaction under equivalent conditions is 80% and $<20\%$, respectively. No relevant change in the percentage Zn- or Ca-binding to pectin occurs when the amount of added zinc and calcium varies (Table 1).

The solubility of calcium and zinc in the buffer is sufficiently high over the whole pH range tested and the maximum precipitate for both elements is $<4\%$ at pH 7·5. The results indicate that in the HCO_3^-–CO_2 buffer at physiological pH ($\sim$pH 6·5–7·5, the pH-range of the jejunum), neither the trace element zinc nor the mineral calcium is bound to pectin to a relevant degree. This applies as well if the metal–pectin proportion changes.

Figure 4 shows the binding of 0·8 µmol of Cu, Zn or Ca to alginate, another polysaccharide, containing carboxyl groups. Generally the same pH-dependent binding for these three elements, as found with pectin can be observed. In contrast to pectin, however, the binding of copper to alginate in diluted NaOH does not decrease at pH $>6·5$ and 10% of the Ca^{2+} ions are bound to alginate in buffer at optimal pH (pH 5). For copper the maximum

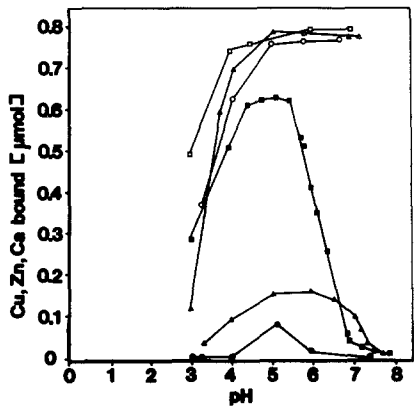

Fig. 4. The pH-dependent binding of 0·8 µmol Cu, Zn, Ca to 1·8 mg alginate in HCO_3^-–CO_2 buffer (Cu, ■——■; Zn, ▲——▲; Ca, ●——●) and in diluted HCl or NaOH (Cu, □——□; Zn, △——△; Ca, ○——○).

binding in buffer is 80% (pH 4·5–5·5) and for zinc 21% (pH 5–6). Decreasing copper and zinc content (0·2 µmol Cu or Zn) does not change considerably the percentage binding of copper and zinc at pH 5 or pH 7 (Table 1). Increasing calcium content (3·0 µmol Ca)—because of higher Ca than trace element concentrations in intestinal contents—leads to enhanced calcium–alginate binding, which obviously is stronger than the calcium–pectin interaction (Table 1). The results indicate that, in buffer, the same order of metal binding exists for alginate as well as for pectin, $Cu \gg Zn > Ca$.

In Fig. 5 the binding of Cu, Zn and Ca to carrageenan is demonstrated. Carrageenan was selected because it is also an acidic polysaccharide, but contains, in contrast to pectin and alginate, sulfate groups.

As with pectin and alginate the copper- and zinc–carrageenan interaction is also pH-dependent in diluted HCl or NaOH, reaching a maximum at pH 5

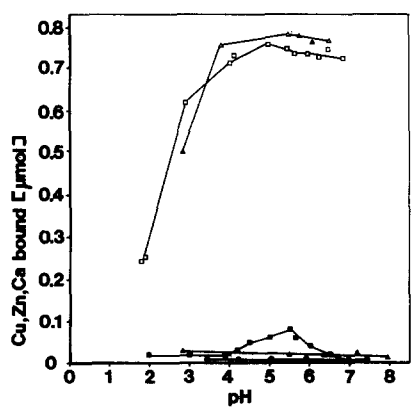

Fig. 5. The pH-dependent binding of 0·8 µmol Cu, Zn, Ca to 1·8 mg carrageenan in HCO_3^-–CO_2 buffer (Cu, ■——■; Zn, ▲——▲; Ca, ●——●) and in diluted HCl or NaOH (Cu, □——□; Zn, △——△).

and pH 5·5, respectively. Under these conditions 98% of Zn^{2+} and 96% of Cu^{2+} ions are bound. In the HCO_3^-–CO_2 buffer an interaction with carrageenan can only be detected for copper with a maximum binding of 11% at pH 5·5. For zinc and calcium, however, no interactions in buffer can be observed. These results demonstrate that, in buffer, the interaction between copper, zinc, calcium and carrageenan is much weaker than the binding to pectin and alginate under equivalent conditions. It is therefore suggested, that carrageenan under the physiological conditions of the intestine will not interact with the tested metals to a nutritionally relevant degree.

Table 2 shows the binding of copper, zinc and calcium to gum guar, a neutral dietary fibre, in HCO_3^-–CO_2 buffer at various pH values (pH 3–7). No interaction between this polysaccharide and the three metals can be detected over the whole pH range tested, not even when the concentration of metals was raised four-fold. Camire & Clydesdale (1981), however, reported bonds between Ca^{2+}, Zn^{2+} and gum guar in the absence of buffer. So it is assumed, that—as demonstrated for pectin, alginate and carrageenan—in aqueous solutions, strong interactions between Cu^{2+}, Zn^{2+}, Ca^{2+} and gum guar exist. In the physiological HCO_3^-–CO_2 buffer, however, the bonds are cleaved or considerably reduced. These differences in binding in both media may either derive from a higher metal affinity of buffer substances than that of the anions of the control solution or from a higher ionic strength of the buffer. Both effects diminish the binding of Cu, Zn and Ca to the dietary

TABLE 2
The Binding of Cu, Zn, Ca to Gum Guar[a] in HCO_3^-–CO_2 Buffer

Total metal (*μmol*)	*Bound metal in % of total metal at various pH*			
	pH 3	*pH 5*	*pH 6*	*pH 7*
Cu				
0·8	0	3	3	2
3·2	0	0	—	—
Zn				
0·8	0	0	0	0
3·2	0	1	1	0
Ca				
0·8	0	0	0	0
3·2	0	0	0	0

[a] 1·35 mg gum guar.

fibres, depending on the binding strength of the respective metal–fibre bonds. Obviously the copper binding to the acid dietary fibres is the strongest, since it is reduced in buffer the least. The calcium binding, however, most diminished in buffer, seems to be the weakest. The results emphasize the strong influence upon the binding exerted by different media in which binding studies are done and illustrate the difficulties, occurring when results of various *in vitro* binding studies are compared with each other.

The presented studies demonstrate that in the physiological $HCO_3^- - CO_2$ buffer the metal binding of the acid dietary fibres is optimal at pH 4–6, which corresponds to the pH range of the duodenum. Metals bound under these conditions, however, will be released again with rising pH, pH ~6–8, the pH range of the jejunum and ileum (Watson *et al.*, 1972) and can still be absorbed effectively in these sections of the small intestine. Although great care is needed in transferring *in vitro* results to *in vivo*, it is concluded from these studies that a nutritionally relevant binding of Cu^{2+}, Zn^{2+} and Ca^{2+} to purified pectin, alginate, carrageenan and gum guar in the intestinal tract, is not very likely.

Additional *in vitro* studies under physiologically relevant conditions are necessary to clarify the binding ability of dietary fibres in the intestine and the complex binding-situation in which many other nutrients may be involved.

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