Cereal Complexes: Binding of Calcium by Bran and Components of Bran[1]

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ABSTRACT

Binding of calcium ions in aqueous media by food-grade white wheat bran, by components of bran, and by constituents of gastrointestinal fluid was studied in vitro at 37°C over the physiological pH range of 5–8. Analysis for free, uncomplexed Ca^{2+} was by means of the metal indicator tetramethylmurexide. No buffers were used. Water-soluble components are responsible for more than half of the binding ability of bran, and the principal soluble chelating agent is phytate. Cellulose, starch, hemicellulose, and pectin (degree of esterification, 61%) have little affinity for Ca^{2+} at neutral pH and, therefore, as bran components, make no important contribution to calcium binding. Affinity of protein for calcium is also very low. At concentrations approximating those found in the human intestine, constituents of gastrointestinal fluid (ie, saliva, sugar, albumin, mucin, amino acids, phosphate ion, hydrogen carbonate ion, and bile acids) bind Ca^{2+} only very weakly. Extent of binding by these constituents was too small to measure accurately.

Commercial wheat bran, composed largely of cellulose, hemicellulose, lignin, pectin, phytate (*myo*-inositol hexaphosphate), protein, and starch, has a marked ability to bind polyvalent metal cations (Ismail-Beigi et al 1977; Reinhold et al 1975), In the small intestines of monogastric animals, complexation of metals with dietary fiber and with nonfiber substances generally associated with dietary fiber is probably the most important cause of diminished bioavailability of these metals. High-fiber diets lead to increased fecal excretion of calcium, zinc, magnesium, and phosphorus, and can thus cause negative balances of these elements in the body (Reinhold et al 1976). Dietary phytate reduces the availability of calcium, zinc, magnesium, iron, manganese, and copper, presumably through the formation of slightly soluble metal-phytate complexes (Davies and Nightingale 1975, Oberleas 1973). Reinhold et al (1975) maintain that, in foodstuffs rich in both phytate and fiber (such as whole meal bread and bran-based products), the fiber rather than the phytate largely determines the degree to which polyvalent metals are absorbed through intestinal walls. However, Davies et al (1977) reached the opposite conclusion from growth studies on rats: phytate rather than fiber appeared to be the major determinant of zinc availability.

Only limited study has been done on interactions of polyvalent cations with bran in vitro. Reinhold et al (1975) investigated the binding of calcium, zinc, and iron by wheat bran and whole meal wheat bread in vitro in aqueous solutions buffered at pH 6.8. Interaction of these ions with foodstuff was assumed to give water-insoluble complexes that could be separated by centrifugation. Quantitative determination of unreacted metal in the clear supernatant was by means of atomic absorption spectrophotometry and radioassay. The results of these studies suggested that the percentage of bound calcium was proportional to the percentage of fiber in the sample. Bran seemed to bind only 59% more Ca^{2+} than did an equal weight of cellulose. Dephytinization of bran with dilute HCl or with phytase at pH 4.5 did not diminish binding ability. In fact, on the basis of equal sample weights, dephytinized bran had a greater binding power than did nondephytinized bran.

Camire and Clydesdale (1981) recently investigated the effect of cooking and pH on the binding of calcium by cellulose, lignin, and wheat bran in buffered solution. Binding by cellulose was weak and not significantly influenced by cooking over pH 5–7. Lignin had a moderately high calcium-binding ability that was not greatly influenced by cooking. The binding ability of wheat bran was diminished by a boiling treatment but was not affected by toasting.

This article presents the results of an in vitro study of the binding of calcium ion by bran, aqueous bran extract, individual components of bran, and certain major constituents of gastrointestinal fluid. Determining whether gastrointestinal substances can compete successfully with bran for calcium was deemed important. However, the primary goal of this work was to determine the relative importance of fiber in the binding of calcium by bran. Lignin was not studied because of the improbability of isolating this bran component in a chemically unaltered state.

All complexation reactions were conducted at 37°C and, in general, within the pH range 5–8. (The average pH of the human small intestine is about 6.8.) No buffers were employed, because of their ability to complex with bivalent metal cations. Adjustment of pH was accomplished by adding KOH or HCl, and ionic strength was kept at 0.165 to maintain a constant activity coefficient for Ca^{2+} that is similar to that in the human gut. Tetramethylmurexide (TMM), a pH-independent metal indicator sensitive to calcium-ion activity, was used to determine the concentration of unreacted Ca^{2+}. Because this indicator responds only to uncomplexed cations, it permitted the study of systems in which the metal complex was either wholly or partially soluble. Total complexed calcium, and not just insolubilized calcium, was therefore determined.

MATERIALS AND METHODS

Materials

The bran was AACC enzyme-deactivated certified food-grade wheat bran R07-3691 (87.3% soft white and 12.7% club white). Analytical data provided by AACC indicated 10.4% moisture, 8.91% crude fiber, 14.3% protein, 5.22% fat, 3.2% lignin, 3.0% pectin, 17.4% starch, 7.04% sugar (as invert sugar), 22.1% pentosan, 3.36% phytic acid, 0.12% Ca (author's analysis showed 0.087%), 0.43% Mg, 0.00545% Zn, 0.0122% Fe, 0.00156% Cu, 1.38% K, 0.10% Na, and 1.04% P. Gastric mucin, bovine serum albumin, bile acids, and all carbohydrates were the purest available and, except where noted, were used without further purification. Amyloglucosidase and pronase were obtained from Sigma Chemical Co. and Calbiochem, respectively. All other chemicals were reagent grade. Water was distilled and deionized.

TMM was synthesized from tetramethyl alloxantine (Cope et al 1941) according to the modified procedure of French et al (1971). TMM is relatively stable over the pH range 4–9.

Hemicellulose suitable for study was isolated from AACC bran as follows: defatted bran (150 g) was stirred with 3 L of deoxygenated 10% NaOH under N_2 for 20 hr at 25°C, according to the procedure of Whistler and Feather (1965). The mixture was then filtered through cheesecloth, and the filtrate was neutralized by cooling in an ice bath to approximately pH 5 with glacial acetic acid. Absolute ethanol (9 L) was added, and the resulting solids were isolated by filtration. These solids were subsequently mixed with 1 L of water in a Waring Blendor and again treated with ethanol (3 L). This operation with water and ethanol was repeated

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[2]The mention of firm names or trade products does not imply that they are endorsed or recommended by the USDA over other firms or similar products not mentioned.

three more times before the solids were dried under vacuum. Starch, an impurity present in considerable amount, was removed by enzymatic hydrolysis with amyloglucosidase at pH 4.5 (sodium acetate buffer). Sodium phytate, another major contaminant, was removed by stirring starch-free hemicellulose (9 g) in 250 ml of 0.15*N* HCl for 5 min, then adding 750 ml of ethanol, filtering, and finally washing the solid with a 3:1 mixture of ethanol and 0.1*N* HCl. This acid-treatment operation was repeated two more times before the solids were washed almost free of acid with 3:1 (v/v) ethanol-water. To remove remaining traces of acid and to eliminate any proteinaceous material that might interfere in the study of metal binding, the hemicellulose was stirred for 20 hr at 25°C in water (1 L) containing 0.1 g of pronase and sufficient KOH to make a pH of 6.0–6.4. Absolute ethanol (1 L) and 5 g of KCl (to facilitate flocculation) were then added; the solid was isolated by centrifugation, washed with 3:1 (v/v) ethanol-water, dried under vacuum, and allowed to equilibrate with atmospheric moisture at 50% rh (11.8% moisture, as determined by weight loss at 100°C in a vacuum; 0.32% Kjeldahl N).

General Procedure for Studying Calcium Binding

A measured sample of bran (or other substrate) and a measured volume of standard $CaCl_2$ solution containing sufficient KCl to make the ionic strength $\mu = 0.165$ were mixed in a Pyrex or polyethylene container. After a pH adjustment with KOH or HCl, administered by a calibrated syringe, the container was capped and agitated for 2–3 hr at 37°C. The reaction mixtures were then centrifuged for 1–2 hr at 37°C, and the resulting clear supernatants were subsequently checked for pH and analyzed for either total soluble calcium (oxalate method) or free, uncomplexed calcium (TMM method), or both. For studies involving acid treatment or cooking of the substrate, some modification of the above procedure was necessary, and the final ionic strength was always 0.165.

If adsorption of calcium ions to the walls of polyethylene containers occurs, it is too small to be detected by TMM analysis. After identical volumes (10 ml) of 3.7 m*M* $CaCl_2$ solution were placed in separate Pyrex and polyethylene containers and left for 24 hr at 25°C, the observed concentrations of calcium were the same.

Analysis for Total Calcium by the Oxalate Method

For analysis of a solid, a weighed sample was pyrolyzed in a platinum crucible for 3 hr at 800–850°C. For analysis of a solution, an aliquot was introduced into a crucible and carefully evaporated to dryness before pyrolysis. Pyrolyzed samples were dissolved in 6*N* HCl and then analyzed according to AACC method 40-21 (1976). Precision was ±1%.

Spectrophotometric Analysis for Free Ca^{2+} by the TMM Method

TMM solution (0.50 ml), prepared fresh daily by dissolving 13.6 mg of TMM in 50 ml of water, was added to a 10-ml aliquot of supernatant from a centrifuged reaction mixture. Absorption measurements on this solution were made with a 1-cm cell at 490 and 530 nm; measurements were also made on a blank composed of 10 ml of supernatant and 0.50 ml of water. The quotient A_{490nm}/A_{530nm} was then used to determine free Ca^{2+} from a standard curve according to the method of Kohn and Furda (1967a, 1967b). The precision was ±0.04m*M* Ca^{2+}, and the deviation of a measurement for one run from the average of the measurements for three separate runs was ±0.04m*M*.

Analysis for Phytate (as *myo*-Inositol) by Gas-Liquid Chromatography

A sample containing approximately 15 mg of phytate ion was heated in 2–6 ml of 1*N* HCl in a sealed tube at 125°C for three days. As a control, 30 mg of sodium phytate (Sigma Chemical Co.) was treated similarly in a separate tube. The content of each tube was then evaporated to dryness in a rotary evaporator at 50–60°C, 1 ml of dry pyridine (containing 6 mg of methyl α-D-mannoside as internal standard) was added to each dry residue, and the mixtures were silylated. Gas-liquid chromatography of the trimethylsilyl derivatives was performed isothermally at 170°C on a stainless steel column 8 ft by ⅛ in. packed with Gas Chrom Q support (100–120 mesh), with 3% OV-1 (methyl silicone) as the liquid phase. Yield of phytate was based upon the observed yield of *myo*-inositol.

RESULTS AND DISCUSSION

Bran and Ca^{2+}

A reaction time of 2 hr was sufficient to achieve equilibrium between bran and calcium ion in aqueous media. Reaction times longer than 3 hr were not desirable because of the likelihood that fermentation would begin before analyses was completed. Fermentation causes a drop in pH, which can not only alter the extent of calcium binding but also can rapidly destroy the metal indicator if the drop is sufficiently large (pH <4).

Preliminary studies of cellulose in $CaCl_2$ solution containing TMM indicated that the cellulose had a significant ability to remove as much as 11% TMM from aqueous solution, possibly through binding. Because of this behavior of cellulose, which could also be typical of other insoluble bran components, TMM was never added directly to reaction mixtures in the binding studies reported. The indicator was always added to samples of clear supernatant after centrifugation. However, in one trial experiment in which bran was treated with $CaCl_2$ solution, the analytical result obtained when TMM was added directly to the reaction mixture was identical to that obtained when TMM was added to clear supernatant.

Except where noted, all studies were conducted with physically unaltered AACC certified bran.

Bran contains a considerable amount of naturally occurring minerals. AACC data on the bran used in these experiments indicated about 21.3 mmol of polyvalent cations per 100 g of bran; of this amount of cations, 3 mmol are Ca^{2+}, and 17.7 mmol are Mg^{2+}. All other polyvalent metals are present in amounts too small to interfere with calcium determination by the TMM method. Furthermore, sensitivity of TMM to either Na^+ or Mg^{2+} is too small for the presence of these ions to cause error. The indicator is totally insensitive to K^+. Thus, only naturally occurring Ca^{2+} is potentially able to introduce error, should this element be incompletely bound by bran when the latter is mixed with water. Fortunately, naturally occurring calcium is so firmly bound that only a minute fraction breaks away as uncomplexed ions. When 4 g of bran was mixed with 100 ml of water at 37°C ($\mu = 0.165$), the aqueous phase was 0.12m*M* in total calcium (free and complexed). To simplify the experiments with bran, we assumed that no free calcium ion was released by bran. Such an assumption should seldom cause more than 1–2% error in the calculated results, because in most of the studies the initial concentration of $CaCl_2$ was relatively high (3.7m*M*).

Effect of Bran Concentration and Ca^{2+} Concentration on Binding

Figure 1 shows that when pH and initial Ca^{2+} concentration are constant, the extent of calcium binding by bran is proportional to bran concentration. The relationship between initial Ca^{2+} concentration and final Ca^{2+} concentration in systems of identical pH and bran content is shown in Fig. 2. The curve obtained by plotting percent calcium loss (bound) against initial calcium concentration is linear over the concentration range 0–3m*M* Ca^{2+}.

Almost all of the bound calcium is in the form of insoluble complexes. When 4 g of bran was stirred in 100 ml of 3.74m*M* $CaCl_2$ at pH 6.1, the final concentration of soluble Ca^{2+} (determined by the oxalate method) was 1.55m*M*. However, analysis by the TMM method showed the solution to be 1.42m*M* in free Ca^{2+}. The difference (1.55m*M* − 1.42m*M* = 0.13m*M*) equalled the concentration of Ca^{2+} in soluble complexed form. Thus, only about 6% of the soluble calcium in the final reaction mixture was in complex form.

Effect of pH on Binding

Perhaps because of its high protein content (14.3%), AACC bran behaves as a buffer. Addition of relatively large amounts of acid or base is required to produce moderate changes of pH in reaction mixtures. Immediately after adjustment of pH at the start of a

reaction, the pH can differ considerably from that observed 2 hr later. Therefore, all reported values for pH are from measurements made after centrifugation of reaction mixtures. Figure 3 shows the relationship between pH and calcium binding for a system prepared by adding appropriate amounts of KOH or HCl to 4 g of bran in 100 ml of 3.74m*M* $CaCl_2$. Over a pH range 5.5–8.3, binding increases with a decrease in pH. However, small or moderate changes in pH have only a slight effect on binding. At pH 7.36, 54% of the Ca^{2+} was bound; at pH 5.55, 67% was bound.

Binding by Cooked Bran and Acid-Treated Bran

Knowledge of the effect of cooking on metal binding is important, because bran ingested by humans is normally in a cooked form. Furthermore, ingested bran remains in the stomach (pH ~2) for several hours before passing into the small intestine, where stomach acid is neutralized by alkaline (bicarbonate) secretions. Experiments were therefore designed to provide information on the influence of acid treatment on binding by raw bran and cooked bran at neutral pH. Cooking consisted of heating a weighed amount of bran for 1 hr at 100°C in a measured volume of water; appropriate amounts of $CaCl_2$, KCl, and water were then added to complete the reaction mixture. Acid treatment consisted of adding sufficient HCl to a mixture of bran and water to make a pH of about 1.6 and then agitating the acidic mixture at 37°C for 2 hr before neutralizing with KOH. Table I summarizes the experimental conditions and results of these studies. Binding power is appreciably increased by cooking. Pretreatment with acid causes an even greater increase. However, the combined influence of cooking followed by acid treatment is essentially the same as acid treatment alone.

A separate experiment in which $CaCl_2$ was excluded showed that no significant amount of free Ca^{2+} is produced when bran is cooked. TMM analysis of the supernatant from a mixture of 4 g of cooked bran in 100 ml of water (ionic strength = 0.165 by addition of KCl) indicated only 0.04m*M* of free Ca^{2+}

The cause of the pronounced increase in calcium binding that occurs when bran is pretreated with acid deserves some speculative comment. Possibly, the treatment produces a larger number of phytate binding sites (phosphate groups) by making available many sites that were masked or occupied before acid treatment by other bran components, such as proteins. A similar argument explains the augmentation of binding power that occurs when pH is only moderately lowered (see Fig. 3).

Binding by Defatted Bran and Water-Washed Bran

AACC bran is 5.3% fat, as determined in our laboratory by extraction with 2:1 (v/v) $CHCl_3$-methanol. (AACC analysis by acid hydrolysis showed 5.2% fat.) Removal of this fat increases calcium-binding power, probably by increasing the number of available binding sites (Table II). Lipids probably act as a physical barrier that prevents metal cations from approaching some of the binding sites.

AACC bran also contains much water-soluble material of which at least one component, phytate, has strong cation-binding power. Thorough washing of a sample of bran with water at pH 6.0 and 37°C removes most of these water-soluble substances, which comprise about 13.9% (by weight) of unwashed bran.

Complete removal of water-soluble complexing agents cannot, however, be accomplished by the method normally used in our work. This method of removal (Table II, footnote d) involved treating a 4-g sample of bran three times with 100-ml portions of water. The appearance of small but significant amounts of complexing agent in the wash liquid after an additional two washes suggested either that the solubility of the water-extractable material is very low, thus hindering extraction, or that the degradation of an insoluble, unstable bran component results in continual production of a water-soluble complexing agent. The metal-binding ability of the wash liquid, or bran supernatant, is not

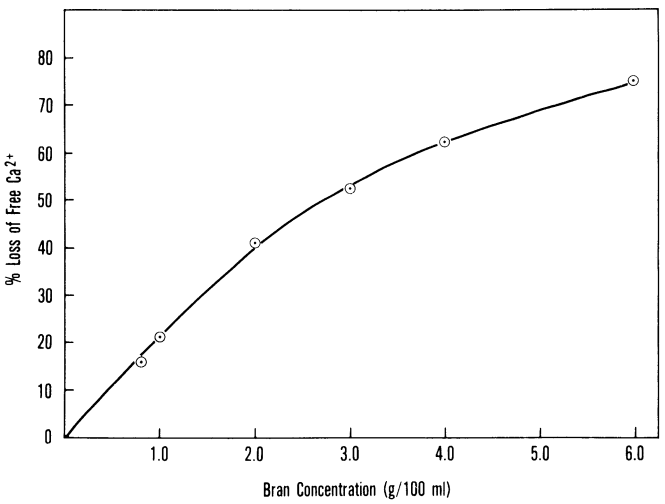

Fig. 1. Effect of bran concentration on loss of free Ca^{2+}. $[Ca^{2+}]_{initial}$ = 3.70m*M*; pH 6.1 (±0.1).

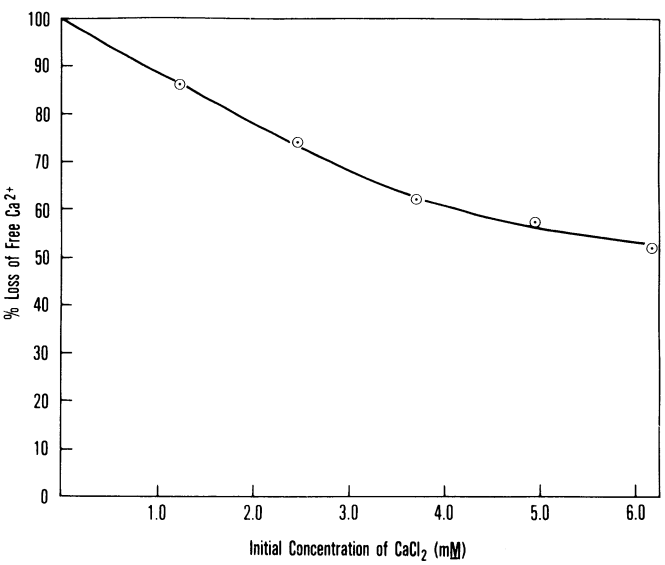

Fig. 2. Effect of initial Ca^{2+} concentration on loss of free Ca^{2+} in a bran-calcium chloride system. Bran concentration, 4 g/100 ml, pH 6.1.

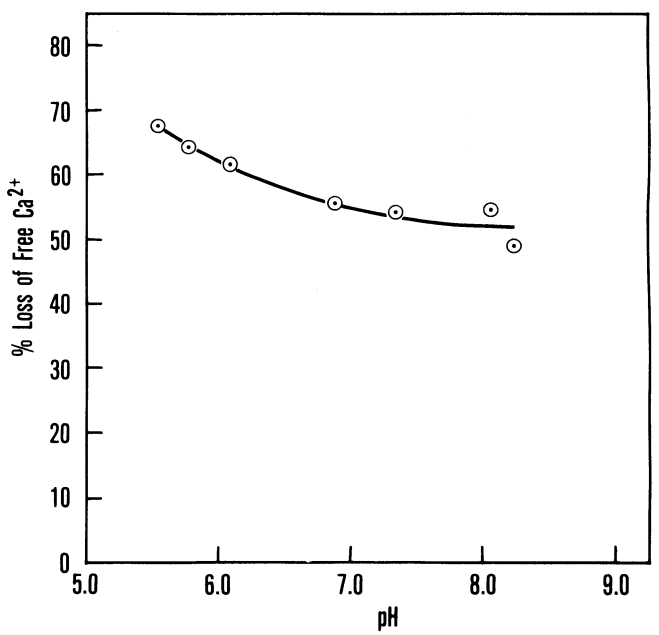

Fig. 3. Influence of pH on binding of Ca^{2+} by bran. $[Ca^{2+}]_{initial}$ = 3.74m*M*; bran concentration, 4 g/100 ml.

significantly influenced by rate of centrifugation. Little or no difference (2% or less) was found in calcium-binding ability between supernatants obtained by centrifugation at 2,000 rpm (810 × *g*) and those obtained at 20,000 rpm (40,000 × *g*).

At pH 6, washed bran (Table II) has slightly less than half the calcium-binding power of unwashed bran (Fig. 3 or Table I). As the pH is decreased to less than 6, the binding ability of washed bran increases, as does that of unwashed bran; lowering the pH influences the binding ability of the washed bran more than that of the unwashed bran. For example, at pH 5.65, washed bran (Table II) has almost as much binding ability as the unwashed bran under similar conditions (Fig. 3 or Table I). This augmentation of binding power with an increase in acidity is possibly caused by the formation of additional amounts of strong, soluble complexing agents. Evidence for this formation came from a study of aqueous bran extract prepared from bran that had been first washed with water in the usual fashion, then acid-treated at pH 1.6 for 2 hr, and finally neutralized with KOH. When 7.5m*M* $CaCl_2$ was added to an equal volume of this neutral extract (phosphorus content, 4.2m*M*), 30% of the Ca^{2+} was lost through binding at pH 6. Replacing the acid treatment with water treatment gave an extract of lower phosphorus content (2.3m*M*) and of lower binding ability (13% loss of Ca^{2+}). Thus, a low pH favors the production of water-soluble complexing agents.

Binding by Water-Soluble Components of Aqueous Bran Extract

Water extracts of AACC bran were prepared by centrifuging mixtures of bran and water (usually 8 g/100 ml) that had been shaken at 37°C for 3 hr. The pH of the resulting extract was approximately 7.0. When water-soluble material from 1, 2, and 4 g of bran was allowed to interact with Ca^{2+} in 100 ml of 3.7m*M* $CaCl_2$ solution, loss of free Ca^{2+} was 18, 28, and 44%, respectively. Over the pH range 6.0–8.0, binding power of bran extract is equal to or slightly less than that of whole bran at the same pH. But unlike whole bran, extract binds more strongly as pH increases (Fig. 4). Heating bran extract at 100°C for 1 hr at pH 6.6 has no detectable influence on binding power. Thus, the augmenting effect that cooking has on the binding ability of whole bran must arise from heat-induced alterations of the insoluble fraction of bran.

To characterize the water-soluble material responsible for calcium binding in bran extract, we prepared the calcium derivative in an amount adequate for quantitative study. An excess of $CaCl_2$

TABLE I
Calcium Binding by Cooked Bran and Acid-Treated Bran

Type of Bran	Weight (g) of Bran 100 ml of $CaCl_2$ in Solution	HCl or KOH[a] (mmols)	pH	[$CaCl_2$] Initial (m*M*)	[$CaCl_2$] Final (m*M*)	Loss (%)	Loss (mmols/g of bran)
Untreated	1	none	5.95	3.70	2.94	20.5	0.076
	2	none	6.01	3.70	2.20	40.5	0.075
	4	0.20 (HCl)	5.78	3.72	1.34	64.0	0.060
	4	none	6.10	3.70	1.42	61.6	0.057
	4	0.29 (KOH)	6.90	3.70	1.65	55.4	0.051
	4	0.47 (KOH)	8.07	3.70	1.68	54.6	0.051
Cooked[b]	4	none	6.00	3.70	1.02	72.4	0.067
Acid-treated[c] (pH ~1.6)	1	none	5.56	3.70	2.49	32.7	0.121
	2	none	5.75	3.70	1.43	61.4	0.114
	4	none	5.70	3.70	0.70	81.1	0.075
	4	none	6.05	3.70	0.53	85.7	0.079
	4	none	5.70	6.17	1.41	77.1	0.119
Cooked + acid-treated[d]	1	none	5.51	3.70	2.64	28.6	0.106
	2	none	5.73	3.70	1.58	57.3	0.106
	4	none	6.01	3.70	0.64	82.7	0.076

[a] Added to each 100 ml of solution for adjusting pH.
[b] Bran was heated in 20 ml of water at 100°C for 1 hr before appropriate amounts of $CaCl_2$, KCl, and additional water were added.
[c] pH ~1.6. Bran was stirred with 28 ml of 0.11*N* HCl for 2 hr and then neutralized with KOH to a pH of about 6.2 before appropriate amounts of $CaCl_2$, KCl, and water were added.
[d] Bran was first cooked and then acid treated and neutralized (as in footnotes b and c) before $CaCl_2$, KCl, and water were added.

TABLE II
Calcium Binding by Defatted Bran and Water-Washed Bran

Type of Bran	Weight (g) of Bran in 100 ml of $CaCl_2$ Solution[a]	HCl or KOH[b] (mmols)	pH	[$CaCl_2$] Initial (m*M*)	[$CaCl_2$] Final (m*M*)	Loss (%)	Loss (mmols/g of bran)
Defatted[c]	1	0.048 (KOH)	6.42	3.70	2.32	37.3	0.138
	2	0.091 (KOH)	6.45	3.70	1.54	58.4	0.108
	4	0.170 (KOH)	6.41	3.70	0.85	77.0	0.071
Water-washed[d]	4	0.160 (HCl)	5.65	3.70	1.72	53.5	0.050
	4	none	6.03	3.70	2.71	26.8	0.025
	4	0.160 (KOH)	6.91	3.70	2.80	24.3	0.022

[a] Weight of bran before solvent treatment to remove fat or water-soluble material.
[b] Added to each 100 ml of solution to adjust pH.
[c] Defatting was accomplished by agitating the bran in 200 ml of 2:1 (v/v) $CHCl_3$-methanol for 24 hr at room temperture followed by washing with the same solvent mixture and finally drying under vacuum at 25°C. The dry residue was then equilibrated at 50% rh (25°C) and subjected to reaction with Ca^{2+} by mixing with 3.70m*M* $CaCl_2$ ($\mu = 0.165$).
[d] Washing was accomplished by agitating the bran for 2 hr with 100 ml of water at 37°C, followed by washing twice with 100-ml portions of water and finally drying under vacuum at room temperature. The pH was approximately 6.0 during washing. The dry residue was equilibrated at 50% rh (25°C) before being subjected to reaction with Ca^{2+} for 2 hr at 37°C. A 4-g sample of whole bran yielded 3.44 g of water-insoluble residue. Moisture content of residue after equilibration was 12.1%.

(4.4 mmol) was added to 200 ml of extract obtained by centrifuging (2,000 rpm) a mixture of bran (32 g) and water (800 ml) that had been agitated for 3 hr at 37°C. The resulting grayish-white precipitate was isolated by centrifugation, washed with water, and allowed to dry at 25°C and 50% rh. Yield of powdery solid was 0.087 g. A reasonable assumption was that this precipitate represented 100% of the calcium complex (or mixture of complexes) formed in the above reaction, because the binding studies with bran at lower concentration of $CaCl_2$ indicated that very little, if any, soluble complex existed in the aqueous phase of the reaction mixture. Analysis of the precipitate showed the following amounts: water of hydration, 13.5%; phosphorus, 14.0%; nitrogen (Kjeldahl), 2.29%; *myo*-inositol, 12.9%; calcium, 16.6%; molar ratio of Ca to inositol, 5.8; and molar ratio of P to inositol, 6.3.

If all *myo*-inositol were derived from Ca_6phytate and all nitrogen were derived from protein, then a possible composition for the precipitate would be: Ca_5phytate, 63.9%; protein (5.7 × N), 13.1%; and water, 13.5% (total, 90.5%). Only 9.5% of the precipitate would be unaccounted for.

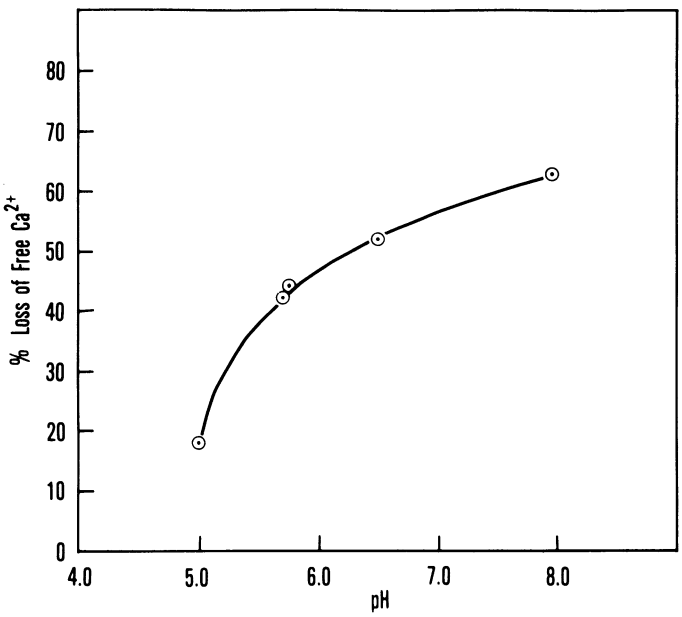

Fig. 4. Influence of pH on binding of Ca^{2+} by bran extract. Concentration of bran solubles is equivalent to that produced by 4 g of bran in 100 ml of water; $[Ca^{2+}]_{initial}$ = 3.74m*M*.

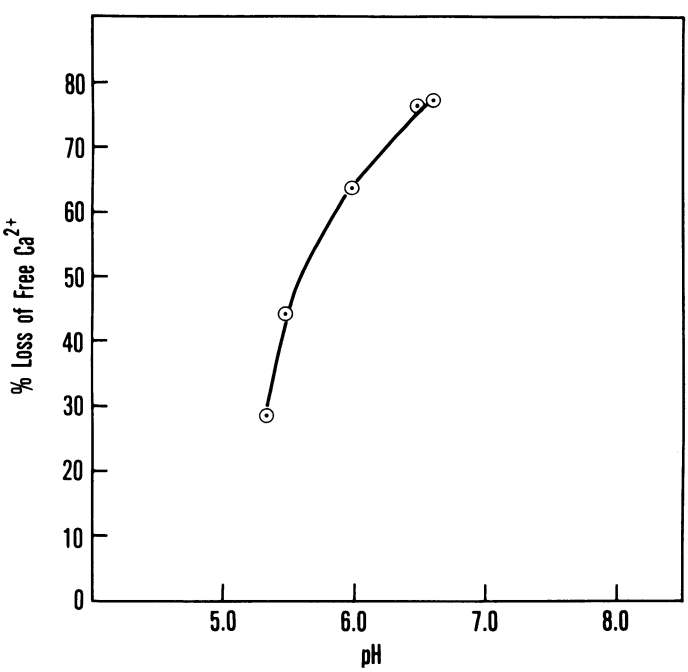

Fig. 5. Influence of pH on binding of Ca^{2+} by phytate ion. $[Ca^{2+}]_{initial}$ = 4.94m*M*; initial concentration of sodium phytate = 0.633m*M*.

Contribution of Individual Components of Bran to Calcium Binding

Phytate. AACC bran is reported to contain 3.36% phytate (calculated as phytic acid) and 1.04% P. If the phytate ion is assumed to be inositol hexaphosphate, then 91% of all the P in bran is phytate P. Only about 31% of the phytate in bran can be removed by water extraction at 37°C and pH 6.1, indicated by P analysis on the extract. A 4-g sample of bran in 100 ml of water at 37°C released 13.0 mg of soluble P into solution. However, when 3.74m*M* $CaCl_2$ (μ = 0.165) was substituted for the water, only 2.5 mg of soluble P was found in solution; calcium ion had removed 81% of the soluble P.

The pronounced effect of pH on binding ability of phytate ion is shown in Fig. 5. The concentration of sodium phytate (0.633m*M*) used in these experiments was the same as the concentration of phytate expected (on the basis of P analysis) in bran extract prepared from 4 g of bran and 100 ml of water. Curiously, the binding power of the extract is appreciably less than that of the sodium phytate solution at the same pH, which would indicate either that not all of the P in bran extract is phytate P or that not all of the phosphate groups on phytate anion in bran extract are available for binding (Figs. 4 and 5). At pH 6.6, the loss of free Ca^{2+} in the sodium phytate solution is 79%, whereas in bran extract at the same initial Ca^{2+} concentration, loss of free Ca^{2+} is approximately 58%.

Normally, when the molar ratio of Ca^{2+} to phytate ion in a reaction mixture is high (>>1) and the pH is close to neutral, the formation of a highly insoluble form of calcium-phytate complex is favored; only indeterminably small amounts of soluble calcium-phytate complex remain in solution. Conditions highly favorable to the formation of insoluble phytate are met when the molar ratio is 6:1 and the pH is 6.8. However, in systems in which a large excess of phytate binding sites is available (eg, when the molar ratio of Ca to phytate is equal to or less than one, an appreciable proportion of soluble calcium-phytate complex can form. For example, when equimolar amounts (0.5*M* each) of Ca^{2+} and phytate ion were allowed to interact at pH 6.8, precipitation occurred, and 96% of the initial free Ca^{2+} was lost through complexation with phytate. However, total calcium analysis of the supernatant showed that only 71% of the initial Ca^{2+} was lost through formation of an insoluble calcium-phytate complex. The difference (25%) was the loss of Ca^{2+} through formation of a soluble calcium-phytate complex, the structure of which was not determined. The molar ratio of calcium to phytate in the soluble complex is probably low, perhaps 1:1, as in soluble monoferric phytate.

Cellulose and Hemicellulose. Contrary to the findings of Reinhold et al (1975), whose data indicated that the calcium-binding power of cellulose is half that of an equal weight of bran and that cellulose must therefore be an important contributor to the binding power of bran, results from this study indicate extremely little interaction between cellulose and Ca^{2+} in the physiological pH range 5–8. The data in Table III indicate that cellulose does not contribute significantly to the binding ability of bran (cellulose content, ~9%). Included in Table III are the results of a binding study on a mixture of cellulose with several selected components of gastrointestinal fluid (albumin, glycine, hydrogen carbonate ion, and phosphate ion). The observed binding in this experiment is caused largely by interaction of Ca^{2+} with gastrointestinal components.

Hemicellulose, composed of sugar units and relatively few uronic acid residues, would be expected to behave like xylan and cellulose, and thus should have very little binding ability. The results of studies with xylan, cellulose, and hemicellulose illustrate the low ability of these polysaccharides to bind Ca^{2+} (Table III). These findings agree with those of Molloy and Richards (1971), who

found that hemicelluloses and cellulose isolated from the grass Yorkshire Fog have little ability to bind either Ca^{2+} or Mg^{2+}. Starch also has low binding power. Studies with pectin indicate that the presence of occasional carboxyl substituents in a basically neutral polymer such as xylan, cellulose, hemicellulose, or starch probably contributes little toward calcium binding.

Pectic Substances. Because of the unavailability of detailed structural information on bran pectin, the structure and degree of esterification of bran pectin was assumed to be the same as that of commercial citrus pectin (d.e. 61%). Table IV shows that citrus pectin binds Ca^{2+} only weakly. In some of these experiments, the weight of citrus pectin (0.12 g/100 ml) was equal to the weight of pectin constituent in 4 g of bran.

Unlike pectin, pure polygalacturonic acid has a relatively high affinity for Ca^{2+} (Table IV). A 0.12-g sample can remove almost half the calcium ions from 100 ml of 3.7m*M* $CaCl_2$. Over the pH range 4.4–7.0, change of pH has little effect on binding. Polygalacturonic acid, however, is not a natural constituent of bran. Its investigation was carried out only for comparative purposes.

Starch. Although it comprises 17.4% of AACC bran, starch makes no detectable contribution (1% or less) to calcium binding. Table V contains the results of studies on corn amylose, corn amylopectin, several potato starches, and one wheat starch. High concentrations of starch (3 g/100 ml) were used because of the low binding capacity of starch. All samples were uncooked.

Neutral Sugars. Soluble saccharides comprise a major part (7%) of bran. However, experiments with D-glucose as the representative sugar showed no significant calcium binding in 3.7m*M* $CaCl_2$ at pH 6.2 (Table VI). The apparent gain of Ca^{2+} in one system containing a high concentration of D-glucose (11 g/100 ml) was probably caused by an increase in calcium-ion activity caused by considerably fewer water molecules per cation.

Proteins. For calcium-binding study, glutenin (from hard red winter wheat) and bovine serum albumin were chosen to represent typical water-insoluble and water-soluble bran proteins, respectively. However, serum albumin might not be strictly representative, because the amino acid profile of water-soluble bran proteins may differ significantly from that of serum albumin. Neither the glutenin nor the albumin exhibited significant metal-binding activity. A 0.6-g sample of glutenin in 100 ml of 3.7m*M* $CaCl_2$ bound an apparent 0.5% of the Ca^{2+} ions at pH 5.3 and 1.6% at pH 5.9. Under identical conditions of concentration, albumin bound an apparent 2.7% of the Ca^{2+} ions at pH 6.0. Thus, although proteins comprise 14% of bran, these data indicate that proteins as bran constituents make no important contribution to calcium binding. However, bran proteins may contribute by joining with phytate in forming a Ca^{2+}-protein-phytate complex, the stability of

TABLE III
Calcium Binding by Cellulose, Hemicellulose, and Xylan

Substrate	Weight (g) of Substrate in 100 ml of $CaCl_2$ Solution	HCl or KOH[a] (mmols)	pH	$[CaCl_2]$ Initial (m*M*)	$[CaCl_2]$ Final (m*M*)	$[CaCl_2]$ Loss (%)
Cellulose						
Avicel	3	0.012 (KOH)	5.21	3.70	3.68	0.5
	3	0.018 (KOH)	5.77	3.69	3.69	0.0
	3	0.036 (KOH)	6.81	3.69	3.55	3.8
	3	0.039 (KOH)	7.96	3.69	3.39	8.1
Whatman	3	0.012 (KOH)	6.70	3.69	3.47	6.0
Mixture		0.88 (HCl)	7.30	3.70	3.52	4.9
Whatman cellulose	1.2					
Albumin	0.0176					
K_2HPO_4	0.0452					
Glycine	0.038					
$KHCO_3$	0.1652					
Hemicellulose[b]	1	0.012 (KOH)	6.44	3.74	3.67	1.9
Xylan[c]	1	0.29 (KOH)	6.10	3.71	3.56	4.0

[a] Added to each 100 ml of solution for the purpose of adjusting pH.
[b] Combined water-soluble and water-insoluble hemicellulose from AACC wheat bran. Water of hydration, 11.8%; Kjeldahl N, 0.32%.
[c] Commercial sample (ICN Pharmaceuticals) isolated from wood gum; treated with 0.15*N* HCl to remove mineral matter. Moisture, 12.6%; Kjeldahl N, 0%.

TABLE IV
Binding of Calcium by Pectic Substances[a]

Substance	Weight (g) of Substance in 100 ml of $CaCl_2$ Solution	KOH[b] (mmols)	pH	$[CaCl_2]$ Initial (m*M*)	$[CaCl_2]$ Final (m*M*)	$[CaCl_2]$ Loss (%)
Pectin[c]	0.136	0.11	6.33	3.69	3.47	6.0
	0.136	0.37	7.50	3.67	3.38	7.9
	0.272	0.26	6.77	3.72	3.30	11.3
Acid-treated pectin[d]	0.136	0.37	7.50	3.67	3.38	7.9
Polygalacturonic acid (PGA)[e]	0.120	none	4.38	3.70	2.11	43.0
	0.120	0.59	6.21	3.65	1.95	46.6
	0.120	0.62	6.85	3.65	1.88	48.5
	0.240	1.17	6.90	3.59	0.73	79.7
Acid-treated PGA[d]	0.120	0.65	7.00	3.64	1.95	46.4

[a] Reaction times were 3 hr except for the acid-treated substances, which were given 5 hr.
[b] Added to each 100 ml of solution for the purpose of adjusting pH.
[c] Commercial sample of citrus pectin (degree of esterification 61%) that was obtained from Sigma Chemical Co. and had the following composition: 76% galacturonic acid, 7.4% methoxy, and 12% water.
[d] Acid treatment consisted of stirring a weighed sample with 14 ml of 0.112*N* HCl for 2 hr at 37° C and then neutralizing with an equivalent amount of KOH. For the binding study, appropriate amounts of $CaCl_2$, KCl, and additional water were then added.
[e] Commercial sample (98% pure) from Sigma Chemical Co. A 0.12-g sample contains 0.68 mmol of carboxyl groups.

which might, under certain pH conditions, be greater than that of a calcium-phytate complex (Saio et al 1967).

Contribution of Gastrointestinal Constituents to Binding

A reasonable assumption is that metal-binding components of gastrointestinal secretions can affect the availability of polyvalent cations in the human intestine. Depending on their concentration and metal-binding ability, these components compete with metal-binding constituents of bran or any other food for the metals in the diet. Conceivably, any gastrointestinal substance that can form a metal complex that is both relatively stable and soluble has a good chance of enhancing the bioavailability of that metal. The bioavailability of certain metals is highly stable, but the soluble complex form is considerably greater than that of the same metals in insoluble form. For example, iron in soluble monoferric phytate is much more available to rats than is iron in insoluble ferric$_{3-4}$phytate (Morris and Ellis 1976); and zinc in its soluble ethylenediaminetetraacetate complex is much more available to turkey poults than is zinc in $Zn_3(PO_4)_2$ (Vohra and Kratzer 1966). On the other hand, any secretory substance that forms either an insoluble metal complex or an insoluble metal salt would probably lower the bioavailability of that particular metal.

Table VI contains the results of calcium-binding studies of some of the major constituents of gastrointestinal fluid. In many of these experiments, the concentration of constituent was chosen to approximate the expected concentration in the human small intestine (Altman and Dittmer 1968). Glycine was chosen for study among the numerous amino acids present in the gut, because it has binding properties typical of most of the amino acids. Little binding occurred by any of the substances listed in Table VI. In most instances, the small losses of calcium were probably only apparent losses caused mainly by experimental error. With the possible

TABLE V
Binding of Calcium by Starch

Type of Starch[a]	Weight (g) of Starch in 100 ml of $CaCl_2$ Solution[b]	KOH[c] (mmols)	pH	$[CaCl_2]$ Initial (m*M*)	$[CaCl_2]$ Final (m*M*)	$[CaCl_2]$ Change (%)
Potato						
Variety A (14.9% H_2O)	3	0.017	6.33	3.71	3.73	+0.8
B (10.0% H_2O)	3	0.019	6.20	3.77	3.57	−5.3
C (16.8% H_2O)	3	0.010	6.00	3.72	3.81	+2.4
Corn						
Amylose (6.1% H_2O)[d]	3	0.0072	6.70	3.85	3.90	+1.3
Amylopectin (10.4% H_2O)	3	0.0053	6.36	4.16	4.01	−3.6
Wheat (12.2% H_2O)	3	none	5.75	3.75	3.82	+1.9
	3	0.062	8.40	3.74	3.68	−1.6

[a] The percent moisture in each starch is shown in parentheses. Nitrogen content ≥0.01%.
[b] Total initial Ca^{2+} includes the small amount of free Ca^{2+} contributed by impurities in the starch sample. The amount of Ca^{2+} contributed by each sample was determined by separate experiment with tetramethylmurexide as metal indicator.
[c] Added to each 100 ml of solution for the purpose of adjusting pH.
[d] Amylose fraction is 0.82 of total starch.

TABLE VI
Binding of Calcium by Constituents of Gastrointestinal Fluid

Constituent	Concentration of Constituent (g/100 ml)	Concentration of Constituent (m*M*)	pH	$[CaCl_2]$ Initial (m*M*)	$[CaCl_2]$ Final (m*M*)	$[CaCl_2]$ Change (%)
Saliva, human	(2.0 ml)	···	6.78	3.63	3.58	−1.4
D-Glucose	0.40	22	6.18	3.70	3.59	−3.0
	11.0	610	5.70	3.70	3.90	+5.4
K_2HPO_4[a]	0.035[b]	2.0	6.83	3.72	3.49	−6.2
$KHCO_3$[c]	0.165[b]	16.5	7.50	3.70	3.48	−5.9
Albumin, serum	0.0452[b]	···	5.80	3.74	3.68	−1.6
	0.6000	···	6.03	3.74	3.64	−2.7
Glycine	0.0380[d]	5.06	6.54	3.70	3.60	−2.7
Mucin, gastric	0.272[b]	···	4.40	3.92[e]	3.80	−3.1
	0.272	···	6.72	3.92[e]	4.05	−3.3
Mixture			6.90	3.70	3.47	−6.2
Albumin	0.452[b]	···				
Glycine	0.380[d]	5.06				
K_2HPO_4	0.0176[b]	1.00				
$KHCO_3$	0.1650[b]	16.50				
Bile acid salts						
Na glycocholate	0.390	8.00	6.35	3.74	3.66	−2.1
Na glycodeoxy-cholate	0.390	8.30	5.99	3.74	3.80	+1.6

[a] The order of mixing the components of the reaction mixture is important: first, a weighed sample of K_2HPO_4 is dissolved in 50 ml of water, then an appropriate volume of 0.4 *N* HCl is added to lower the pH into the range of 6.0–6.5, and finally, 50 ml of 7.4m*M* $CaCl_2$ is added. KOH is subsequently introduced to raise the pH to the desired level. In an aqueous solution of K_2HPO_4, the predominant species of phosphate ion are $H_2PO_4^-$ and HPO_4^{-2}. The molar ratio of $H_2PO_4^-/HPO_4^{-2}$ is 2.1 at pH 6.8.
[b] This amount approximates that present in 100 ml of human gastrointestinal fluid (Altman and Dittmer 1968).
[c] In an aqueous solution of $KHCO_3$, the species HCO_3^- and H_2CO_3 are in equilibrium with each other. At pH 6.9 and 7.5, the ratio of HCO_3^-/H_2CO_3 is 3.8 and 15.6, respectively.
[d] This weight of glycine equals approximately the average weight of total amino acid in 100 ml of human gastrointestinal fluid (Altman and Dittmer 1968).
[e] The initial concentration has been corrected for the small amount of free Ca^{2+} introduced as an impurity in the mucin sample. Mucin contributed 4.6% of the total calcium in the reaction mixture.

exception of the loss incurred in the mixture study, the calculated losses fell too close to the limits of experimental error to be trustworthy reflections of calcium binding. One might conclude, therefore, that the gastrointestinal constituents listed in Table VI bind Ca^{2+} too weakly to have more than a minor effect on bioavailability.

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