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Effect of Iron(III) Chitosan Intake on the Reduction of Serum Phosphorus in Rats

JOSEPH BAXTER, FUKI SHIMIZU, YASUYUKI TAKIGUCHI, MASAHIRO WADA* AND TATSUAKI YAMAGUCHI

*Chiba Institute of Technology, Department of Industrial Chemistry, 2-17-1 Tsudanuma, Narashino-shi, Chiba 275 and *Department of Nutrition, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156, Japan*

Abstract

Because of the widespread use of aluminium- and calcium-containing phosphate binders for the control of hyperphosphataemia in patients with end-stage renal failure, an iron(III) chitosan complex was synthesised and fed to rats to measure its effect on serum phosphorus and calcium, intestinal phosphate binding and phosphate absorption.

Thirty-six Wistar rats were randomly selected and distributed into a baseline group (n = 6), a control group (n = 8 (days 0–15), n = 8 (days 16–30)) and a treatment group (n = 8 (days 0–15), n = 8 (days 16–30)). The control groups ingested AIN-76 diet mix with a 1% w/w fibre content; however, the treatment groups had the fibre content completely substituted with iron(III) chitosan. The mean weights of the treated rats were slightly lower from 15 days (not significant); but overall, rat growth was not stunted in the treatment groups. The serum phosphorus levels of the treated group (n = 8) were significantly reduced after 15 days ($P = 0{\cdot}004$; control: $5{\cdot}7 \pm 0{\cdot}9\,mg\,dL^{-1}$; treatment: $4{\cdot}4 \pm 0{\cdot}5\,mg\,dL^{-1}$; 95% CI of difference: 0·5–2·2) and 30 days ($P = 0{\cdot}002$; control: $5{\cdot}5 \pm 0{\cdot}9\,mg\,dL^{-1}$; treatment = $4{\cdot}1 \pm 0{\cdot}6\,mg\,dL^{-1}$; 95% CI of difference: 0·6–2·3) as compared with the respective control group. The serum calcium-phosphorus product was $62{\cdot}0 \pm 12{\cdot}1\,mg^2\,dL^{-2}$ for the control and $45{\cdot}1 \pm 6{\cdot}6\,mg^2\,dL^{-2}$ for the treatment group after 30 days ($P = 0{\cdot}004$). The serum iron concentration of the treatment group did not differ from the baseline value after 15 and 30 days, but the treatment group was significantly higher than the control group ($P < 0{\cdot}05$) after 30 days. The faeces phosphorus levels ($mg\,day^{-1}$) were higher ($P < 0{\cdot}01$) and its iron content was much higher ($P < 0{\cdot}01$) for the treated group. The urine phosphorus ($mg\,kg^{-1}$) was not significantly reduced for the treated group, but the mean was consistently less. The kidney and liver weights of both groups were similar, but the phosphorus content of the kidney ($mg\,(g\,kidney)^{-1}$) was higher for the treated group after 30 days ($P = 0{\cdot}041$; control, $4{\cdot}2 \pm 1{\cdot}2\,mg\,g^{-1}$ vs treatment, $5{\cdot}6 \pm 1{\cdot}4\,mg\,g^{-1}$. Because iron(III) chitosan had a high phosphorus-binding capacity of 308 (mg P) per gram of Fe^{3+} for both the in-vitro (pH 7·5) and in-vivo studies, which is greater than nearly all commonly used phosphate binders, and a small net phosphorus absorption difference of $3{\cdot}7\,mg\,day^{-1}$, it is an efficient phosphate binder for lowering serum phosphate levels without increasing serum calcium levels.

Hyperphosphataemia, due to chronic renal failure, is associated with secondary hyperparathyroidism, osteodystrophy and metastatic calcification (Goodman 1985; Alfrey & Zhu 1991). Normal treatment regimes for control of chronic hyperphosphataemia require control of dietary phosphorus intake, use of phosphate binders to help control intestinal phosphate (P_i, inorganic phosphate) absorption, haemodialysis or CAPD to reduce serum phosphate levels below $1{\cdot}8\,mmol\,L^{-1}$ ($5{\cdot}4\,mg\,P_i\,dL^{-1}$) to within normal levels of $0{\cdot}6–1{\cdot}3\,mmol\,L^{-1}$ (Coburn & Salusky 1989). In addition to preventing dietary phosphate absorption, intestinal phosphate binders disrupt the reabsorption of endogenous phosphate, which is excreted normally via the parotid glands and pancreatic fluid.

Correspondence: T. Yamaguchi, Chiba Institute of Technology, Department of Industrial Chemistry, 2-17-1 Tsudanuma, Narashino-shi, Chiba 275, Japan.
E-Mail: cityamag@pf.it-chiba.ac.jp

For prevention of hyperphosphataemia in patients undergoing chronic dialysis, aluminium hydroxide gel and various aluminium-containing antacids have been used because of their excellent phosphate-binding ability (Spencer et al 1982). Unfortunately, long-term use of aluminium compounds results in serious aluminium toxicity (Swartz et al 1987). Calcium-containing compounds such as calcium carbonate are also excellent in controlling the level of serum phosphorus, but calcium accumulation often leads to hypercalcaemia with possible side effects including soft-tissue calcification, hypercalcaemic nephropathy, metabolic alkalosis, polyuria and constipation (Gonella et al 1985; Beall & Scofield 1995). In our previous papers (Jing & Yamaguchi 1992; Yoshimoto et al 1995a, b; Yamaguchi et al 1999), we have shown that iron-containing complexes are effective phosphate binders in-vitro and in-vivo in rats.

Chitosan, which is chemically similar to cellulose, is a non-toxic natural polysaccharide of β-1,4-D-glucosamine residues obtained from the deacetylation of chitin. Its uniqueness is attributed to its cationic polymer-containing amino group, which greatly differs from other dietary fibres. Its adsorption of acid metabolites such as uric acid and bile acid (Jing & Yamaguchi 1992; Yoshimoto et al 1995a, b) and its hypocholesterolaemic effect in humans (Sugano et al 1978; Maezaki et al 1993; Jing et al 1997) have been its most notable properties. Furthermore, chitosan-coated dialdehyde cellulose in acute bilaterally nephrectomized rats (Nagano et al 1995a), chronic renal failure (CRF) rats induced by adriamycin (Nagano et al 1995b) and normal rats (Yoshimoto et al 1995c), showed marked prolongation of the survival period and improved pharmacological properties.

Incorporating the strengths of iron and chitosan compounds, iron(III) chitosan was synthesised to primarily study its phosphate-binding capacity in rats, as noted by the effluent phosphorus and serum phosphorus fluxes. Secondary studies involved observing the effect the amino nitrogen in chitosan exerts on cholesterol lowering by suppressing its reactivity through binding to iron(III).

Materials and Methods

Materials

Iron(III) chitosan was synthesised from reagent-grade iron(III) sulphate hydrate, $[Fe_2(SO_4)_3]\cdot nH_2O$ (60–80% purity, Kanto Kagaku Co.), and chitosan 10B with an average molecular weight of 200 000 Daltons (Funakoshi Co.). Potassium dihydrogen phosphate (KH_2PO_4, Wako Pure Chemical Industries) and Tris buffer (Wako, 99%) were used for all phosphate adsorption studies. For spectral characterisations, 1,10-phenanthroline monohydrate (Wako, assay 99%) and hexaammonium heptamolybdate tetrahydrate (Wako) were used. Other chemicals were of spectroscopic grade and purchased from commercial sources.

Analysis

At pH 2 and pH 7·5, iron dissolution of the iron(III) chitosan complex and the iron concentration in rat faeces were measured by the 1,10-phenanthroline spectrophotometric method at 510 nm according to Japan Industrial Standard (JIS), as previously reported (Jing & Yamaguchi 1992). Also, the phosphate concentrations were determined spectrophotometrically using a standard molybdate assay (Taussky & Shorr 1953). The FT-IR spectrum of the iron(III) chitosan complex was measured on a JASCO 400 spectrometer using KBr pellets. Mössbauer spectra were measured on an Austin Science controller (S-600) with a Norland analyser (5500) at room temperature. Both the chitosan (10 B) and iron(III) chitosan (10 B) were analysed by thermal gravimetric analysis and differential thermal analysis for water content and characteristic iron patterns.

Preparation

Chitosan (1 g) was added to 40 mL of deionized water with 9 mL of acetic acid (1·0 M acetic acid) followed by addition of sodium acetate to make a pH 6 solution. Deionized water was added to bring the solution to 100 mL followed by addition of iron(III) sulphate hydrate (3·00 g) and stirring for 24 h. The colloidal suspension was filtered, washed three times with deionized water and twice with ethanol, and dried under reduced pressure for 24 h to give iron(III) chitosan in near quantitative yield.

In-vitro phosphate adsorption studies

Into 500 mL of deionized water, 71·62 mg of desiccated KH_2PO_4 (16·31 mg of elemental phosphorus) was dissolved to afford a phosphate concentration of 1·05 mmol L^{-1} (3·26 mg P_i dL^{-1}). The phosphate binder (10·0 mg) was added to a quantity of phosphate solution at pH 2·0 or pH 7·4 to give a final volume of 20 mL. After addition of binder, the phosphate solution was adjusted back to either pH 2 or pH 7·4 with HCl (0·1 M), NaOH (0·1 M), or Tris (0·1 M, pH 7·4). The mixture was stirred with a magnetic stirrer at 100 rev min^{-1} for 2 min. The

solutions were kept covered in a shaker bath (20 cycles per min) at 37°C, for a 180-min adsorption period to reach maximum binding, and adjusted again to the appropriate pH. The suspensions were centrifuged at 10 000 *g* for 30 min and the supernatants were filtered (0·2-μm, Milipore Corp., Medford, MA). Spectrophotometric controls were first measured before measuring the phosphate and iron concentrations of the reaction solutions. The decrease in phosphate concentration from the original concentration in the standard phosphate solution to that of the filtrate represents the amount of bound phosphate.

Phosphate-binding effect of iron(III) chitosan in normal rats

White male Wistar rats (n = 38, Clea Japan Co.), ~6 weeks old, 175·5 ± 5·6 g, were randomised in a placebo-controlled, double-blind, parallel-design trial that was divided into a control group (n = 16), a treatment group (n = 16) and a baseline group (n = 6). The control group had free access to tap water and were fed powdered rodent chow (AIN-76 rodent diet, Clea Japan Co.; ~20–25 g day^{-1}), containing 0·40% phosphorus, 0·52% calcium, 100 000 int. units vitamin D_3, 0·0035% iron and 1·0% fibre. The treatment group received the same diet, but the 1% fibre was substituted with 1·0% iron(III) chitosan, providing 0·054% iron by weight of diet. All rats were housed in individual metabolic cages (24 cm × 20 cm × 15 cm) with a food container attached to the outside so that the rat was unable to bring food into the cage, to prevent contamination of urine and stool. The air-conditioned breeding room (22 ± 2°C, ~60% humidity) had a fixed 12-h artificial light–dark cycle (0700–1900 h).

Samples of urine and stool were collected from each rat on days 2, 5, 7, 9, 11, 13, 15, 16, 18, 20, 22, 23, 25, 28 and 30 at the same time (1000 h). The collected sample was the total excreted since the previous collection. For example, if a sample was collected on day 5, this sample represented the total of day 3, 4 and 5; therefore, all of the calculated values were divided by three and expressed as the average per day. After collection, the urine samples were stored at −30°C until analysed. The faeces were stored at room temperature until analysed. The rats' weight, diet intake amount, wet and dry faeces weight and urine volume were measured.

Faeces and urine analysis

All phosphate determinations were measured and expressed as phosphorus. The stools (0·5 g) were heated at 115°C for 5 h and ashed at 550°C for 5 h. The phosphorus was extracted from the ash for 24 h with 15 mL of 10% HCl, filtered, diluted with distilled water and followed by reaction with 1,10-phenanthroline to measure the iron concentration at 510 nm.

The urine vials were defrosted with water and 0·5 mL of urine was transferred to each test tube, followed by addition of 4·5mL of 5% trichloroacetic acid to degrade the protein. This solution stood for 10 min before being centrifuged at 3000 rev min^{-1} at 5°C for 15 min. Phosphate concentrations were determined spectrophotometrically using a standard molybdate assay (Taussky & Shorr 1953).

Serum, kidney and liver measurements

At the beginning of the experiment six rats were randomly selected, killed and anaesthetised with sodium pentobarbital (50 mg kg^{-1}). At the end of the study period, blood (1·5 mL) was obtained from the aorta of non-fasted and anaesthetised rats in the morning (1000–1100 h). Blood was collected, by cardiac puncture, in heparinized plastic tubes. The kidneys and livers were removed, rinsed in normal saline solution and weighed. They were stored at −30°C until further analysis. This was repeated on days 15 and 30 at the same time. After the experiment, 1·0 g each of liver and kidney were heated at 115°C for 5 h to remove water, and ashed at 550°C for 5 h. The phosphorus was extracted from the ash for 24 h with 15 mL of 10% HCl, filtered and diluted with distilled water. Phosphorus was assayed using a standard molybdate assay (Taussky & Shorr 1953).

The sera of the rats were analysed for phosphorus, calcium, iron, glucose, total protein, adenine/guanine ratio (A/G), creatinine, BUN, uric acid, total cholesterol, triglycerides, GOT, ALP and phospholipids at the commencement of the experiment (day 0), day 15 (n = 8 from each group) and day 30 (n = 8 from each group). The serum was sent to a clinical laboratory (Hoken Kagaku Kenkyujyo) for measurement of the above parameters beside the phosphate. The blood was treated in the same way as the urine method and the phosphorus was assayed using a standard molybdate assay (Taussky & Shorr 1953).

Statistical analysis

Groups of data were first tested for normality by Levene's test, Shapiro-Wilk, Kolmogorov-Smirnov and detrended normal probability plots. When the data differed significantly from normal it was

characterised by the median and interquartile limits (25%, 75%), otherwise continuous variables are expressed as the mean ± s.d., including 95% confidence intervals for primary results. If assumptions of normality were met, the data was analysed by the independent (unpaired) two-tailed Student's *t*-test or analysis of variance with $\alpha < 0{\cdot}05$ considered significant. For nonparametric data, the Mann-Whitney *U*-test was used. The effect of iron(III) chitosan on serum phosphorus and other laboratory values was assessed by comparing the difference in serum values with those in control rats on the same experimental day. For the calculation of the Pearson's product-moment correlation the assumptions of a bivariate normal distribution were verified, otherwise, the Spearman's rank-order correlation was used. The assumptions were verified for the simple-linear regression analysis, which was validated, with $\alpha < 0{\cdot}01$ considered significant. Statistical analyses were conducted using the SPSS 9.0 software package (Chicago, IL).

Results

Iron(III) chitosan analysis

The iron content of iron (III) chitosan, was 5·1% w/w as determined by elemental analysis and a water content of 19·23% w/w as determined by TG (thermo-gravimetric analysis, not shown). Therefore, with a composition of 1% w/w of iron(III) chitosan in the diet mix the resulting iron content is only 0·054%, which includes 0·003% w/w of iron initially in the mix. Chitosan had a much lower water content of 7%, but iron (II) chitosan (synthesis not reported) had the highest water content of 23%.

The FT-IR (not shown) of iron(III) chitosan indicated a loss of the amine (NH_2) of the polyglucosamine compound with a decrease of its band at $1590\,cm^{-1}$ together with a decrease of the amide band ($1650\,cm^{-1} \rightarrow 1629\,cm^{-1}$) and protonated amine bands ($1560\,cm^{-1} \rightarrow 1530\,cm^{-1}$). The Mössbauer spectra (not shown) of the iron(III) chitosan complex is characterised by a symmetrical and broad quadrupole doublet with an isomer shift (IS) value of $0{\cdot}35\,mm\,sec^{-1}$ and a quadrupole splitting (QS) value of $0{\cdot}69\,mm\,s^{-1}$.

Phosphate adsorption (in-vivo and in-vitro) and iron dissolution

The in-vitro phosphate adsorption or phosphate binding capacity (mg (g adsorbent)$^{-1}$) of iron(III) chitosan at pH 2 and pH 7·5 were nearly identical, $17{\cdot}0\,mg\,g^{-1}$ ($333\,mg\,(g\,Fe^{3+})^{-1}$) and $15{\cdot}6\,mg\,g^{-1}$ ($308\,mg\,(g\,Fe^{3+})^{-1}$), respectively (Table 1). The in-vivo values were the same as the in-vitro values at pH 2, indicating consistent results. Adsorption was immediate and the adsorption values fluctuated only $5\,mg\,g^{-1}$ over a period of 500 min, whereas the other binders listed in Table 1 usually require a longer time to reach equilibrium.

Table 1. Comparison of phosphate binders on phosphorus absorption, and their phosphorus binding capacity under in-vivo and in-vitro conditions.

Compound	Average net P absorption vs control diet	Phosphorus binding capacity			
		In-vivo		In-vitro	
	(mg day^{-1})	(mg (g compound)$^{-1}$)	(mg (g Fe^{3+}, Ca^{2+} or Al^{3+})$^{-1}$)	(mg (g compound)$^{-1}$)	(mg (g Fe^{3+}, Ca^{2+} or Al^{3+})$^{-1}$)
Iron(III) chitosan	3·7	16·6	308	17·0 (pH 2) 15·7 (pH 7·5)	333 (pH 2) 308 (pH 7·4)
Iron(III) hydroxide[a]	NA	NA	NA	58 (pH 2) 21 (pH 7·4)	152 (pH 2) 56 (pH 7·4)
Ferric citrate[b]	18·6	19·1	84·4	NA	NA
Ferric chloride[b]	33·0	36·9	181·2	NA	NA
Iron(III) dextran[c]	NA	20	112	23 (pH 2–3) 16 (pH 5)	126 (pH 2–3) 89 (pH 5)
Calcium acetate	174	27	103 ± 23	5 (pH 4) 55 (pH 7)	21 (pH 4) 213 (pH 213 (pH 7)
Calcium carbonate	112	17	43 ± 39	68 (pH 5) 16 (pH 7)	170 (pH 5) 42 (pH 7)
Aluminium chloride[e]	NA	NA	NA	93 (pH 5–7)	460 (pH 5–7)

The in-vitro experiments are not under the same conditions of phosphate concentration, binder concentration, pH and time (1–4 h); therefore, only a general approximation for comparing different compounds should be made. NA, Information not available from the report. [a]Yamaguchi et al (1999); [b]Hsu et al (1999); [c]Spengler et al (1996); [e]Sheikh et al (1989). Note: The average net P absorption is for patients.

The exact preparation of the iron(III) chitosan is very important in achieving the highest possible phosphate adsorption and iron content. For example, if iron(III) sulphate is added at pH 5, instead of the preferred pH of 6, the resulting phosphate adsorption of this iron(III) chitosan is only $10\,mg\,g^{-1}$ at pH 7·5, even though the iron content increases slightly to $53\,mg\,g^{-1}$. At pH 4, the iron content is reduced to $6\,mg\,g^{-1}$ with a phosphate adsorption of $8\,mg\,g^{-1}$ at pH 7·5.

Iron(III) chitosan was robust to acid conditions whereas under similar conditions chitosan was completely dissolved. Iron(III) chitosan at pH 2 released 5% by weight of iron into the solution after 300 min while at pH 7·5 no iron was detected. As reported in Jing & Yamaguchi (1992), iron (II) chitosan dissolved six times more readily than iron(III) chitosan at pH 2.

In-vivo experiment

There were no significant differences among the rats at the commencement of the study. The body-weights were similar and remained so throughout the study as shown in Figure 1. The body-weights of the iron(III) chitosan-treated rats were consistently less than those of the control group after 20 days, but the differences were not significant. As shown in Figure 2, the average food intake of the treatment group was slightly less than the control group after 16 days, especially on days 18 ($P<0·01$), 20 ($P<0·05$) and 23 ($P<0·05$). The 95% confidence intervals (95% CI) for the treatment group were very broad from the eighteenth day.

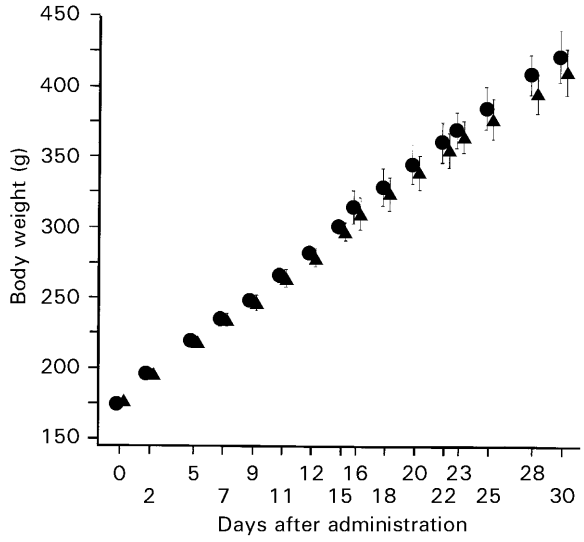

Figure 1. The effect of iron(III) chitosan (▲) at 1% of food weight on body-weight of rats compared with a control group (●), which received AIN-76 rat feed with a 1% fibre content. Values are means ± confidence intervals (95%) of 16 observations in each group until day 15, and afterwards there are 8 observations in each group until day 30.

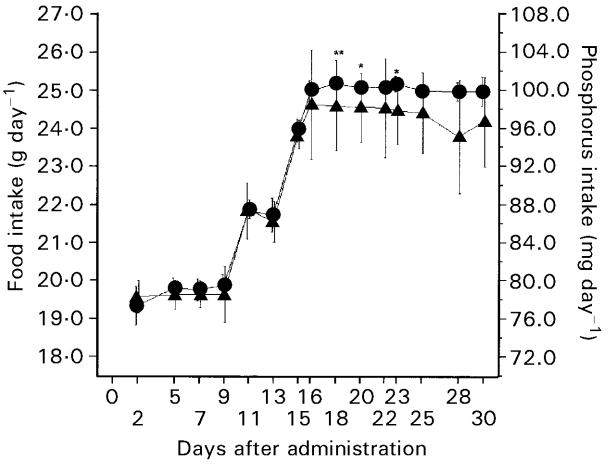

Figure 2. The effect of iron(III) chitosan (▲) at 1% of food weight on food and phosphorus intake of rats compared with a placebo group (●). Values are means ± confidence intervals (95%) of 16 observations in each group until day 15, and afterwards there are 8 observations in each group. $*P<0·05$, $**P<0·01$, compared with control group.

The amount of iron ingested and excreted, faeces weight, faeces phosphorus, urine phosphorus and urine volume are summarised in Table 2. The iron values of the control and treated groups differed greatly ($P=0·004$). Less than 30% of the iron ingested was excreted. However, the serum iron levels for the treatment group (Table 4) were not significantly different from baseline values, even though the treatment group was $116\,\mu g\,dL^{-1}$ higher after 15 days (not significant) and $86\,\mu g\,dL^{-1}$ higher ($P<0·05$) after 30 days. The mean faeces weight during the first 15 days of treatment did not significantly differ ($P=0·056$) as shown in Table 2, but the treatment group was 11% higher than the control. During the second period the mean faeces weights were nearly identical ($P=0·899$). The faeces phosphorus was consistently higher for the treated group throughout the study and was even more pronounced during the second period ($P=0·001$). The urine volume of the treated group was consistently higher throughout the study but there was no statistical significant difference. The urine phosphorus content ($mg\,kg^{-1}$ body-weight) was also consistently less for the treated group, but it was not significant (see Table 2). The difference in mean urine phosphorus concentration was $5·5\,mg\,dL^{-1}$ during the second period ($P=0·061$) and the 95% confidence interval had a wide range ($-0·2$–$11·2\,mg\,dL^{-1}$).

The primary outcome of interest was the change in serum phosphorus concentration and calcium-phosphorus product in relation to treatment with iron(III) chitosan compared with placebo. Table 3

Table 2. Mean metabolic parameters in the control and treated (1% iron chitosan) rats during the initial 15-day and final 15-day experimental period.

Parameters (per 24 h)	Control 0–15 days	Treatment 0–15 days	*P*	95% CI of difference		Control	Treatment	*P*	95% CI of difference	
				Lower	Upper	16–30 days	16–30 days		Lower	Upper
Fe intake (μg)[c]	703 (699, 840)	10917 (10831, 12034)	0·004[b]	NA	NA	879 (875, 882)	13589 (12400, 13648)	0·004[b]	NA	NA
Faeces										
Weight (mg)	501·4 ± 49·8	563·4 ± 49·5	0·0056	−124·2	0·2	735·5 ± 56·9	739·3 ± 45·1	0·899	−64·6	56·9
Iron (μg)[c]	276 (221, 333)	3102 (2234, 3727)	0·004[b]	NA	NA	395 (317, 447)	4102 (3582, 4776)	0·004[b]	NA	NA
P (mg)	4·8 ± 0·7	6·3 ± 0·9	0·008	−2·60	−0·5	7·4 ± 0·4	9·0 ± 0·7	0·001	−2·4	−0·91
Urine										
V (mL)	12·6 ± 2·1	14·6 ± 1·9	0·118	−4·6	0·6	16·1 ± 2·8	17·4 ± 1·1	0·289	−4·1	0·4
P (mg kg^{-1}/day)	7·9 = 1·6	6·4 ± 1·7	0·149	−0·6	3·6	7·3 ± 1·4	6·3 ± 1·6	0·311	−1·0	2·9
P (mg dL^{-1})	14·6 ± 2·6	11·5 ± 4·6	0·084	−0·5	6·7	19·7 ± 5·0	14·2 ± 3·8	0·061	−0·2	11·2

All values are mean ± s.d. unless noted. NA, not available. [a]Independent two-tailed Student's *t*-test for n = 8 rats for averaged data collected throughout each 15-day period. [b]Mann-Whitney *U*-test. [c]Median (interquartile range) for non-normal data. [d]Milligrams of phosphorus per kg of body-weight.

summarises the control and treatment results for eight rats in each group after 15 and 30 days of administration. The baseline values are taken from a random sample of six rats before treatment and separation into groups. Bonferroni's correction was used to compare the treatment and control groups with the baseline group because four comparisons were made. At the end of the initial 15-day treatment, the calcium-phosphorus product and phosphorus concentrations decreased from the baseline concentration by a mean of 12 mg^2 dL^{-2} ($P = 0{\cdot}27$) and 1·0 mg dL^{-1} ($P = 0{\cdot}15$) in the control group, respectively, compared with a mean decrease of 27 mg^2 dL^{-2} ($P = 0{\cdot}0009$) and 2·4 mg dL^{-1} ($P < 0{\cdot}0002$), respectively, for the treatment group. The calcium-phosphorus product and phosphorus levels of the treatment group were 15 mg^2 dL^{-2} and 1·3 mg dL^{-1} lower than the control group ($P = 0{\cdot}004$ for both), respectively. After 30 days, different rats (n = 8) from the same two groups had similar calcium-phosphorus product and phosphorus concentrations, but the phosphorus difference was more significant, 5·5 ± 0·9 mg dL^{-1} (control) vs 4·1 ± 0·6 mg dL^{-1} ($P = 0{\cdot}002$).

The serum phosphorus concentrations for the 15- and 30-day measurements of the control and treatment groups were combined into a histogram (Figure 3) because the values did not differ much between these days. Values range from 3·4 to 5·0 mg dL^{-1} for the treatment group (n = 16) at 15 and 30 days, whereas those of the control group (n = 16) range from 4·2 to 6·8 mg dL^{-1}. The treated group (right side) values are clustered in a narrower region with a sharper normal curve compared with the control.

To better understand how the serum phosphorus concentration was lowered by the supposed binding of iron(III) chitosan to phosphate, we attempted to

Table 3. Mean serum calcium-phosphorus products and phosphorus contents in the control and treatment (1% iron chitosan) rats at baseline after 15 and 30 days.

Time	Serum	Control	Treatment	*P*[a]	95% CI of difference	
					Lower	Upper
Baseline[b,c]	Ca-P product	76·5 ± 10·6		NA	65	88
	P (mg dL^{-1})	6·8 ± 0·6		NA	6·1	7·4
15 Days	Ca-P products (mg^2 dL^{-2})	64·6 ± 10·1	49·7 ± 7·2**	0·004	5·5	24·3
	P (mg dL^{-1})	5·7 ± 0·9	4·4 ± 0·5**	0·004	0·5	2·2
30 Days	Ca-P product (mg^2 dL^{-2})	62·0 ± 12·1	45·1 ± 6·6**	0·004	6·4	27·3
	P (mg dL^{-1})	5·5 ± 0·9*	4·1 ± 0·6**	0·002	0·6	2·3

NA, not available. [a]Independent two-tailed Student's *t*-test for n = 8 rats for comparison of respective control and treatment data. [b]n = 6 rats at baseline. [c]95% Confidence interval of mean. *$P < 0{\cdot}05$, **$P < 0{\cdot}001$ vs baseline using analysis of variance with Bonferroni's correction for 5 groups.

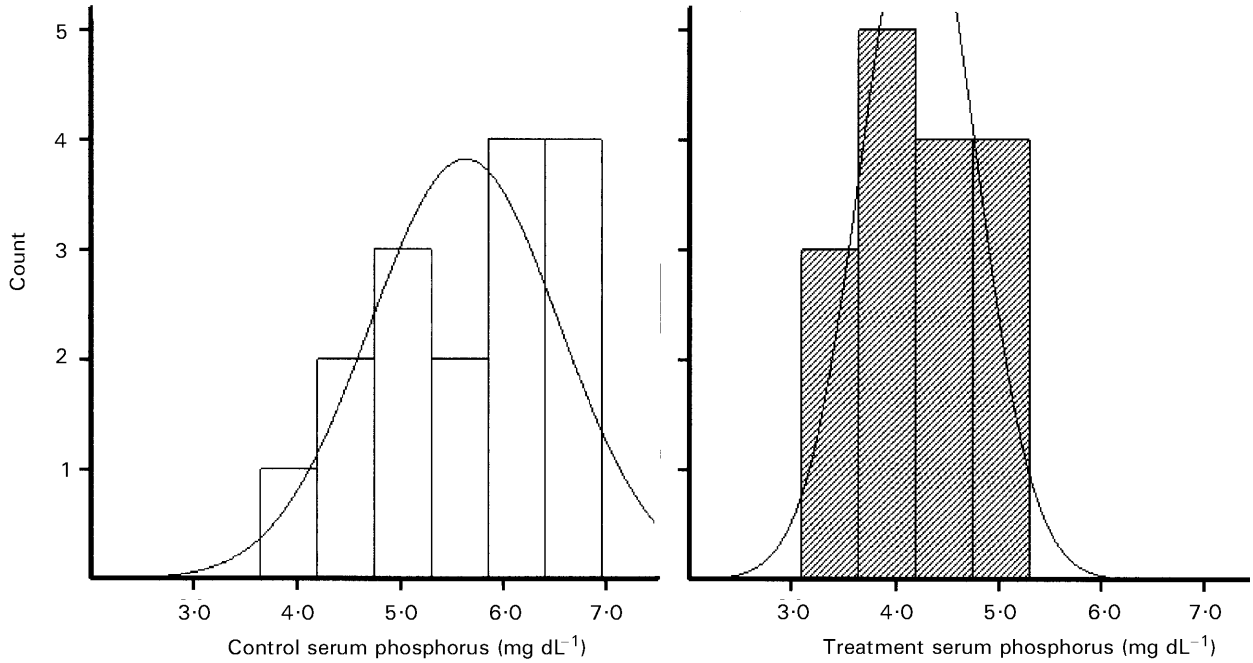

Figure 3. The effect of iron(III) chitosan (▨) at 1% food weight on the serum phosphorus concentration (mg dL^{-1}) of rats compared with a control group (□). Values are a combination of the 15-day (n = 8) and 30-day (n = 8) group measurements. A normal curve is traced over the histogram.

predict and correlate the faeces phosphorus concentration (mmol (g faeces)$^{-1}$) from the faeces iron concentration (μmol (g faeces)$^{-1}$) using simple linear regression analysis and Pearson's product-moment correlation coefficient (r). The data of the first two days for both groups were not entered into the calculation because the excreted iron did not yet reach a plateau for the treatment group. As shown in Figure 4, the slope of the regression line for the treated group was significantly greater than zero, indicating that faeces phosphorus tends to increase as faeces iron increases (slope $= 4{\cdot}1 \times 10^{-3}$; 99% CI = 0·0035–0·0046; $t_{83} = 15{\cdot}0$; $P = 0{\cdot}0003$; $Y = -0{\cdot}034 + 0{\cdot}0041X$; $r^2 = 0{\cdot}74$). The control group data for the faeces iron did not vary in a bivariate normal distribution, nor was it linear, and it was quite affected by only one outlier, because the iron intake varied over a small range ($r^2 = 0{\cdot}12$ and Pearson's $r = 0{\cdot}35$). Moreover, the amount of faeces iron correlated well with the amount of faeces phosphorus, indicating that faeces with a higher content of iron has a higher content of phosphorus (n = 83; Pearson's $r = 0{\cdot}87$; $P < 0{\cdot}001$); however, Figure 4 shows that the data is not ideally distributed in a bivariate normal distribution.

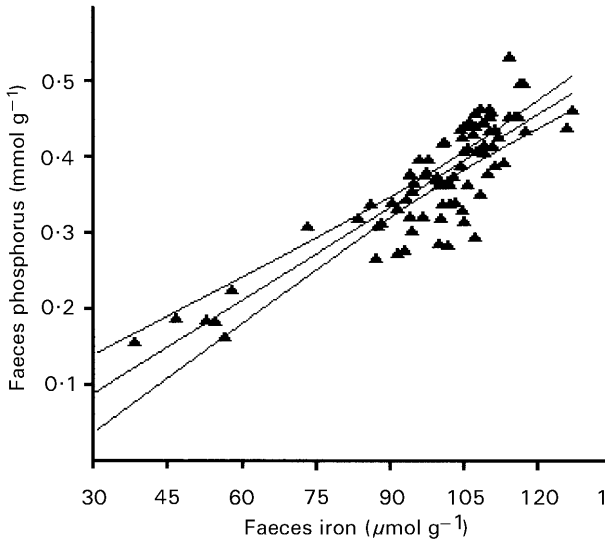

Figure 4. The relation of the faeces phosphorus concentration (mmol (g faeces)$^{-1}$) vs faeces iron concentration (μmol (g faeces)$^{-1}$) in iron(III) chitosan-treated rats (▲; n = 83) from days 3 to 30, first using simple linear regression analysis increases (slope $= 4{\cdot}1 \times 10^{-3}$; 99% CI = 0·0035–0·0046; $t_{83} = 15{\cdot}0$; $P = 0{\cdot}0003$; $Y = -0{\cdot}034 + 0{\cdot}0041X$; $r^2 = 0{\cdot}74$) and Pearson's product-moment correlation coefficient ($r = 0{\cdot}87$; $P < 0{\cdot}001$).

The serum concentrations of a variety of biochemical markers are displayed in Table 4. The calcium levels remained identical for both groups along with the creatinine and uric acid levels. For both groups the triglycerides increased dramatically from baseline values ($P < 0{\cdot}01$) although there was no difference among the groups. Unexpectedly, the total cholesterol levels were statistically higher for the treated group throughout the study (15 days:

$P = 0{\cdot}013$; 30 days: $P = 0{\cdot}044$), but the difference was presumably not clinically significant. After 30 days, the HDL cholesterol level was markedly higher for the treated group ($P = 0{\cdot}008$), yet unchanged from baseline values. The phospholipids were higher for the treated group after 15 days ($P = 0{\cdot}021$), but after 30 days both groups were not significantly different. Finally, alkaline phosphatase levels decreased equally and dramatically over the treatment period for both groups.

The kidneys and livers of the rats were investigated for any visible abnormalities and unusual mass and phosphorus differences (Table 5). The livers were not different among the groups for the entire period; however, within 30 days the kidneys in the treated group had 4·27 mg more phosphorus ($P < 0{\cdot}01$) and a 1·49 mg g^{-1} higher phosphorus mass concentration ($P < 0{\cdot}05$) than in the control group.

Discussion

Over the past two decades researchers have made great advances in the understanding of the pathogenesis and treatment of uraemic secondary hyperparathyroidism. Dietary counselling, appropriate prescription of phosphorus binders and the use of vitamin D metabolites have significantly reduced the severity of hyperparathyroidism concomitantly with the reduction of hyperphosphataemia.

For many years, aluminium hydroxide and other aluminium compounds have been widely used to

Table 4. Mean serum parameters during the baseline, 15 days and 30 days in rats on the AIN-76 diet (control) compared with those given AIN-76 diet with 1% iron chitosan (treatment).

Serum concns	0 Days Baseline[a]	15 Days		30 Days	
		Control	Treatment	Control	Treatment
Iron (μg dL^{-1})	286 ± 75	164 ± 67	241 (139, 469)[b]	154 (111, 238)[b]	265 ± 72*
Calcium (mg dL^{-1})	11·3 ± 0·8	11·3 ± 0·2	11·3 ± 0·6	11·2 ± 0·4	11·1 ± 0·3
Total protein (g dL^{-1})	5·1 ± 0·1	5·6 (5·5, 5·6)[b]	5·7 ± 0·4	5·8 ± 0·2	5·8 ± 0·3
Albumin/globulin	2·40 ± 0·14	2·11 ± 0·15	2·28 ± 0·25	1·96 ± 0·14	1·92 ± 0·11
BUN (mg dL^{-1})	10·8 ± 1·7	11·2 ± 3·0	11·9 ± 3·5	15·4 ± 3·6	18·0 ± 3·1
Creatinine (mg dL^{-1})	0·43 ± 0·05	0·44 ± 0·07	0·43 ± 0·05	0·44 ± 0·05	0·46 ± 0·05
Uric acid (mg dL^{-1})	2·2 ± 0·7	1·0 ± 0·2	1·1 ± 0·2	1·1 ± 0·3	1·0 ± 0·2
Total cholesterol (mg dL^{-1})	86·3 ± 8·8	73·1 ± 11·1	97·6 ± 21·8*	88·9 ± 25·1	118·5 ± 28·4*
HDL-C (mg dL^{-1})	50·7 ± 8·0	35·9 ± 8·3	39·9 ± 6·6	30·8 ± 10·2	47·4 ± 11·4**
Triglycerides (mg dL^{-1})	38·0 ± 19·5	140·5 ± 51·9	134·9 ± 43·9	144 (112, 252)[b]	159·2 ± 51·1
Phospholipids (mg dL^{-1})	153 ± 4	163 ± 22	206 ± 41*	194 ± 47	224 ± 38
GOT (U L^{-1})	82 (75, 114)[b]	72 ± 7	75 ± 13	76 (68, 96)[b]	75 ± 8
GPT (U L^{-1})	42 ± 3	22 ± 2	21 (20, 23)[b]	24 (22, 31)[b]	27 ± 6
Alkaline Pase (U L^{-1})	836 (729, 927)[b]	435 ± 89	446 ± 93	326 ± 113	315 ± 76
Glucose (mg dL^{-1})	190 ± 26	177 ± 21	168 ± 26	188 ± 27	173 ± 25

All values are mean ± s.d. unless noted. [a]n = 6 for baseline, whereas n = 8 for 15 days and 30 days. [b]Median (interquartile range) for non-normal data. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, vs corresponding control. GOT, glutamic-oxaloacetic transaminase; GPT, glutamic-pyruvic transaminase.

Table 5. Mean kidney mass, liver mass and phosphorus content in rats at 0, 15 and 30 days.

Contents	0 Days Baseline	15 Days		30 Days	
		Control	Treatment[a]	Control	Treatment[a]
Kidney					
Mass (g)	1·35 ± 0·17	1·77 ± 0·59	2·08 ± 0·42	2·68 ± 0·13	2·73 ± 0·17
P (mg)	6·4 ± 1·7	8·9 ± 3·4	11·4 ± 2·4	11·1 ± 3·1	15·4 ± 3·6*
P (mg g^{-1})	4·7 ± 0·8	5·0 ± 0·7	5·5 ± 0·7	4·2 ± 1·2	5·6 ± 1·4*
Liver					
Mass (g)	6·32 ± 0·88	11·94 ± 1·58	12·87 ± 2·37	16·29 ± 2·07	16·57 ± 2·07
P (mg)	2·2 ± 0·4	4·3 ± 2·1	4·7 ± 1·4	5·6 ± 1·4	5·7 ± 1·4
P (mg g^{-1})	0·4 ± 0·1	0·4 ± 0·2	0·4 ± 0·1	0·4 ± 0·1	0·4 ± 0·1

Values are given as mean ± s.d., n = 6 for baseline, n = 8 for 15 days and 30 days. [a]One percent iron(III) chitosan substituted for fibre in diet mix (AIN-76). *$P < 0{\cdot}05$ vs corresponding control.

control hyperphosphataemia in dialysis patients. Studies indicate that accumulation of aluminium in the body leads to vitamin D refractory osteomalacia (Smith et al 1986), microcytic anaemia and encephalopathy (Wills & Savory 1983). Furthermore, excessive aluminium intake may cause taste intolerance, anorexia, constipation and nausea (Slatopolsky 1987).

Similar undesirable results have been reported with calcium-containing binders such as calcium carbonate, which reduce gastrointestinal absorption of phosphorus. However, their use has been limited by the occurrence of hypercalcaemia, reported in approximately one-third of dialysis patients, and by the subsequent possibility of metastatic calcifications (Moriniere et al 1983).

The measured live-phase parameters such as body-weight and urine and faeces output were not significantly different in the control and iron(III) chitosan-treated groups, although the food and phosphorus intakes of the treated rats tended to be less than that of the controls from the 16th day onward (see Figure 2). The food and phosphorus intakes were significantly less for the treatment group on days 18, 20 and 23 because the number of rats decreased from sixteen to eight after 15 days. Also, omission of a lower extreme value from the calculations due to an underweight rat in the treatment group eliminated the significant differences on days 20 and 23. The observed $\sim 25\%$ lower phosphorus serum of the treatment group at days 15 and 30 is many times more than what would be expected from lowering the mean food (phosphorus) intake by only 2·5%. In Spengler's study with iron(III) dextran (Spengler et al 1996), it was noted that the control rat group had a 20% higher food and phosphorus intake than the treatment group, but this difference was not considered significant enough to have much of an effect on the serum phosphate levels.

In a real restriction meal, a phosphate difference of 35% or more between the control and treatment group was necessary to observe a significant lowering of serum phosphorus levels in dogs with advanced renal insufficiency that was induced by 5/6 nephrectomy (Lopez-Hilker et al 1990). However, in patients with moderate renal insufficiency with normal serum phosphorus levels, a phosphorus intake restriction of 70% was insufficient to lower serum phosphorus levels, but it did suppress parathyroid hormone secretion independent of the levels of calcitriol or plasma ionized calcium (Portale et al 1984). In many patients with chronic renal failure, phosphate retention cannot be controlled by dietary phosphate restriction alone since inadequate nutrition may result. Dialysis may also be unsatisfactory (Hou et al 1991) because wider swings in plasma phosphorus will occur as the glomerular filtration rate declines. Thus, the additional use of phosphate binders is necessary while maintaining adequate nutrition, which is of utmost importance since malnutrition is a common consequence of advanced renal failure (Block et al 1998).

In normal adult rats most of the dietary phosphorus that is absorbed is eliminated through the urine and a fraction is used for growth. The excess phosphorus that is not absorbed through the intestines and the endogenous phosphorus that is not reabsorbed are eliminated in the faeces. In our study, the faeces phosphorus of the iron(III) chitosan complex increased by about 25% relative to the control, but the urine phosphorus content was reduced, though not significantly, by 10% during the first 15-day period and by 20% thereafter. In addition, the extra phosphorus excreted in the treated group in comparison with the control does not seem clinically significant since this amount corresponds to about 4% of the amount of phosphorus ingested. However, the net phosphorus absorption difference of $3{\cdot}7\,\mathrm{mg\,day^{-1}}$ (Table 1, $P < 0{\cdot}001$) seemed to have an effect on lowering the serum phosphorus levels. Therefore, since the phosphorus content of the rodent chow was only 0·4% it can be concluded that more than 90% of the ingested phosphorus was digested.

The amount of iron excreted is directly related to the amount of iron-containing phosphate binder ingested, but more importantly it relates to the insolubility, or iron dissolution, of the binder in the stomach's acidic environment. A common symptom of iron toxicity is constipation, but this was not observed in the treated group, which had borderline significance ($P = 0{\cdot}056$) for greater mean faecal weight during the first 15 days. During the first 15 days, the treated rats excreted 26% of the ingested iron and 31% during the second 15 days. The control groups excreted 37% and 44% of the ingested iron in the first and second periods, respectively, due to a much lower iron intake. Furthermore, the excess iron intake did not hinder the function of the alimentary canal since the weights of the rats neither decreased at the beginning of the experiment nor significantly differed throughout the 30 days. Most importantly, the serum iron for the treatment group was not significantly raised above the baseline values, but the serum iron of the control was significantly lowered ($P < 0{\cdot}05$) from baseline values at 15 and 30 days; however, two rats in the treatment group had high serum levels of iron ($\sim 500\,\mu\mathrm{g\,dL^{-1}}$) at 15 days and a significantly higher level after 30 days ($P < 0{\cdot}05$) as compared with the corresponding control group.

Although our study has demonstrated that iron-(III) chitosan can effectively bind dietary phosphate, long-term safety has not been fully addressed, partially because the haematocrit, haemoglobin and ferritin levels, including a complete blood count, were not measured. The iron intake was only 0·054% of the food intake, which is remarkably lower than in other reported experiments that showed minimal or none of the toxic effects due to iron (Spengler et al 1996; Hsu et al 1999). Therefore, it was realistically considered that the low iron content would produce none of the symptoms associated with iron toxicity. For example, no iron toxicities were noted in rats that ingested iron(III) dextran (Spengler et al 1996) and ferric citrate (Hsu et al 1999) that received twenty-eight times and eighteen times higher iron dosages, respectively, than in the iron(III) chitosan experiment. Hsu et al (1999) reported that the haematocrit and plasma concentration of serum iron were higher in treated azotaemic rats than in the control group, suggesting that some degree of intestinal iron absorption took place, but these were considered beneficial symptoms for ameliorating the problems of renal failure, including iron deficiency and anaemia. Jacobs (1965) has shown that toxicity in humans was associated with ingestion of large quantities of iron. Acute iron poisoning usually manifests with serum iron concentrations above $500\,\mu g\,dL^{-1}$ (Chyka & Butler 1993), which is twice the mean levels observed in this study; but, as mentioned, two of the rats had abnormally high levels after 15 days. Iron toxicity can sometimes include gastrointestinal haemorrhage, coagulation defects, shock, metabolic acidosis, hypoparathyroidism and haemosiderosis (Sherman et al 1970; Witten & Brough 1971; McCarthy et al 1991). Granick (1946) reported, however, that oral administration of iron does not cause haemosiderosis. Moreover, if iron is given with a meal, the pH of the gastric juice increases, the dissociation of iron is inhibited, and the iron combines with phosphoric compounds in the meal, converting it to an inabsorbable compound (Hegsted et al 1949; Moore 1955). Therefore, iron absorption decreases with a meal. Furthermore, the concentrations of serum iron, TIBC (total iron binding capacity) and ferritin decreased in 74 haemodialysis patients receiving erythropoietin and oral iron administration for 6 months (Raja et al 1993).

The principal outcome of interest was the lowering of the serum phosphorus and the serum calcium-phosphorus product. As shown in Figure 3, the serum phosphorus concentrations of the treatment group are distinctly lower and constrained within a smaller range than those of the control group. The difference in mean serum phosphorus concentrations between the control group and treatment group was $1{\cdot}3\,mg\,dL^{-1}$ (95% CI = 0·5–$2{\cdot}2\,mg\,dL^{-1}$) after 15 days (see Table 3), and after 30 days the difference was nearly the same, $1{\cdot}4\,mg\,dL^{-1}$ (95% CI = 0·6–$2{\cdot}3\,mg\,dL^{-1}$). Similar reductions were also observed for the serum calcium-phosphorus product since the serum calcium levels did not change; therefore, the decrease in the calcium-phosphorus product was due to the low serum phosphorus levels. A different group of rats in the control and treatment groups were measured at 15 and 30 days to confirm that the differences in serum phosphorus were experimentally reproducible. Also, the continuation of the study to 30 days demonstrated that the low phosphorus levels achieved could be maintained. In addition, the high coefficient of determination ($r^2 = 0{\cdot}76$) and correlation coefficient ($r = 0{\cdot}87$) for the treatment group adds credence to the assumption that the reduction in serum phosphorus is principally due to the binding of iron with phosphate, because as the concentration of iron in the faeces increases the concentration of phosphate increases. However, further studies with a greater range in iron intake are needed to verify this result, besides observing calcitriol and parathyroid hormone levels for any secondary effects.

To better understand the reaction of the body to lower P_i stores, which might have an effect on the reabsorption of P_i in the kidney tubules, the phosphorus content of the kidneys was measured. During dietary P_i restriction, P_i can disappear from the urine. This phenomenon is associated with an increase (adaptation) of the overall tubular capacity to reabsorb P_i (Bonjour & Caverzasio 1984; Mizgala & Quamme 1985). In the treatment group, the concentration of phosphorus in the kidneys did not increase relative to the control after 15 days, but it significantly increased after 30 days (see Table 5). The storing of phosphate in the kidneys seems to be kinetically slow. The higher phosphorus content is another indication of the great capability of iron-(III) chitosan to lower serum phosphorus. The reasoning is that as the blood levels of phosphorus become lower the kidneys switch mechanisms to capture the perceived dwindling levels of phosphorus in the body.

Secondary effects in the other measured serum parameters (Table 4) were similar except for the cholesterol values. It is unknown why the total cholesterol levels increased in the treatment group as other chitosan reports have shown a hypocholesterolaemic effect in humans (Sugano et al 1978; Maezaki et al 1993; Jing et al 1997). The cholesterol levels were not closely studied in this report,

so the exact LDL-C amount is unknown. The beneficial HDL-C was significantly higher, however, but there was no clinical significance compared with the baseline values. The HDL-C was lowered from baseline values for the control group and this is probably due to the low 1% fibre content of the modified AIN-76 rat feed. The lowering of the fibre content for both groups presumably led to an increase in the LDL-C content, as would be expected. Under normal conditions, with administration of 5% fibre the results would likely have been different, but incorporating iron into chitosan had an opposite effect to that reported for chitosan alone. The binding of iron is principally to the amino group of the glucosamine polymer of chitosan, therefore, it can be speculated that previous studies showed a hypocholesterolaemic effect due to the availability of the amino group to bind LDL-C either through ionic bonding or through some other bonding mechanism.

The dose of the iron(III) chitosan complex used in our animal experiments was equivalent to 60 g daily for a 70-kg adult person, four times less than what would be required for other ferric compounds such as ferric citrate and ferric chloride (Hsu et al 1999). This is not only due to the difference in their phosphate binding ability, but also to many factors, including the compound solubility, gastrointestinal motility, food mixing characteristics and pH of the gastrointestinal tract (Sheikh et al 1989). The reason that iron(III) chitosan had a lower net phosphorus absorption difference of $3{\cdot}7\,mg\,day^{-1}$ as compared with other binders (Table 1), is the very low concentration of iron in the diet. In addition, the lowered net phosphorus absorption of the treatment group exactly corresponded with the in-vitro phosphorus binding capacity of iron(III) chitosan at pH 2 (Table 1), since the phosphorus binding capacities are identical at pH 2. Moreover, this very high binding capacity of 308 mg of phosphorus per gram of elemental iron in both the in-vivo and in-vitro studies is about three times greater than calcium acetate, nearly four times greater than ferric citrate and seven times greater than calcium carbonate (Table 1). As a result, only a 1% addition of iron(III) chitosan to the rat diet was needed to decrease the serum phosphorus of the iron(III) chitosan treated rats.

The reason for the lowering of serum phosphorus is not completely understandable because the parathyroid hormone and calcitriol concentrations were not measured. Although serum phosphorus does not itself directly affect acute release of parathyroid hormone, it plays an important part in the regulation of parathyroid hormone secretion by inducing reciprocal changes in the serum concentrations of ionized calcium and $1,25(OH)_2D_3$ (Vitamin D) (Tanaka & DeLuca 1973). During dietary P_i restriction, P_i can disappear from the urine (Biber 1989), and in theory this is sometimes associated with a decrease in parathyroid hormone in renal patients (Portale et al 1984). It has clearly been demonstrated, however, that the ability of the renal (proximal) cell to respond to P_i availability is independent of parathyroid hormone (Tenenhouse et al 1988). Nevertheless, in our study the rats had neither renal failure nor high serum phosphorus levels, so a reduction in parathyroid hormone would not be expected to be significant, especially since the urine phosphorus levels were not significantly decreased. Furthermore, 1% chitosan without iron was fed to rats in a current study of ours and we found no significant reduction or increase in serum parathyroid hormone. This shows that ingestion of chitosan does not alter serum parathyroid hormone levels, but the effect with iron(III) chitosan is unknown.

In conclusion, iron(III) chitosan seems to be useful for lowering serum phosphorus levels, with a greater elemental binding capacity than the problematic calcium and aluminium phosphate binders. Additional toxicity studies, dosage effects, and parathyroid hormone- and phosphorus-level measurements in renal failure rats would be necessary before assuming that iron(III) chitosan can be safely and effectively used for hyperphosphataemia and secondary hyperparathyroidism in patients with uraemia. Iron(III) chitosan merits further investigation as a phosphate-binding agent.

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