

# Inositol phosphates with different numbers of phosphate groups influence iron absorption in humans<sup>1-3</sup>

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## ABSTRACT

**Background:** Inositol hexaphosphate (IP<sub>6</sub>) is a well-known inhibitor of iron absorption, whereas the effects of the less-phosphorylated derivatives of IP<sub>6</sub> are less known.

**Objectives:** The objective was to investigate the effects of inositol tri-, tetra-, and pentaphosphates (IP<sub>3</sub>, IP<sub>4</sub>, and IP<sub>5</sub>, respectively) on iron absorption in humans.

**Design:** Iron absorption was measured in 5 experiments from single meals by extrinsic labeling with <sup>55</sup>Fe and <sup>59</sup>Fe and determination of whole-body retention and the erythrocyte uptake of isotopes. In experiments 1–3 the meals contained white-wheat rolls to which 10 mg P as IP<sub>5</sub>, IP<sub>4</sub>, or IP<sub>3</sub>, respectively, was added. Inositol 1,2,6-triphosphate [Ins(1,2,6)P<sub>3</sub>] and a mixture of isomers of IP<sub>4</sub> and IP<sub>5</sub> were studied. White-wheat rolls contained 10 mg P as IP<sub>3</sub> + IP<sub>4</sub> and 2 mg P as IP<sub>5</sub> + IP<sub>6</sub> in experiment 4 and 20 mg P as IP<sub>3</sub> + IP<sub>4</sub> and 3 mg P as IP<sub>5</sub> + IP<sub>6</sub> in experiment 5; inositol phosphates were obtained via fermentation of sodium phytate. Each experiment had 8–11 subjects.

**Results:** In experiment 1, iron absorption was reduced by 39%, whereas there was no significant effect on iron absorption in experiments 2 and 3. In experiments 4 and 5, iron absorption was reduced by 54% and 64%, respectively, suggesting that IP<sub>3</sub> and IP<sub>4</sub> contributed to the inhibitory effect.

**Conclusions:** IP<sub>5</sub> has an inhibitory effect on iron absorption, whereas IP<sub>3</sub> and IP<sub>4</sub> in isolated form have no such effect. IP<sub>3</sub> and IP<sub>4</sub> in processed food contribute to the negative effect on iron absorption, presumably by binding iron between different inositol phosphates. To improve iron absorption from cereals and legumes, degradation of inositol phosphates needs to be to less-phosphorylated inositol phosphates than IP<sub>3</sub>. *Am J Clin Nutr* 1999;70:240–6.

**KEY WORDS** Phytate, inositol phosphates, iron absorption, humans

## INTRODUCTION

*myo*-Inositol hexaphosphate (IP<sub>6</sub>), also known as phytate, is a recognized inhibitor of iron and zinc absorption in humans (1). During food processing, such as soaking, malting, and fermenting, phytate is hydrolyzed (2–5) because of the activation of intrinsic plant phytases, extrinsic microbial phytases, or both. Phytase hydrolyzes phytate to *myo*-inositol and inorganic phosphate via intermediate *myo*-inositol phosphates: inositol mono-

bi-, tri-, tetra-, and pentaphosphates (IP<sub>1</sub>, IP<sub>2</sub>, IP<sub>3</sub>, IP<sub>4</sub>, and IP<sub>5</sub>, respectively) (6). Thermal processing can lead to a partial nonenzymatic hydrolysis of phytate (7–9).

No effect on zinc and calcium absorption of aqueous solutions of pure fractions of inositol phosphates with <5 phosphate groups, produced via acid-catalyzed hydrolysis, was observed in suckling rats (10). Added separately to white bread, IP<sub>4</sub> produced via acid-catalyzed hydrolysis had no effect on zinc absorption in humans, whereas IP<sub>5</sub> and IP<sub>6</sub> depressed zinc absorption (11). In vitro addition of isolated IP<sub>5</sub> and IP<sub>6</sub> to a white-wheat roll under simulated physiologic conditions had a strong negative effect on iron solubility (12). The results suggested that the inhibitory effect of IP<sub>5</sub> was five-sixths that of equimolar amounts of IP<sub>6</sub>, thus corresponding to the number of phosphate groups. The addition of IP<sub>3</sub> and IP<sub>4</sub> produced via acid-catalyzed hydrolysis slightly increased the iron solubility under simulated physiologic conditions, but it was found to be strongly dependent on pH, in contrast with the addition of IP<sub>5</sub> and IP<sub>6</sub>. Iron solubility decreased markedly when the pH was increased from 6 to 7, making the effect of IP<sub>3</sub> and IP<sub>4</sub> on iron absorption difficult to predict. In a human study of iron absorption from different bread meals, a strong negative correlation was found between iron absorption and the sum of IP<sub>3</sub>, IP<sub>4</sub>, IP<sub>5</sub>, and IP<sub>6</sub>, suggesting that IP<sub>3</sub> and IP<sub>4</sub> also contributed to the negative effect on iron absorption (13). In the present study, the effect of inositol phosphates on iron absorption was investigated in humans.

## SUBJECTS AND METHODS

### Subjects

Forty-eight subjects participated in experiments 1–5. All subjects were healthy volunteers aged 18–54 y and each group of

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8–11 subjects included both men and women. Some of the subjects in each group were regular blood donors, which provided a reasonable range of intersubject variation in iron absorption. None of the subjects were taking antibiotics, diuretics, antacids, or mineral-vitamin supplements. Blood donation during the study was not allowed and no volunteer had donated blood within the 2 mo before the study began. Hemoglobin and other hematologic determinations were made in duplicate on venous blood drawn without stasis from an antecubital vein with the subjects in the supine position for 15 min. The values given were determined from blood samples drawn in the morning after the subjects had fasted overnight. Subjects had hemoglobin values of 120–169 g/L ( $\bar{x} \pm \text{SEM}$ :  $139 \pm 4$  g/L), a transferrin saturation of 12–54% ( $\bar{x} \pm \text{SEM}$ :  $28.9 \pm 2.4\%$ ), and a serum ferritin concentration of 10–127  $\mu\text{g/L}$  ( $\bar{x} \pm \text{SEM}$ :  $39 \pm 9$   $\mu\text{g/L}$ ). The volunteers were given oral and written information about the aims and procedures of the study. The project was approved by the Ethical Committee of the Medical Faculty of the University of Göteborg.

## Experimental design

### Experiments 1–5

Each experiment included 8–11 subjects and iron absorption was measured in each subject by using 2 different radioiron tracers, 1 in the control meal and 1 in the test meal. Thus, each subject served as his or her own control. The control meal served in each experiment consisted of 2 white-wheat rolls, butter, and water. The test meals in the 5 experiments consisted of 2 white-wheat rolls, butter, and water, with added inositol phosphates as described below.  $\text{IP}_5$  (experiment 1),  $\text{IP}_4$  (experiment 2), and  $\text{IP}_3$  (experiment 3) were dissolved in a small amount of water and injected into the rolls after they were baked. The amount of inositol phosphates added to these 3 test meals was 10 mg P as inositol phosphates (Table 1). In experiments 4 and 5, 1.00 and 1.71 mg P as phytate/g flour, respectively, were added as sodium phytate (Sigma Chemical Co, St Louis) to the dough before fermentation for 1.3 and 3 h, respectively. As a result,  $\approx 10$  and 20 mg P as  $\text{IP}_3$  and  $\text{IP}_4$ , and 2 and 3 mg P as  $\text{IP}_5$  and  $\text{IP}_6$  were obtained in the respective rolls.

Test rolls (A) and control rolls (B) were served on alternate mornings after an overnight fast on 4 consecutive days in the order ABBA or BAAB. No food or drink was allowed for 3 h after the meals. The rolls were labeled with 2 different radioiron isotopes:  $^{55}\text{Fe}$  and  $^{59}\text{Fe}$ . A blood sample (20 mL) was drawn 2 wk after the last roll was served to determine the content of  $^{55}\text{Fe}$  to  $^{59}\text{Fe}$  in red blood cells.  $^{55}\text{Fe}$  and  $^{59}\text{Fe}$  in blood were analyzed with a liquid-scintillation spectrometer (Tri-Carb; Packard Instruments, Downers Grove, IL) according to a modification of the method described by Eakins and Brown (14). Relative absorption of  $^{55}\text{Fe}$  and  $^{59}\text{Fe}$  was calculated from blood samples. The total retention of  $^{59}\text{Fe}$  was measured by whole-body counting (15). The whole-body counter used in this study was a plastic scintillator unit with 4 large scintillator blocks arranged in a floor-roof configuration and shielded from background radiation in a room with 0.15-m thick walls, in which the maximum counting efficiency was 89% for photons in the energy range of 1.0–1.5 MeV. The total retention of  $^{55}\text{Fe}$  was calculated from the ratio of  $^{55}\text{Fe}$  to  $^{59}\text{Fe}$  in red blood cells and the total retention of  $^{59}\text{Fe}$ . An oral reference dose (see below) was then given to fasting subjects and a second reference dose was given on the following morning. Absorption of the reference doses was measured by

whole-body counting 2 wk later (see below). All procedures and methods of calculation were described previously by Björn-Rasmussen et al (16) and Hallberg (17).

### Experiment 6

$\text{IP}_1$ ,  $\text{IP}_2$ ,  $\text{IP}_3$ ,  $\text{IP}_4$ ,  $\text{IP}_5$ , and  $\text{IP}_6$  were analyzed and the isomeric forms of  $\text{IP}_3$  and  $\text{IP}_4$  were quantified in rye rolls containing native inositol phosphates in amounts comparable with those in experiment 5. These rye rolls were used in a previous iron-absorption study in which the test meal consisted of sourdough-fermented rye rolls and the control meal consisted of white-wheat bread (13).

### Meals, roll making, and meal labeling

Each control or test meal consisted of 2 white-wheat rolls. In all experiments, the rolls were served with 20 g butter and 150 mL demineralized water. The calculated energy content of the meals was 1.8 MJ. Iron (3.7 mg) as ferrous sulfate was added to the dough of all rolls (Table 1). The white-wheat rolls were made from 40 g unfortified white-wheat flour (55% extraction), yeast, sugar, table salt, and demineralized water. The amounts of yeast, sugar, salt, and water were 65, 35, 10, and 750 g/kg flour, respectively. The control and test doughs in experiments 1–3 were fermented for 3 h at 23°C to reduce the natural inositol phosphate content to undetectable concentrations. The test doughs in experiments 4 and 5 were fermented for 1.3 and 3 h, respectively, at 23°C after addition of 1.00 and 1.71 g P as phytate/g flour, respectively, to give 10 and 20 mg P as  $\text{IP}_3$  +  $\text{IP}_4$ . The control and test doughs in experiments 1–5 were then kneaded again and weighed amounts were transferred to small aluminum forms, which were left standing for 20 min for further fermentation. The rolls were then baked at 250°C for 15 min.

In all studies, each meal was labeled with 46.2 kBq  $^{59}\text{Fe}$  or  $^{55}\text{Fe}$  by adding the liquid portion of the dough mixture. The isotope solutions of  $^{55}\text{Fe}$  (specific activity: 626 MBq/mg) and  $^{59}\text{Fe}$  (specific activity: 1127 MBq/mg) (Amersham International, Amersham, Buckinghamshire, United Kingdom), as ferric chloride in 0.1 mol/L, were prepared at the beginning of the study and checked for activity at the Radiation Physics Department, Sahlgren's Hospital, Göteborg, Sweden, before being used. In experiments 1–5, the test rolls were labeled with  $^{55}\text{Fe}$  and the control rolls with  $^{59}\text{Fe}$ .

### Oral reference doses

A solution of 10 mL of 0.01 mol HCl/L containing 3 mg Fe as ferrous sulfate and 30 mg ascorbic acid labeled with  $^{59}\text{Fe}$  was used as a reference in all studies. The 10-mL vials containing the iron solution were rinsed twice with water and this rinse water was also consumed. Each subject received 2 reference doses on 2 consecutive mornings after an overnight fast. No food or drink was allowed for 3 h after the reference dose. Each subject received a total of 55.5 kBq  $^{59}\text{Fe}$ .

### Preparation of inositol tri-, tetra-, and pentaphosphates

$\text{IP}_4$  and  $\text{IP}_5$  were prepared according to the method of Sandberg et al (12). Sodium phytate (1.5 g; BDH Chemicals, Poole, United Kingdom) was hydrolyzed with 100 mL of 0.5 mol HCl/L for 7 h. The hydrolysate was evaporated to dryness at reduced pressure and a temperature of 40°C and then dissolved in 10 mL deionized water. The inositol phosphates formed were separated by anion-exchange chromatography. Glass columns (1 × 70 cm) containing 10 mL resin (AG 1-X8, 200–400 mesh;



TABLE 1

Iron absorption from meals with and without (control) inositol phosphates<sup>1</sup>

Experimental meals	Iron status indexes			Nonheme-iron content <i>mg</i>	Iron absorption from meal <i>%</i>	Iron absorption		
	Hemoglobin <i>g/L</i>	Transferrin saturation <i>%</i>	Serum ferritin <i>μg/L</i>			From reference dose <i>%</i>	Individual absorption corrected for reference dose <sup>2</sup> <i>%</i>	Individual absorption ratios
Previous study ( <i>n</i> = 8) <sup>3</sup>	143 ± 7.9	34 ± 5.8	49 ± 40.1	4.0	—	39.0 ± 3.4	—	0.41 ± 0.04
Control rolls <sup>6</sup>	—	—	—	—	17.4 ± 2.8	—	18.8 ± 4.8	—
Test rolls: 10 mg P as IP <sub>6</sub>	—	—	—	—	7.7 ± 1.1 <sup>4</sup>	—	8.4 ± 2.4	—
Experiment 1 ( <i>n</i> = 11)	149 ± 6.3	36 ± 6.6	73 ± 30.5	4.0	—	31.3 ± 3.1	—	0.61 ± 0.06
Control rolls	—	—	—	—	21.3 ± 4.1	—	24.3 ± 3.2	—
Test rolls: 10 mg P as IP <sub>5</sub>	—	—	—	—	12.3 ± 2.0 <sup>5</sup>	—	15.2 ± 1.6	—
Experiment 2 ( <i>n</i> = 10)	151 ± 8.6	37 ± 9.9	81 ± 35.5	4.1	—	35.9 ± 4.1	—	0.90 ± 0.07
Control rolls	—	—	—	—	16.9 ± 3.1	—	20.0 ± 3.2	—
Test rolls: 10 mg P as IP <sub>4</sub>	—	—	—	—	15.8 ± 3.1	—	18.0 ± 3.2	—
Experiment 3 ( <i>n</i> = 9)	159 ± 7.0	39 ± 5.8	110 ± 40.3	3.8	—	17.8 ± 1.9	—	0.98 ± 0.06
Control rolls	—	—	—	—	7.7 ± 1.1	—	17.6 ± 2.8	—
Test rolls: 10 mg P as IP <sub>3</sub>	—	—	—	—	6.8 ± 0.8	—	16.8 ± 2.8	—
Experiment 4 ( <i>n</i> = 10)	155 ± 6.9	37 ± 8.8	66 ± 45.1	4.4	—	38.2 ± 5.8	—	0.46 ± 0.04
Control rolls	—	—	—	—	20.4 ± 4.9	—	20.8 ± 2.8	—
Test rolls: 1.6 mg P as IP <sub>6</sub> , 0.6 mg P as IP <sub>5</sub> , 2.9 mg P as IP <sub>4</sub> , and 6.9 mg P as IP <sub>3</sub>	—	—	—	—	9.8 ± 2.8 <sup>4</sup>	—	10.0 ± 2.0	—
Experiment 5 ( <i>n</i> = 8)	148 ± 9.9	36.5 ± 7.5	67 ± 48.3	4.0	—	35.1 ± 3.8	—	0.36 ± 0.04
Control rolls	—	—	—	—	29.0 ± 5.3	—	29.1 ± 4.8	—
Test rolls: 2.2 mg P as IP <sub>6</sub> , 0.9 mg P as IP <sub>5</sub> , 6.2 mg P as IP <sub>4</sub> , and 15.1 mg P as IP <sub>3</sub>	—	—	—	—	9.3 ± 1.5 <sup>4</sup>	—	9.8 ± 1.2	—
Experiment 6 ( <i>n</i> = 10) <sup>6</sup>	157 ± 10.3	38 ± 7.7	92 ± 65.3	4.0	—	25.1 ± 4.4	—	0.32 ± 0.03
Control rolls	—	—	—	—	16.3 ± 3.58	—	25.6 ± 4.0	—
Rye rolls: 2.3 mg P as IP <sub>6</sub> , 2.6 mg P as IP <sub>5</sub> , 14.9 mg P as IP <sub>4</sub> , and 10.9 mg P as IP <sub>3</sub>	—	—	—	3.4	6.1 ± 1.9 <sup>4</sup>	—	9.2 ± 2.8	—

<sup>1</sup> $\bar{x} \pm \text{SEM}$ , except for the iron-status indexes ( $\bar{x} \pm \text{SD}$ ). IP<sub>3</sub>, IP<sub>4</sub>, IP<sub>5</sub>, and IP<sub>6</sub>, inositol tri-, tetra-, penta-, and hexaphosphates.<sup>2</sup>Mean individual absorption ratios (test roll:control roll) were multiplied by 40 to obtain the percentage absorption of iron corresponding to a 40% reference dose absorption.<sup>3</sup>Results from a previous study (with a similar design) of iron absorption from a single meal (22).<sup>4,5</sup>Significantly different from control rolls (unpaired two-sided *t* test): <sup>4</sup>*P* < 0.001, <sup>5</sup>*P* < 0.005.<sup>6</sup>Results from a previous study of iron absorption from bread meals (13).

Bio-Rad Laboratories, Hercules, CA) were used. Fractions of 30 mL were eluted by a linear gradient from 0.05 to 0.5 mol HCl/L. The initial concentration of 0.05 mol HCl/L (2 × 30 mL) was used to separate IP<sub>1</sub> and IP<sub>2</sub> from the hydrolysate. IP<sub>3</sub>, but not IP<sub>4</sub> and IP<sub>5</sub>, start to elute at a concentration >0.05 mol/L. The collected fractions of IP<sub>4</sub> and IP<sub>5</sub> were evaporated to dryness and dissolved in 10 mL mobile phase or water. The inositol phosphates were identified and quantified by high-performance ion-pair chromatography (12, 18). IP<sub>3</sub> was prepared from sodium phytate and baker's yeast and the inositol 1,2,6-triphosphate [Ins(1,2,6)P<sub>3</sub>] isomer was separated by ion-exchange chromatography on an Aminex column (19). This fraction was prepared by Perstorp Pharma, Perstorp, Sweden.

#### Analysis of inositol phosphates and their isomers

The contents of IP<sub>3</sub>, IP<sub>4</sub>, IP<sub>5</sub>, and IP<sub>6</sub> in white-wheat rolls; of isomers of IP<sub>4</sub> and IP<sub>5</sub> produced via acid-catalyzed hydrolysis; and of isomers of IP<sub>3</sub>, IP<sub>4</sub>, and IP<sub>5</sub> in the white-wheat rolls used in experiments 4 and 5 and in the rye rolls used in experiment 6 were determined according to the method of Skoglund et al (20) after improvements to the anion exchanger were made (21). This

method measures IP<sub>2</sub>, IP<sub>3</sub>, IP<sub>4</sub>, IP<sub>5</sub>, and IP<sub>6</sub> in system 1 and IP<sub>1</sub>, IP<sub>2</sub>, and IP<sub>3</sub> in system 2 with good separation of isomers. The method includes extraction of inositol phosphates from rolls with hydrochloric acid and separation of the inositol phosphates from the crude extract by anion-exchange chromatography. The inositol phosphate isomers were separated on high-performance ion-exchange columns with gradient elution (acidic gradient in system 1 and alkaline gradient in system 2) and were detected by using postcolumn reaction and ultraviolet detection (in system 1) or suppressed conductivity detection (in system 2). Furthermore, this improved method effectively separated the Ins(1,2,3)P<sub>3</sub> and DL-Ins(1,2,6)P<sub>3</sub> isomers in the white-wheat and rye rolls (21).

#### Analysis of iron

Rolls were freeze-dried and ground in a porcelain mortar. Duplicate samples (2.0 g) were analyzed for their iron content. All glassware was washed in 2.5 mol HCl/L and rinsed in deionized water before used. Iron was determined by atomic absorption spectrophotometry (model 360; Perkin-Elmer Co, Norwalk, CT) after dry-ashing at 450°C.

## Statistical methods

All statistical analyses were made by using the STATVIEW II computer program (Abacus Concepts, Inc, Berkeley, CA). For statistical comparisons, means and SEs of the individual absorption ratios in each experiment were used. The possible statistical significance of the difference between the mean absorption and 1 was examined with an unpaired, two-sided *t* test. Differences were considered significant at  $P < 0.05$ . The recalculation of values to 40% of the absorption of the reference dose was used to compare the results of different iron-absorption experiments. The conclusion from each experiment was based on the results of pairwise comparisons in the same subjects without use of the reference dose results.

## RESULTS

### Analysis of inositol phosphates and their isomers

#### Experiments 1–5: white-wheat rolls

The control white-wheat rolls used in experiments 1–5 did not contain detectable amounts of IP<sub>3</sub>, IP<sub>4</sub>, IP<sub>5</sub>, or IP<sub>6</sub> after baking.

#### Experiments 1 and 2: pure fractions of IP<sub>4</sub> and IP<sub>5</sub>

During acid-catalyzed hydrolysis of sodium phytate, a mixture of several isomers was formed as shown in **Figure 1A**. A similar sample was used to prepare the IP<sub>4</sub> and IP<sub>5</sub> fractions. The IP<sub>5</sub> fraction was found to contain 24% Ins(1,3,4,5,6)P<sub>5</sub>, 41% DL-Ins(1,2,4,5,6)P<sub>5</sub>, 28% DL-Ins(1,2,3,4,5)P<sub>5</sub>, and 8% Ins(1,2,3,4,6)P<sub>5</sub>. The IP<sub>4</sub> fraction was found to contain 7% DL-Ins(1,4,5,6)P<sub>4</sub>, 5% Ins(2,4,5,6)P<sub>4</sub>, 21% DL-Ins(1,2,5,6)P<sub>4</sub>, 14% DL-Ins(1,3,4,5)P<sub>4</sub>, 11% DL-Ins(1,2,4,5)P<sub>4</sub>, 6% Ins(1,3,4,6)P<sub>4</sub>, 23% DL-Ins(1,2,3,4)P<sub>4</sub>, 10% DL-Ins(1,2,4,6)P<sub>4</sub>, and 3% Ins(1,2,3,5)P<sub>4</sub>.

#### Experiments 4 and 5: white-wheat rolls with added phytate

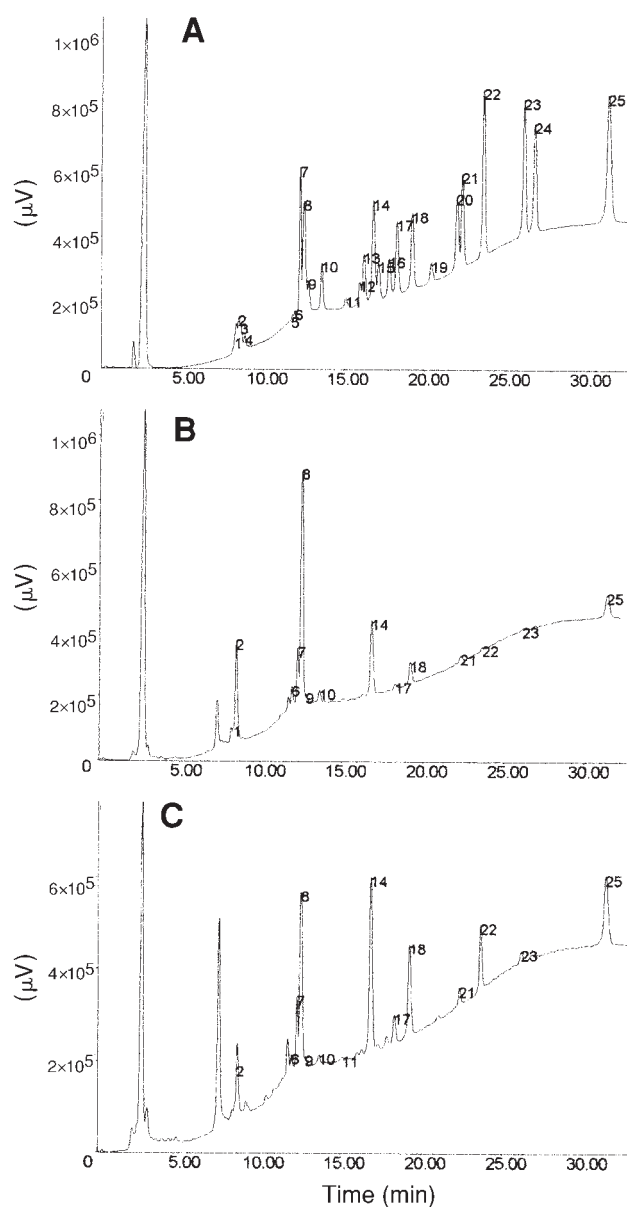
The white-wheat rolls in which sodium phytate was added to the dough before fermentation contained 6.9 and 15.1 mg P as IP<sub>3</sub>, 2.9 and 6.2 mg P as IP<sub>4</sub>, 0.6 and 0.9 mg P as IP<sub>5</sub>, and 1.6 and 2.2 mg P as IP<sub>6</sub> in experiments 4 and 5, respectively, calculated as milligrams phosphorus as inositol phosphates/meal. The chromatographic profile of a white-wheat roll from experiment 5 is shown in **Figure 1B**. The main IP<sub>4</sub> isomers in the white-wheat roll were DL-Ins(1,2,3,4)P<sub>4</sub> (73%), DL-Ins(1,2,5,6)P<sub>4</sub> (19%), and DL-Ins(1,3,4,5)P<sub>4</sub> (4%); the IP<sub>3</sub> isomers Ins(1,2,3)P<sub>3</sub> and DL-Ins(1,2,6)P<sub>3</sub> accounted for more than two-thirds of the total IP<sub>3</sub> isomers. Furthermore, isomeric separation showed that there were almost equal amounts of these 2 IP<sub>3</sub> isomers in the white-wheat rolls (21) (**Figure 2C**). Considerable amounts of less-phosphorylated inositol phosphates were found in the white-wheat rolls.

#### Experiment 6: rye rolls

Three IP<sub>4</sub> isomers that formed after acid-catalyzed hydrolysis of sodium phytate were found in the rye rolls: 29% DL-Ins(1,2,5,6)P<sub>4</sub>, 8% DL-Ins(1,3,4,5)P<sub>4</sub>, and 55% DL-Ins(1,2,3,4)P<sub>4</sub> (**Figure 1C**). Ins(1,2,3)P<sub>3</sub> and DL-Ins(1,2,6)P<sub>3</sub> were present in equal amounts and accounted for 72% of total IP<sub>3</sub> (**Figure 2B**). The latter isomer was used in experiment 3. The rye rolls were also found to contain considerable amounts of less-phosphorylated inositol phosphates.

## Iron absorption

Individual mean iron-absorption ratios (test rolls:control rolls) are given in **Table 1**. In experiment 1, iron absorption was significantly lower (by 39%) from the test roll, to which 10 mg P as IP<sub>5</sub> was added, than from the control roll. Results from a previous study by Hallberg et al (22), who fed subjects a similar meal



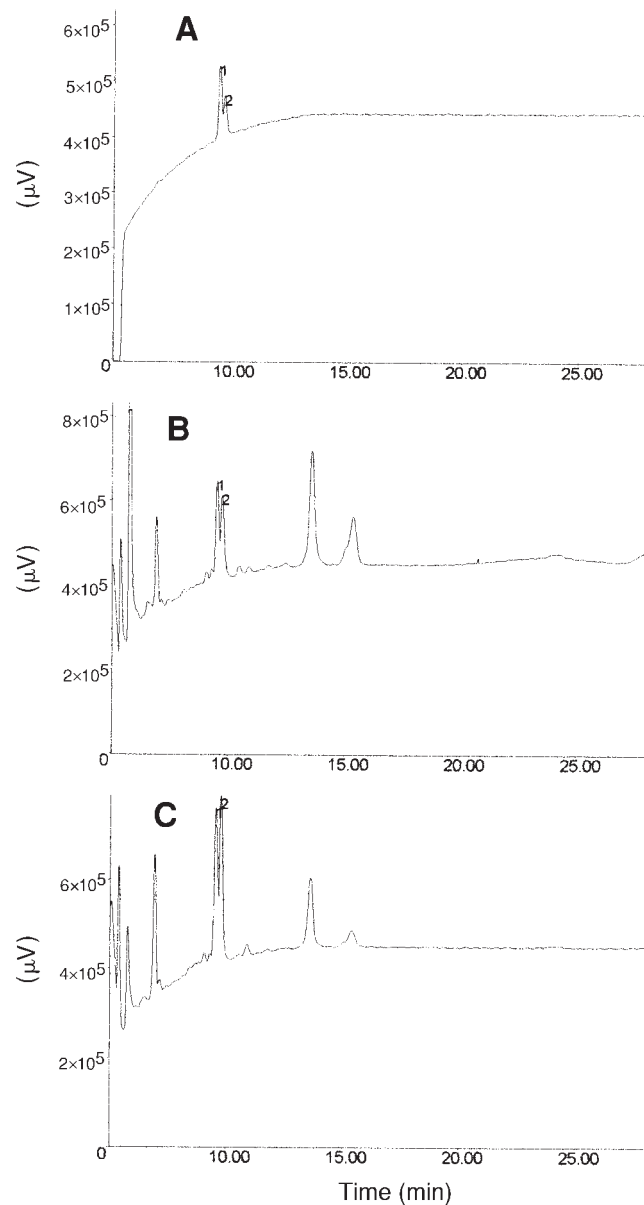
**FIGURE 1.** Chromatographic profiles of hydrolyzed sodium phytate similar to that used to isolate the tetra- and pentaphosphate fractions of inositol phosphate (IP<sub>4</sub> and IP<sub>5</sub>, respectively) (A), the fermented white-wheat roll in which sodium phytate had been added to the dough (B), and a fermented rye roll (C). The samples were analyzed according to the method of Skoglund et al (20) with some improvements (21). Peaks 1: DL-inositol 1,6-diphosphate [DL-Ins(1,6)P<sub>2</sub>]; 2: DL-Ins(1,2)P<sub>2</sub>; 3: DL-Ins(1,4)P<sub>2</sub> and DL-Ins(2,4)P<sub>2</sub>; 4: DL-Ins(4,5)P<sub>2</sub>; 5 and 6: unidentified; 7: DL-inositol 1,2,4-triphosphate [DL-Ins(1,2,4)P<sub>3</sub>], DL-Ins(1,3,4)P<sub>3</sub>, and Ins(1,2,3)P<sub>3</sub>; 8: DL-Ins(1,2,6)P<sub>3</sub> and Ins(1,2,3)P<sub>3</sub>; 9: DL-Ins(1,4,5)P<sub>3</sub>; 10: DL-Ins(1,5,6)P<sub>3</sub>; 11: DL-Ins(4,5,6)P<sub>3</sub>; 12: Ins(1,2,3,5)P<sub>4</sub>; 13: DL-Ins(1,2,4,6)P<sub>4</sub>; 14: DL-Ins(1,2,3,4)P<sub>4</sub>; 15: Ins(1,3,4,6)P<sub>4</sub>; 16: DL-Ins(1,2,4,5)P<sub>4</sub>; 17: DL-Ins(1,3,4,5)P<sub>4</sub>; 18: DL-Ins(1,2,5,6)P<sub>4</sub>; 19: Ins(2,4,5,6)P<sub>4</sub>; 20: DL-Ins(1,4,5,6)P<sub>4</sub>; 21: Ins(1,2,3,4,6)P<sub>5</sub>; 22: DL-Ins(1,2,3,4,5)P<sub>5</sub>; 23: DL-Ins(1,2,4,5,6)P<sub>5</sub>; 24: Ins(1,3,4,5,6)P<sub>5</sub>; and 25: IP<sub>6</sub>.

was significantly lower (by 39%) from the test roll, to which 10 mg P as IP<sub>5</sub> was added, than from the control roll. Results from a previous study by Hallberg et al (22), who fed subjects a similar meal

to which 10 mg P as  $IP_6$  had been added, showed 59% less iron absorption from the test meal than from the control meal. The inhibitory effect on iron absorption of  $IP_5$  was thus less marked than that of  $IP_6$ . No significant effect on iron absorption was found when 10 mg P as  $IP_4$  (experiment 2) or  $Ins(1,2,6)P_3$  (experiment 3) was added to the test roll. In experiments 4 and 5, iron absorption was significantly lower (by 54% and 64%, respectively) from the test roll than from the control roll.

## DISCUSSION

When  $IP_5$  produced via acid-catalyzed hydrolysis was added to a white-wheat roll containing no inhibitors, iron absorption was



**FIGURE 2.** Chromatographic profiles of samples of inositol 1,2,3- and inositol 1,2,6-triphosphate [ $Ins(1,2,3)P_3$ , peak 1; and  $Ins(1,2,6)P_3$ , peak 2; respectively] (A), a fermented white-wheat roll in which sodium phytate had been added to the dough (B), and a fermented rye roll (C) isolated according to the method of Skoglund et al (20) with improvements (21). Peaks 1:  $Ins(1,2,3)P_3$ ; and 2:  $DL-Ins(1,2,6)P_3$ .

impaired, whereas addition of  $IP_3$  and  $IP_4$  had no significant effect. These results agree with those of in vitro studies of the effect of isolated inositol phosphates produced via nonenzymatic hydrolysis on iron solubility at simulated physiologic digestion conditions (12). The capacity of a certain inositol phosphate to bind metal ions was found to be a function of the number of phosphate groups on the molecule (23). If the inhibitory effect on iron absorption was related to the number of phosphate groups present as inositol phosphates, a similar reduction in absorption should have been obtained when isolated  $IP_3$ ,  $IP_4$ ,  $IP_5$ , or  $IP_6$  was added because the same amount of phosphorus as inositol phosphates ( $\approx 10$  mg) was added to the test rolls in experiments 1–3. A similar reduction in absorption was not found. The inhibiting effect on iron absorption was less for  $IP_5$  than that found previously by Hallberg et al (22) for  $IP_6$ , and no significant inhibiting effect on absorption was seen with  $IP_4$  and  $IP_3$ .

Thus, the results suggest that  $\geq 5$  of the 6 possible sites on inositol needed to be phosphorylated for the inositol to exert an inhibitory effect. It is possible that this many phosphate groups are required to form the strong association between mineral ions and inositol phosphates that would be necessary to inhibit iron absorption. The inhibitory effect on absorption was due to the formation of an insoluble precipitate that cannot be absorbed. There is some evidence suggesting that mineral-binding capacity becomes progressively lower when phosphate groups are removed from  $IP_6$  (23, 24). In addition, the complexes formed by these derivatives become more soluble as the number of phosphate groups per molecule decreases (25). Thus, it is conceivable that no effects were observed when  $IP_3$  or  $IP_4$  was added to the test rolls because these inositol phosphates are more soluble and have a lower mineral-binding capacity for inositol phosphates than do  $IP_5$  and  $IP_6$ .

In our previous study of iron absorption from many bread meals containing different amounts of various inositol phosphates, a strong negative correlation was found between the amounts of  $IP_3$ ,  $IP_4$ ,  $IP_5$ , and  $IP_6$  and iron absorption (13), suggesting that these inositol phosphates contribute to the negative effect on iron absorption. In the present study, this effect was shown in experiment 6 from rolls baked from whole-rye flour and white-wheat flour containing 85% of the whole-wheat grain. Iron absorption from these rolls was  $\approx 68\%$  lower than that from the control roll despite the fact that there was only 4.9 mg P as  $IP_5 + IP_6$  remaining after fermentation and baking. These rolls contained large amounts of  $IP_3$  and  $IP_4$  (25.8 mg P), with an approximate molar ratio of  $IP_3 + IP_4$  to iron of 3.9:1. In experiment 5, iron absorption from the white-wheat test roll, which contained a mixture of inositol phosphates in amounts comparable with those in the rolls used in experiment 6 (21 mg P as  $IP_3 + IP_4$  and 3 mg P as  $IP_5 + IP_6$ ) and a molar ratio of  $IP_3 + IP_4$  to iron of 3:1, was 64% lower than that from the control roll. This 64% lower absorption was likely not a result of the  $IP_5$  and  $IP_6$  content of the rolls alone.

The inhibition of nonheme-iron absorption by phytate was shown to be dose dependent (22). This relation between iron absorption and phytate content was composed of 2 regression lines with a point of intersection at 10 mg P as phytate. At this point of intersection, iron absorption was 60% less from the white-wheat test rolls than from the control rolls. On the basis of these regression lines, a test meal containing 3 mg P as  $IP_6$  would be expected to reduce iron absorption by 20–30%.


In addition, we found a 54% lower absorption of iron from the test roll with 10 mg P as  $IP_3 + IP_4$  and a molar ratio of  $IP_3 + IP_4$  to iron of 1:1 than from the control roll (experiment 4). The observed reduction in iron absorption was not a result of the content of 2 mg

P as IP<sub>5</sub> + IP<sub>6</sub> in the roll only or of the content of the isolated form of 10 mg P as IP<sub>3</sub> + IP<sub>4</sub>. In previous absorption studies with a similar design, 2 mg P as IP<sub>6</sub> reduced iron absorption by 18% (22).

Hence, in the present study, IP<sub>3</sub> and IP<sub>4</sub> contributed to the negative effect on iron absorption from the rolls when IP<sub>5</sub> and IP<sub>6</sub> were also present, but not when isolated fractions of the inositol phosphates were added. One explanation for the results could be that certain isomers of IP<sub>3</sub> and IP<sub>4</sub> in processed foods other than those in the isolated fractions may have had an inhibitory effect, ie, the position of the phosphate groups on the inositol molecule influences its mineral-binding capacity and inhibitory effect. The isolated IP<sub>4</sub> fraction added to the rolls in experiment 2 contained a mixture of isomeric forms, whereas one specific isomer of IP<sub>3</sub>, which predominates when sodium phytate is hydrolyzed by baker's yeast, was added to the rolls in experiment 3. Three of the 4 isomers in the isolated IP<sub>4</sub> fraction were found to be formed in the rolls in experiments 4 and 5 and in the rye rolls used in the previous study (experiment 6; 13) as a result of phytate degradation by cereal phytase and yeast. The Ins(1,2,6)P<sub>3</sub> isomer that was added to the rolls in the present study was 1 of the 2 predominant isomers formed in the white-wheat and rye rolls containing high concentrations of IP<sub>3</sub> and IP<sub>4</sub>; the other predominant isomer was Ins(1,2,3)P<sub>3</sub>.

With use of potentiometric titration it was found that Ins(1,2,6)P<sub>3</sub> forms stable complexes with Fe<sup>3+</sup> at a pH of 7.0 (26). The 1,2,3-triphosphate grouping has, as well, been shown to be an orientation forming strong complexes with Fe<sup>3+</sup>. The iron uptake as a function of various isomeric forms of IP<sub>3</sub> and IP<sub>4</sub> in vitro was studied in Caco-2 cells, a highly differentiated enterocyte-like cell line (27). No effect on absorption due to different isomeric forms of inositol phosphates was found. Therefore, it does not seem likely that the isolated forms of the predominant isomers of IP<sub>3</sub> and IP<sub>4</sub> in the rolls had an inhibitory effect on iron absorption.

On the other hand, it is conceivable that a mixture of various inositol phosphates has effects different from those of the pure fractions alone. It is possible that IP<sub>3</sub> and IP<sub>4</sub>, and the less-phosphorylated inositol phosphates (IP<sub>1</sub> and IP<sub>2</sub>) that were found in considerable amounts (although not quantified in this study) in the rolls, contributed to the negative effect on iron absorption by interacting with the higher-phosphorylated inositol phosphates, resulting in an increase in mineral-binding capacity. One iron molecule presumably binds, with a high degree of covalency, 2 or 3 phosphate groups located on 1, 2, or 3 different inositol phosphate molecules. This presumption is strongly supported by the negative effect on iron absorption of the test rolls that contained a mixture of inositol phosphates with small amounts of IP<sub>5</sub> and IP<sub>6</sub> (2 or 3 mg P as inositol phosphates) and large amounts of IP<sub>3</sub> and IP<sub>4</sub> (10 or 21 mg P as inositol phosphates). The observed inhibition of iron absorption from these test rolls was related to the sum of the tri-, tetra-, penta-, and hexaphosphate groups.

In conclusion, addition of IP<sub>5</sub> to white-wheat rolls inhibits iron absorption to a significantly lesser degree than does IP<sub>6</sub>, whereas IP<sub>4</sub> produced via acid-catalyzed hydrolysis and Ins(1,2,6)P<sub>3</sub> does not inhibit iron absorption significantly. Furthermore, the results suggest that IP<sub>3</sub> and IP<sub>4</sub> contribute to the negative effect on iron absorption of processed foods by interacting with the higher-phosphorylated inositol phosphates, resulting in an increase in mineral-binding capacity. These findings suggest that phytate degradation of not only IP<sub>5</sub> and IP<sub>6</sub>, but also of IP<sub>3</sub> and IP<sub>4</sub> in cereals and soy products, for example, must occur in the home or in the manufacturing process to improve the iron absorption of such food products. 

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