

## Distribution of Added Iron and Polyphosphate Phosphorus in Cow's Milk

### Abstract

The distribution of iron and polyphosphate phosphorus added to cow's milk was investigated by both analytical and radiochemical techniques. Whole milk was separated isoelectrically and/or centrifugally into three major fractions, cream, casein, and whey, after the addition of ferripolyphosphate and other iron or polyphosphate compounds. Casein, a phosphoprotein, had a greater affinity for iron binding than for phosphorus binding: 85 to 95% of the iron and 50 to 55% of the phosphorus was bound to acid-precipitated casein; and when the casein was obtained by centrifugation, 60 to 70% of the iron was micellar-bound while 50 to 55% of the phosphorus was bound by micelles.

### Introduction

Cow's milk is nearly an ideal food, but it is deficient in some essential trace elements, notably iron (11). Pla and Fritz (13) emphasized the need to select assimilable sources of iron for fortification of foods. In keeping with this recommendation, Jones et al. (7) investigated the use of ferripolyphosphate as a nutritional supplement for iron fortification of foods and, in addition, as a protein precipitant for recovery of food-quality protein from industrial discharges such as whey, tannery wastes, potato wastes, etc. They also explored the use of ferripolyphosphate-whey protein complex for iron fortification of foods.

Ferripolyphosphate, a soluble complex of ferric ions and a long-chain polyphosphate, was formulated according to the method of Hazel et al. (5). Previous reports have shown that polyphosphate anion (3) and  $\text{Fe}^{+3}$  (2) precipitate proteins from whey at low pH. The use of ferripolyphosphate and ferripolyphos-

phate-whey protein complexes (hereafter referred to as FIPP) for fortification of whole milk was discussed and reported by Jones et al. (8). The same report indicated that feeding FIPP restored hemoglobin of iron-depleted rats with relatively high efficiency (90+%).

Our report describes the distribution in cow's milk of iron and phosphorus derived from ferripolyphosphate, as studied by both standard analytical and radiochemical techniques. For comparison, the distribution of iron and phosphorus from other compounds is included. The distribution of iron in cow's milk after addition of  $\text{Fe}^{59}$  as ferric chloride was studied by King et al. (9), who found that essentially all of the added iron was associated with the skim milk and that casein accounted for most of the activity in the original milk.

### Materials and Methods

*Preparation of ferripolyphosphates.* Ferripolyphosphate (FIP) was prepared in a Waring Blendor<sup>1</sup> by mixing 100 ml ferric chloride, .5 M, with 200 ml Calgon (glassy sodium polyphosphate), 3 M in monomeric  $\text{NaPO}_3$  units. The soluble FIP complex had an Fe/P mole ratio equal to 1/12 and was .167 M in  $\text{Fe}^{+3}$ , containing 9.3 mg Fe/ml.

For radioactive experiments, FIP was prepared singly-labeled with  $\text{Fe}^{59}$  (half-life 45.1 days, specific activity 1 mc/49.5  $\mu\text{g}$  Fe) and singly-labeled with  $\text{P}^{32}$  (half-life 14.3 days, specific activity 1.07 mc/26.6 mg sodium polymetaphosphate). The isotopes were purchased from Amersham/Searle as ferric chloride- $\text{Fe}^{59}$  and sodium polymetaphosphate- $\text{P}^{32}$ .

$\text{Fe}^{59}$ -labeled ferric chloride solution was prepared by diluting the isotope solution (as received) to 10 ml with deionized water and mixing .1 ml of the dilution with 3.0 ml .5 M  $\text{FeCl}_3$ . FIP- $\text{Fe}^{59}$  was then obtained by flash mixing 2.1 ml of labeled  $\text{FeCl}_3$  with 4.0 ml 3 M Calgon.

$\text{P}^{32}$ -labeled Calgon solution was prepared by dissolving approximately 4.6 mg labeled sodium polymetaphosphate in 10 ml deionized  $\text{H}_2\text{O}$  and mixing 120  $\mu\text{l}$  of the solution with 10 ml 3 M Calgon. Four milliliters of Calgon- $\text{P}^{32}$

Received December 10, 1973.

<sup>1</sup> Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

were then flash-mixed with 2 ml .5 M  $\text{FeCl}_3$  to form the desired complex. For one experiment, Calgon- $\text{P}^{32}$  was prepared as above and added directly to whole milk except that 2.0 ml deionized water replaced the  $\text{FeCl}_3$ .

*Addition of ferripolyphosphate to milk.* Nonhomogenized whole milk, pasteurized and raw, was obtained locally from three commercial dairies.

In the nonradioactive experiments, 2 ml of FIP solution (.167 M in  $\text{Fe}^{+3}$ ) were added per liter whole milk (18.6 mg Fe/liter). Variations introduced into the experimental procedure included pasteurization at 60 C for 30 min before or after addition of iron and using aged (15 days old) FIP as well as freshly made FIP. It had been previously demonstrated (7) that aged FIP is a more effective whey protein precipitant than freshly prepared FIP.

FIP- $\text{Fe}^{59}$  was added at 3.3 ml/liter (30.7 mg Fe/liter). In one experiment, iron added as FIP- $\text{Fe}^{59}$  was increased to 111.7 mg/liter by adding 16 ml of FIP- $\text{Fe}^{59}$ .

FIP- $\text{P}^{32}$  was added at 2.0 ml/liter (62 mg P/liter).

Labeled .5 M  $\text{Fe}^{59}\text{Cl}_3$ , 1.1 ml, was added directly to whole milk in one experiment, giving 29.7 mg Fe/liter. Labeled Calgon, diluted to 2 M, was also added directly giving 62 mg P/liter.

Since the amount of iron in milk is small and much less than the minimum daily requirement (about 11 mg Fe per day for an adult), a realistic fortification for dairy products is in the range 10 to 20 mg Fe/liter of fluid milk. In the experiments described here, added iron was selected to fall in the upper part of this range and above.

*Fractionation of the milk.* After stirring for 2 to 3 h at ambient temperature, the iron-supplemented milk was warmed to 40 C, and the cream was separated in a DeLaval cream separator. The skim milk was cooled to ambient temperature, adjusted to pH 4.6 with concd HCl, and filtered. The precipitated casein (also called isoelectric casein) was suspended in water and lyophilized. The whey proteins were precipitated by addition of 80 g  $(\text{NH}_4)_2\text{SO}_4$  to 100 ml whey, collected by filtration, suspended in water, and lyophilized.

Milk fractions were obtained alternatively by centrifugation (9, 10). The cream was removed at low speed ( $900 \times g$  for 20 min at 30 C in an International Model SBV cen-

trifuge), and the skim milk was separated by decantation after the cream was solidified by chilling the centrifuge bottles in an ice bath for about 10 min. Casein micelles were separated at  $25,000 \times g$  for 3 h at 15 C, according to Kitchen et al. (10), or  $36,000 \times g$  for 4 h at 15 C in a Beckman Model L2-65B centrifuge in a type 19 rotor. In all cases, casein fractions were lyophilized before iron content was determined. Continuous dialysis in tap water for 24 h, followed by dialysis in distilled water, did not change the iron content of the isolated fractions when they subsequently were lyophilized and assayed.

The fractionation procedure was the same throughout the radioisotope experiments. After the addition of the radioisotope to 1 liter of whole milk, the low-speed centrifugation method was employed to separate cream from skim milk. Isoelectric casein was obtained by acid precipitation at pH 4.6, and centrifugal casein was obtained by high-speed centrifugation at  $36,000 \times g$  for 4 h at 15 C. The centrifugal whey was acidified to pH 4.6 to precipitate the remaining soluble casein (isoelectric centrifugal casein).

In one experiment aliquots of selected iron salts (ferrous sulfate, ferrous ammonium sulfate, ferric chloride, and ferric ammonium citrate) were added to 4.0-ml samples of commercial whole milk to achieve final concentrations of approximately 40 ppm of iron. After mixing, the samples were centrifuged at 5 C in a Beckman Model L-4 ultracentrifuge in an SW-39 rotor at  $100,000 \times g$  for 25 min. Samples were then frozen; cream, serum, and casein fractions were separated by slicing through the cellulose nitrate test tubes. Cream and casein pellets were hydrolyzed overnight in 5.7 N HCl. Serum samples were acidified with  $\text{H}_2\text{SO}_4$  and centrifuged to remove most of the protein. All samples were diluted to 5.0 ml volumes and analyzed for iron.

*Analytical procedures.* Iron content in each nonradioactive fraction was determined by atomic absorption spectrophotometry (12) or by the AOAC bipyridyl method (1). In the chemical analysis, 5 ml of liquid sample (except the casein and whey protein fractions) were diluted to 25 ml, and the iron content of the sample was determined directly by comparison with appropriate standards. Standards were prepared by adding known quantities of iron to milk fractions having the same composition as the one to be assayed except that they were obtained from whole milk to which no

iron had been added. Dried samples of casein and whey protein (about 1.5 g each) were ashed at 550 C, dissolved in 5 ml concd HCl, and diluted to 25 ml. Ten milliliter aliquots of this solution were diluted to 50 ml and then read in the Perkin-Elmer Model 303 atomic absorption spectrophotometer. Ten milliliter aliquots of the final dilution for each sample were used in the AOAC colorimetric method.

In the radioactive experiments, fractions obtained by isoelectric precipitation and centrifugation methods and samples of each liquid or colloidal fraction were dissolved directly in the scintillator (Aquisol, New England Nuclear) for counting. Samples of the lyophilized proteins were first solubilized with Unisol overnight, and then Complement was added as scintillator (Unisol-Complement System for protein and tissue solubilization, Isolab, Inc.) for radioassay of protein by liquid scintillation in a Nuclear-Chicago Mark I liquid scintillation counter. Radioactivity was expressed as counts per min (cpm). Correction for decay was not needed because samples and standards were always counted together.

#### Results and Discussion

The distribution of added iron in milk when whole milk was fractionated by a DeLaval cream separator, acid precipitation of casein, and salt fractionation of whey proteins is in Table 1. Iron contents of the milk fractions (both in milligrams and as percentage of total added iron) for four experiments are

shown. Each experiment in Table 1 employed slightly different conditions. Experiments 1 and 2 used pasteurized milk, and the use of aged and fresh FIP was compared. Experiments 3 and 4 used raw milk and compared the effect of pasteurization after addition of FIP.

Table 1 shows that recovery of added iron was quantitative and 90% or more of the iron was bound to the casein fraction. The cream fraction contains an average 7% of the iron added, probably due to skim milk in the cream which was incompletely removed by centrifugal separators. This was verified by later experiments of low-speed centrifugation and chilling to separate the cream from the skim milk. The whey fraction contained the least iron. The overall distribution of iron is not appreciably dependent on the age of the FIP, on prior heat treatment of the milk (raw or pasteurized), or on the order of addition of the FIP (before or after pasteurization).

Table 2 shows the iron distribution determined chemically when pasteurized milk was fractionated by centrifugation after addition of iron from aged FIP. In both cases, the same percentage of iron (about 70%) was in the micellar fraction. This value is lower than the 90 to 110% reported by King et al. (9). Since they found 21 to 23% of the added iron (from FeCl<sub>3</sub>) in the centrifuged whey, their total iron recovery was 111 to 133%. A further observation with respect to the iron content of the centrifuged whey is that when the pH

TABLE 1. Distribution of added iron in milk when casein was acid-precipitated.

Milk fraction <sup>a</sup>	1		2		3		4	
	Pasteurized FIP (fresh)		Pasteurized FIP (aged)		Raw FIP (fresh)		Raw FIP (fresh) pasteurized	
	(mg) <sup>b</sup>	(%)	(mg)	(%)	(mg)	(%)	(mg)	(%)
Total Fe added <sup>c</sup>	69.6	100.0	69.6	100.0	29.7	100.0	42.3	100.0
Cream	5.2	7.5	5.2	7.5	1.9	6.4	2.7	6.4
Skim milk	65.6		51.6		20.0		30.4	
Casein	61.8	88.8	63.7	91.5	29.5	99.3	39.2	92.7
Whole whey	.6		2.8		.9		2.0	
Whey proteins <sup>d</sup>	1.5	2.2	.7	1.0	.3	1.0	.3	.7
Supernatant <sup>d</sup>	.4	.6	.3	.4	Trace	...	Trace	...
Total Fe in milk fractions <sup>e</sup>	68.9	99.1	69.9	100.0	31.7	106.7	42.2	99.8

FIP = ferrilpolyphosphate.

<sup>a</sup> Fractions obtained by DeLaval cream separation, acid precipitation of casein, and salt fractionation of whey proteins.

<sup>b</sup> Analyses were by atomic absorption or AOAC method.

<sup>c</sup> Concentration of Fe in all experiments was 18 mg/liter.

<sup>d</sup> Whey proteins and supernatant were fractions of whole whey.

<sup>e</sup> The sum of four fractions which are cream, casein, whey proteins, and supernatant.

TABLE 2. Distribution of added iron in milk when casein was centrifugally precipitated.

Milk fraction <sup>a</sup>	1		2	
	FIP (aged) Centrifuged casein 25,000 × g, 3 h, 15 C		FIP (aged) Centrifuged casein 36,000 × g, 4 h, 15 C	
	(mg) <sup>b</sup>	(%)	(mg)	(%)
Total Fe added	18.6	100.0	30.1	100.0
Cream	1.3	7.0	.5	1.7
Skim milk	15.6		27.8	
Casein	13.2	71.0	20.6	68.4
Whole whey	2.8	15.1	7.9	26.2
Total Fe in milk fractions <sup>c</sup>	17.3	93.1	29.0	96.3

FIP = ferripolyphosphate.

<sup>a</sup> Fractions obtained by low speed centrifugation of cream and high speed centrifugation of skim milk in Beckman Model L2-65B centrifuge.

<sup>b</sup> Analyses by AOAC method.

<sup>c</sup> The sum of the three fractions which are cream, casein, and whole whey.

of this fraction was reduced to 4.6, a precipitate, presumably of soluble casein and noncentrifuged micelles, was obtained. This precipitate contained 4.6 mg of the 7.9 mg Fe reported for whole whey (Table 2, experiment 2). The percent iron in the casein may thus be revalued to 83.5%. Interestingly, .53 and .51 mg Fe/g casein were found for the casein precipitated from centrifuged whey and for the centrifuged micellar casein.

Table 3 shows the percentage distribution of added iron when the additives were ferric or ferrous salts. The oxidative state of iron does not appear to play an important role in its distribution in the milk fractions. Further, the conditions of centrifugation do not seem to influence the amount of iron bound to the casein micelles. Data in Tables 2 and 3 show that about 70% of the added iron is concentrated in the centrifuged casein fraction. King et al. (9), as noted previously, have reported higher values (about 90 to 110%) when Fe<sup>59</sup>Cl<sub>3</sub> was added. They also showed that the results were

TABLE 3. Distribution of iron from four iron salts in major fractions of milk.<sup>a</sup>

Salt	Distribution of iron <sup>b</sup> (% added iron)		
	Cream	Casein	Whey
Ferrous sulfate	4.6	66.8	15.8
Ferrous ammonium sulfate	6.0	70.0	20.0
Ferric chloride	4.6	71.2	14.4
Ferric ammonium citrate	5.8	69.6	29.6

<sup>a</sup> Fractions obtained by centrifugation in Beckman Model L-4 at 100,000 × g for 25 min.

<sup>b</sup> Analyses by atomic absorption.

independent of temperature and length of time the salt was in contact with the milk.

Table 4 shows the distribution of Fe<sup>59</sup> (as ferric chloride and FIP) in milk fractions. After the iron was added and the cream was separated, one portion of skim milk was fractionated by isoelectric precipitation and another portion by centrifugation followed by isoelectric precipitation of the centrifugal whey at pH 4.6. The empirical iron content of each liter of milk is indicated by the numbers in parentheses. Iron in experiment 3 is nearly four times that in experiments 1 and 2. The percentages of iron (71.4 and 63.4 in experiments 1 and 2) in the centrifugal caseins are similar to values for caseins in Table 2 (71.0 and 68.4%). The percentage sum of the two fractions, isoelectric centrifugal casein and isoelectric centrifugal whey, are almost identical to the values for centrifugal whey. Also, the sum of the percentages of iron in the centrifugal casein and whey are similar to values for skim milk reported in each experiment in Table 4. In experiment 3 the percentage of iron in centrifugal casein decreased from about 70 to 10% while the values for centrifugal whey and isoelectric centrifugal casein increased. The quantity of the centrifugal casein in experiment 3 was only about half that in experiment 2. This could indicate that when iron was nearly quadrupled, the formation of casein micelles was inhibited. Acid precipitation of the centrifugal whey (experiment 3) gave precipitates containing 85.6% of the iron activity. The sum of the iron contents of the centrifugal casein and isoelectric centrifugal casein was 95.5% (similar to the sum of 98.6% in experiment 2).

TABLE 4. Distribution of iron-59 in milk fractions.

Milk fraction	1 Fe <sup>59</sup> Cl <sub>3</sub> (29.7 mg Fe) <sup>a</sup>		2 FIP-Fe <sup>59</sup> (30.7 mg Fe) <sup>a</sup>		3 FIP-Fe <sup>59</sup> (111.7 mg Fe) <sup>a</sup>	
	(cpm × 10 <sup>6</sup> )	(%)	(cpm × 10 <sup>6</sup> )	(%)	(cpm × 10 <sup>6</sup> )	(%)
Total Fe <sup>59</sup> added	3.800	100.0	.984	100.0	13.342	100.0
Cream	.116	3.05	.0310	3.15	.458	3.43
Skim milk	3.775	99.3	1.066	108.3	13.778	103.3
Isoelectric casein	n.a.		.867	88.1	9.752	73.1
Isoelectric whey	n.a.		.0990	10.1	1.498	11.2
Centrifugal casein	2.713	71.4	.624	63.4	1.317	9.87
Centrifugal whey	.781	20.6	.443	45.0	12.733	95.4
Isoelectric centrifugal casein <sup>b</sup>	.419	11.0	.346	35.2	11.422	85.6
Isoelectric centrifugal whey <sup>b</sup>	.313	8.24	.0965	9.81	1.582	11.9
Total Fe <sup>59</sup> in milk fractions:						
I <sup>c</sup>	n.a.		.997	101.3	11.708	87.7
II <sup>d</sup>	3.610	95.0	1.098	111.5	14.508	108.7

FIP = ferripolyphosphate.

n.a. — not available.

<sup>a</sup> Empirical iron content per liter milk.<sup>b</sup> Isoelectric centrifugal casein and isoelectric centrifugal whey were fractions of centrifugal whey.<sup>c</sup> I. The sum of the three fractions which are cream, isoelectric casein, and whey.<sup>d</sup> II. The sum of the three fractions which are cream, centrifugal casein, and whey.

The distribution in cow's milk of phosphorus-32 from ferripolyphosphate and Calgon is reported in Table 5. The purpose of this work with P<sup>32</sup> was to study the fate of phosphorus when the Calgon or FIP was added to whole milk and to determine any correlation with the iron distribution in milk fractions (Table 4). About 22% (Table 5, isoelectric centrifugal whey) of the phosphorus

remains apparently unbound to casein, a phosphoprotein, whereas data in Tables 1, 2, and 4 indicate a high affinity of casein for iron binding. In Table 5, the phosphorus recovered in isoelectric casein was approximately the same as that recovered in centrifugal casein (49.5 vs. 48.5% in experiment 1; 55.3 vs. 54.1% in experiment 2). Similarity of results in experiments 1 and 2 indicates that the interaction

TABLE 5. Distribution of phosphorus-32 in milk fractions.

Milk fraction	1 Calgon-P <sup>32</sup>		2 FIP-P <sup>32</sup>	
	(cpm)	(%)	(cpm)	(%)
Total P <sup>32</sup> added	206,514	100.0	205,650	100.0
Cream	8,586	4.16	6,780	3.30
Skim milk	195,980	94.9	193,660	94.2
Isoelectric casein	102,262	49.5	113,812	55.3
Isoelectric whey	76,832	37.2	57,638	28.0
Centrifugal casein	100,127	48.5	111,256	54.1
Centrifugal whey	64,820	31.4	69,930	34.0
Isoelectric centrifugal casein <sup>a</sup>	17,605	8.52	21,743	10.6
Isoelectric centrifugal whey <sup>a</sup>	44,849	21.7	45,059	21.9
Total P <sup>32</sup> in milk fractions:				
I <sup>b</sup>	187,680	90.9	178,230	86.6
II <sup>c</sup>	173,533	84.0	187,966	91.4

FIP = ferripolyphosphate.

<sup>a</sup> Isoelectric centrifugal casein and isoelectric centrifugal whey were fractions of centrifugal whey.<sup>b</sup> I. The sum of the three fractions which are cream, isoelectric casein, and whey.<sup>c</sup> II. The sum of the three fractions which are cream, centrifugal casein, and whey.

of phosphate with milk was not affected by iron.

Proteins in milk, other than casein, which bind iron have been reported. Lactoferrin (4), catalase, peroxidase, and xanthine oxidase (6) may account for some of the iron recovered in the whey fractions. However, the low concentrations of these proteins in milk would preclude their importance in binding iron to the extent reported here. When FIP is added to whey alone, a precipitate of the principal whey proteins containing up to 15% iron can be obtained (7). Of course, much more FIP is added to induce the precipitation of whey proteins (~2 g Fe/liter whey).

In summary, when FIP was added to milk, about 90% of the added iron was bound to the acid-precipitated casein in a form which was not dialyzable, and about 50% of the added phosphorus was bound to the isoelectric casein. When the casein was obtained by centrifugation, about 70% of the added iron and about 50% of the added phosphorus were bound to micelles. These values were increased to about 85 and 60% for the added iron and phosphorus if the casein of centrifugal whey was removed by acid precipitation. Results with FIP were not different from those with other ferrous or ferric salts when centrifugation was the method for fractionating milk. In addition, results of experiments with Fe<sup>59</sup> are not inconsistent with results of King et al. (9) using Fe<sup>59</sup> as ferric chloride. The experiments with P<sup>32</sup> show that caseins bind less phosphorus than iron.

#### References

- (1) Association of Official Agricultural Chemists. 1965. Official methods of analysis, 10th ed. 192-13.011.
- (2) Block, R. J., and D. Bolling. 1955. U.S. Pat. 2,710,858. June 14, Borden Co.
- (3) Gordon, W. G. 1945. U.S. Pat. 2,377,624. June 5, Smith, Kline, and French Laboratories.
- (4) Groves, M. L. 1971. Minor milk proteins and enzymes. Page 367 in *Milk proteins*, vol. 2. H. A. McKenzie, ed. Academic Press, Inc., New York.
- (5) Hazel, J. F., W. H. McNabb, and M. K. McElroy. 1968. U.S. Pat. 3,403,971. October 1, Research Corp.
- (6) Jenness, R., and S. Patton. 1959. *Principles of dairy chemistry*. John Wiley and Sons, Inc., New York.
- (7) Jones, S. B., E. B. Kalan, T. C. Jones, and J. F. Hazel. 1972. Ferripolyphosphate as a whey protein precipitant. *J. Agr. Food Chem.* 20:229.
- (8) Jones, S. B., E. B. Kalan, T. C. Jones, J. F. Hazel, L. F. Edmondson, and A. N. Booth. 1971. Composition and properties of ferripolyphosphate-whey protein powders. 162nd Amer. Chem. Soc., Abstr. Papers, Agr. Food Chem. Div., Abstr. AGFD 13.
- (9) King, R. L., J. R. Luick, I. I. Litman, W. G. Jennings, and W. L. Dunkley. 1959. Distribution of natural and added copper and iron in milk. *J. Dairy Sci.* 42:780.
- (10) Kitchen, B. J., G. C. Taylor, and I. C. White. 1970. Milk enzymes — their distribution and activity. *J. Dairy Res.* 37:279.
- (11) Murthy, G. K., and U. S. Rhea. 1971. Cadmium, copper, iron, lead, manganese, and zinc in evaporated milk, infant products, and human milk. *J. Dairy Sci.* 54:1001.
- (12) Perkin-Elmer Corp. 1971. *Analytical methods for atomic absorption*. Norwalk, Conn.
- (13) Pla, G. W., and J. C. Fritz. 1970. Vitamins and other nutrients: Availability of iron. *J. Ass. Offic. Anal. Chem.* 53:791.