Of all the enzymes assayed, the peroxidases were the most heat sensitive. After dry heat at 110°, only a trace of activity was observed. LAP showed one active band up to 120° in the dry-roasted seed. Results from *in vitro* heating of the dormant peanut extracts also indicated that this particular band was the most heat stable of the four bands and retained very weak activity at 65° (Thomas and Bright, 1972). This band reproducibly stains more densely than the remaining three bands, suggesting that the enzyme plays a dominant role in peptidase activity.

Reasons for the nonspecific staining in soluble protein extracts tested for LAP activity and counterstained with bromophenol blue stain are somewhat obscure. The diffused nonspecific banding appeared more densely stained with bromophenol blue after the gel was immersed in LAP stain (Figure 6B, arrow 1). The bands were also observed in acid phosphatase stain in acetate buffer (pH 4.6), and in α-EST stain (pH 6.0), but not consistently.

In summary, the electrophoretic separations of general proteins in starch gels reported here showed less difference in banding patterns after wet heat than after dry heat. The enzyme data suggested that dry seeds give better heat protection to these functional proteins than do imbibed seeds. Some of the isoenzymes were differentially sensitive to heat, suggesting possible differences in both their structure and function.

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Effect of Processing on Availability of Iron Salts in Liquid Infant Formula Products. Experimental Milk-Based Formulas

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Pilot plant batches of liquid milk-based infant formula were prepared without iron and with eight iron salts added. Portions of the formulas containing three of the iron salts were frozen rather than sterilized. These three salts were also added to lyophilized formula processed without iron. Iron availability was calculated from the hemoglobin responses of anemic rats fed measured amounts of lyophilized formula added to a milk-free basal diet. The relative iron availability of ferrous sulfate incorporated into the formula in these three ways was 114 to 129, expressed as a percentage of the hemoglobin response to standard ferrous sulfate added to the milk-free basal diet. Sterilization increased the relative iron availability of ferric pyrophosphate from 75 to 125, and of sodium iron pyrophosphate from 40 to 60. Formulas containing five other iron salts had relative iron availabilities of 118 to 148. Milk-based formulas containing ferrous sulfate and produced in production equipment had relative iron availabilities of 136 to 143.

The relative availability of the iron of various iron salts, when added to milk or to processed milk-based liquid products, is controversial. For instance, Niccum *et al.* (1953) added either ferrous sulfate or ferric ammonium citrate to high-protein milk-based formulas fed to infants and found higher hemoglobin values in those infants fed the formula supplemented with ferrous sulfate. In contrast, Pla *et al.* (1971) reported that the iron of ferric ammonium citrate added to milk before or after pasteurization was utilized as well as standard ferrous sulfate by iron-depleted rats and chicks.

These conflicting reports suggest that many factors affect the biological availability of iron in milk and in processed milk-based liquid products. Duration of exposure of the iron salt to the liquid milieu is a factor. Hodson (1970) reported chemical evidence that ferric orthophosphate in liquid dietaries dissolves over a period of time and the iron therein is reduced to the ferrous state during a 2- to 5-month storage period. An effect of processing itself is suggested by our previous report that processing liquid soy isolate infant formula products markedly increased the biological availability of the iron of ferric pyrophosphate and sodium iron pyrophosphate, two sources of iron found to have mediocre or poor availability when added as dry salts to a basal diet (Theuer *et al.*, 1971).

In the present study we determined the availability of the iron of eight iron salts incorporated into a milk-based infant formula. With three of these salts, we also attempted to separate the effects of the two major steps in the processing of a liquid infant formula (physical mixing of ingredients and heat sterilization) on the availability of

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Table I. Hemoglobin and Hematocrit Responses and Gastrointestinal Organ Weights of Anemic Rats Fed Graded Levels of Iron as Ferrous Sulfate

Added iron, mg/day	0.00	0.10	0.20	0.30	F[a]	Significant difference[b] $p < 0.05$	$p < 0.01$
Hemoglobin increase, g/100 ml	−0.05 ± 0.6[c]	4.0 ± 0.8	7.1 ± 0.7	8.7 ± 1.3	180	1.1	1.4
Hematocrit increase, % P.C.V.	0.5 ± 2	15 ± 3	23 ± 2	27 ± 3	192	3	4
Gastrointestinal organ weights[d]							
Stomach, mg	345 ± 26	347 ± 32	341 ± 37	346 ± 25	0.34	37	46
Small intestine, mg	1446 ± 194	1373 ± 122	1253 ± 118	1242 ± 111	9.89	172	214
Cecum, mg	174 ± 80	188 ± 53	160 ± 29	194 ± 44	0.75	67	83
Colon plus rectum, mg	230 ± 51	239 ± 38	276 ± 27	217 ± 60	0.34	56	70
Final body weight, g	208 ± 28	234 ± 14	244 ± 15	236 ± 15	6.60	23	29

[a] Statistical significance is as follows: $F > 2.88$, $p < 0.05$; $F > 4.41$, $p < 0.01$. [b] Significant differences were calculated by Tukey's Procedure (Steel and Torre, 1960). [c] Mean given with standard deviation. [d] Dry basis.

iron by taking portions before as well as after sterilization. Iron availability was determined by measuring hemoglobin regeneration in iron-depleted rats. Ferrous sulfate added to a milk-free basal diet served as the standard.

MATERIALS AND METHODS

Preparation of Milk-Based Formulas. A sufficient amount of experimental concentrated milk-based infant formula (134 kcal/100 ml) was homogenized and separated into nine batches. Various iron salts were added and homogenized into eight of the batches to provide 25 mg of iron per liter. Product was filled into 390-ml (13 fl oz) cans. A sufficient number of cans of the three batches containing sodium iron pyrophosphate, ferric pyrophosphate, and ferrous sulfate were segregated and frozen. The remaining cans of formula were then sterilized by standard commercial techniques in a pilot plant. A sufficient amount of each batch of formula was lyophilized 9 days after preparation and subsequently incorporated into one or more experimental diets.

The average proximate composition of the concentrated formula was: protein, 3.0%; fat (soy, corn, and coconut oils), 7.5%; carbohydrate (lactose), 14%; and ash, 0.68%. The iron salts incorporated into each portion were those studied previously in soy isolate infant formulas (Theuer *et al.*, 1971) and are identified in Tables II and III. Lyophilized experimental product O made without added iron contained 0.58 mg of iron per 100 g on a dry basis.

Table II. Effect of Processing on Iron Availability in Milk-Based Infant Formula Products

Iron salt added	Experimental formula fed[a]	Formula sterilized with added iron	Iron from added iron salt, mg/day	Hemoglobin increase, g/100 ml	Hematocrit increase, % P.C.V.	Relative iron availability[b]
None	Product O		0	0.3 ± 0.8[c]	3 ± 3	
Ferrous sulfate	Product O[d]	No[d]	0.234	8.7 ± 1.1	26 ± 2	126
	Product A	No	0.236	8.2 ± 0.7	27 ± 2	114
	Product A	Yes	0.248	9.1 ± 1.3	27 ± 3	129
	Commercial formula[e]	Yes	0.137	6.7 ± 1.0	24 ± 2	138
	Product MJ 3218-C[f]	Yes	0.250	9.4 ± 0.7	30 ± 2	136
	Product MJ 3224-C[f]	Yes	0.181	8.1 ± 0.8	28 ± 2	143
Ferric pyrophosphate	Product O[d]	No[d]	0.186	5.4 ± 1.3**[g]	19 ± 3**	78
	Product B	No	0.215	5.6 ± 1.4**	19 ± 3**	71
	Product B	Yes	0.196	7.8 ± 1.0	24 ± 2	125
Sodium iron pyrophosphate	Product O[d]	No[d]	0.236	3.8 ± 0.8*	17 ± 2**	42
	Product C	No	0.212	3.4 ± 1.3	15 ± 2	39
	Product C	Yes	0.130	3.2 ± 0.8	13 ± 3	60

[a] Two grams of lyophilized product were fed daily. [b] Percent of ferrous sulfate added to the milk-free basal diet. [c] Mean given with standard deviation. [d] The added iron salt was dry-blended into lyophilized Product O. [e] Enfamil with Iron Concentrated Liquid, Mead Johnson Laboratories, Evansville, Ind., a milk-based iron-fortified concentrated infant formula containing lactose and corn, oleo, and coconut oils. [f] Experimental milk-based iron-fortified concentrated infant formulas. [g] Asterisks indicate that the mean for rats fed the unsterilized product or the Product O with iron was statistically significantly different from the mean for rats fed the product sterilized with added iron by "t" test (Steel and Torre, 1960); *, $p < 0.05$; **, $p < 0.01$.

Table III. Availability of Iron in Milk-Based Infant Formula Products Made with Various Iron Salts

Iron salt added	Experimental formula fed[a]	Iron from added iron salt, mg/day	Hemoglobin increase g/100 ml	Hematocrit increase, % P.C.V.	Relative iron availability[b]
Ferrous citrate	Product D	0.250	10.0 ± 0.8[c]	30 ± 2	148
Ferric citrate	Product E	0.306	10.0 ± 0.5	32 ± 2	122
Ferric gluconate	Product F	0.215	8.7 ± 0.9	28 ± 3	139
Ferrous lactate	Product G	0.178	7.2 ± 0.8	26 ± 3	118
Ferric glycerol phosphate	Product H	0.196	8.2 ± 0.9	28 ± 2	135

[a] Two grams of lyophilized product were fed daily. [b] Percent of ferrous sulfate. [c] Mean given with standard deviation.

Sufficient amounts of three commercially processed iron-fortified concentrated milk-based infant formulas of the same proximate composition as the experimental formula also were lyophilized and evaluated in this study. These products contained 17–25 mg of iron per liter by analysis, as ferrous sulfate.

Animals and Diets. The rats and diets used have been described previously (Theuer *et al.*, 1971). The low-iron lactose-free diet was fed for 39 days, at which time hemoglobin levels averaged 4.5 g/100 ml. Rats with hemoglobin levels from 2.9 to 5.7 g/100 ml were selected for the hemoglobin regeneration portion of this study. Each animal was fed a weighed 10 g portion of the diet daily in the four-week hemoglobin regeneration portion of the study. Standard supplements of ferrous sulfate were 0, 0.1, 0.2, and 0.3 mg of iron, as $FeSO_4 \cdot 7H_2O$, in 10 g of the basal diet. The animals in the other groups received 8 g of basal diet and 2 g of lyophilized milk-based formula. The formulas studied are shown in Tables II and III. The treatment of the animals, methods, and calculation of relative iron availability have been described (Theuer *et al.*, 1971).

RESULTS AND DISCUSSION

Response to Standard Ferrous Sulfate. There was a significant ($p < 0.01$) increase in hemoglobin response with each increase in iron intake from 0 to 0.3 mg daily (Table I). The highest level of standard ferrous sulfate provided 0.3 mg of iron daily, which was more iron than was contributed by 2 g of all the lyophilized milk-based formulas but one. The relative availability of the iron in all of the lyophilized formulas was estimated from the standard hemoglobin response curve, with extrapolation to provide for the four groups of animals whose hemoglobin responses were greater than that of the animals fed the highest level of standard ferrous sulfate. Gastrointestinal organ weights responded to iron supplementation as reported previously (Theuer *et al.*, 1971), with small intestine dry weight being 15% higher in the most anemic animals.

Processing and Iron Availability in Infant Formulas. When ferrous sulfate was added in the dry state to Product O made without added iron, iron availability was 126% of that of standard ferrous sulfate added to the milk-free basal diet (Table II). The relative availability of iron from ferrous sulfate in unsterilized Product A was calculated to be 114%, compared to 129% for fully processed Product A. The hemoglobin increase of animals fed unsterilized Product A was slightly less than those of the animals fed either sterilized Product A or Product O supplemented with ferrous sulfate, but hematocrit responses were virtually identical for the three groups. The commercially processed milk-based infant formulas containing ferrous sulfate also had relative availabilities substantially greater than 100% of standard ferrous sulfate (Table II).

Product B contained ferric pyrophosphate. Rats fed the supplement of sterilized Product B had significantly ($p < 0.01$) greater hemoglobin increases and hematocrit increases than rats fed unsterilized Product B or Product O to which ferric pyrophosphate was added in the dry state (Table II). Heat sterilization increased the availability of the iron of ferric pyrophosphate in milk-based formula from about 75% up to 125% of standard ferrous sulfate. In a soy isolate formula, the relative availability of the iron of ferric pyrophosphate was increased from 39 to 93 by processing (Theuer *et al.*, 1971).

Product C contained sodium iron pyrophosphate. The hemoglobin responses of animals fed lyophilized Product O to which sodium iron pyrophosphate had been added, unsterilized Product C, and sterilized Product C are shown in Table II. Processing increased the availability of the iron of sodium iron pyrophosphate in this milk-based formula from 40 to 60. We previously reported that the relative availabilities of the iron of sodium iron pyrophosphate in a soy isolate formula were increased from 15 to 66 by processing (Theuer *et al.*, 1971).

The formulas were lyophilized 9 days after preparation, allowing little time for the physical and chemical changes in iron that Hodson (1970) found in a liquid dietary evaluated after 0, 1, and 2 months of storage. Consequently, heat sterilization of the milk-based formulas containing ferric pyrophosphate and sodium iron pyrophosphate increased iron availability substantially and rapidly.

Availability of Iron in Formulas Made with Other Salts. The hemoglobin responses of anemic rats fed supplements of lyophilized Products D through H are shown in Table III. Products processed with ferrous citrate, ferric citrate, ferric gluconate, ferrous lactate, and ferric glycerol phosphate had iron availabilities greater than 100. The values are greater than those observed previously when these salts were incorporated into soy isolate infant formula products (Theuer *et al.*, 1971).

Availabilities of 114 to 129% of standard ferrous sulfate for the iron in a milk-based formula made with ferrous sulfate appear high but are consistent with other observations in this study which indicate that adding 2 g of lyophilized milk-based infant formula to 8 g of basal diet improves iron availability. For instance, animals fed 0.25 mg of iron daily from lyophilized formulas with added ferrous sulfate had greater hemoglobin responses than animals fed 0.3 mg of iron as ferrous sulfate added to the basal diet. The hemoglobin and hematocrit responses to graded levels of ferrous sulfate added to the basal diet were virtually identical to those reported previously (Theuer *et al.*, 1971). In this previous report we demonstrated that the availability of ferrous sulfate processed in a soy isolate infant formula was essentially the same as that of standard ferrous sulfate (Theuer *et al.*, 1971), indicating that infant formulas *per se* are not responsible for this effect. This suggests that milk solids are responsible, possibly because of lactose. It has long been recognized that lactose increases the absorption of mineral elements, including, for instance, calcium and strontium (Wasserman and Lengemann, 1960). Amine and Hegsted (1971) reported larger cecas in the gastrointestinal tract of anemic rats fed a skim milk diet compared to anemic rats fed a casein diet.

The hypothesis that milk solids or lactose improves iron absorption makes our present findings consistent with those of Fritz *et al.* (1970). They reported that the relative availabilities of the iron of ferrous sulfate dissolved in skim milk or evaporated milk were 95 and 110%, respectively, of the iron of ferrous sulfate added to their basal diet containing 40% dried skim milk. Our basal diet, in contrast, contains no milk and no added lactose. If our results for iron in sterilized and unsterilized Product A containing ferrous sulfate are expressed relative to ferrous sulfate added in the dry state to Product O, the relative availabilities of the ferrous sulfate in milk-based formula become 102 and 90%, respectively, virtually the same as those found by Fritz *et al.* (1970) for ferrous sulfate in milk.

The high availability of iron in the commercially processed formulas evaluated in this test situation is consistent with clinical findings. Gross (1970) reported that infants 3 to 5 months of age absorbed 40 to 54% of the iron when this milk-based formula contained 3 mg of iron as ferrous sulfate per liter (1.4 mg of iron per 640 kcal). Fritz *et al.* (1970) calculated that anemic rats absorbed 53% of the iron of standard ferrous sulfate added to their milk-containing basal diet.

Forty to fifty percent absorption of iron from milk-based infant formulas by infants and anemic test animals indicates that these dietary sources of iron are essentially different from other food sources of iron, for which only 10% absorption is assumed (NAS–NRC, 1968) and is usually found (Council on Foods and Nutrition, 1968). This substantial advantage of formula iron is further supported

by comparing the report of Fritz *et al.* (1970) which says that the iron in enriched breakfast cereal and enriched flour had relative availabilities of 43 to 32%, respectively, with the present findings that the iron in commercially processed milk-based liquid infant formulas has an availability of 138 to 143%. This suggests a substantially lower dietary iron requirement when the dietary iron source is an iron-fortified processed infant formula, compared to when it is other food sources of iron.

With information on the biological availability of a variety of iron salts in processed infant formulas now available, it can be concluded that iron salts other than ferrous sulfate can be used in infant formula products and other milk-based and soy isolate liquid nutrition products with the assurance that they furnish absorbable iron.

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trans-2-Nonenal: Coffee Compound with Novel Organoleptic Properties

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A compound which imparts a fresh-brewed woody character to roasted and ground coffee was isolated by gas chromatography and shown to be *trans*-2-nonenal. Organoleptic evaluation was utilized extensively during the fractionation to follow the desired component. The organoleptic properties of 2-nonenal in soluble coffee and in water are described and the importance of this compound to the flavor of other natural materials is discussed.

Although the volatile composition of coffee has been studied for many years, great strides have been made in the last few years. The advent of new instrumental methods of analysis and the reduction in required sample size have led to the identification of an impressive list of chemicals (Friedel *et al.*, 1971; Gianturco, 1967; Stoffelsma *et al.*, 1968; Stoll *et al.*, 1967), which extends our knowledge of the chemistry of coffee.

For some time, coffee tasting experts have agreed that there is a note in brewed coffee, absent in soluble coffee, which contributes an effect described as "woody." None of the previously identified coffee aroma chemicals possess this characteristic. This note is found in steam distillates of roasted coffee beans; however, the complexity of coffee aroma concentrates hampered earlier efforts to characterize and isolate the compound responsible. In the course of our efforts to identify this woody flavor in coffee aroma we became aware that a similar flavor note existed in the volatile fraction of bell peppers. Since the volatiles of peppers are a much less complex material than roasted coffee, initial efforts were directed toward isolating this note from a distillate of bell peppers. After the compound responsible for the "woody" effect had been identified in peppers, similar techniques were used in the present study on coffee. This report describes the isolation and characterization of the compound responsible for the "woody" flavor in coffee and gives a description of its organoleptic properties.

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PROCEDURE

Preparation of Aroma Concentrate. Eighteen kilograms of coarsely ground roasted Arabica coffee beans were steam distilled at atmospheric pressure. A total of 13 l. of aqueous distillate was collected and this was extracted with a total of 10 l. of diethyl ether in small portions. The ethereal extract was concentrated by distillation through a 20-cm Vigreux column to a volume of about 5 ml.

Separation Scheme. Gas chromatography was used throughout in the purification of the desired component.

A preliminary crude separation was made by preparative scale gas chromatography using an Aerograph Autoprep Model 713 equipped with a flame ionization detector. Gas chromatographic collections were made on an equal time basis as described previously (Parliment, 1971) using the Aerograph 5-ml collector bottles and collecting at 2-min intervals. Gas chromatographic conditions are described in Table I, Purification Step No. 1. Each of the fractions obtained was evaluated by mixing a small quantity of the trapped material in soluble coffee and the samples were tasted. By this procedure, the desired effect was shown to be associated with the gas chromatographic fraction which eluted at 7 to 9 min. This fraction was still quite complex, as shown by rechromatography on other gas chromatographic columns containing dissimilar liquid phases.

By trial and error it was shown that rechromatography on three successive liquid phases was required to yield the chemically pure component with the desired effect. The procedures used for each of these three further purification steps were similar. In each case the separation was