Chemical characteristics and relative bioavailability of supplemental organic zinc sources for poultry and ruminants[1,2]

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ABSTRACT: Eight commercially available organic Zn products and reagent-grade $ZnSO_4 \cdot 7H_2O$ (Zn Sulf) were evaluated by polarographic analysis, and solubility in .1 *M* K_2HPO_4-KH_2PO_4 buffer (pH 5), .2 *M* HCl-KCl buffer (pH 2), and deionized water. Fractions from these solubility tests were evaluated by gel filtration chromatography for structural integrity. Degree of chelation was generally positively related to chelation effectiveness determined by polarography. The organic sources were Zn methionine complex A (Zn MetA), Zn methionine complex B (Zn MetB), Zn polysaccharide complex (Zn Poly), Zn lysine complex (Zn Lys), Zn amino acid chelate (Zn AA), Zn proteinate A (Zn ProA), Zn proteinate B (Zn ProB), and Zn proteinate C (Zn ProC). Three experiments were conducted to estimate the relative bioavailability of Zn from the organic Zn supplements for chicks and lambs when added at high dietary levels to practical diets. Bone Zn concentration increased ($P < .001$) as dietary Zn increased in both experiments. When Zn Sulf was assigned a value of 100% as the standard, multiple linear regression slope ratios of bone Zn from chicks fed 3 wk regressed on dietary Zn intake gave estimated relative bioavailability values of 83 ± 14.6 and 139 ± 16.9 for Zn AA and Zn ProA, respectively, in Exp. 1 and 94 ± 11.6, 99 ± 8.8, and 108 ± 11.4 for Zn Poly, Zn ProB, and Zn ProC, respectively, in Exp. 2. In Exp. 3, 42 lambs were fed diets containing Zn Sulf, Zn ProA, Zn AA, or Zn MetB for 21 d. Based on multiple linear regression slope ratios of liver, kidney, and pancreas Zn and liver metallothionein concentrations on added dietary Zn, bioavailability estimates relative to 100% for Zn Sulf were 130, 110, and 113 for Zn ProA, Zn AA, and Zn MetB, respectively. Except for Zn ProA, which was greater, the organic Zn supplements had bioavailability values similar to that of Zn Sulf for chicks and lambs. Bioavailability of organic Zn products was inversely related to solubility of Zn in pH 5 buffer in chicks ($r^2 = .91$) and pH 2 buffer in lambs ($r^2 = .91$), but not to an estimate of degree of chelation.

Key Words: Bioavailability, Chick Production, Lambs, Solubility, Zinc

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Introduction

Some researchers (Spears, 1989; Wedekind et al., 1992) have reported greater bioavailability for organic Zn sources than that observed for inorganic forms, including Zn oxide and Zn sulfate; consequently, organic forms of the element have been used with increasing frequency by the feed industry. In general, variable bioavailability values have been reported with the trace mineral chelates and complexes, indicating no advantage to the use of organic forms of this element (Ammerman et al., 1995).

One of the characteristics considered important to the physiological function of chelated and complexed metals is the degree to which the organic ligands remain bound to the metal under physiological pH conditions. Unfortunately, neither the Association of Official Analytical Chemists (AOAC, 1995) nor the Association of American Feed Control Officials (AAFCO, 2000) has definitive methods to test the degree of chelation or complexation of the mineral element to organic ligands in commercial samples. Several different chemical methods have been used to evaluate strength of organic

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mineral chelation. Only three reports were found in the literature that evaluated any of these techniques in conjunction with results from animal trials (Kerley and Ledoux, 1992; Matsui et al., 1996) or tissue cultures (Beutler et al., 1998).

The main objective of our studies was to evaluate eight commercial organic Zn sources in the laboratory and correlate these data with relative bioavailability estimates for Zn obtained using tissue Zn concentrations following high dietary additions of Zn in both chicks and lambs. In addition, synthesis of metallothionein (**MT**) in several tissues in response to Zn over time was investigated as a functional response variable to use as a bioassay criterion.

Materials and Methods

Characterization of Zn Sources

Eight commercial organic Zn products from several manufacturers and reagent-grade inorganic $ZnSO_4 \cdot 7H_2O$ (**Zn Sulf**) were evaluated in the laboratory. Organic sources included four complexes: Zn methionine A (**Zn MetA**), Zn methionine B (**Zn MetB**), Zn polysaccharide (**Zn Poly**), and Zn lysine (**Zn Lys**); and four chelated products: Zn amino acid chelate (**Zn AA**), Zn proteinate A (**Zn ProA**), Zn proteinate B (**Zn ProB**), and Zn proteinate C (**Zn ProC**). All sources were obtained from independent distributors, rather than the manufacturers of the products. The Zn Poly was a sequestered mineral product using dried kelp as a source of organic ligands.

Mineral and Nitrogen. Except for P, mineral concentrations in Zn sources were determined by flame atomic absorption spectroscopy (Model 5000 with AS-50 autosampler, Perkin-Elmer, Norwalk, CT; Perkin-Elmer, 1982). Phosphorus was determined by a colorimetric method (Harris and Popat, 1954). Approximately 1 g of each Zn source was weighed in triplicate and dried at 105°C for 12 h, ashed at 550°C for 16 h, solubilized in HCl, and filtered through ashless Whatman 42 paper.

Samples for N analysis were digested in duplicate with sulfuric acid and hydrogen peroxide at 420°C for 45 min in 250-mL calibrated tubes using the Technicon BD-20 system (Technicon Industrial Systems, Tarrytown, NY). Digestates were analyzed for N content on a Technicon AutoAnalyzer II (Technicon Industrial Systems.

Polarographic Analysis. The polarography study was conducted with a hanging mercury drop electrode (Ag/AgCl reference electrode; Bioanalytical Systems, West Lafayette, IN). Saturated Zn solutions for each organic Zn source were prepared in 50 mL of deionized H_2O, and final pH was measured. To prepare saturated solutions, the Zn products were added to the H_2O until excess was observed. The solution was stirred overnight, then osmotic pressure was measured. More Zn product was added and stirring continued for 4 h, and osmotic pressure was measured again. This procedure was repeated until no further change in osmotic pressure was observed. Total molar concentration of all dissolved substances, including cations, anions, and neutral molecules, was measured by vapor pressure osmometry with a Wescor model 5500 osmometer (Wescor, Inc., Logan, UT). The saturated solution was diluted 1:100 in pH 6.0, .1 *M* 4-morpholinoethanesulfonic acid, a noncomplexing buffer for anaerobic electrochemical measurements (N_2 purge). Molar metal concentration was determined from the cathodic wave height (.1 *M* Zn sulfate standard). The shift in half-wave potential, $\Delta E_{1/2}$, was measured and used to calculate the formation quotient, Q_f, which is a quantitative measure of chelation effectiveness (Holwerda et al., 1995). The principle is that the applied potential E (voltage) is scanned to negative values until a current is drawn from the reduction of Zn^{2+} ions to amalgamated Zn. When a chelating or complexing agent is added to aqueous Zn sulfate, an uncomplexed metal salt, the entire voltametric wave is shifted to more negative potential. This shift, quantified through the parameter $\Delta E_{1/2}$, can be understood by realizing that strongly bound ligands enhance the difficulty of reducing Zn(II) to Zn(Hg), in which all complexation or chelation is lost. Theoretically, the more positive the $\Delta E_{1/2}$, the more stable a chelate.

Zinc and Nitrogen Solubility in Neutral Ammonia Citrate, Citric Acid, HCl, and Deionized H_2O. Zinc and N solubility of all Zn sources was determined in duplicate by mixing an approximately .1-g sample with 100 mL of neutral ammonia citrate (**NAC**), 2% citric acid (**CA**), .4% HCl, or deionized H_2O. Mixtures were stirred constantly while incubating at 37°C for 1 h and then filtered through Whatman 42 filter paper (Watson et al., 1970). Filtrates were analyzed for N (except for the NAC solutions) and Zn content after proper dilution with deionized H_2O. The Zn and N in the filtrates were assumed to be soluble, and the values obtained were expressed as a percentage of the total Zn or N in the source.

Soluble and insoluble fractions of Zn and N in the Zn sources were also determined in deionized H_2O as described by Leach and Patton (1997). A 2.0-g sample of each source was weighed in quadruplicate into 250-mL Nalgene bottles. Samples were mixed with 150 mL of deionized H_2O, shaken at 25°C in a water bath for 30 min, and then passed through Whatman 541 filter paper into 200-mL volumetric flasks and rinsed with deionized H_2O to bring the flask to volume. Duplicate samples of filter paper or filtrate were used for both Zn and N as described previously, except that N was determined on an Alpkem 300 Series Autoanalyzer (Alpkem Corp., Clackamas, OR 97015). These values are presented by weight rather than percentage of total Zn and N in the source so that Zn:N ratios may be calculated for the original sources and both fractions as suggested by Leach and Patton (1997).

Solubility in pH 2 and pH 5 Buffers. Solubility of all Zn sources was also measured at concentrations of .125, .25, .50, 2.5, and 12.5 mg of product/mL in .1 *M* K_2HPO_4-

KH_2PO_4 buffer (pH 5) and in .2 *M* HCl-KCl buffer (pH 2). Products were weighed in duplicate, mixed with 100 mL of buffer and incubated at 39°C in a water bath with constant shaking for 12 h and then filtered through Whatman 42 paper for mineral analysis. Either .1 *M* K_2HPO_4 or HCl was used to adjust pH values if the pH of buffer was altered when Zn products were dissolved (Brown and Zeringue, 1994). The average solubility values from the range of concentrations in each buffer are given in the present paper, whereas individual data can be found in Cao (1998).

Gel Filtration Chromatography. Soluble fractions from solubility tests (deionized H_2O [Watson et al., 1970], pH 2 and 5 buffers) were evaluated by gel filtration chromatography for structural integrity as described by Brown and Zeringue (1994). The chromatographic column (1.5 cm × 19 cm) was packed with Biogel P-2, a polyacrylamide size exclusion gel with a fractionation range of 100 to 1,800 Da (Bio-Rad, Rockville Center, NY) according to instructions from the manufacturer. Aliquots (.20 mL) of all filtrates were loaded onto the column and eluted with the same buffer that was used in the solubility test. Eluent fractions (.9 mL) were collected with a Gilson micro fraction collector (Gilson Inc., Middleton, WI) and analyzed for Zn by atomic absorption spectroscopy. The presence of AA or related proteinaceous material was detected qualitatively using a modified ninhydrin procedure (Sigma Chemical Co., St. Louis, MO; Moore and Stein, 1954). The capacity of this chromatographic column to separate free metal ions, AA and small peptides, and metal chelates or complexes, based on molecular size was verified as described by Cao (1998). Chromatograms from the column test solutions and the organic sources solubilized in deionized H_2O can be found in Cao (1998). The percentage of Zn remaining in the chelated or complexed form in deionized H_2O is presented herein.

Experiment 1

A total of 432 1-d-old Avian × Avian broiler chicks was assigned randomly to six pen replicates for each of eight treatment combinations. The basal corn-soybean meal diet (Table 1) containing 59 mg of Zn/kg DM was formulated to meet or exceed requirements for starting chicks (NRC, 1994). Dietary treatments included the basal diet supplemented with 0, 200, 400, or 600 mg/kg added Zn as reagent-grade Zn Sulf or 200 or 400 mg/kg added Zn as Zn AA or Zn ProA. Chicks were housed in electrically heated, thermostatically controlled brooders with stainless steel feeders, waterers, floors, and gates and were maintained on a 24-h constant-light schedule. The birds were allowed ad libitum access to feed and to tap water that contained no detectable Zn. Chicks were managed according to guidelines approved by the University of Florida Animal Care and Use Committee. At 1, 2, and 3 wk of age, feed consumption was recorded per pen and three chicks from each pen were selected randomly, weighed individually, and killed by cervical dislocation. The right legs were excised and frozen in individual heat-sealed polyethylene bags. The duodenal loop was excised, opened longitudinally and washed with isotonic saline to remove contents. Mucosal cells were collected by carefully scraping the intestinal loop with a glass slide and the sample was divided into two aliquots. One aliquot was frozen immediately in a polypropylene tube for Zn analysis. The section of remaining serosa after mucosal cells were removed is referred to as intestine and was frozen in a heat-sealed bag. Liver samples were obtained from the left lobe. Zinc concentration was determined in bone, intestine, and mucosal cells. Metallothionein was determined in the second aliquot of mucosal cells and liver.

Table 1. Composition of basal diet, Exp. 1, 2, and 3

	%		
Item	Exp. 1	Exp. 2	Exp. 3
Ingredient composition[a]			
Ground yellow corn	55.42	55.42	58.95
Soybean meal (48%)	37.2	37.2	12.0
Corn oil	2.5	2.5	2.0
Cottonseed hulls	—	—	21.0
Alfalfa meal	—	—	3.0
Dicalcium phosphate	1.72	1.72	—
Ground limestone	1.01	1.01	.55
Microingredients[b]	.5	.5	.5
Iodized salt	.4	.4	1.0
D,L-Methionine	.25	.25	—
Cornstarch + zinc[c]	1.0	1.0	1.5
Chemical composition[d]			
Dry matter, %	88.9	87.9	88.7
Calcium, %	1.0	1.0	.47
Phosphorus, %	.68	.71	.34
Zinc, mg/kg	59	100	58
Copper, mg/kg	19	16	13
Iron, mg/kg	366	345	103
Manganese, mg/kg	115	121	34

[a]As-fed basis.
[b]Ingredients supplied per kilogram of diet in Exp. 1 and 2: vitamin A palmitate, 6,600 IU; cholecalciferol, 2,200 IU; menadione dimethylpyrimidinol bisulfite, 2.2 mg; riboflavin, 4.4 mg; pantothenic acid, 13 mg; niacin, 40 mg; choline chloride, 500 mg; vitamin B_{12}, 22 μg; ethoxyquin, 125 mg; iron, 50 mg ($FeCO_3$); zinc, 40 mg (ZnO); copper, 6 mg (Cu_2O); manganese, 60 mg (MnO); iodine, 1.1 mg (KI); selenium, .2 mg (Na_2SeO_3); in Exp. 3, Zn, 30 mg ($ZnSO_4 \cdot 7H_2O$); Cu, 10 mg ($CuSO_4 \cdot 5H_2O$); Fe, 50 mg ($FeSO_4 \cdot 7H_2O$); Mn, 30 mg (MnO); Co, .2 mg ($CoCO_3$); I, .8 mg (KI); Se, .2 mg (Na_2SeO_3); vitamin A, 2200 USP units; vitamin D_3, 440 IU; vitamin E, 15 IU; ethoxyquin, 187 mg.
[c]Zinc supplement added in place of equivalent weights of corn starch.
[d]DM basis, determined by analysis.

Experiment 2

A total of 288 1-d-old Avian × Avian broiler chicks was assigned randomly to eight pen replicates for each of six treatment combinations. The basal corn-soybean meal diet (Table 1) containing 100 mg of Zn/kg DM was formulated to meet or exceed requirements for starting chicks (NRC, 1994). Dietary treatments included the basal diet supplemented with 0, 200, or 400 mg/kg added Zn as reagent-grade Zn Sulf or 200 mg/kg added

Zn as Zn Poly, Zn ProB, or Zn ProC. Chicks were fed diets for 3 wk and then weighed and killed by cervical dislocation. The right leg was removed and frozen individually from all chicks. Other management practices were as described for Exp. 1.

Experiment 3

Forty-two crossbred wether lambs averaging 37.4 kg initially were assigned to seven treatment groups in a completely randomized design. Before the experiment, animals were group-fed a commercial corn-soybean meal diet at an amount to supply each lamb with 800 g daily, and bermudagrass (*Cynodon dactylon*) hay was available for ad libitum consumption. The basal experimental corn-soybean meal-cottonseed hulls diet (58 mg of Zn/kg DM by analysis; Table 1) was formulated to meet requirements for growing lambs (NRC, 1985). The experimental treatments were the basal diet supplemented with 0, 700, 1,400, or 2,100 mg/kg added Zn from reagent-grade Zn Sulf, or 1,400 mg/kg added Zn from either Zn ProA, Zn AA, or Zn MetB added in place of an equivalent weight of cornstarch. Lambs were housed in individual wooden pens with expanded metal floors in an open-sided barn and managed in a manner that was approved by the University of Florida Animal Care and Use Committee. Feed intake was restricted to 1.0 kg daily (as-fed basis) and tap water (containing no detectable Zn) was available for ad libitum consumption. Lambs were fed the basal diet for 7 d and then switched to their treatment diets and fed for 21 d. At the end of the experiment, lambs were stunned with a captive-bolt shot and killed by exsanguination. Liver, both kidneys, and pancreas were excised and frozen in plastic bags for Zn analyses. A second sample of liver was prepared for MT analysis.

Chemical Analyses

Zinc concentrations in Zn sources, diets, and tissues were determined by flame atomic absorption spectrophotometry (Model 5000 with AS-50 autosampler, Perkin-Elmer; Perkin-Elmer, 1982). Samples of diets and Zn sources were dried at 105°C for 12 h, dry-ashed at 550°C for 16 h, and then solubilized in HCl and filtered through Whatman 42 paper. Bones were boiled for approximately 10 min in deionized water, cleaned of all soft tissues, dried for 12 h at 105°C, and then extracted in a Soxhlet apparatus with petroleum ether for 48 h before drying and ashing as indicated above. Soft tissue (liver, kidney, pancreas, and small intestine) was preashed in 9 *M* HNO_3 on a hot plate before dry ashing in a muffle furnace, and then solubilized and filtered. Standards were matched for macroelement (salt) and acid concentrations as needed. Validation of the mineral analyses was done using citrus leaves-1572, bovine liver-1577a, and bone ash-1400 standard reference materials (National Institute of Standards and Technology, Gaithersburg, MD). Samples of mucosal cells were weighed, dried at 105°C, and charred in a muffle furnace at 450°C for 12 h (Reeves, 1995). Each of the charred samples was suspended in 2 mL of 1:1 (vol:vol) concentrated HCl and HNO_3 and heated to dryness on a hot plate. The samples were returned to the furnace and heated at 450°C for another 12 h. The mineral residue was dissolved in 1 mL of the above concentrated acid solution and diluted appropriately with deionized H_2O to determine Zn.

About 1 g of hepatic tissue or mucosal cells was homogenized with 3 volumes of cold 10 m*M* Tris·HCl buffer (pH 7.4) with a Potter-Elvehjem glass-Teflon tissue grinder and centrifuged (40,000 × *g*; 30 min; 4°C). The supernatant was heated (100°C; 5 min), centrifuged (10,000 × *g*; 5 min), and stored in small centrifuge tubes at –70°C. Metallothionein (MT) in liver and mucosal cells was measured by the $^{109}Cd^{2+}$ hemoglobin affinity assay (Eaton and Toal, 1982) with a gamma spectrometer (Model 4000, Beckman Instruments, Palo Alto, CA). The concentration of MT was calculated using ^{109}Cd-MT binding stoichiometry of 7:1.

Statistical Analysis

Data in Exp. 1 were analyzed by three-way ANOVA using the General Linear Models (GLM) procedure of SAS (1988) with a model that included Zn source, dietary Zn concentration, and chick age as main effects and their interactions. Pen was the experimental unit. Relative bioavailability values were determined using Zn Sulf as the standard source by slope ratio comparisons from multiple linear regression (Littell et al., 1995). Due to the differences in feed intake among treatments, regressions were calculated using total dietary Zn intake (mg) as the independent variable rather than dietary Zn concentration. Slope ratios and their standard errors were estimated using the method of error propagation as described by Kempthorne and Allmaras (1965). Differences among sources were determined by differences in their respective regression coefficients.

In Exp. 2, the data were analyzed by one-way ANOVA and multiple linear regression using the GLM procedure (SAS, 1988). Relative bioavailability values and slope ratios and their standard errors were estimated as described for Exp. 1. Although feed intake did not differ in this experiment, total Zn intake was also used as the independent variable to be able to compare values derived from both chick trials.

In Exp. 3, data were analyzed by one-way ANOVA and then multiple linear regressions were calculated using added dietary Zn concentration as the independent variable because feed intake did not differ among treatments. There was notable heterogeneity among treatment variances, so a $\log_{10}$ transformation of all dependent variables was used in the regression equations. Slope ratios and their standard errors were estimated as described for Exp. 1. Stepwise regression (Proc Stepwise) and all possible subset regression (Proc Rsquare) procedures of SAS (1988) were used to com-

Table 2. Chemical composition of zinc sources on an as-fed basis[a]

Source	DM, %	N, %	Zn, %	Ca, %	P, %	Mg, %	Cu, mg/kg	Fe, mg/kg	Mn, mg/kg
Zn Methionine A	90.7	.97	3.78	2.99	.027	.10	13.3	5,456	158
Zn Methionine B	91.3	1.81	9.29	3.20	.009	.05	32.0	12,166	181
Zn Polysaccharide	87.6	.45	19.02	.61	.079	.25	31.4	334	37
Zn Lysine	91.7	3.10	9.43	2.30	.009	.07	39.0	747	608
Zn Amino acid chelate	86.0	5.79	9.42	.17	.318	.22	144.7	191	312
Zn Proteinate A	93.7	7.19	13.63	.28	.262	.14	93.3	378	962
Zn Proteinate B	79.7	1.79	13.65	.16	.454	.22	28.2	752	339
Zn Proteinate C	83.8	4.14	13.01	.17	.277	.09	6.5	1,033	132
Zn Sulfate	62.7	ND[b]	20.8	ND	ND	ND	.7	ND	.5

[a]Each value based on triplicate determinations except for N content which had duplicate determinations.
[b]Not detectable.

pare relative bioavailability estimates from the animal experiments with chemical characteristics. Proc Stepwise was also used to correlate various chemical characteristics with each other.

Results

Characterization of Zn Sources

Mineral and Nitrogen Contents. Zinc content of the organic Zn sources varied considerably, ranging from 3.78% for Zn MetA to 19.02% for Zn Poly on an as-fed basis (Table 2). Other minerals were found in relatively small quantities with the exception of Fe in Zn MetB, which was 12,166 mg/kg. The Zn MetA, Zn MetB, and Zn Lys sources had higher Ca contents than other sources, probably due to the carrier material. Other organic sources contained relatively low Ca but greater P concentrations. This was probably related to the hydrolyzed proteins used as ligands in the chelated products.

Polarographic Analysis. Results of the electrochemical assay of organic Zn sources by polarography are presented in Table 3. All eight organic Zn sources showed different degrees of acidity. When saturated solutions were prepared in 50 mL of deionized H_2O, pH values decreased below the neutral point. The pH values of saturated solutions of these products ranged from as low as 4.41 for Zn ProC to as high as 6.73 for Zn ProA. The Zn AA, Zn ProB, and Zn ProC were the most acidic of products tested, with pH values of their saturated solutions of only 4.51, 4.64, and 4.41, respectively. The Zn Lys and Zn ProA showed only slight acidity with pH values of 6.37 and 6.73, respectively.

In saturated solutions, the total amount of organic Zn product that could be dissolved in deionized H_2O differed among sources (Table 3), ranging from .588 *M* for Zn MetB to 1.595 *M* for Zn Poly. Total Zn metal concentration in saturated solutions varied from as low as .101 *M* for Zn ProA to .840 *M* for Zn Poly. Chelation effectiveness of the organic Zn sources varied (Table 3) with chelation formation quotients ranging from 1.5 to 180. The Zn MetA, Zn MetB, Zn Poly, and Zn Lys complexes displayed relatively weak chelation, with Q_f values below 10. The Zn AA and Zn ProC contained strongly chelated Zn, with chelation quotients greater than 100. Moderately strong chelation (values from 10 to 100) was shown by Zn ProA and Zn ProB chelates.

Zinc and Nitrogen Solubility in Neutral Ammonium Citrate, Citric Acid, HCl, and Deionized H_2O. At concentrations of 1 mg of Zn product/mL of solvent combined with physiological temperatures, Zn from all products was nearly 100% soluble in NAC, CA, and HCl (Table 4). Nitrogen solubility varied in CA and HCl with Zn ProA having the lowest N solubility in these two solutions. Unlike Zn solubility in these solvents, solubility of N in the organic sources in water varied considerably at concentrations of 1 mg of product/mL, ranging from 11 for Zn ProA to 92% for Zn MetB. Nitrogen solubility in deionized H_2O for the eight organic products was not correlated with Zn solubility in deionized H_2O. Nitrogen from Zn MetA, Zn MetB, and Zn Lys was highly soluble in deionized H_2O.

Solubility of Zn in the Zn sources at 10 mg/mL with shaking at room temperature (Table 5) was similar or somewhat greater than values found at 1 mg/mL. A similar low value (17%) was found for soluble Zn in Zn ProA and the Zn in Zn Lys was 75% soluble at the greater concentration. The small insoluble fraction observed for Zn Sulf probably represented a residue that was not washed from the filter paper due to a limitation of the volume of water used to make a quantitative transfer from the bottles. All samples were handled similarly so that this error should be constant for all sources.

All of the N in the Zn Poly sample remained in the insoluble fraction on the filter paper (Table 5). Only a small portion of N from the three specific amino acid complexes appeared in the insoluble fraction. In contrast, approximately 60% of the N in Zn AA and Zn ProC and 75% from Zn ProA and Zn ProB was found in the insoluble fraction on the filter paper. Consequently, there were considerable differences among Zn:N ratios calculated for the dry sources as included in animal diets and their subsequent soluble and insoluble fractions (data not shown). Considerable amounts of both

Table 3. Chelation effectiveness of zinc sources

Source	pH[a]	[total], M[b]	[metal], M[c]	$E_{½}$, V[d]	$\Delta E_{½}$, V[e]	Q_f [f]
Zn Methionine A	5.83	.613	.107	–1.002	.008	1.9
Zn Methionine B	5.44	.588	.198	–.999	.005	1.5
Zn Polysaccharide	5.49	1.595	.840	–1.011	.017	3.8
Zn Lysine	6.37	.847	.219	–1.013	.019	4.4
Zn Amino acid chelate	4.51	1.026	.313	–1.061	.067	180
Zn Proteinate A	6.73	.631	.101	–1.027	.033	13
Zn Proteinate B	4.64	.764	.274	–1.052	.058	91
Zn Proteinate C	4.41	.609	.165	–1.056	.062	120
Zn Sulfate	—[g]	—	.100	–.994	—	—

[a]The acidity of an organic Zn saturated solution.
[b]Total concentration of all soluble species in the saturated solution.
[c]Total Zn concentration (chelated and free) in the saturated solution.
[d]Half-wave reduction potential of a soluble Zn sample (volts relative to Ag/AgCl reference electrode). Half-wave potentials and Q_f values pertain to samples diluted 1:100 in .1 *M* 4-morpholinoethanesulfonic acid buffer, pH 6.0.
[e]Shift in $E_{½}$ = $E_{½}$ (free Zn^{2+}) – $E_{½}$ (Zn complex).
[f]Chelation formation quotient. It is calculated from $\log(Q_f) = (n)(\Delta E_{½})/.05916$, where n = 2, the number of electrons transferred to Zn(II) in the polarography experiment.
[g]$ZnSO_4$ (.10 *M*) used as the blank.

the Zn and N in Zn ProA were insoluble, so the ratio did not change greatly among the fractions. The considerable variation in the Zn:N ratios between the sources and their respective fractions indicated that little of the original material remained chelated under conditions of this test for all sources with the exception of Zn ProA.

Solubility in pH 2 and pH 5 Buffers. In pH 5.0 buffer, solubility was decreased for all Zn sources as product concentration increased from .125 to 12.5 mg product/mL buffer (Cao, 1998). The average of the solubility across the concentration range, which was used in regressions, is presented in Table 5. The solubility of organic Zn sources decreased at concentrations of 2.5 mg product/mL or greater. At a concentration of 12.5 mg product/mL buffer, solubilities were 12, 29, 45, 5, 50, 1, 45, 43, and 97% for Zn MetA, Zn MetB, Zn Poly, Zn Lys, Zn AA, Zn ProA, Zn ProB, Zn ProC, and Zn Sulf, respectively. In pH 2.0 buffer, organic Zn sources were at least 80% soluble even at 12.5 mg of product/mL of buffer, except for Zn ProA, which was only 40.0% soluble (Cao, 1998). The average solubility across the range of added concentrations in pH 2 buffer used in regressions is presented in Table 5.

Gel Filtration Chromatography. The capacity of the gel filtration column to separate molecules of different sizes such as free metal ions and metals in intact chelates was confirmed (Cao, 1998). Ionic Zn eluted in fractions 25 to 34. Zinc as Zn-EDTA chelate eluted primarily in fractions 14 to 20, with a small amount of Zn that was not chelated eluted in fractions 26 to 30. Mixing ionic Zn with methionine did not cause chelation to occur. Methionine eluted in fractions 23 to 27, whereas Zn in a mixture of Zn + methionine eluted in fractions 28 to 34 (data not shown; Cao, 1998). Chromatographs

Table 4. Zinc and nitrogen solubility of zinc sources in deionized water (dH_2O), neutral ammonium citrate (NAC), 2% citric acid (CA), and .4% HCl[a]

	Zn Solubility, %[b]				N Solubility, %[b]		
Source	dH_2O	NAC	CA	HCl	dH_2O	CA	HCl
Zn Methionine A	66	99	95	98	96	88	89
Zn Methionine B	92	100	96	100	94	96	96
Zn Polysaccharide	84	100	96	100	5	100	96
Zn Lysine	53	99	95	99	94	72	86
Zn Amino acid chelate	67	98	91	96	42	96	99
Zn Proteinate A	11	99	89	99	38	22	22
Zn Proteinate B	73	98	100	92	11	44	51
Zn Proteinate C	72	95	85	90	39	55	71
Zn Sulfate	100	100	100	100	ND[c]	ND	ND

[a]Duplicate .1-g samples were mixed with 100 mL of solvents at 37°C for 1 h with constant stirring (Watson et al., 1970).
[b]Value expressed as a percentage of total Zn and N content.
[c]Not detectable.

Table 5. Solubility of zinc and nitrogen in zinc sources in deionized water and average zinc solubility in .1 *M* K_2HPO_4-KH_2PO_4 buffer, at pH 5.0 and .2 *M* HCl-KCl buffer, pH 2.0

	Zinc, mg[a]		Nitrogen, mg[a]		.1 *M* K_2HPO_4-KH_2PO_4 buffer, at pH 5.0[b]	.2 *M* HCl-KCl buffer, pH 2.0[b]
Source	Soluble	Insoluble	Soluble	Insoluble	Soluble, %	
Zn Methionine A	60	5	14	6	63.5	98.2
Zn Methionine B	164	3	31	3	69.3	95.6
Zn Polysaccharide	348	33	—	7	79.6	97.6
Zn Lysine	127	43	66	4	59.7	95.6
Zn Amino acid chelate	149	17	48	77	81.1	96.4
Zn Proteinate A	41	206	45	131	54.1	84.7
Zn Proteinate B	200	26	11	31	74.6	94.2
Zn Proteinate C	216	10	40	58	76.7	97.8
Zn Sulfate	423	2	—	—	98.2	98.6

[a]Duplicate 2.0-g samples of products were mixed with 150 mL of dH_2O and incubated at 25°C with constant shaking for 30 min, and then brought to a final volume of 200 mL (Leach and Patton, 1997).
[b]Duplicate samples (various concentrations, see Cao, 1998) of products were mixed with 100 mL of buffer and incubated at 39°C with constant shaking for 12 h. Solubility is expressed as a percentage of total Zn.

revealed that all Zn from organic products was eluted in the same range of fractions as was Zn from Zn Sulf for filtrates from pH 2.0 and pH 5.0 buffers (data not shown), indicating that none of the material remained chelated under these pH conditions. Filtrates from deionized H_2O (Watson et al., 1970; Table 4), however, displayed a small peak for all eight organic Zn products before the peak for dissociated Zn in the range of fractions found for Zn from Zn Sulf. The percentages of chelated Zn in total Zn in filtrate calculated from these peaks were 2.5, 2.2, 4.1, 5.7, 12.2, 10.2, 11.3, and 11.7% for Zn MetA, Zn MetB, Zn Poly, Zn Lys, Zn AA, Zn ProA, Zn ProB, and Zn ProC, respectively. The percentage of chelated Zn remaining was positively related to $\log_{10}$ of the chelation effectiveness as determined by polarography (r = .96). The complexed products dissociated to a greater extent than did the chelated products.

Experiment 1

Feed intake decreased ($P < .0001$) with increasing dietary Zn supplementation from an average of 29 g daily in chicks given the basal diet to 26.4 g daily in those given 600 mg/kg Zn as Zn Sulf (Table 6). There was no difference in feed intake ($P > .10$) in chicks fed the two organic Zn sources; however, birds fed Zn Sulf had lower ($P < .0001$) overall feed intake than birds fed the organic sources. Feed intake increased ($P < .0001$) with age. Daily gain also increased with age and Zn source ($P < .0001$) and decreased with dietary Zn intake ($P < .001$; Table 6).

Bone Zn concentration increased ($P < .0001$) as added dietary Zn increased for all three Zn sources (Table 7). Bone Zn concentration was also affected by Zn source ($P < .0001$) with birds fed Zn ProA having the greatest ($P < .05$) response to dietary Zn. There was no effect of age ($P > .10$) on bone or intestinal Zn concentrations; however, birds fed Zn treatments for 1 wk had greater ($P < .01$) concentrations of Zn in mucosa than those fed for 2 or 3 wk (Table 7). As dietary Zn increased, intestinal ($P < .05$) and mucosal ($P < .0001$) Zn concentrations increased (Table 7). The main effect of Zn source observed for intestinal ($P < .01$) and mucosal ($P < .0001$) Zn concentrations was a difference between birds fed supplemented vs unsupplemented Zn diets rather than a difference among the various sources of the element.

Hepatic MT content (Table 8) was affected by Zn source ($P < .05$); however, this was a difference between birds fed supplemented vs unsupplemented diets rather than a difference among Zn sources. Elevation of dietary Zn concentrations tended ($P < .10$) to increase concentration of liver MT. Mucosal MT increased with increasing dietary Zn concentration ($P < .0001$). The main effect of source for mucosal MT ($P < .0001$) resulted from addition of Zn to the diet compared with the unsupplemented control group and not a difference among the supplemental sources. Metallothionein in liver generally increased up to 3 wk ($P < .0001$), but mucosal MT increased from an overall average of 28.6 μg/g at 1 wk to 45.2 μg/g at 2 wk and then decreased to 30.3 μg/g at 3 wk ($P < .0001$).

Regressions were calculated based on total Zn intake during the experimental period because feed intake varied with dietary treatment (Table 9). At 1 and 2 wk, respectively, the coefficient of determination (R^2) for fit to a linear model was greater for bone Zn (R^2 = .83 and .81) than for mucosal Zn (R^2 = .51 and .56). At 3 wk, the R^2 value was greater for mucosa Zn (R^2 = .87) than for bone Zn (R^2 = .64). Intestinal Zn was not a good indicator of dietary Zn supplementation, with a R^2 value less than .30 for all time periods (Table 10). Liver ($R^2 \leq .26$) and mucosal ($R^2 \leq .53$) MT concentrations were not as well correlated to supplemental Zn intake as were tissue Zn concentrations (Table 10) and were consequently not used to calculate relative bioavailability estimates.

Relative bioavailability values were only calculated based on bone and mucosal Zn concentrations regressed on total Zn intake during each experimental period (Table 9). The standard, Zn Sulf, was assigned a value of

Table 6. Effect of Zn sulfate (ZnSulf), Zn amino acid chelate (ZnAA), and Zn proteinate A (ZnProA) and added dietary Zn concentration on daily feed intake and gain in chicks fed 1, 2, or 3 wk, Exp. 1[a]

Added Zn, mg/kg[b]	Daily feed intake[c], g			Daily gain[d], g		
	ZnSulf	ZnAA	ZnProA	ZnSulf	ZnAA	ZnProA
	Age, 1 wk					
0	18	—	—	13.6	—	—
200	17.5	19.2	18.5	13.2	15.0	14.9
400	17.2	17.8	18.3	12.5	14.5	14.0
600	15.8	—	—	12.4	—	—
	Age, 2 wk					
0	28.4	—	—	22.5	—	—
200	30.1	31.8	30.1	22.3	22.4	22.4
400	26.3	29.3	30.5	17.6	20.9	22.8
600	26.6	—	—	18.1	—	—
	Age, 3 wk					
0	40.7	—	—	28.2	—	—
200	40.9	44.5	42.9	28.1	32.7	30.3
400	37.8	41.3	41.0	25.9	29.7	28.9
600	36.9	—	—	25.1	—	—
Pooled SE		.49			.49	

[a]Each value represents the mean of six pens of nine chicks at 1 wk, six chicks at 2 wk, and three chicks at 3 wk.
[b]Basal diet contained 59 mg of Zn/kg DM.
[c]Main effects ($P < .0001$) of source, added Zn, and week.
[d]Main effects of source, week ($P < .0001$), and added Zn ($P < .001$).

Table 7. Effect of Zn sulfate (ZnSulf), Zn amino acid chelate (ZnAA), and Zn proteinate A (ZnProA) and added dietary Zn concentration on Zn concentration in bone, intestine, and mucosa (DM basis) of chicks fed for 1, 2, or 3 wk, Exp. 1

Added Zn mg/kg[a]	Bone Zn, mg/kg[b]			Intestinal Zn, mg/kg[c]			Mucosal Zn, mg/kg[d]		
	ZnSulf	ZnAA	ZnProA	ZnSulf	ZnAA	ZnProA	ZnSulf	ZnAA	ZnProA
	Age, 1 wk								
0	391	—	—	59.5	—	—	86.7	—	—
200	450	455	463	71.1	78.9	79.1	128.1	120.2	120.3
400	483	492	503	81.6	84.2	87.6	149.9	130.1	129.8
600	523	—	—	89.8	—	—	160.2	—	—
	Age, 2 wk								
0	396	—	—	68.5	—	—	93.3	—	—
200	445	447	468	76.3	84.5	77.3	115.3	107.3	112.4
400	476	484	511	81.8	84.8	85.4	123.8	124.7	130.8
600	513	—	—	87.0	—	—	124.5	—	—
	Age, 3 wk								
0	404	—	—	67.7	—	—	97.6	—	—
200	455	452	490	77.7	76.5	82.6	107.8	106.9	114.3
400	480	481	521	77.9	83.8	89.1	121.2	119.6	137.6
600	534	—	—	85.0	—	—	140.5	—	—
Pooled SE		4.23			3.41			2.39	

[a]Basal diet contained 59 mg of Zn/kg DM.
[b]Ash weight basis. Main effects of source and added Zn ($P < .0001$). Each value represents the mean of six pens of three chicks.
[c]Main effects of source ($P < .01$) and added Zn ($P < .05$). Each value represents the mean of six pens of three chicks.
[d]Main effects of source and added Zn ($P < .0001$) and week ($P < .01$). Each value represents the mean of three pens of three chicks.

Table 8. Effect of Zn sulfate (ZnSulf), Zn amino acid chelate (ZnAA), and Zn proteinate A (ZnProA) and added dietary Zn concentration on liver and mucosal metallothionein (MT) content (fresh tissue basis) of chicks fed for 1, 2, or 3 wk, Exp. 1

Added Zn mg/kg[a]	Liver MT, μg/g[b]			Mucosal MT, μg/g[c]		
	ZnSulf	ZnAA	ZnProA	ZnSulf	ZnAA	ZnProA
	Age, 1 wk					
0	10.6	—	—	14	—	—
200	15.4	12.6	15.3	27.4	21.4	32.0
400	17.4	15.2	16.5	34.9	25.0	35.5
600	14.7	—	—	38.2	—	—
	Age, 2 wk					
0	19.1	—	—	30.8	—	—
200	19.4	16.2	20.5	42.1	42.8	42.3
400	20.3	18.4	25.6	45.8	52.7	49.8
600	21.2	—	—	55.0	—	—
	Age, 3 wk					
0	17.8	—	—	18.2	—	—
200	17.4	18.3	21.6	23.6	26.2	32.7
400	19.0	22.3	29.8	33.2	36.6	38.1
600	24.1	—	—	33.4	—	—
Pooled SE		1.23			1.75	

[a]Basal diet contained 59 mg of Zn/kg DM.
[b]Main effects of week ($P < .0001$) and source ($P < .05$). Each value represents the mean of six pens of three chicks.
[c]Main effects ($P < .0001$) of source, added Zn, and week. Each value represents the mean of six pens of three chicks.

100%. The estimated values based on bone Zn were 104 and 116 at 1 wk, 100 and 135 at 2 wk, and 83 and 139% at 3 wk for Zn AA and Zn ProA, respectively. The slopes did not differ in the first week, but bioavailability values were greater ($P < .05$) for Zn ProA than for Zn Sulf and Zn AA at 2 and 3 wk. When bioavailability was estimated based on mucosal Zn, relative values for Zn AA and Zn ProA were approximately 65% at 1 wk but were similar to values based on bone Zn at 2 or 3 wk.

Experiment 2

Feed intake and gain were not influenced by dietary Zn at dietary concentrations up to 400 mg/kg (Table 11). Bone Zn concentration increased linearly ($P < .0001$) with added Zn in the diet. The estimated bioavailability values based on bone Zn concentration regressed on dietary Zn intake relative to 100% for Zn Sulf were 94, 99, and 108 for Zn Poly, Zn ProB, and Zn ProC, respectively (Table 12). The slope for Zn Poly was lower ($P < .05$) than that for Zn ProC, but other slopes did not differ.

Experiment 3

All lambs consumed their entire feed allotment of 1.0 kg daily, so regressions were calculated based on added dietary Zn concentration rather than on total Zn intake as in the chick trials. Hepatic, kidney, and pancreatic Zn and hepatic MT increased linearly ($P < .0001$) with additions of dietary Zn (Table 13). Liver Zn had the poorest fit to the model in the ANOVA with an R^2 value of .61. The fits for kidney and pancreas Zn and liver MT were .73, .74, and .77, respectively. Due to high variance heterogeneity, a $\log_{10}$ transformation was made on the dependent variables before regressions were calculated (Littell et al., 1995). Multiple linear regression equations of $\log_{10}$ tissue Zn and MT concentrations on added dietary Zn concentration are shown in Table 14. The fit to the linear model was greater for liver MT and kidney and pancreas Zn than for liver Zn. For kidney and pancreas Zn and liver MT, the slope for Zn ProA was greater ($P < .05$) than that for Zn Sulf. The overall average bioavailability estimates based on all four criteria relative to 100% for Zn Sulf were 130, 110, and 113 for Zn ProA, Zn AA, and Zn MetB, respectively.

Comparison of Bioavailability Estimates and Chemical Characteristics

Regression procedures were used to calculate which of the chemical characteristics best predicted the bioavailability estimates of the organic Zn sources relative to that for reagent-grade Zn Sulf calculated for chicks and lambs. Because some of these chemical tests used Zn Sulf as a blank rather than a sample, it was not used in subsequent regression calculations. The bioavailability estimates used were values based on bone Zn in chicks fed 3 wk as reported in Tables 9 and 12 (Exp. 1 and 2) and an overall average value based on all criteria for lambs reported in Table 14 (Exp. 3). The

Table 9. Estimated relative bioavailability (RBV) of Zn sources based on multiple linear regression slope ratios of bone and mucosal Zn concentrations on total dietary Zn intake (mg), Exp. 1

Age, wk	Zn source	Regression coefficient slope ± SE	RBV ± SE, %
		Bone[a]	
1	Zn Sulfate	1.774 ± .13	100
	Zn Amino acid chelate	1.852 ± .18	104 ± 8.7
	Zn Proteinate A	2.051 ± .18	116 ± 8.9
2	Zn Sulfate	.482 ± .041[c]	100
	Zn Amino acid chelate	.482 ± .056[c]	100 ± 9.9
	Zn Proteinate A	.650 ± .054[d]	135 ± 10.8
3	Zn Sulfate	.241 ± .031[c]	100
	Zn Amino acid chelate	.199 ± .042[c]	83 ± 14.6
	Zn Proteinate A	.335 ± .043[d]	139 ± 16.9
		Mucosa[b]	
1	Zn Sulfate	.938 ± .211	100
	Zn Amino acid chelate	.605 ± .261	64 ± 23.4
	Zn Proteinate A	.614 ± .261	65 ± 23.5
2	Zn Sulfate	.138 ± .031	100
	Zn Amino acid chelate	.144 ± .042	104 ± 37.8
	Zn Proteinate A	.180 ± .041	130 ± 37.3
3	Zn Sulfate	.092 ± .009[d]	100
	Zn Amino acid chelate	.070 ± .012[e]	76 ± 11.0
	Zn Proteinate A	.122 ± .012[c]	133 ± 12.7

[a]Bone Zn expressed as mg/kg ash weight. The R^2 values were .83, .81, and .64 for 1 , 2, and 3 wk, respectively. The SD was 17.6, 17.9, and 28.5, respectively.
[b]Mucosa Zn expressed as mg/kg DM. R^2 values were .51, .56, and .87 for 1, 2, and 3 wk, respectively. The SD was 18.5, 10.0, and 6.0, respectively.
[c,d,e]Within an age group, slopes lacking a common superscript letter differ ($P < .05$).

single-variable model r^2 values from all possible subset regressions are found in Table 15 with separate comparisons for the two animal species, and then again with all data combined. The best fit to a linear model with the chick bioavailability estimates was found for Zn solubility in pH 5 buffer, although the relationship was an inverse response. The insoluble fraction of Zn remaining after stirring at 25°C for 30 min, solubility of Zn in water after stirring for 1 hr at 37°C, and solubility of Zn in pH 2 buffer had coefficients of determination that were greater than those for the remaining chemical characteristics. There was little correlation between bioavailability estimates and solubility in HCl, NAC, or CA in the tests conducted at 37°C. Neither the chelation formation quotient from polarography (Q_f values) nor the percentage of Zn in the source remaining chelated in water-soluble samples following gel chromatography was an indicator of the chicks' response to supplemental sources. For the availability values estimated in the lamb study, several of the chemical characteristics had fits to a single-variable linear model with r^2 in excess of .90. These independent variables included Zn solubility in pH 2 or pH 5 buffers, and the soluble and insoluble Zn fractions following solubilization in water at 25°C with 30 min stirring. Solubility in water at 37°C for 1 h was also high, but the other three solvents used in that test showed little correlation with bioavailability. The fits for the chelation estimates based on polarography and gel chromatography were low, similar to those found with chick data. When bioavailability estimates for both chicks and lambs were included in the regression, the best single predictor was Zn solubility in pH 5 buffer as observed with the chick data alone and the relationship was again the inverse. Decreasing solubility in the buffer increased bioavailability in the animal. The single-variable best fit equation for chick data was

$$\text{Zn bioavailability} = 240 - 1.848\ (\text{pH 5 solubility});\ r^2 = .91;\ P = .0113$$

The equation for lambs was

$$\text{Zn bioavailability} = 269 - 1.645\ (\text{pH 2 solubility});\ r^2 = .99;\ P = .0498.$$

The expanded sequential best fit models from all possible subset regressions are presented in Table 16. For bioavailability estimates based on chick experiments,

Table 10. Multiple linear regression of tissue Zn and metallothionein (MT) concentrations with respect to total dietary Zn intake (mg) by age, Exp. 1

Dependent variable[a]	Age, wk	Intercept	Regression coefficients: Zn Sulfate	Zn Amino acid chelate	Zn Proteinate A	R^2	SD	*P*
Intestinal Zn	1	62	.429	.469	.538	.15	22.9	.0156
	2	70	.0753	.112	.0898	.27	9.0	.0012
	3	69	.0347	.0422	.0618	.20	12.4	.0085
Liver MT	1	12	.0643	.048	.0914	.10	4.58	.0529
	2	19	.0109	–.0082	.0341	.08	7.67	.6167
	3	16	.0145	.0183	.0394	.26	5.94	.0173
Mucosal MT	1	18	.321	.1306	.379	.40	9.45	.0001
	2	32	.1071	.13	.11	.53	7.32	.0002
	3	19	.0348	.0473	.06	.41	7.72	.0001

[a]Tissue Zn concentration expressed as mg/kg DM. Tissue MT concentration expressed as micrograms per milligram of fresh tissue.

Table 11. Effect of Zn sources and added dietary Zn concentration on feed intake, body weight, and bone Zn concentrations in chicks fed 3 wk, Exp. 2[a]

Zn source	Added Zn, mg/kg[b]	Daily feed intake, g	Daily gain, g	Bone Zn, mg/kg (ash basis)[c]
Control	0	42.5	32.3	341
Zn Sulfate	200	43.8	33.6	380
	400	43.4	32.3	416
Zn Polysaccharide	200	43.5	32.9	369
Zn Proteinate B	200	42.6	33.6	381
Zn Proteinate C	200	45.0	33.9	376
Pooled SE		.38	.29	1.44

[a]Each value represents the mean of eight pens of six chicks.
[b]Basal diet contained 100 mg of Zn/kg DM.
[c]Effect of treatment ($P < .0001$).

a fit of $R^2 = 1.0$ was achieved with a four-variable model. The fit of the single-variable model that had Zn solubility in pH 5 buffer as the independent variable increased to .9996 when Zn solubility at pH 2 entered the model. The addition of variables three and four gave insignificant ($P > .05$) improvement in the fit. A similar phenomenon was observed when a second variable entered the single-variable model based on lamb bioavailability estimates. Only the highest 40 fits were requested from all possible subset regressions because there were 13 independent variables included. For the data set based on the chick studies, 24 of the two-factor models gave R^2 values greater than .80 and 39 of the three-factor models gave values greater than .98 (data not shown). All 40 of the four-factor models had an R^2 value of 1.00 (data not shown). With lambs, all 40 regressions had a perfect fit to a linear model. Inclusion of additional variables in these higher-order equations resulted in no significant improvement in fit and were, therefore, not confirmed in the stepwise regression models.

Discussion

The degree of chelation of an organic mineral source and its behavior under physiological conditions have been suggested by marketing literature as the key points in determining the value of products used as supplements in animal nutrition. A simple analysis of the mineral and AA or N composition provides no indication of the amount of the product that is chelated or the strength of the bonds. A product consisting of a simple mixture of meat meal with totally unavailable cupric oxide would give N and Cu concentrations similar to a highly chelated Cu proteinate but would be totally unavailable as a source of Cu for nonruminant animals (Leach and Patton, 1997).

Chelation chemistry is a very sophisticated and complex subject (Kratzer and Vohra, 1986; Hynes and Kelly, 1995). Several solid-state laboratory techniques, including infrared spectroscopy, nuclear magnetic resonance, and x-ray power diffractometry (Hynes and Kelly, 1995) are available to assess degree of chelation in dry mineral products, but these techniques have, as yet, provided no information as to how the mineral sources react under conditions present in the animal's gastrointestinal tract. In principle, however, solution-phase nuclear magnetic resonance may be a useful technique for further investigation. The stability of any chelation product is influenced by the metals and ligands involved in the process. There is, however, a tradeoff in animal nutrition in that a highly stable chelate, which will not be prone to dissociation and loss in the gastrointestinal tract, must be able to dissociate within the animal's body and allow use of the metal in normal metabolism without the ligand rebinding and creating a loss of other needed metal ions from within the body. Any chemical differences found among sources by various laboratory procedures are irrelevant unless concurrent, measurable differences in animal performance can also be detected.

All organic Zn sources tested in the present study showed different degrees of acidity. When saturated solutions were prepared in deionized H_2O, the pH of the solutions decreased. The consistency of pH readings can be used as one criterion to test product uniformity from batch to batch (Holwerda et al., 1995). The amount of organic Zn that could be dissolved in deionized H_2O varied from source to source in the present experiments, indicating different degrees of solubility. Concentration of the compound in the solution and temperature affected solubility, so these values must be applied cautiously to simulate conditions within the animal's body.

The different organic products examined in the present study showed different degrees of chelation effectiveness as determined by polarography. According to the classification suggested by Holwerda et al. (1995), two organic Zn sources were strong "chelates" (Zn AA and Zn ProC), two were moderate "chelates" (Zn ProA and Zn ProB), and four were weak "chelates" (Zn MetA, Zn MetB, Zn Poly, and Zn Lys). Holwerda et al. (1995) found that most commercial proteinates are weaker chelates than corresponding glycine complexes, when dissolved in H_2O. Chelation quality has been shown to differ even for the same product among different batches from the same commercial source (Holwerda et al., 1995). In addition, these authors reported that

Table 12. Estimated relative bioavailability (RBV) of Zn sources based on multiple linear regression slope ratios of bone Zn concentration (mg/kg ash basis) on total dietary Zn intake (mg) in chicks fed 3 wk, Exp. 2

Zn source	Regression coefficient[a] slope ± SE	RBV ± SE, %
Zn Sulfate	.2045 ± .032[bc]	100
Zn Polysaccharide	.1711 ± .047[c]	94 ± 11.6
Zn Proteinate B	.2025 ± .044[bc]	99 ± 8.8
Zn Proteinate C	.2199 ± .052[b]	108 ± 11.4

[a]Intercept = 321; R^2 = .82; SD = 10.8.
[b,c]Slopes lacking a common superscript letter differ ($P < .05$).

Table 13. Effect of dietary Zn on tissue Zn and metallothionein (MT) concentration of lambs, Exp. 3[a]

Treatment	Added Zn, mg/kg[b]	Tissue Zn, mg/kg (DM basis)			Liver MT, μg/g[c]
		Liver	Kidney	Pancreas	
Basal	0	113	108	84	18
Zn Sulfate	700	255	528	305	677
	1,400	374	1,164	1,080	1924
	2,100	436	1,519	1,157	2441
Zn Proteinate A	1,400	415	1,394	1,129	2332
Zn Amino acid chelate	1,400	324	1,114	842	1603
Zn Methionine B	1,400	342	980	924	1749
Pooled SE		13.7	47.2	39.8	84.1
$P <$		.0001	.0001	.0001	.0001

[a]Each value represents the mean of six lambs fed for 21 d.
[b]Basal diet contained 58 mg Zn/kg DM.
[c]Expressed as fresh tissue.

drying methods were shown to affect metal proteinate quality. The authors also suggested that dramatic improvements in the bioavailability of transition metals cannot be expected when only AA and proteins are used as chelating agents because, in general, they are only moderately strong chelating ligands.

The results from polarographic tests should, therefore, be interpreted with caution. Chelation effectiveness decreased at lower pH values. The percentage of chelated metal for a typical chelate may drop from 90% at pH 6 to only 10% at pH 4 (Holwerda et al., 1995). Chelation effectiveness tests in the present studies were conducted for saturated organic Zn solutions, which had been diluted 1:100 in buffer so that Zn concentrations ranged from 1.01 to 8.40 m*M*, rather than the more concentrated solutions that are unlikely to exist in the animal's gastrointestinal tract because Zn is normally supplemented at about 50 to 60 mg/kg in the diet. Chelation effectiveness in the gastrointestinal tract of animals should be lower in more dilute solutions and a more acidic environment than the values determined here. Holwerda et al. (1995) demonstrated that, as initial complexation concentration decreased, the percentage of dissociation into free Zn and free ligand increased.

Solubility tests have been used to assess the quality of mineral products (Leach and Patton, 1997), and the results may sometimes be misleading. Solubility is pH-

Table 14. Estimated relative bioavailability (RBV) of Zn sources based on multiple linear regression of $\log_{10}$ transformed tissue Zn (DM basis) and liver metallothionein (MT, fresh basis) concentration on added dietary Zn concentration for lambs, Exp. 3

Dependent variable	Zn source	Regression coefficient (slope ± SE)	RBV ± SE, %
Liver Zn[a]	Zn Sulfate	.000264 ± .0000316	100
	Zn Proteinate A	.000345 ± .0000460	131 ± 16.4
	Zn Amino acid chelate	.000271 ± .0000460	103 ± 15.3
	Zn Methionine B	.000284 ± .0000460	108 ± 15.5
Kidney Zn[b]	Zn Sulfate	.000541 ± .0000440[e]	100
	Zn Proteinate A	.000691 ± .0000641[f]	128 ± 11.0
	Zn Amino acid chelate	.000611 ± .0000641[ef]	113 ± 10.6
	Zn Methionine B	.000579 ± .0000641[ef]	107 ± 10.5
Pancreas Zn[c]	Zn Sulfate	.000562 ± .0000443[e]	100
	Zn Proteinate A	.000736 ± .0000646[f]	131 ± 10.8
	Zn Amino acid chelate	.000641 ± .0000646[ef]	114 ± 10.3
	Zn Methionine B	.000672 ± .0000646[ef]	120 ± 10.5
Liver MT[d]	Zn Sulfate	.000536 ± .0000374[e]	100
	Zn Proteinate A	.000705 ± .0000545[f]	131 ± 9.6
	Zn Amino acid chelate	.000596 ± .0000545[ef]	111 ± 9.7
	Zn Methionine B	.000625 ± .0000545[ef]	116 ± 9.2

[a]Intercept = 2.13, R^2 = .70, SD = .121.
[b]Intercept = 2.17, R^2 = .84, SD = .168.
[c]Intercept = 2.01, R^2 = .85, SD = .170.
[d]Intercept = 1.36, R^2 = .88, SD = .143.
[e,f]Within a dependent variable slopes lacking a common superscript letter differ ($P < .05$).

Table 15. Linear regression r^2 values for fits of estimated bioavailability values (relative to reagent-grade Zn sulfate) by chicks and lambs with chemical characteristics of organic Zn sources for models with a single independent variable

Independent variable	r^2 Value for bioavailability estimates used as dependent variables[a]		
	Chicks (n = 5)[b]	Lambs (n = 3)[b]	Chicks and lambs
Zn Solubility pH 5 buffer	.913	.906	.791
Zn Solubility pH 2 buffer	.734	.994	.686
Zn Solubility at 37°C for 1 hr			
Water	.744	.811	.575
2% Citric acid	.164	.393	.175
.4% HCl	.053	.279	.138
Neutral ammonium citrate	.006	.167	.048
Zn Solubility at 25°C for 30 min			
Soluble Zn fraction	.386	.939	.509
Insoluble Zn fraction	.788	.960	.649
Q_f from polarography	.323	.324	.272
Chelation from chromatography	.005	.037	.000

[a]Bioavailability estimates used were based on bone Zn in chicks fed 3 wk as reported in Tables 9 and 12 (Exp. 1 and 2) and an overall average value based on all criteria for lambs in Table 14 (Exp. 3).
[b]The number of bioavailability estimates (sources) used in regressions for each species.

and buffer-dependent as well as time- and temperature-dependent. Effect of pH and buffer on mineral solubility was evident in the present study. The concentrations of products used in the study with the two buffers varied widely. Manufacturers of most of these supplements recommend a range from 2.5 to 10 g/d of product for cattle. This would result in concentrations ranging from .05 to .2 mg/mL assuming a ruminal volume capacity of 50 L. The lower end of the range used in the present experiments would represent similar concentrations. In the gastrointestinal tract, the initially insoluble fraction will be increasingly solubilized and pH changes in the tract should apply to both soluble and insoluble fractions (Leach, 1995). The pH of gastric juice ranged from about .5 to 2.5 and intestinal pH ranged from about 3.0 to 7.2. Dintzis et al. (1995) indicated that pH in the stomach of pigs was 4.5 ± .3. The pH in the rumen ranged from around 7.0 on forage diets to about 4.6 when animals were fed high-grain diets (Hoover and Miller, 1991). The digesta leaving the abomasum of cattle is approximately pH 2 to 3 (Church, 1969). The organic Zn sources must become soluble before reaching the absorption sites. Most metal chelates involving AA or proteins dissociated at a pH less than 3 or greater than 9. At this point, they would become similar to inorganic minerals in their behavior. It can be assumed that very few free ions would be found in the gastrointestinal tract because there are so many ligands available for (re)chelation. This is one reason that results from in vitro experiments with ligated gut segments should be applied very conservatively with regard to a living animal that is on a normal feeding regimen. Unless food is in the gastrointestinal tract at the same time as the test mineral source, the inference space may be extremely limited.

A problem encountered during the present experiments was the inability to determine free dissociated Zn from soluble Zn that was still bound to the respective ligands. A Zn-selective electrode to determine free ionic Zn could not be found commercially. A divalent electrode manufactured to test water hardness was tried, but it was not sufficiently sensitive to detect free Zn at the concentrations necessary.

Gel filtration chromatography was used to assess the structural integrity of organic Zn sources. The hypothesis in this study was that if Zn remained bound to ligand(s), because of their larger molecular size, this chelated Zn would be eluted from the column earlier than would a free metal ion. The methods used in gel filtration in our study were similar to those used by Brown and Zeringue (1994). Another technique that

Table 16. Sequential best-fit models from all possible subset regressions of estimated bioavailability values (relative to reagent-grade Zn sulfate) by chicks and lambs with chemical characteristics of organic Zn sources as independent variables

R^2	Independent variables in model
	Chicks (n = 5)
.9126	Zn solubility pH 5 buffer
.9996	Zn solubility pH 5 buffer + Zn solubility pH 2 buffer
.9999	Zn solubility pH 5 buffer + Zn solubility pH 2 buffer + soluble Zn fraction at 25°C
1.0000	Zn solubility pH 5 buffer + Zn solubility pH 2 buffer + soluble Zn fraction at 25°C + Q_f from polarography
	Lambs (n = 3)
.9939	Zn solubility pH 2 buffer
1.0000	Zn solubility pH 2 buffer + soluble Zn fraction at 25°C
	Chicks and lambs (n = 8)
.7909	Zn solubility pH 5 buffer
.8210	Zn solubility pH 5 buffer + citric acid solubility
.8279	Zn solubility pH 5 buffer + citric acid solubility + insoluble Zn fraction at 25°C
.8282	Zn solubility pH 5 buffer + citric acid solubility + insoluble Zn fraction at 25°C + neutral ammonium citrate solubility

could be useful to determine strength of chelation was demonstrated by Caroli et al. (1993). They used high-performance liquid chromatography/inductively coupled plasma-atomic emission spectrometry for the speciation of proteic Fe. In the present study, soluble Zn in filtrates of organic sources dissolved in pH 2.0 HCl-KCl buffer and pH 5.0 potassium phosphate buffer eluted in the same range of fractions as did free metals from Zn Sulf. The chromatography study suggested that no intact organic Zn was present in the soluble filtrates when organic products were dissolved in pH 2.0 and pH 5.0 buffer. This was in agreement with results of Brown and Zeringue (1994). These authors used gel filtration to evaluate 15 commercially complexed or chelated mineral supplements (five Cu, five Zn, and five Mn). In their study, .25 mL filtrate from solubility at pH 5.0 buffer was loaded onto the column and eluted with the same buffer. The .5-mL eluent fractions were analyzed quantitatively for metals and qualitatively for the presence of ninhydrin reactants to test the presence of AA or related proteinaceous material. The authors found that ninhydrin reactants and metals in filtrates eluted in the same general ranges of fractions as did ninhydrin reactants from pure AA and atomic absorption standards. They concluded that the minerals solubilized were no longer chelated in pH 5 K_2HPO_4-KH_2PO_4 buffer. However, they did not test for ninhydrin reactants in the original filtrate before loading onto the column. Ninhydrin reactants were detected in the filtrate from all solubility tests in the present study. Ninhydrin may react with not only free AA or proteinaceous materials but also that bound to metals (Matsui et al., 1996). We concluded that ninhydrin reactants are not a valid test for identifying the integrity of organic Zn sources. The Zn elution patterns should provide a good estimation of the integrity of the organic metals. Our results seemed to differ with those of Matsui et al. (1996). These authors used the same method and found that Zn in a Zn amino acid chelate eluted faster than that in Zn Sulf at pH 4; however, at pH 2, the retention time of Zn from the chelated Zn was comparable to that from the inorganic Zn. The authors concluded that the chelated Zn was stable at pH 4 but was dissociated at pH 2. These authors used a different amino acid chelate than that used in our studies. Also, they did not determine chelation effectiveness by polarography for comparative purposes.

Based on the Brown and Zeringue (1994) method, we further tested structural integrity of organic Zn sources of filtrate from solubility tests in deionized H_2O. Chromatographic data confirmed that small portions of Zn were still chelated in filtrates when these organic sources were solubilized in deionized H_2O at a concentration of 1 mg product/mL. The amount of chelated Zn can be calculated from chromatographic data. The results indicated that less than 13% of Zn was still chelated in all samples. If the Zn is separated in a mild solvent like H_2O, it is likely to separate in the rumen or nonruminant stomach in which the pH is low. The amount of Zn chelated in these organic Zn products was in agreement with their chelation effectiveness. The higher the chelation quotient, the more Zn was still chelated in H_2O. The correlation coefficient between the $\log_{10}$ of the Q_f value from polarography and the percentage of each source remaining chelated following a test of solubility in water was .96. The amount of Zn remaining bound in the complexed products was not as great as that in the chelated products.

Bioavailability was defined as the degree to which an ingested nutrient in a particular source is absorbed in a form that can be utilized in metabolism by the normal animal (Ammerman et al., 1995). It should, however, be kept in mind that bioavailability is not an inherent characteristic of a specific source of any mineral element (Fairweather-Tait, 1987). It is an experimentally determined value that is dependent on conditions during a specific test situation. Therefore, there is no single, correct value to assign to any particular source of an element, although this is a common perception among researchers and the feed industry. Earlier reports from our laboratory (Sandoval et al., 1997a,b) have discussed the advantages of using plethoric vs low dietary concentrations of Zn in bioavailability assays, as well as compared the similar estimates of bioavailability in various sources found with the two different bioassay methods, so that will not be discussed again herein.

Bone Zn has been used primarily as the criterion for bioavailability assays with nonruminants (Baker and Ammerman, 1995). Bone Zn concentrations in the present studies were similar to those reported in other studies with chicks fed similar amounts of Zn (Henry et al., 1987; Sandoval et al., 1997a, 1998, 1999). Henry et al. (1997) reported no difference in bone Zn concentrations when yearling wethers were supplemented with 700, 1,400, or 2,100 mg/kg added Zn as the sulfate for 10, 20 or 30 d. These researchers, along with Sandoval et al. (1997b), reported that liver and kidney were the most sensitive tissues to dietary Zn in more mature ruminants; however, they did not collect samples of pancreas.

Mucosal Zn accumulation was also found in the present study to be a useful estimate of bioavailability of Zn sources not examined previously in our laboratory. Bioavailability estimates were similar based on bone or mucosal cell Zn at 2 and 3 wk of supplementation, but not at 1 wk. Accumulation of the element in mucosal cells is a reflection of Zn absorption and homeostasis rather than actual Zn utilization as is bone deposition. Some of the Zn found in mucosa will be in the form of MT that will eventually be lost from sloughing of the gastrointestinal tract to protect the animal from an overabundance of the element (Kelly et al., 1996). Bone also acts as a long-term storage sink for the element (Foster et al., 1984) but has a slower turnover than tissue MT and can be resorbed for use in metabolic processes. The difference in estimated bioavailability values observed with very young chicks obtained by

either bone Zn or mucosal MT may result, in part, from a delay in synthesis of MT in response to dietary Zn. Use of a highly dynamic compound such as MT can be highly dependent on time and amount of the element consumed so that comparisons of results among studies must be made carefully. Another potential problem with use of MT as a bioassay criterion is the fact that the animal is able to adapt to high dietary Zn consumption and eventually decreases MT production even when intake of the element remains high. This mechanism has been investigated in rats but is still not well understood (Reeves, 1995). There was evidence of a similar phenomenon in chicks in that the MT content of mucosal cells decreased from 2 to 3 wk of age in chicks supplemented with all levels of Zn, especially Zn Sulf at 600 mg/kg.

Experiments reported herein indicated that Zn ProA was more available as a source of Zn than reagent-grade Zn Sulf for chicks and lambs, but that the other organic sources were similar in degree of utilization to the inorganic form. The literature reports conflicting results regarding bioavailability of organic Zn sources. Supplemental Zn from a Zn amino acid chelate resulted in growing more hair of higher Zn content in dogs than when they were fed Zn from either Zn oxide or Zn polysaccharide (Lowe et al., 1994). Rojas et al. (1995) reported that Zn from Zn methionine and Zn lysine had equal or greater availability than Zn from Zn sulfate for lambs when tissue Zn and MT concentrations were used as indices of Zn bioavailability. The same authors found no differences between organic and inorganic sources when fed to cattle (Rojas et al., 1996). In other studies with ruminants (Greene et al., 1988; Kerley and Ledoux, 1992;) no differences in utilization among sources were reported. Wedekind et al. (1992) used multiple linear regression analysis on bone Zn accumulation and found that Zn was more bioavailable from Zn methionine than from Zn sulfate for chicks. Results from studies with swine have been quite variable. Zinc from organic forms was less available compared with Zn sulfate (Schell and Kornegay, 1996), was more available (Matsui et al., 1996), or was equal in value (Hill et al., 1986).

A radiolabeled sample of ^{65}[Zn] methionine similar to that used in the present experiments was eluted from a gel column earlier than Zn chloride or a commercial Zn-propionate compound (Beutler et al., 1998). No differences were found, however, among these three sources of Zn as measured by incorporation into various cell culture systems. In an experiment with a Zn amino acid chelate that was not used in the present studies, the chelated zinc eluted from a gel column faster than ionic Zn at a pH of 4, but the rate of elution was similar when the buffer was pH 2 (Matsui et al., 1996). In a 30-d trial in which pigs were fed these products at 100 mg/kg added Zn, the authors reported bone Zn was greater in pigs fed the chelated product than in those fed Zn sulfate. Concentrations of Zn in liver, kidney, spleen, and plasma were similar for the organic and inorganic sources.

In the present studies, it did not appear that many of the chemical indicators of chelation effectiveness have any influence on the manner in which chicks or lambs are able to absorb the element. There was a high negative correlation, however, between bioavailability and solubility of the Zn in organic products in buffers with a physiological pH. In the present experiments, Zn ProA, the only organic source with greater bioavailability than that of Zn Sulf, had the lowest solubility in either pH 2 or 5 buffers, but it did not have the greatest amount of Zn remaining in a chelated form following gel chromatography. The results reported herein suffer from a lack of numbers and have a lack of range in relative bioavailability values. They do, however, show that use of chemical characteristics as an indicator of bioavailability should be approached with discretion.

Implications

Bioavailability of the Zn in commercial organic Zn products was most related to negative solubility of Zn in pH 5 buffer in chicks and pH 2 buffer in lambs. Of the six organic products tested, only Zn proteinate A had a bioavailability estimate in chicks or lambs greater than that of reagent-grade $ZnSO_4 \cdot 7H_2O$. Although numerous differences were found in the chemical characterization among the organic Zn products, animal feeding trials failed to distinguish among products with regard to absorption and tissue deposition of Zn from the various organic sources. The results indicate that the organic Zn compounds tested are generally equal to $ZnSO_4 \cdot 7H_2O$ as supplemental sources of the element for domestic animals.

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