ELSEVIER

Early and advanced glycation end-products are increased in dietary copper deficiency

Jack T. Saari and Gwen M. Dahlen

United States Department of Agriculture, Agricultural Research Service, Grand Forks Human Nutrition Research Center, Grand Forks, ND USA

The hypothesis that nonenzymatic glycosylation of proteins (glycation) contributes to damage associated with dietary copper deficiency has depended largely on indirect evidence. Thus far, the observation of an elevated percentage of glycated hemoglobin in copper-deficient rats has provided the only direct evidence of an increase in glycation. We sought further direct evidence of increased glycation in copper deficiency. Male weanling rats were fed a copper-adequate (CuA, 6.4 mg Cu/kg diet) or copper-deficient diet (CuD, 0.4 mg Cu/kg diet) for 5 weeks. Rats fed the CuD diet were copper deficient as judged by depressed organ copper concentrations and a variety of indirect indices. Measurements of hemoglobin A_1 and serum fructosamine (both early glycation end-products) as well as serum pentosidine (an advanced glycation end-product) indicated that all three compounds were elevated in CuD rats relative to CuA rats. This finding further supports the view that glycation is enhanced and thus may contribute to defects associated with dietary copper deficiency. (J. Nutr. Biochem. 10:210–214, 1999) *Published by Elsevier Science Inc.*

Keywords: copper; glycation; hemoglobin A_1; fructosamine; pentosidine

Introduction

Nonenzymatic glycosylation (glycation) is the deleterious binding of sugars to protein that is commonly observed in diabetes and aging.[1,2] The definition of glycation comprises a series of reactions that includes the binding of the acyclic form of a sugar to specific amino acids on a protein to form a Schiff base, rearrangement of the Schiff base to form an Amadori product (so-called early products), and crosslinking and subsequent degradation of proteins to form advanced glycation end-products.[1,2]

Previously, we proposed that glycation may contribute to the deleterious effects of dietary copper deficiency.[3,4] A variety of indirect evidence pointed to this possibility. The observation of reduced glucose tolerance in copper-deficient rats[5–8] provided evidence for the hyperglycemia required for glycation. Evidence that dietary enrichment with fructose, a better glycator than glucose,[9] enhanced the defects of copper deficiency[4,10,11] suggested exaggeration of an existing effect. Further, amelioration of signs of copper deficiency by food restriction,[4,12,13] which reduces blood glucose and glycation,[14] and by treatment with aminoguanidine,[3] an inhibitor of advanced glycation,[15] reinforced the view that glycation contributes to defects of copper deficiency.

Despite the compelling indirect evidence, thus far the only direct evidence that glycation occurs in copper deficiency has been the observation of an elevated percentage of glycated hemoglobin (Hb A_1) in copper-deficient rats.[3,4,16] Hb A_1 is an early glycation end-product.[17] The objective of the present study was to corroborate the enhancement of early glycation by measurement of another early glycation end-product and to determine whether the glycation hypothesis could be supported further by measurement of an advanced glycation end-product. For that purpose we measured concentrations of serum fructosamine, an early (Amadori) product of protein glycation,[18] and pentosidine, a

Address correspondence to Jack T. Saari, Ph.D., USDA, ARS, Grand Forks Human Nutrition Research Center, Grand Forks, ND 58202-9034.

Received August 31, 1998; accepted November 30, 1998.

0955-2863/99/$–see front matter
PII S0955-2863(98)00100-4

fluorescent condensation product of ribose, lysine, and arginine that represents protein crosslinking,[19] in copper-adequate and copper-deficient rats. In addition, we attempted to determine whether the previously observed inhibition of signs of copper deficiency by aminoguanidine[3] could be correlated with a depressed production of advanced glycation end-products and, further, whether treatment with the antioxidant n-acetylcysteine[20] might demonstrate a synergy between oxidative stress and glycation.

Methods and materials

Animals and diets

Experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*.[21]

Fifty-six male weanling Sprague-Dawley rats (Sasco, Lincoln, NE USA) were divided into eight weight-matched groups. Four of the groups were fed a copper-adequate (CuA) diet and the other four a copper-deficient (CuD) diet. Rats in each of the four subgroups within each dietary group were given daily intraperitoneal injections of, respectively, (1) normal saline, (2) aminoguanidine (as the hemisulfate; 50 mg/kg; Sigma, St Louis, MO USA), (3) n-acetylcysteine (12.5 mg/kg body weight; Sigma), or (4) both aminoguanidine and n-acetylcysteine.

Diets were composed of 940.0 g/kg of basal diet (catalog #TD84469, Teklad Test Diets, Madison, WI USA), 50.0 g/kg of safflower oil (Hollywood Foods, Los Angeles, CA USA), and 10.0 g/kg of mineral mix. The mineral mix contained cornstarch (Best Foods, Englewood Cliffs, NJ USA) and iron with or without copper and was designed to provide 0.22 g of ferric citrate (16% Fe; Baker, Phillipsburg, NJ USA) and either 0.02 g of or no $CuSO_4 \cdot 5H_2$ (Baker) per kilogram of diet. The final CuA diet contained 200 g/kg of casein, 386 g/kg of sucrose, 304 g/kg of cornstarch, 50 g/kg of safflower oil, minerals equivalent to adding 35 g/kg of AIN-76 mineral mix (catalog #170915, Teklad), and vitamins equivalent to adding 10 g/kg of Teklad vitamin mix (catalog #40060, Teklad).[22] Diet analysis by atomic absorption spectrophotometry indicated that the CuA diet contained 6.4 mg of copper per kilogram of diet and the CuD diet contained 0.4 mg of copper per kilogram of diet. Parallel analysis of National Institute of Standards and Technology (NIST, Gaithersburg, MD USA) reference samples (#1515, apple leaves) yielded a value (5.41 mg Cu/kg) within the specified range (5.40–5.88 mg Cu/kg).

Copper status indicators

After consumption of their respective diets for 5 weeks, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg body weight; Sigma). Blood was withdrawn from the inferior vena cava and divided into aliquots for erythrocyte counting (EDTA-treated) and serum assays (samples allowed to clot at room temperature). Hearts were collected for copper assays and determination of copper, zinc-superoxide dismutase (Cu, Zn-SOD) activity, and livers were collected for copper and iron assays.

Hematocrits were determined by using a Cell-Dyn cell counter (model 3500CS, Abbott, Santa Clara, CA USA).

For mineral assays, samples of heart and liver were lyophilized and then digested with nitric acid and hydrogen peroxide.[23] Mineral concentrations of digested samples were measured by inductively coupled argon plasma emission spectroscopy (Fisons-ARL, model 3560B, Thermo-Jarrell-Ash, Franklin, MA USA).

Heart Cu,Zn-SOD activity was measured spectrophotometrically using a Bioxytech SOD-525 kit (OXIS, Portland, OR USA). A unit of activity is that amount of enzyme required to double the rate of autoxidation of a proprietary reagent, the product of which is a chromophore with maximal absorbance at 525 nm.

Assays for early glycation products

Hb A_1 was measured by using the glycated hemoglobin (Hb A_1) kit (procedure no. 441; Sigma Diagnostics, St. Louis, MO USA), which utilizes a cation exchange resin to separate Hb A from Hb A_1 and spectrophotometric measurement (415 nm) of each fraction. Hb A_1 is expressed as a percentage of total Hb A.

Serum fructosamine was measured by using the fructosamine kit (procedure no. 465; Sigma Diagnostics), a colorimetric test based on the ability of glycated serum proteins to reduce nitroblue tetrazolium.[24]

Pentosidine assay

Pentosidine was measured by high pressure liquid chromatography (HPLC) that utilized column switching to enhance separation. Sample preparation followed the technique of Odetti et al.[25] Fifty milligrams of protein from a serum sample were precipitated on ice with an equal volume of 10% trichloroacetic acid (TCA; Eastman Kodak, Rochester, NY USA). Protein pellets were washed twice with 5% TCA and acid hydrolyzed. Hydrolysis was accomplished in borosilicate tubes by adding 2 mL of degassed 6N HCl to the tubes, purging with nitrogen, capping the tubes, and heating for 16 hours at 110°C in an aluminum heating block. Residual acid was evaporated (AS160 Speed Vac, Savant, Farmingdale, NY USA) and samples were reconstituted in purified water (Millipore, Milford, MA USA) containing 0.01 mol/L of n-heptafluorobutyric acid (HFBA; Sigma). The sample was filtered through a 0.45-μm spin filter and a volume containing 1.4 to 1.7 mg protein was injected onto column 1 of the HPLC system.

The use of column switching in the HPLC isolation and detection of pentosidine followed the protocol of Takahashi et al.[26] The HPLC system consisted of a system controller (model SCL-6B, Shimadzu, Columbia, MD USA), two pumps (model LC-10AD, Shimadzu), an autosampler (model SIL-7A, Shimadzu), an ultraviolet detector (detector 1, model 166, Beckman, Fullerton, CA USA), a fluorescence detector (detector 2, model RF-551, Shimadzu), and a six-port valve with a two position activator (model EHMA, Valco, Houston, TX USA). Column 1 was a gel-filtration column [TSK precolumn PW (4.6 mm × 3.5 cm); TosoHaas, Montgomeryville, PA USA] and column 2 was an octadecylsilyl column [TSK-GEL ODS-80T (4.6 mm × 15 cm), TosoHaas]. The mobile phase for column 1 was 50 mL/L acetonitrile (Baker), containing 30 mmol/L HFBA; the mobile phase for column 2 was 200 mL/L acetonitrile containing 30 mmol/L HFBA. The flow rate of through each column was 1.0 mL/min.

The initial valve setting provided for column 1 and detector 1 to run in parallel with column 2 and detector 2. Sample was injected onto column 1 and passage of the fraction containing pentosidine was identified by detector 1 (absorbance peak at 297 nm). The eluant containing this fraction was then diverted to column 2 by switching the valve position and pentosidine was measured by detector 2 via fluorometry (emission 385 nm, excitation 335 nm). After transfer of the pentosidine-containing fraction from column 1 to column 2 (duration of transfer, 1.6 minutes), the valve was switched back to its original position and was ready for injection of the next sample. All operations were performed automatically by the autosampler and system controller.

Pentosidine standard was prepared[27] and provided by Dr. V.M. Monnier (Institute of Pathology, Case Western Reserve University, Cleveland, OH USA).

Table 1 Characteristics [mean ± SEM (*N*)] of rats fed copper-adequate (CuA) and copper-deficient (CuD) diets and treated by daily intraperitoneal injection of normal saline, aminoguanidine (AG), n-acetylcysteine (NAC), or AG plus NAC

		Variable						
Diet	Treatment	Body weight (g)	Heart weight (mg/g body wt)	Hematocrit	Liver Cu (nmol/g dry wt)	Heart Cu (nmol/g dry wt)	Liver Fe (μmol/g dry wt)	Heart Cu,Zn-SOD (U/μg protein)
CuA	Saline	268 ± 21 (7)	3.64 ± 0.15 (7)	0.39 ± 0.02 (6)	342 ± 26 (7)[a]	298 ± 9 (7)	4.59 ± 0.21 (7)	280 ± 12 (7)
	AG	299 ± 9 (7)	3.49 ± 0.06 (7)	0.41 ± 0.01 (7)	227 ± 13 (7)[b]	293 ± 4 (7)	3.51 ± 0.19 (7)	279 ± 20 (7)
	NAC	285 ± 14 (7)	3.32 ± 0.10 (7)	0.42 ± 0.01 (7)	198 ± 17 (7)[b]	296 ± 9 (7)	3.92 ± 0.12 (7)	254 ± 7 (7)*
	AG + NAC	307 ± 7 (7)	3.40 ± 0.09 (7)	0.41 ± 0.01 (7)	278 ± 29 (7)[b]	285 ± 6 (7)	4.26 ± 0.32 (7)	302 ± 8 (7)
CuD	Saline	259 ± 12 (7)	5.33 ± 0.24 (7)	0.20 ± 0.01 (7)	62 ± 18 (5)[c]	78 ± 9 (6)	6.25 ± 0.91 (7)	128 ± 14 (7)
	AG	251 ± 10 (7)	5.78 ± 0.46 (7)	0.20 ± 0.02 (7)	80 ± 18 (7)[c]	72 ± 4 (7)	7.55 ± 0.75 (7)	93 ± 12 (7)
	NAC	261 ± 4 (7)	5.69 ± 0.41 (7)	0.19 ± 0.01 (5)	72 ± 17 (7)[c]	77 ± 5 (7)	7.33 ± 1.00 (7)	61 ± 12 (7)*
	AG + NAC	255 ± 5 (7)	5.40 ± 0.26 (7)	0.21 ± 0.02 (6)	55 ± 13 (7)[c]	75 ± 6 (7)	6.87 ± 0.71 (7)	103 ± 14 (7)
Source of variation		Analysis of variance, *P*-values						
Diet		0.0002	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Treatment		NS	NS	NS	0.02	NS	NS	0.003
Diet × treatment		NS	NS	NS	0.002	NS	NS	NS

[a,b,c] Values in this column not sharing a common superscript are different ($P < 0.05$).
*Rats treated with NAC have significantly lower values than those treated with saline, AG, or AG+NAC.
Cu,Zn-SOD–copper, zinc superoxide dismutase.

Statistics

Two-way analysis of variance (ANOVA)[28] was used to test for effects of diet and treatment on the measured variables. If ANOVA showed significant interactions between diet and treatment, individual means were compared by using the Student-Newman-Keuls test.[28] Main effects, interactions, or differences in means were regarded as significant if the *P*-value was less than 0.05.

Results

Evidence of copper deficiency in rats fed a CuD diet is provided in *Table 1*, which lists characteristics often associated with copper deficiency. ANOVA indicated that body weight was lower, heart weight relative to body weight was higher, hematocrit was lower, organ copper concentrations were lower, liver iron was higher, and activity of heart Cu,Zn-SOD was lower in rats fed a CuD diet than in those fed a CuA diet. ANOVA showed an interaction effect between diet and treatment on liver copper concentration, which, after comparison of means, was accounted for by a higher copper concentration in CuA saline-treated rats than in other CuA rats. A main effect of treatment on Cu,Zn-SOD activity was caused by a depression of activity by n-acetylcysteine treatment relative to other treatments. The inhibition by aminoguanidine of defects associated with copper deficiency that was observed in a prior study[3] was not evident in this study.

The effects of diet and treatment on products of glycation are shown in *Table 2*. ANOVA indicated that rats fed a CuD diet had a higher percentage of Hb A_1 and higher concen-

Table 2 Products of glycation [mean ± SEM (*N*)] in rats fed copper-adequate (CuA) and copper-deficient (CuD) diets and treated by daily intraperitoneal injection of normal saline, aminoguanidine (AG), n-acetylcysteine (NAC), or AG plus NAC

		Product		
Diet	Treatment	Hemoglobin A_1(%)	Serum fructosamine (nmol/mg protein)	Serum pentosidine (pmol/mg protein)
CuA	Saline	2.15 ± 0.04 (7)	21.1 ± 0.4 (5)[a]	ND (3)
	AG	2.07 ± 0.08 (7)	21.0 ± 0.5 (7)[a]	ND (5)
	NAC	2.03 ± 0.04 (7)	21.9 ± 1.2 (6)[a,b]	ND (5)
	AG + NAC	2.04 ± 0.07 (7)	22.6 ± 0.3 (7)[a,b]	ND (4)
CuD	Saline	3.33 ± 0.12 (7)	25.8 ± 1.2 (6)[b,c]	7.4 ± 1.8 (3)
	AG	3.44 ± 0.17 (7)	24.7 ± 0.7 (7)[a,c]	9.1 ± 2.7 (5)
	NAC	3.37 ± 0.20 (7)	26.6 ± 1.8 (7)[c]	5.9 ± 1.3 (5)
	AG + NAC	3.09 ± 0.12 (6)	32.1 ± 1.1 (7)[d]	3.4 ± 2.3 (5)
Source of variation		Analysis of variance, *P*-values		
Diet		0.0001	0.0001	0.0001
Treatment		NS	0.0004	NS
Diet × treatment		NS	0.03	NS

[a,b,c,d] Values in this column not sharing a common superscript are different ($P < 0.05$).
ND–not detectable.

trations of serum fructosamine and serum pentosidine than did rats fed a CuA diet. An interaction between diet and treatment on fructosamine production was caused primarily by its relatively higher elevation in copper-deficient, aminoguanidine + n-acetylcysteine-treated rats. The expected inhibition of pentosidine production by aminoguanidine did not occur.

Discussion

The new findings of this study—that fructosamine and pentosidine are elevated in the serum of copper-deficient rats—provide additional direct evidence of the occurrence of glycation in dietary copper deficiency.

The possible consequences of enhanced glycation are extensive. Because the current observations involved blood samples, the most explicit possibility is that blood-related functions are impaired. Dietary copper deficiency alters platelet function and aggregation, enhances inflammation, and impairs vasodilation.[29] It also alters immune function[30] and causes anemia.[31,32] Proper function in all of these areas requires adequately functioning blood cells and proteins, each of which may be altered by glycation. That glycation may contribute, for instance, to anemia, is illustrated by the finding that hematocrit is inversely correlated with Hb A_1 when copper status is varied[4] and that aminoguanidine can ameliorate the anemia of copper deficiency.[3] Altered erythrocyte membrane protein[22,33] and altered osmotic fragility[4,34] in copper-deficient rats also may be reflections of glycation damage. Although oxidative stress has been implicated in some of this pathology, glycation and oxidation have been shown to be so interdependent[35–37] that we believe it is beneficial to study the two mechanisms concurrently as possible causes of copper deficiency-induced damage.

Direct observation of glycated protein in tissues beyond the circulation has yet to be made. We have shown indirectly that glycation may be involved in the organ enlargement of copper deficiency by use of manipulations that affected glycation and concurrently affected the enlargement.[4] In these studies it was not possible to rule out a contribution of oxidative damage and, as stated above, future studies should consider both glycation and oxidation in order to clarify the respective roles of the two mechanisms.

The occurrence of glycation in dietary copper deficiency very likely involves endocrine pancreatic dysfunction. Although pancreatic insulin production is elevated, fasting plasma insulin concentration is lower in copper-deficient rats,[38,39] particularly in males.[40] Following a glucose load, release of insulin is either reduced[38,41] or delayed.[7] This manifests itself as a reduced glucose tolerance.[5–8] The reduced glucose tolerance thus produces the hyperglycemia requisite for enhanced glycation. Aside from pancreatic dysfunction *per se*, insulin resistance and altered insulin binding also have been proposed as contributors to altered carbohydrate handling.[42,43]

Treatments aimed at more closely examining the roles of glycation and oxidative stress in the defects of copper deficiency were ineffective. One possible explanation is that the treatment doses were too small. In the prior study where an effect of aminoguanidine was observed,[3] a different diet formulation (preliminary, but similar, to AIN-93) was used, which, although dietary copper was similar, caused a more severe deficiency that was perhaps more susceptible to improvement. The absence of an effect of aminoguanidine on pentosidine production in this study corroborates our view that the dosage of aminoguanidine was too small. We feel that examination of the relationship between advanced products and defects of copper deficiency must be pursued more aggressively in future studies.

Acknowledgments

The authors wish to acknowledge V.M. Monnier and D.R. Sell, The Institute of Pathology, Case Western Reserve University (Cleveland, OH USA) for providing pentosidine standard and for useful technical discussions regarding the pentosidine assay. The technical assistance of Peter Leary, animal care by Jackie Nelson and Deb Raasakka, and diet preparation by Karin Tweton also was appreciated.

References

1 Reiser, K.M. (1991). Nonenzymatic glycation of collagen in aging and diabetes. *Proc. Soc. Exp. Biol. Med.* **196,** 17–29
2 Ruderman, N.R., Williamson, J.R., and Brownlee, M. (1992). Glucose and diabetic vascular disease. *FASEB J.* **6,** 2905–2914
3 Saari, J.T. (1994). Implication of nonenzymatic glycosylation as a mode of damage in dietary copper deficiency. *Nutr. Res.* **14,** 1689–1699
4 Saari, J.T., Bode, A.M., and Dahlen, G.M. (1995). Defects of copper deficiency in rats are modified by dietary treatments that affect glycation. *J. Nutr.* **125,** 2925–2934
5 Keil, H.L. and Nelson, V.E. (1934). The role of copper in carbohydrate metabolism. *J. Biol. Chem.* **106,** 343–349
6 Cohen, A.M., Teitelbaum, A., Miller, E., Ben-Tor, V., Hirt, R., and Fields, M. (1982). Effects of copper on carbohydrate metabolism in rats. *Isr. J. Med. Sci.* **18,** 840–844
7 Hassel, C.A., Marchello, J.A., and Lei, K.Y. (1983). Impaired glucose tolerance in copper-deficient rats. *J. Nutr.* **113,** 1081–1083
8 Fields, M., Ferretti, R.J., Smith, J.C., and Reiser, S. (1984). Impairment of glucose tolerance in copper deficient rats: Dependency on the type of dietary carbohydrate. *J. Nutr.* **114,** 393–397
9 Bunn, H.F. and Higgens, P J. (1981). Reaction of monosaccharides with proteins: Possible evolutionary significance. *Science* **213,** 222–224
10 Reiser, S., Ferretti, R.J., Fields, M., and Smith, J.C. (1983). Role of dietary fructose in the enhancement of mortality and biochemical changes associated with copper deficiency in rats. *Am. J. Clin. Nutr.* **38,** 214–222
11 Fields, M., Ferretti, R.J., Reiser, S., and Smith, J.C. (1984). The severity of copper deficiency is determined by the type of dietary carbohydrate. *Proc. Soc. Exp. Biol. Med.* **175,** 530–537
12 Saari, J.T., Johnson, W.T., Reeves, P.G., and Johnson, L.K. (1993). Amelioration of effects of severe dietary copper deficiency by food restriction in rats. *Am. J. Clin. Nutr.* **58,** 891–896
13 Werman, M.J. and Bhathena, S.J. (1993). Restricted food intake ameliorates the severity of copper deficiency in rats fed a copper-deficient, high-fructose diet. *Med. Sci. Res.* **21,** 309–310
14 Masoro, E.J., Katz, M.S., and McMahan, C.A. (1989). Evidence for the glycation hypothesis of aging from the food-restricted rodent model. *J. Gerontol.: Biol. Sci.* **41,** B20–B22
15 Brownlee, M., Vlassara, H., Kooney, A., Ulrich, P., and Cerami, A. (1986). Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking. *Science* **232,** 1629–1632.
16 Klevay, L.M. (1982). An increase in glycosylated hemoglobin in rats deficient in copper. *Nutr. Rep. Int.* **26,** 329–334
17 Koenig, R.J., Blobstein, S.H., and Cerami, A. (1977). Structure of carbohydrate of hemoglobin A_{1c}. *J. Biol. Chem.* **252,** 2992–2997

18 Armbruster, D.A. (1987). Fructosamine: Structure, analysis and clinical usefulness. *Clin. Chem.* **33,** 2153–2163

19 Sell, D.R. and Monnier, V.M. (1989). Structure elucidation of a senescence cross-link from human extracellular matrix. Implication of pentoses in the aging process. *J. Biol. Chem.* **264,** 21597–21602

20 Aruoma, O.I., Halliwell, B., Hoey, B.M., and Butler, J. (1989). The antioxidant action of N-acetylcysteine: Its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Radic. Biol. Med.* **6,** 593–597

21 National Research Council (1996). *Guide for the Care and Use of Laboratory Animals*, National Academy Press, Washington, DC USA

22 Johnson, W.T. and Kramer, T.R. (1987). Effect of copper deficiency on erythrocyte membrane proteins of rats. *J. Nutr.* **117,** 1085–1090

23 Nielsen, F.H., Zimmerman, T.J., and Shuler, T.R. (1982). Interactions among nickel, copper and iron in rats. Liver and plasma contents of lipids and trace elements. *Biol. Trace Elem. Res.* **4,** 125–143

24 Johnson, R.N., Metcalf, P.A., and Baker, J.R. (1982). Fructosamine: A new approach to the estimation of serum glycosylprotein. An index of diabetic control. *Clin. Chim. Acta* **127,** 87–95

25 Odetti, P., Fogarty, J., Sell, D.R., and Monnier, V.M. (1992). Chromatographic quantitation of plasma and erythrocyte pentosidine in diabetic and uremic subjects. *Diabetes* **41,** 153–159

26 Takahashi, M., Hoshino, H., Kushida, K., Kawana, K., and Inoue, T. (1996). Direct quantification of pentosidine in urine and serum by HPLC with column switching. *Clin. Chem.* **42,** 1439–1444

27 Grandhee, S.K. and Monnier, V. (1991). Mechanism of formation of the Maillard protein cross-link pentosidine. *J. Biol. Chem.* **266,** 11649–11653

28 Kleinbaum, D.G. and Kupper, L.L. (1978). *Applied Regression Analysis and Other Multivariable Methods*, Duxbury, North Scituate, MA USA

29 Schuschke, D.A. (1997). Dietary copper in the physiology of the microcirculation. *J. Nutr.* **127,** 2274–2281

30 Lukasewycz, O.A. and Prohaska, J.R. (1990). The immune response in copper deficiency. *Ann. N.Y. Acad. Sci.* **587,** 147–159

31 Evans, J.L. and Abraham, P.A. (1973). Anemia, iron storage and ceruloplasmin in copper nutrition in the growing rat. *J. Nutr.* **103,** 196–201

32 Cohen, N.L., Keen, C.L., Hurley, L.S., and Lonnerdal, B. (1985). Determinants of copper-deficiency anemia in rats. *J. Nutr.* **115,** 710–725

33 Sukalski, K.A., LaBerge, T.P., and Johnson, W.T. (1997). In vivo oxidative modification of erythrocyte membrane proteins in copper deficiency. *Free Radic. Biol. Med.* **22,** 835–842

34 Jain, S.K. and Williams, D.M. (1988). Copper deficiency anemia: Altered red blood cell lipids and viscosity in rats. *Am. J. Clin. Nutr.* **48,** 637–640

35 Hicks, M., Delbridge, L., Yue, D.K., and Reeve, T.S. (1988). Catalysis of lipid peroxidation by glucose and glycosylated collagen. *Biochem. Biophys. Res. Commun.* **151,** 649–655

36 Wolff, S.P. and Dean, R.T. (1987). Glucose autoxidation and protein modification. The potential role of 'autoxidative glycosylation' in diabetes. *Biochem. J.* **245,** 243–250

37 Hunt, J.V., Bottoms, M.A., and Mitchinson, M.J. (1993). Oxidative alterations in the experimental glycation model of diabetes mellitus are due to protein-glucose adduct oxidation. Some fundamental differences in proposed mechanisms of glucose oxidation and oxidant production. *Biochem. J.* **291,** 529–535

38 Recant, L., Voyles, N.R., Timmers, K., Zalenski, C., Fields, M., and Bhathena, S.J. (1986). Copper deficiency in weanling rats, increased pancreatic contents of enkephalin-containing peptides and insulin. *Peptides* **7,** 1061–1069

39 Fields, M. and Lewis, C.G. (1997). Low dietary iron restores functional capabilities of both exocrine and endocrine pancreas in copper-deficient rats. *J. Trace Elem. Exp. Med.* **10,** 1–8

40 Fields, M. and Lewis, C.G. (1997). Impaired endocrine and exocrine pancreatic functions in copper-deficient rats: The effect of gender. *J. Am. Coll. Nutr.* **16,** 346–351

41 Cohen, A.M. and Miller, E. (1986). Effect of copper on insulin release by the intact rat pancreas and the perfused rat pancreas. *Pancreas* **1,** 309–316

42 Fields, M., Ferretti, R.J., Smith, J.C. and Reiser, S. (1983). Effect of copper deficiency on metabolism and mortality in rats fed sucrose or starch diets. *J. Nutr.* **113,** 1335–1345

43 Fields, M., Reiser, S., and Smith, J.C. (1983). Effect of copper and zinc on insulin binding and glucose transport by isolated rat adipocytes. *Nutr. Rep. Int.* **29,** 163–169