collagen and to leave behind the remaining noncollagenous protein and elastin. The very high level of hydroxyproline and hydroxylysine indicated that these preparations were not contaminated with noncollagenous proteins. The amino acid composition of insoluble collagen in uteri from several species is shown in Table I. For easy comparison, the amino acid composition of soluble collagens in uteri from pigs and human beings are listed at the end. The principal difference in amino acid composition was a higher content of hydroxyproline and hydroxylysine in insoluble as compared to soluble collagen. More hydroxyproline was present in the insoluble collagen of guinea pigs and rabbits than in that of the other species studied. The insoluble collagen of rats showed the highest content of hydroxylysine and the lowest content of lysine. In agreement with the findings of Piez (5), despite the variations which may occur in the amounts of lysine and hydroxylysine present in the collagen of different organs of rats or in the uteri of various species, the summation of these two amino acids always represents a constant. Serine in the insoluble collagen of the rat and the guinea pig was higher than in any other species studied. Uterine insoluble collagen was found to contain more glycine and aspartic acid and less proline, alanine, and valine than the soluble collagen of the same organ. The cysteic acid content, which was observed as 1 residue/1000 residues in soluble collagen of uteri of pig and human beings, was absent from the insoluble collagen of these two species, but 1 residue/1000 residues were found in the insoluble collagen of the rabbit and cow. The present study is in agreement with our early findings (1,2) that there is a higher content of hydroxyproline and hydroxylysine in uterine collagen than in dermal collagen.

Summary. Insoluble collagen was prepared from the uteri of rat, guinea pig, rabbit, pig, cow, and human being, after extracting the salt- and acetic acid-soluble collagens. The insoluble collagen of uterus contained a significantly higher content of hydroxyproline and hydroxylysine than the soluble collagen from uterus. The highest content of hydroxyproline, 115 residue/1000 residues, was found in the insoluble collagen of rabbit uterus. The highest content of hydroxylysine, 16 residues/1000 residues, was found in rat uterine insoluble collagen.

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Production of Copper Deficiency in the Rat by an Egg Albumin Diet*
(32781)

R. S. MORRIS,[1] WILLARD D. HUBBARD AND FAYE S. GIBSON (Introduced by M. R. Spivey Fox)
Division of Nutrition, Food and Drug Administration, Washington, D. C. 20204

Copper deficiency in experimental animals is characterized by impaired growth, anemia, and lower copper levels in certain tissues. Hair pigmentation is a sensitive indicator of the copper status of the animal, and achromotrichia is a characteristic symptom of copper

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[1] Present address: Experimental Nutrition Laboratory, Human Nutrition Research Division, ARS, USDA, Beltsville, Md. 20705.

deficiency (1). Zinc status and tissue zinc levels of swine and man are known to be related to zinc content of hair (2–5), suggesting that the copper content of hair may be a reliable and easily measured index of the copper status of the individual.

Copper levels of liver, kidney, brain, and blood are reduced by feeding a diet deficient in copper, commonly a diet containing dried whole milk or skim milk powder. Few studies have been made with other protein sources. Long-Evans rats fed a casein diet containing 14.6 mg copper per kg of diet developed achromotrichia but showed no growth retardation or anemia (6). Mills and Murray (7) also used a casein-containing diet to attain copper deficiency in the rat. They collected milk and prepared casein from it under conditions where trace element contamination was minimal, and the dietary salts were purified in the laboratory before use. Animals fed this diet showed retarded growth and anemia.

Egg albumin is low in many trace elements including copper and should be a good source of protein for copper-deficient diets. In this study, a diet containing egg albumin and other commercially available materials was used without further purification to attain copper deficiency in the black rat. This diet meets the NRC requirements for growth and maintenance of the rat (8). These animals were compared to animals fed a dried skim milk diet. Since dietary ascorbic acid intensifies copper deficiency symptoms in chicks and rabbits fed skim milk diets as judged by growth, anemia, and myocardial and aortic histopathology, and arterial rupture (9–11), the effect of adding ascorbic acid to the egg albumin diet was studied in rats. Growth, achromotrichia, hematocrit values, and copper levels of blood plasma, liver, kidney, brain, and hair were used as indices of copper status.

Materials and Methods. Weanling black rats of both sexes of the Division of Nutrition strain, which is derived from Long-Evans and Osborne and Mendel strains, were housed in suspended wire cages made of type 304 stainless steel. Environmental contamination was reduced by covering the rack with a curtain of polyethylene and nonwoven cloth (Pellon). Food and demineralized water were provided *ad libitum*. Animals were weighed weekly. At the end of the experimental period, they were fasted overnight (16–18 hours) and anesthetized with sodium pentobarbital. Blood was withdrawn by cardiac puncture into a heparinized syringe. Plasma, obtained by centrifugation, was stored at —4°C in plastic tubes. Tissues were weighed immediately, put into polyethylene envelopes, sealed and stored at —4°C. Hair was shaved from the back and stored in plastic envelopes. Before analysis, the hair was washed with 0.1% soap (Ivory) solution, rinsed thoroughly with deionized water and finally washed twice with diethyl ether (5). All glassware was acid-washed and thoroughly rinsed in deionized water. Every effort was made to eliminate contamination with trace elements.

Tissues were wet ashed with sulfuric-nitric–perchloric acids and were analyzed for their content of copper by the oxalyldihydrazide color reaction as described by Markowitz *et al.* (12). Removal of interfering iron was necessary only for liver. Plasma protein patterns were obtained by disc electrophoresis.

The compositions of the diets are shown in Table I. The egg albumin diet contained 1.00 mg of copper/kg of diet and the skim milk diet contained 0.40 mg/kg of copper. The salt mixture, with copper sulfate omitted, was prepared from reagent grade chemicals. Copper was added to the diets of copper-supplemented groups as copper sulfate.

Results and Discussion. The rats fed a copper-deficient diet containing egg albumin developed a copper deficiency similar to that obtained by feeding the unsupplemented skim milk diet. Performance of copper-supplemented animals on the two diets was comparable. No mortality was observed in any group. After 6 weeks, the average weight of male rats fed the egg albumin diet containing copper (4 mg/kg) was 176 gm while that of female rats was 156 gm. Male rats fed the skim milk diet containing copper (20 mg/kg) had an average weight of 190 gm while female rats had an average weight of 135 gm. Growth on these diets was similar to that of animals fed a stock diet. On both copper-deficient diets, hematocrit values and growth were not affected by the deficiency but achromotrichia was evident in the animals after 4 weeks.

TABLE I. Composition of Diets.

Component	Egg albumin (gm/kg)	Skim milk (gm/kg)	Soybean protein (gm/kg)
Egg albumin[a]	200	—	—
Dried skim milk[b]	—	550	—
Soybean protein[c]	—	—	350
Glycine	—	—	10
DL-Methionine	—	5	6
Dextrose[d]	686.8	377.3	521.8
Salt mixture[e]	60	—	60
Corn oil[f]	40	40	40
Supplementary salts[g]	—	15.46	—
Vitamin mixture[h]	10	10	10
Choline chloride	2	2	2
Ethoxyquin[i]	0.2	0.2	0.2
D-Biotin powder[j]	1	—	—

[a] General Biochemicals, Chagrin Falls, Ohio. Contains 0.85 mg/kg of Cu.

[b] Nonfat dry milk solids, spray process, Chippewa Brand, Chippewa County Co-op Dairy, Bloomer and Cameron, Wisconsin. This amount of solids provides 197 gm of protein.

[c] Assay protein C-1, Skidmore Enterprises, Cincinnati, Ohio. Contains 31–33 mg/kg of Cu.

[d] Dextrose, hydrous, USP, Merck.

[e] Fox, M. R. S. and Briggs, G. M., J. Nutr. 72, 243 (1960). $CuSO_4$ was omitted from the mixture used in this study.

[f] Contains added vitamin A acetate, 6 mg; vitamin D_3, 0.02 mg; DL-α-tocopherol, 25 mg; DL-α-tocopheryl acetate, 25 mg; menadione, 1 mg.

[g] Contains $CaHPO_4$, 10 gm; NaCl, 5 gm; $MnSO_4 \cdot H_2O$, 0.25 gm; ferric citrate, 0.2 gm; KIO_3, 0.01 gm.

[h] Contains thiamine·HCl, 8 mg; riboflavin, 8 mg; Ca pantothenate, 20 mg; nicotinic acid, 100 mg; pyridoxine·HCl, 8 mg; D-biotin, 0.3 mg; folic acid, 3 mg; vitamin B_{12}, 0.02 mg; and dextrose to 10 gm.

[i] Added as 4% solution in ethanol. Monsanto Co., St. Louis, Mo.

[j] Provides 0.6 mg of D-biotin.

Table II shows the tissue copper levels. Values from animals of both sexes are included in the averages, as there are no sex differences in tissue copper levels (1). Plasma, liver, brain, and kidney copper levels of deficient animals were markedly lower after 8 weeks on the egg albumin diet, as compared to those of the copper-supplemented animals. Kidney copper levels of animals fed the skim milk diet were elevated over those fed the egg albumin diet at the same dietary supplement of copper (20 mg/kg), but the levels in plasma, liver, and brain of supplemented animals on the two diets were similar. The copper levels of hair were reduced in copper deficiency; this measurement therefore appears to be a good index of the copper status of the animal.

Soybean protein diets commonly used to produce zinc deficiency were completely ineffective in producing copper deficiency in rats as shown by tissue copper levels (Table II). The zinc in these diets is largely unavailable, but apparently the copper is available.

The use of skim milk diets has the disadvantage that dietary levels of many important nutrients cannot be controlled. The casein diet described by Mills and Murray (7) contained only 0.3–0.4 mg/kg of copper and permitted rapid growth and reproduction for one generation when supplemented with copper. This diet has several disadvantages: special precautions are needed in the preparation of casein from milk to minimize contamination with trace elements; and the components of the salt mixture must be purified by extraction of either the sodium diethyldithiocarbamate or 8-hydroxyquinoline complexes. Both these processes are time consuming. The egg albumin diet described in this study has the advantage that all components are available commercially and that no special purification procedures are necessary to attain a low copper content in the diet.

In this study, none of the animals on either the copper-deficient skim milk or the egg albumin diet had reduced hematocrit values. The copper requirement of the rat for hemoglobulin production is only 1 mg/kg of diet but the requirement for growth is 3 mg/kg and for hair pigmentation is 8–10 mg/kg of diet (7). Furthermore, Gallagher *et al.* (13) observed that in rats fed a milk diet, anemia was a late sign of deficiency; hemoglobin concentrations did not begin to fall until after 10 weeks of feeding the diet. In earlier studies when anemia was obtained on copper-deficient diets, there may have been borderline deficiencies in other factors required for hematopoiesis. The diets used in this study were adequate in all nutrients except copper: the level of dietary copper in this study ap-

TABLE II. Tissue Copper Levels of Black Rats Fed Copper-Deficient Diets and Diets Supplemented with Copper.

Copper supplement (mg/kg)	Time (weeks)	No. of animals	Plasma copper level (μg/ml)	Tissue copper levels (μg/gm)[a]			
				Liver[b]	Kidney[b]	Brain[b]	Hair
Skim milk diet							
0	12	12	0.12 ± 0.03	3.62 ± 0.19	3.20 ± 0.09	1.84 ± 0.07	—
20	12	12	1.43 ± 0.08	7.01 ± 0.22	20.0 ± 1.88	2.85 ± 0.12	—
Egg albumin diet, no ascorbic acid							
0	8	10	0.13 ± 0.03	3.41 ± 0.35	4.16 ± 0.33	1.94 ± 0.08	5.84 ± 0.66
	12	4	0.58 ± 0.27	3.73 ± 0.86	3.59 ± 0.33	1.73 ± 0.05	5.94 ± 0.78
4	8	6	1.60 ± 0.21	5.69 ± 0.23	9.61 ± 0.43	2.32 ± 0.06	9.75 ± 0.34
	12	2	2.20	5.57	9.59	2.34	9.41
20	8	6	1.03 ± 0.05	9.43 ± 0.46	8.35 ± 0.96	2.43 ± 0.02	—
Egg albumin diet, 1% ascorbic acid							
0	8	5	0.41 ± 0.09	1.73 ± 0.04	3.05 ± 0.16	1.64 ± 0.16	4.95 ± 0.90
4	8	6	1.73 ± 0.24	6.10 ± 0.18	6.66 ± 0.57	2.46 ± 0.15	9.62 ± 0.68
Soybean protein diet							
0	9	9	1.46 ± 0.06	5.89 ± 0.24	—	—	12.3 ± 0.39
4	9	11	1.40 ± 0.21	6.32 ± 0.15	—	—	11.1 ± 0.76

[a] Mean ± SE.

[b] Values based on fresh weight of tissue.

parently was not sufficiently low to induce anemia in the rat in the experimental periods used.

In early copper deficiency, after 4 weeks on the diet, liver copper levels had fallen to half the control values. In moderate copper deficiency, after 10 weeks on the diet when anemia began to appear, liver copper levels were one-fifth of the control values. In extreme copper deficiency, liver copper levels had fallen to one-fifteenth of the control values. Dempsey *et al.* (14) found that adult male rats fed a low copper diet for 9 weeks had liver copper levels depressed to half the control values and serum copper levels depressed to one-fourth the control levels. Hemoglobin values were not depressed. These results are consistent with the results obtained with the egg albumin diet, that is, animals fed the copped-deficient diet had lower tissue copper levels but did not have anemia.

Ascorbic acid supplementation of the egg albumin diet did not accentuate copper deficiency in the rat; however, the liver and kidney copper levels of copper-deficient rats fed ascorbic acid tend to be lower than those of copper-deficient rats not fed ascorbic acid (Table II). Averages for the latter group include values from an experiment in which the effects of ascorbic acid were not studied. Tissue copper levels of the deficient animals in this experiment were somewhat higher than in the experiment in which 1% ascorbic acid was fed. Ascorbic acid has previously been shown to have no effect on tissue copper levels of copper-deficient chicks (10). There is no apparent explanation for the failure of ascorbic acid to accentuate copper deficiency in the rat. The mechanism of dietary ascorbic acid in accentuating the other effects of copper deficiency in the chick and rabbit is not known. Carlton and Henderson (9) suggest that in the chick, ascorbic acid interferes with copper assimilation, producing a "conditioned" deficiency; findings in rabbits support this view (11). Hill and Starcher (10) suggest that ascorbic acid exerts its effects on copper metabolism by influencing the movement of copper throughout the body. The failure of dietary ascorbic acid to influence tissue copper levels in the rat suggests that ascorbic acid does not prevent copper absorption. Our data do not permit conclusions about the influence of ascorbic acid on copper mobilization. It is possible that ascorbic acid could have accentuated copper deficiency at the lower levels of dietary copper in the skim milk diet. Ascorbic acid supplementation induced anemia in chicks fed skim milk diets supplemented with 8 mg/kg of copper (9). This suggests that the response of the rat to ascorbic acid is different from that of the chick or that there is something specific about the skim milk diet which enhances the effect of ascorbic acid in copper-deficient animals.

Plasma protein patterns obtained by disc electrophoresis showed no marked changes in copper deficiency. Ceruloplasmin is the major if not the only copper protein of plasma under fasting conditions. Changes in ceruloplasmin content of plasma would not result in noticeable changes in the electrophoretic patterns of these animals. These results do not suggest a role of copper in protein metabolism other than as a component of copper metalloproteins. Gallagher *et al.* (13) found that total plasma proteins and urinary amino acid excretion were not affected by copper deficiency in rats. Although protein synthesis did not decrease in copper deficiency, some rats showed diminished incorporation of glycine-^{14}C into plasma proteins. When only the incorporation of glycine into total liver and plasma protein was measured, no effect of copper deficiency was observed. When only incorporation into plasma gamma globulin or albumin was measured, incorporation of glycine was markedly depressed in copper deficiency (15).

Summary. Copper deficiency as judged by achromotrichia and lower levels of copper in blood, plasma, liver, kidney, brain, and hair was induced in black rats by feeding a copper-deficient diet containing egg albumin. Results obtained with this diet were comparable to those obtained with a skim milk copper-deficient diet. Ascorbic acid, at 1% of the diet, had no effect on the development of copper deficiency in rats fed the egg albumin diet. Soybean protein diets were ineffective in producing copper deficiency. Plasma protein patterns obtained by disc electrophoresis were not influenced by copper deficiency.

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