Copper deficiency in chicks: effects of ascorbic acid on iron, copper, cytochrome oxidase activity, and aortic mucopolysaccharides

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1. Copper deficiency was induced in newly hatched chicks by feeding on a milk-based diet for 12 d; effects of supplementation with ascorbic acid were studied.

2. Cu deficiency alone resulted in 30% mortality from aortic rupture. This was associated with a 20% increase in total acid mucopolysaccharides in the aorta, manifested as an increase in chondroitin sulphate and a relative decrease in hyaluronic acid. Cytochrome oxidase activity of liver and heart was less than half that of the controls.

3. Supplementing the Cu-deficient diet with 0·5% L-ascorbic acid increased mortality to 40%, raised total aortic acid mucopolysaccharides to a higher level, and increased liver iron by 36%.

4. Supplementing the control diet with ascorbic acid decreased liver Cu by 30% and significantly reduced total aortic acid mucopolysaccharides.

5. The enhancement of the Cu-deficiency effect by ascorbic acid probably results from interactions between ascorbic acid and absorption or metabolism of Cu; untoward effects of supplementing the control diet with ascorbic acid may be interpreted as manifestations of ascorbic acid toxicity *per se*.

Chicks have been used extensively as an experimental model for copper-deficiency studies, principally because eggs contain little Cu and day-old chicks reared on a Cu-deficient diet rapidly develop the signs and lesions of deficiency. Elvehjem & Hart (1929) first studied the effect of Cu on anaemia in the chick. More recently, other workers have investigated the Cu requirement of this species and confirmed its need in haematopoiesis (Carlton & Henderson, 1964*b*; Hill & Matrone, 1961). O'Dell, Hardwick, Reynolds & Savage (1961) reported that Cu is essential for the development and maintenance of the cardiovascular system of chicks. Shortly thereafter, Carlton & Henderson (1963) published a histopathological evaluation describing a defect in elastin in the major vessels of Cu-deficient chicks. Subsequently, results of several studies have implicated Cu in the formation of the elastin cross-links, desmosine and isodesmosine (Hill, Starcher & Kim, 1967; Partridge, 1966). Other components of connective tissue are also altered in aortas from Cu-deficient animals (Linker, Coulson & Carnes, 1964; O'Dell, Bird & Ruggles, 1966; Weissman, Shields & Carnes, 1963). In addition to the above changes, bone lesions (Carlton & Henderson, 1964*a*) and reduced cytochrome oxidase activity in heart (Hill & Matrone, 1961) have been observed in Cu-deficient chicks.

The severity of Cu deficiency in chicks is increased when the diet is supplemented

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with ascorbic acid (Carlton & Henderson, 1965; Hill & Starcher, 1965). We have investigated this apparent interrelationship and report here the effects of Cu and ascorbic acid on tissue iron and Cu, cytochrome oxidase activity, and aortic mucopolysaccharides (MPS) in the chick.

EXPERIMENTAL

For these studies we used pigmented, sex-linked cockerels (Barred Plymouth Rock) purchased from a commercial hatchery (Hall Brothers Hatchery, Wallingford, Conn.). Groups of birds were received at 14 d intervals; within 12–24 h after hatching they had been weighed and distributed at random into experimental groups in electrically

Table 1. *Composition of the copper-deficient diet*

Component	Amount (g/kg)
Dried skim milk	600
Glycine	4
DL-Methionine	3
L-Arginine	4
Sucrose	133
Starch	133
Maize oil*	50
Mineral mix†	50
Vitamin mix‡	20
Choline chloride	3
Total	1000 g

* Mazola oil; Best Foods Division, Corn Products Co., New York, NY.

† Special Cu-deficient salt mix purchased from General Biochemicals, Inc., Chagrin Falls, Ohio. The mix provided the following elements (mg/kg diet): Mg, 300; Fe, 20; Mn, 100; Zn, 15; I, 0·19.

‡ The mix provided the following vitamins (mg/kg diet): thiamine hydrochloride, 20; riboflavine, 20; pyridoxine hydrochloride, 20; calcium pantothenate, 40; nicotinic acid, 30; inositol, 50; folic acid, 2; biotin, 0·15; menaphthone, 4; DL-α-tocopherol acetate, 50; vitamin A acetate and ergocalciferol, 9750 and 975 USP units respectively. The following were added separately (per kg diet): cyanocobalamin in aqueous solution, 20 μg, and crystalline cholecalciferol in oil, 250 USP units.

heated battery brooders. Feed and distilled water were supplied *ad lib.* in stainless steel containers. The diet was based on dried skim milk supplemented with all vitamins, minerals, and amino acids necessary for chick growth (Table 1). The basal diet consistently contained less than 1 μg Cu/g. Cu, as reagent-grade cuprous chloride (CuCl), was added in a starch premix to the basal diet to provide a control diet that contained 6 μg Cu/g. Four experimental groups were studied: control, Cu-deficient, control plus 0·5% L-ascorbic acid, and Cu-deficient plus 0·5% L-ascorbic acid. There were four identical experiments; each included twenty-five birds per group.

The chicks were killed by decapitation after 12 d on the experiment. Chicks that were crippled or otherwise incapacitated were rejected for analyses. Whole blood was collected in polyethylene containers for haemoglobin and haematocrit determinations. Haemoglobin was measured as oxyhaemoglobin, and the packed cell volume was measured with heparinized micro-haematocrit capillary tubes. Samples of blood and liver were frozen for mineral analyses at a later date; at the time of analysis

they were digested in a mixture of concentrated nitric acid and 70% perchloric acid (1:1, v/v). All glassware was washed, rinsed once in 10% aqueous hydrochloric acid, then rinsed three times in deionized distilled water. The digested samples of blood and liver were analysed for Fe and Cu by atomic absorption spectrophotometry using individual cathode lamps (Perkin–Elmer Model 303; Perkin–Elmer Corp., Norwalk, Conn.).

Portions of the thoracic aorta, heart, liver, spleen, duodenum, and pancreas were fixed in freshly prepared, cold, buffered 10% formalin. Tissues were trimmed, paraffin-embedded, and cut at 6 μm for staining with haematoxylin and eosin. Selected sections of arteries were stained for elastic tissue by the method of Verhoeff & Hale (Armed Forces Institute of Pathology, 1960) and for acid MPS by colloidal iron. Liver, spleen, duodenum, and pancreas were stained for Fe by the Gomori technique (Armed Forces Institute of Pathology, 1960).

Cytochrome oxidase (ferrocytochrome *c*:oxygen oxidoreductase, *EC* 1.9.3.1) activity was measured spectrophotometrically on 5% homogenates of liver and heart. The substrate, cytochrome *c* (Sigma Chemical Co., St Louis, Mo.), was prepared as described by Neufeld, Levay, Lucas, Martin & Stotz (1960). Liver and heart were chilled immediately in phosphate buffer (pH 7·4), weighed, minced with stainless steel scissors, and homogenized in an Omni-Mixer (Ivan Sorvall, Inc., Norwalk, Conn.). The rate of oxidation of reduced cytochrome *c* was determined by measuring the decrease in optical density at 550 nm. Protein was estimated according to the method of Lowry, Rosebrough, Farr & Randall (1951).

Aortas with attached carotid arteries were preserved in acetone (AR) after removal of the adventitia. Extraction and identification of acid MPS were performed according to methods described by Gore, Tanaka, Fujinami & Goodman (1965).

Standard error of the mean was calculated and Student's *t* test used to determine significance of values. These values are recorded in the results section.

RESULTS

Visible evidence of Cu deficiency was apparent in about 7 d. The most prominent signs were lameness and respiratory distress. Those animals with respiratory distress often exhibited internal haemorrhage and haemopericardium at post-mortem examination. Weight gains were poorest in birds given the Cu-deficient diet supplemented with 0·5% L-ascorbic acid. The mortality rate in this group usually approached 40% by 12 d; mortality in the Cu-deficient chicks did not exceed 30% during the same period. In both groups death was generally associated with massive haemorrhage into the thorax or peritoneal cavity. The Cu content of the diet was a critical factor with respect to mortality and varied from 0·56 to 0·87 μg/g in these experiments. To test the effects of ascorbic acid in a practical diet, we included the vitamin at a level of 5·0% for a 42 d period. Compared with the controls, growth, haemoglobin, and haematocrit were not affected.

Livers and blood from five chicks were pooled, and twelve such composite samples from each treatment group were analysed for Fe and Cu. These results are summarized

in Fig. 1. Ascorbic acid had a greater effect than Cu on the concentration of Fe in the tissues. In fact, Cu deficiency altered the concentration of Fe in the liver only when the diet was supplemented with the vitamin. In birds fed on the Cu-deficient diet plus ascorbic acid the concentration of liver Fe was 36% greater than in birds fed on the control diet supplemented with ascorbic acid ($45{\cdot}3 \pm 2{\cdot}6$ compared with $33{\cdot}3 \pm 1{\cdot}6$, $P < 0{\cdot}005$). Supplementary ascorbic acid also was associated with an increase in blood Fe (Fig. 1).

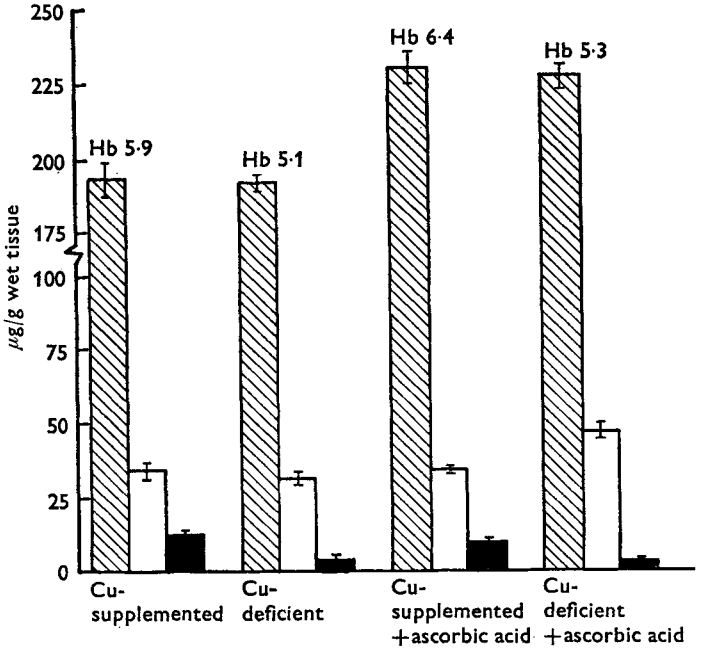

Fig. 1. Copper and iron values in whole blood and livers of chicks (mean values with their standard errors for twelve samples, each from five chicks). Values for haemoglobin Fe (g/100 g whole blood; mean values for ten chicks), determined as oxyhaemoglobin, are shown above the columns for each group (SE of a mean = 0·21). ▨, blood Fe; □, liver Fe; ■, liver Cu.

The Cu content of the liver was affected mainly by dietary Cu and its concentration was three times greater in the livers of birds from the Cu-supplemented than in those from the corresponding Cu-deficient group (Fig. 1). Addition of ascorbic acid to the Cu-supplemented diet decreased liver Cu by 30% ($12{\cdot}4 \pm 1{\cdot}6$ compared with $8{\cdot}8 \pm 1{\cdot}8$, $P < 0{\cdot}005$). Blood Cu varied slightly among treatment groups and, in all instances, was less than 45 μg/100 ml whole blood.

Total acid MPS were determined in the thoracic aorta together with the first 1·3 cm of the carotid arteries. As shown in Table 2, a 20% increase in aortic acid MPS was associated with Cu deficiency. The increase was considerably greater (46%) when ascorbic acid was given. Addition of ascorbic acid to the control diet resulted in a decrease in acid MPS. Upon separation and identification of the acid MPS, we found that the percentage of hyaluronic acid was decreased in aortas of Cu-deficient birds, with a resulting increase in the chondroitin sulphate group (Table 2). The effect was

reversed in aortas from birds given ascorbic acid together with the control diet, i.e. chondroitin sulphate was decreased.

Cytochrome oxidase activity was reduced significantly in homogenates of liver and heart from Cu-deficient chicks (Table 3). Results were similar in both liver and heart from birds given the diets supplemented with ascorbic acid. Differences in cytochrome oxidase activity which could be attributed only to ascorbic acid were not significant for the number of birds in this study.

Table 2. *Acid mucopolysaccharides in chick aorta (mean values with their standard errors for twelve samples, each of five aortas)*

Diet	Total (mg/g)	Hyaluronic acid (%)	Chondroitin sulphate group (%)
Control	10·95 ± 0·23	11·9 ± 0·5	88·1 ± 0·5
Copper-deficient	13·30 ± 0·37*	6·6 ± 0·4	90·1 ± 0·4
Control + ascorbic acid	9·68 ± 0·26†	13·2 ± 0·8	86·8 ± 0·8
Copper-deficient + ascorbic acid	14·23 ± 0·33‡	11·3 ± 0·7	88·7 ± 0·7

* Significantly greater than control ($P < 0·001$).
† Significantly less than control ($P < 0·005$).
‡ Significantly greater than control + ascorbic acid ($P < 0·001$).

Table 3. *Cytochrome oxidase activity* of chick tissues (mean values with their standard errors for three samples, each from two birds)*

Diet		Liver		Heart	
Ascorbic acid added	Copper status	Activity/ mg dry weight	Activity/ mg protein†	Activity/ mg dry weight	Activity/ mg protein‡
None	Supplemented	6·3 ± 0·4	11·6 ± 0·4	8·2 ± 0·2	23·3 ± 0·7
None	Deficient	2·2 ± 0·6	3·4 ± 1·1	3·5 ± 0·7	8·0 ± 1·7
0·5%	Supplemented	5·5 ± 0·3	11·0 ± 0·7	8·2 ± 1·4	24·7 ± 4·0
0·5%	Deficient	2·6 ± 0·2	4·5 ± 0·3	3·4 ± 0·5	7·7 ± 1·1

* Change in optical density × 10^2.
† Livers from Cu-deficient chicks significantly less active than those from Cu-supplemented chicks ($P < 0·025$).
‡ Hearts from Cu-deficient chicks significantly less active than those from Cu-supplemented chicks ($P < 0·01$).

DISCUSSION

The level of Fe in the liver and blood of chicks did not appear to be affected by Cu intake alone. This finding contrasts with studies on rats (Chase, Gubler, Cartwright & Wintrobe, 1962) and pigs (Gubler, Cartwright & Wintrobe, 1957) in which there was a marked reduction in liver and total body Fe of Cu-deficient animals. Nacht, Lee, Cartwright & Wintrobe (1967) observed increased amounts of Fe in the duodenal epithelium of Cu-deficient pigs and concluded that, while uptake of Fe by duodenal mucosa was normal, transfer of Fe to the blood was blocked. In our studies Fe did not accumulate in the duodenal mucosa of Cu-deficient chicks (as judged by Prussian-blue staining). The difference between our results and those of Nacht *et al.* (1967) may be partly attributable to species variation. However, it is probably best explained by length of study and Fe content of the diet: our experiments were of short duration

(12 d) and the diet was marginal in Fe (Hill & Matrone, 1961). Using the level of haemoglobin as an index, Hill & Matrone found a reciprocal relationship between dietary Fe and Cu in the chick, i.e. as Fe was increased, less Cu was needed and vice versa. Perhaps the inclusion of higher levels of Fe in our diets for a longer period of time would reveal a relationship between Fe absorption and Cu intake in the chick. Such a study would require determination of mucosal and total body Fe.

It is well established that ascorbic acid promotes Fe absorption in the intestine; this is probably the result of its reducing action and chelation (Hopping & Ruliffson, 1966). In our studies, blood and liver Fe were increased in birds given ascorbic acid in a Cu-deficient diet. In other groups of chicks almost all of the blood Fe can be accounted for as haemoglobin on the basis of 3·35 mg Fe/g haemoglobin. Haemolysis would be expected to increase liver Fe, and in Cu-deficient pigs the life span of red cells is decreased (Bush, Jensen, Athens, Ashenbrucker, Cartwright & Wintrobe, 1956). We stained sections of liver and spleen for Fe but found no increase in the Fe content of the reticuloendothelial cells. Consequently, we assume that the increased concentration was present in hepatic cells. Furthermore, the anaemia associated with Cu deficiency in chicks is the result of a decreased production of red cells rather than of haemolysis (Carlton & Henderson, 1964*b*; Hill & Matrone, 1961). Under the conditions of our experiments, ascorbic acid may have promoted Fe absorption. Perhaps, in the Cu-deficient chicks that were given the vitamin, low levels of Cu in the liver prevented movement of Fe from this organ.

Liver Cu was reduced significantly in chicks given ascorbic acid. This may have resulted from decreased absorption of Cu in the presence of the vitamin. The results of Hill & Starcher (1965) indicate that the role of reducing agents, including ascorbic acid, in increasing the severity of Cu deficiency is related to the intermediate metabolism of Cu rather than to an inhibitory effect on absorption. These investigators noted that there was no significant reduction in concentration of liver Cu as a result of a diet containing 0·1% ascorbic acid. However, after dosing with radioactive Cu, they found less radioactivity in livers of chicks given additional ascorbic acid than in livers from those given the control diet. The question is still unsettled, but we feel that ascorbic acid may affect either absorption or utilization of Cu.

The absolute increase in acid MPS in aortas from Cu-deficient chicks observed in our studies is in accord with results reported by other investigators, in which the hexosamine content was increased in aortas from Cu-deficient chicks (O'Dell *et al.* 1966) and pigs (Weissman *et al.* 1963). Furthermore, the ratio of hyaluronic acid to chondroitin sulphate was altered, with a resulting proportional increase in the latter. Although we did not identify and measure the individual chondroitin sulphates this observation seems to parallel that reported by Linker *et al.* (1964) that chondroitin sulphate B was increased in aortas from Cu-deficient pigs. The primary defect which leads to formation of dissecting aneurysms and rupture of major vessels in Cu-deficient chicks and pigs has been defined biochemically as a failure in elastin synthesis (Hill *et al.* 1967; Partridge, 1966). Therefore our results agree with the conclusion of Coulson & Linker (1968) that the increase in aortic acid MPS is a secondary manifestation of Cu deficiency.

The effects of ascorbic acid on MPS in chick aortas are sufficiently interesting to warrant further investigation. When the vitamin was added to the Cu-deficient diet, the changes in MPS associated with uncomplicated Cu deficiency were exacerbated. If our interpretation is correct, this may be an attempt to compensate for the greater reduction in aortic elastin under these conditions (Hill & Starcher, 1965). We did not expect to observe exactly the opposite effect in aortas from birds given ascorbate in the control diet, i.e. total MPS were decreased and the chondroitin sulphates were reduced relative to hyaluronic acid. These findings may correlate with those of Kofoed & Robertson (1966) in guinea-pig cartilage. Their results suggest that ascorbic acid may inhibit synthesis of chondroitin sulphate. Chicks given our experimental diet supplemented with ascorbic acid and adequate Cu always grew at least as well as the controls; therefore, our results were not due to inanition.

Cytochrome oxidase activity is reduced in tissues of various mammalian species made deficient in Cu, and we wanted to determine the effect of ascorbic acid on the activity of this enzyme under our experimental conditions. Cu deficiency caused a significant reduction in cytochrome oxidase activity in liver and heart, but this effect was not accentuated by dietary ascorbic acid. The results contrast with those obtained in rabbits given a Cu-deficient diet supplemented with the vitamin (Hunt, Carlton & Newberne, 1970). Cytochrome oxidase activity was reduced to a greater degree in hearts from these animals than in hearts from those which were simply Cu-deficient. Activity in liver was the same in both groups. Cu and haemin *a* (1:1 ratio) are the functional prosthetic groups of cytochrome oxidase (Griffiths & Wharton, 1961; Morrison, Horie & Mason, 1963) and the effect of Cu deficiency on the activity of this enzyme may be related to the absence of one or both of these constituents. The haemin *a* content is reduced greatly in hearts from Cu-deficient rats (Gallagher, Judah & Rees, 1956) and pigs (Lemberg, Newton & Clarke, 1962). Consequently, the observed loss of activity of cytochrome oxidase may be ascribed to an inhibition of enzyme synthesis as a result of insufficient haemin *a*.

Our results provide additional evidence that the severity of Cu deficiency is increased by including ascorbic acid in a purified diet. Further studies are needed to elucidate the reasons for the effects we observed and to determine in what way(s) ascorbic acid influences Cu absorption or metabolism, or both, in chicks.

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