Interrelationships between copper deficiency and dietary ascorbic acid in the rabbit

By C. E. HUNT, W. W. CARLTON AND P. M. NEWBERNE

Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 *USA*

(*Received* 18 *March* 1969—*Accepted* 28 *July* 1969)

1. Copper deficiency was induced in growing rabbits and the effects of ascorbic acid supplementation were studied.

2. Signs of Cu deficiency, including reduced growth, achromotrichia and alopecia, anaemia, and gross alterations in the bones of the forelimbs, developed most rapidly in those animals fed ascorbic acid.

3. Microscopic lesions in ossification centres were seen only in bones of rabbits which had received the vitamin.

4. Calcium and phosphorus contents of ash from cortical bone were not changed.

5. Compared with the controls, the concentration of liver Cu decreased and that of iron increased (> 50%) in Cu-deficient animals.

6. Cytochrome oxidase activity was reduced in liver and heart in Cu-deficient animals; this effect was accentuated in heart preparations from animals fed ascorbic acid.

Dietary ascorbic acid is known to increase the severity of copper-deficient symptoms in chicks. Carlton & Henderson (1964) found that bone lesions resulting from Cu deficiency were accentuated by adding ascorbic acid to the diet, and the vitamin depressed growth and increased the mortality rate in Cu-deficient chicks in experiments performed by Carlton & Henderson (1965) and Hill & Starcher (1965). These observations have been confirmed in our laboratory (Hunt, Landesman & Newberne, 1967). The increased mortality rate as a result of aortic rupture is at least partially explained by the observation that the content of aortic elastin is decreased to a greater extent in chicks whose diet has been supplemented with ascorbic acid than in those with uncomplicated Cu deficiency (Hill & Starcher, 1965). Furthermore, the activity in chick aorta of amine oxidase, which is a Cu-containing enzyme that may be involved in the formation of elastin cross-links, is inhibited by ascorbic acid in vitro (Bird, Savage & O'Dell, 1966).

In earlier experiments (Hunt & Carlton, 1965) we observed a number of adverse effects when ascorbic acid was added to the diet of rabbits given a purified Cu-deficient diet and noted gross changes in the bones of the forelimbs of Cu-deficient animals that received the vitamin. The present study was designed to further examine the interrelationship between Cu deficiency and dietary ascorbic acid in this species.

EXPERIMENTAL

Dutch Belted rabbits raised from the same parental stock as rabbits used in earlier experiments (Hunt & Carlton, 1965) were distributed at random among four treatment groups at the time of weaning: control (which received 6 ppm Cu in the basal diet);

basal diet, as control, plus 1% L-ascorbic acid; Cu-deficient plus 1% L-ascorbic acid; and Cu-deficient. The experimental conditions and the basal diet (in agar-gel form) were identical to those described previously (Hunt & Carlton, 1965). Feed and glass-distilled water were supplied *ad lib.* and replenished twice daily. The animals were observed daily, and their weights recorded at weekly intervals.

Haemoglobin and haematocrit determinations were made at 4-week intervals commencing on the 4th week of the experiment. Haemoglobin was determined by the cyanmethaemoglobin method and the packed cell volume was measured with heparinized micro-haematocrit capillary tubes.

Cytochrome oxidase (ferrocytochrome *c*: oxygen oxidoreductase, *EC* 1.9.3.1) activity was assayed in liver biopsy samples at 8 weeks and in liver and heart *post mortem*. The excised tissues were immediately chilled, weighed, and placed in 0·06 M-phosphate buffer (pH 7·4) to prepare a 5% homogenate. Liver was minced with stainless steel scissors and heart muscle was passed through a Delepine tissue press (A. H. Thomas Co., Philadelphia, Pa.) before being homogenized in a modified Potter-Elvehjem homogenizer consisting of a Teflon pestle and glass tube. All operations were performed at 0–5°. The homogenate was transferred to chilled centrifuge tubes and spun for 20 min at 1000 *g* in a refrigerated centrifuge. The supernatant fraction was assayed for cytochrome oxidase activity. The substrate (cytochrome *c*, Sigma Type III) was prepared as described by Neufeld, Levay, Lucas, Martin & Stotz (1958). The rate of oxidation of reduced cytochrome *c* was determined spectrophotometrically by measuring the decrease in optical density at 550 nm. The basic procedure followed was that of Smith (1955). Reaction rates were first order with respect to cytochrome *c* concentration for at least 60 sec. The results are expressed as the reaction rate constant per min per mg nitrogen. Nitrogen was determined by a modification of the micro-Kjeldahl method, as described by Scales & Harrison (1920).

Animals surviving after 12 weeks on the experiment were killed, and all animals were surgically examined *post mortem*. Tissues were fixed in freshly prepared, cold, buffered, 10% formalin. Bones were decalcified with DECAL (Omega Chemical Co., New York, NY). Bones and soft tissues were trimmed, paraffin-embedded, and cut at 6 μm for staining with haematoxylin and eosin.

Bones (femurs) for chemical analysis were removed from the animals immediately after death and frozen in sealed polyethylene bags. Before ashing, the adherent muscle was scraped off and the ends were sawed off at the metaphyseal-epiphyseal junction. The remaining diaphysis was split, the marrow removed, and the cortical bone placed in a drying oven for 24 h at 60°. The bone was extracted with reagent grade absolute methanol for 20 h in a Soxhlet extractor; it was then dried and weighed. The fat-free bone was ashed to constant weight in a muffle furnace at 550°. After acid digestion of the ash it was analysed for calcium by atomic absorption spectrometry (Perkin–Elmer Model 303; Perkin–Elmer Corp., Norwalk, Conn.) and for phosphorus by the method of Fiske & Subbarow (1925).

Frozen samples of plasma and liver were digested in a mixture of concentrated nitric and 70% perchloric acids (1:1, v/v) in preparation for mineral analysis. The samples were diluted with double-distilled water and assayed for Cu and iron using

the atomic absorption spectrometer. Individual cathode lamps were purchased from Perkin–Elmer Corp. (Norwalk, Conn.) for these analyses.

Where indicated, the results were evaluated statistically by Student's *t* test. Comparisons were made between the control and Cu-deficient groups and between the control + ascorbic acid and Cu-deficient + ascorbic acid groups.

RESULTS

We began with seven rabbits per group but unfortunately lost several animals during the early weeks of the study. They died following an acute course of diarrhoea with concomitant dehydration. At autopsy considerable mucus was observed in the large intestine but no significant lesions were observed on the microsections. We assume this was mucoid enteritis, a disease of uncertain aetiology that sometimes occurs in young rabbits. Some animals in each group were affected, but after the first 4–5 weeks on experiment no new cases developed.

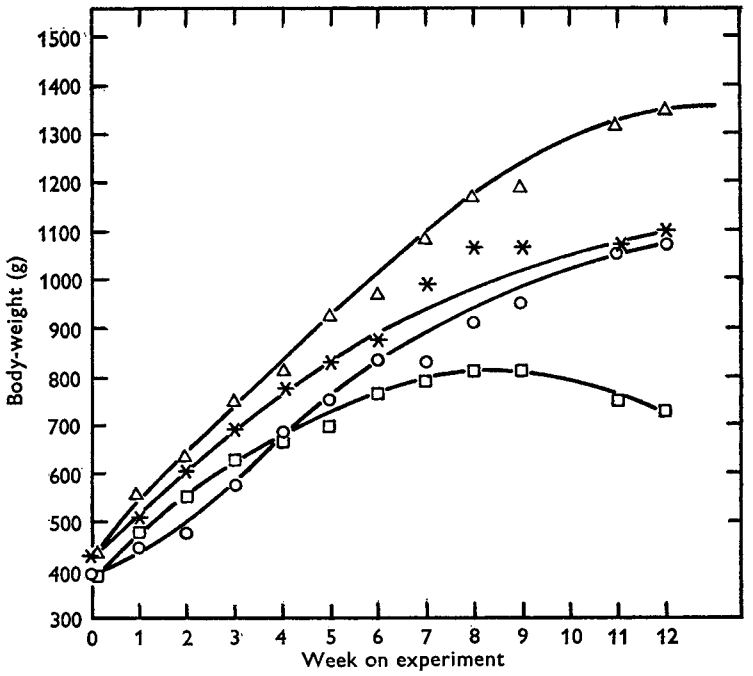


Fig. 1. Effect of Cu deficiency and ascorbic acid supplementation on the body-weight of growing rabbits. Because of deaths there were only five animals/group beyond week 7 (initially there were seven/group). △, Cu-supplemented; *, Cu-supplemented + ascorbic acid; □, Cu-deficient + ascorbic acid; ○, Cu-deficient.

The clinical course followed during development of Cu deficiency was similar to that observed in our earlier studies (Hunt & Carlton, 1965). The rabbits given the control diet grew considerably better than those in the other three groups (Fig. 1). The poorest weight gains were observed in the Cu-deficient + ascorbic acid group,

and some of these animals lost weight after week 9. At 8 weeks the average haemoglobin values were (in g/100 ml): control, 11·6; control + ascorbic acid, 11·0; Cu-deficient + ascorbic acid, 9·0; and Cu-deficient, 9·9. The values for three of the animals in the Cu-deficient + ascorbic acid group were 8 g/100 ml or less. The terminal haematological values are presented in Table 1. It should be noted here that, although there was an effect of ascorbic acid on haemoglobin values after 8 weeks, no such effect was observed after 12 weeks on experiment. The explanation for this is not obvious but may be related to changes in growth rate and presumably a decrease in the demand for Cu between 8 and 12 weeks. The Cu-deficient groups were significantly anaemic

Table 1. *Haematological values of rabbits fed the various diets for 12 weeks*

(Mean values and standard errors for five animals/group)

Diet	Haematocrit (%)	Haemoglobin (g/100 ml)
Control	40·7 ± 2·3	12·4 ± 1·2
Control + ascorbic acid	40·8 ± 2·5	12·1 ± 1·5
Copper-deficient + ascorbic acid	26·4 ± 2·7	7·7 ± 1·5
Copper-deficient	26·4 ± 2·6	7·8 ± 1·5

($P < 0{\cdot}01$) compared to their respective Cu-supplemented groups. Achromotrichia and alopecia began to appear in the Cu-deficient + ascorbic acid group at 5 weeks. These changes became apparent in some animals receiving supplemental Cu and ascorbic acid (control + ascorbic acid) at about the same time. Eight weeks elapsed before these signs were seen in rabbits in the basal Cu-deficient group. Easily seen bone deformities became noticeable at 5 weeks in one rabbit in the Cu-deficient + ascorbic acid group. The forelimbs were rotated medially and the radii and ulnas were curved. Five of the animals in this group eventually developed these changes and some became frankly lame in the forelimbs. A rabbit in the control + ascorbic acid group also developed the alterations in the forelimbs, but such changes were not observed in animals in the control and Cu-deficient (without ascorbic acid) groups.

Differences in cytochrome oxidase activity were apparent in liver biopsy samples taken at 8 weeks. The activity in both ascorbic acid-supplemented groups was lower than that of the control group (mean ± standard error): control, 92·8 ± 1·4; control + ascorbic acid, 79·0 ± 2·4; Cu-deficient + ascorbic acid, 57·9 ± 2·8. The terminal values for liver and heart preparations are summarized in Table 2. Terminal liver values for both Cu-deficient groups were about equal and were less than 50% of the activity of the control groups ($P < 0{\cdot}005$ for the control *v.* the Cu-deficient). Activity of the enzyme in heart was generally ten times greater than that observed in liver. The activity in hearts from animals given the Cu-deficient + ascorbic acid diet was 40% less than that in animals in the Cu-deficient group. Values for animals in the control + ascorbic acid group are not presented because the samples for N analysis were lost.

The results of analyses for Fe and Cu in liver are presented in Table 3. The concentration of Cu in livers of animals on the Cu-deficient diets was about half that of the respective Cu-supplemented groups. Liver concentration of Fe in the Cu-

deficient groups was more than 50% above that of the respective Cu-supplemented groups. Dietary ascorbic acid seemed to lower the amount of liver Fe when Cu was not limiting.

Plasma Fe values were determined but are not presented because they were excessively high, probably as a result of haemolysis. Plasma and liver Cu were decreased to about the same degree. Levels in the plasma of the Cu-supplemented groups

Table 2. *Cytochrome oxidase activities in rabbit tissues* after 12 weeks on the experimental diets*

Diet	Liver	Heart
Control	89·4 ± 2·2	37·9 ± 2·9
Copper-deficient + ascorbic acid	43·1 ± 3·0	11·9 ± 2·1
Copper-deficient	41·3 ± 4·1	29·1 ± 3·1

* The values are given as the means for three animals ± SE. Each value is the rate constant/min per mg nitrogen ($\times 10^3$ for liver, $\times 10^2$ for heart).

Table 3. *Iron and copper values in rabbit liver after 12 weeks on the experimental diets*

(Mean values and standard errors for five animals)

Diet	Iron (μg/g wet tissue)	Copper (μg/g wet tissue)
Control	143·7 ± 11·1	4·5 ± 1·6
Control + ascorbic acid	123·5 ± 5·0	3·8 ± 2·0
Copper-deficient + ascorbic acid	228·8 ± 6·1	2·2 ± 0·8
Copper-deficient	238·0 ± 11·3	2·4 ± 0·6

Table 4. *Composition of dry, fat-free cortical bone from rabbits given the experimental diets for 12 weeks*

Diet	Unashed weight (femoral shaft) (g)	Calcium (% ash)	Phosphorus (% ash)	Ca:P weight ratio
Control	1·19	36·60 ± 0·60	17·50 ± 0·30	2·10 ± 0·06
Control + ascorbic acid*	0·73	36·70	16·90	2·17
Copper-deficient + ascorbic acid	0·78	36·50 ± 0·40	17·40 ± 0·40	2·11 ± 0·02
Copper-deficient	0·85	36·50 ± 0·30	17·20 ± 0·50	2·14 ± 0·01

* These values are for one animal. Values for the other groups are the mean values and standard errors for five animals.

averaged approximately 200 μg/100 g compared to 70 μg/100 g in the deficient animals. Dietary ascorbic acid did not significantly alter plasma Cu concentration.

Histopathological changes in bones were observed only in animals that received supplemental ascorbic acid in their diet. We found lesions in the distal ossification centres of the humerus, radius, and ulna; they were most severe in the latter two. The epiphyseal plates were wider than normal and lacked precise organization throughout the various cartilaginous zones (compare Pl. 1 *a* and *b*). Whereas bones from Cu-

supplemented or simply Cu-deficient animals were normal, one of the rabbits given the diet supplemented with Cu and ascorbic acid had uneven epiphyseal lines with peripheral lipping, less calcified cartilage, and fewer bone trabeculae in the metaphyses. These changes were also present in bones from several animals in the Cu-deficient + ascorbic acid group.

The results of Ca and P analyses on ash from femurs of rabbits given the various diets are summarized in Table 4. The femoral shafts of the control animals were slightly heavier than those of the other groups. There were no significant differences in Ca or P content of the ash from the various groups or in the Ca:P ratios.

DISCUSSION

The results of this study and of that reported previously (Hunt & Carlton, 1965) indicate that dietary ascorbic acid accentuates the effects of experimental Cu deficiency in the rabbit. Compared with the other groups of animals, those given the Cu-deficient + ascorbic acid diet gained the least weight, developed a more severe anaemia in the first 8 weeks, and presented the first outward signs of Cu deficiency (including skeletal changes which were never observed in the merely Cu-deficient rabbits). Histological evidence of bone disease was observed only in animals that received ascorbic acid in their diet. Furthermore, the activity of cytochrome oxidase was considerably lower in homogenates of heart from rabbits given the Cu-deficient + ascorbic acid diet than in those from either the Cu-deficient or the control group.

It is well documented that Cu deficiency causes a reduction in cytochrome oxidase activity in various tissues in a number of species of animal. The activity of this enzyme is decreased in hearts from Cu-deficient rats, pigs, and chicks (Elvehjem, 1935; Schultze, 1939; Gallagher, Judah & Rees, 1956; Gubler, Cartwright & Wintrobe, 1957; Hill & Matrone, 1961) and in mammalian liver, bone marrow, and brain (Gubler *et al.* 1957; Howell & Davison, 1959; Schultze, 1941). Copper and haemin *a* (1:1 ratio) are established as the functional prosthetic groups of cytochrome oxidase (Griffiths & Wharton, 1961; Morrison, Horie & Mason, 1963), and it appears that these units are attached to separate specific proteins in the cytochrome oxidase complex (MacLennan & Tzagoloff, 1965). The haemin *a* content of rat (Gallagher *et al.* 1956) and pig (Lemberg, Newton & Clarke, 1962) heart is decreased greatly in Cu deficiency, and Gallagher *et al.* (1956) and Lemberg *et al.* (1962) contend that it is possible to ascribe the loss of activity of cytochrome oxidase in Cu deficiency entirely to this observation (i.e. the effect of Cu deficiency is an inhibition of the synthesis of the enzyme, resulting from a reduced production of the haemin *a* prosthetic group). The haemin-proteins myoglobin, cytochrome *c*, and catalase are not decreased in moderate Cu deficiency in pigs (Gubler *et al.* 1957; Lahey, Gubler, Chase, Cartwright & Wintrobe, 1952), but the porphyrin prosthetic group in these protein complexes is not identical to haemin *a*.

To our knowledge no findings have been published on the cytochrome oxidase activity in tissues of Cu-deficient rabbits. In this experiment we observed a relative decrease in the activity of the enzyme in rabbit liver and heart. Myocardial necrosis and

calcification have been observed in Cu-deficient rabbits maintained on these same diets for a longer period of time (Hunt & Carlton, 1965). It is conceivable that, in a metabolically active tissue such as heart, death of myofibrils *in situ* could occur as a result of loss of activity of this important respiratory enzyme. Also, the work load of the heart would be expected to be increased in Cu-deficient rabbits because of the severe induced anaemia. Thus, the myocardial lesions observed may be related to these two factors: namely, anaemia and reduced cytochrome oxidase activity of the heart muscle. Suggestions of an analogy in other species have been reported. Cattle grazing on Cu-deficient pasture develop focal necrosis of the myocardium with subsequent fibrosis (Bennetts, Beck & Harley, 1948) and these animals often die suddenly as a result of exertion or excitement. Gallagher (1964) has observed deaths in Cu-deficient rats which were forced to exercise. He suggested that the lesions in the hearts of Cu-deficient cattle, and deaths occurring in deficient cattle or rats forced to exercise, were related to reduced cytochrome oxidase activity in the myocardium.

Why ascorbic acid in vivo should accentuate the effect of Cu on heat cytochrome oxidase is a matter for speculation. Several possibilities may be considered. We mentioned above that Cu deficiency results in a reduction of the haemin *a* content of the myocardium in some species. Perhaps ascorbic acid enhances this effect. In fact, production of the haemin which is incorporated into haemoglobin may also be more severely affected because rabbits given ascorbic acid in a diet marginal in Cu developed anaemia whereas litter-mates given the same diet without the vitamin remained normal (Hunt & Carlton, 1965).

In in vitro studies ascorbic acid has been shown to inhibit catalase (Orr, 1966) and the Cu-containing enzyme, amine oxidase (Bird *et al.* 1966). The vitamin may inhibit cytochrome oxidase in the myocardium in vivo. The assay system was checked for the effect of ascorbic acid on enzyme activity. There was no effect on activity when ascorbate was added to the system after the pellet was cleaned up. Furthermore, the centrifugation and washing of the pellet is such that any ascorbate present in the homogenate would be washed away when cleaning up the preparation. We did not measure tissue levels of ascorbate but it is commonly utilized as a reductant in cytochrome oxidase assays in which oxidation of ascorbic acid is used as a measure of enzyme activity (Pearl, Cascarano & Zweifach, 1963).

The fact that Cu is one of the prosthetic groups of cytochrome oxidase suggests the possibility that ascorbic acid in vivo may reduce the quantity of this element in the heart or may alter its valency state in the enzyme complex. There is some evidence that the vitamin influences the movement of Cu throughout the body (Hill & Starcher, 1965). However, it seems unlikely that the Cu content of the heart would be selectively reduced and in fact the Cu content of the liver was sharply decreased (Table 3). More likely there is a general decrease in Cu content of most tissues.

Analysis of livers from animals given the various diets indicates that dietary Cu affects the concentration of Fe in this tissue. When dietary Cu was limiting, liver Fe was increased. Our rabbits were anaemic, and haemolysis of red cells could account for part of this increased Fe. Bush, Jensen, Athens, Ashenbrucker, Cartwright & Wintrobe (1956) have found that the life span of red blood cells is decreased in Cu-

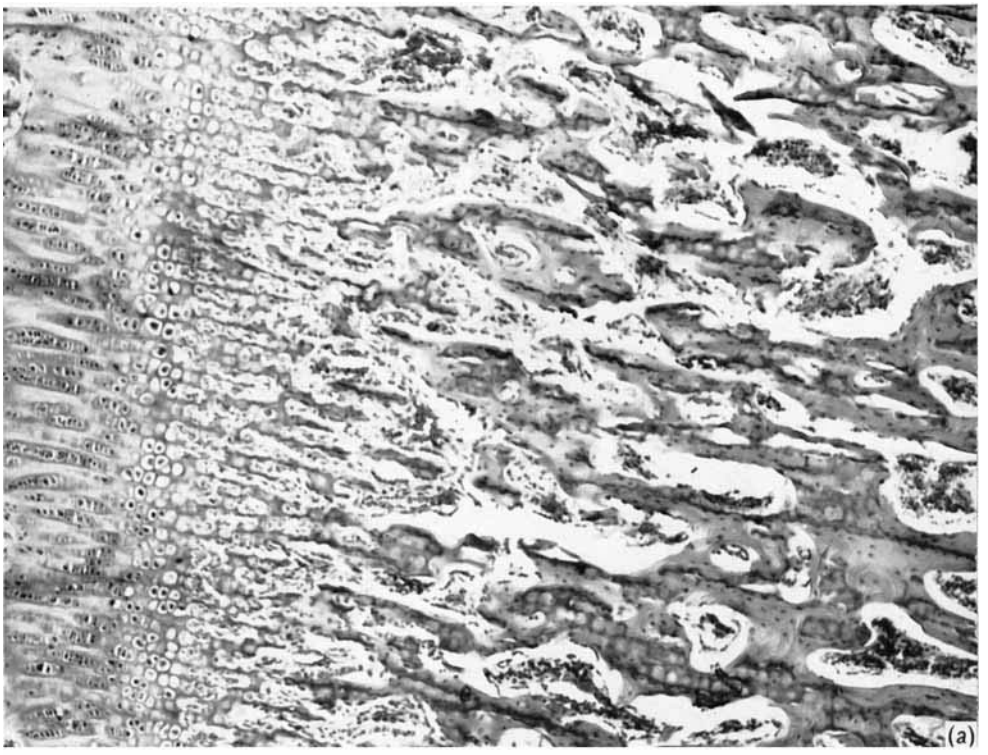
deficient pigs. Another factor that may be involved is a defect in movement or utilization of Fe. Signs of Fe deficiency observed in Cu-deficient pigs (Lahey *et al.* 1952) and rats (Chase, Gubler, Cartwright & Wintrobe, 1952) could not be corrected simply by increasing dietary Fe. Studies by Nacht, Lee, Cartwright & Wintrobe (1967) in Cu-deficient pigs have shown that dietary Fe enters the mucosal cells of the duodenum and remains there. These workers have postulated a role for a Cu-containing protein in the transport of Fe from the mucosal cells into the blood. Our results are not helpful with respect to Fe absorption. However, interference with utilization of body Fe may have contributed to the development of anaemia in the Cu-deficient animals.

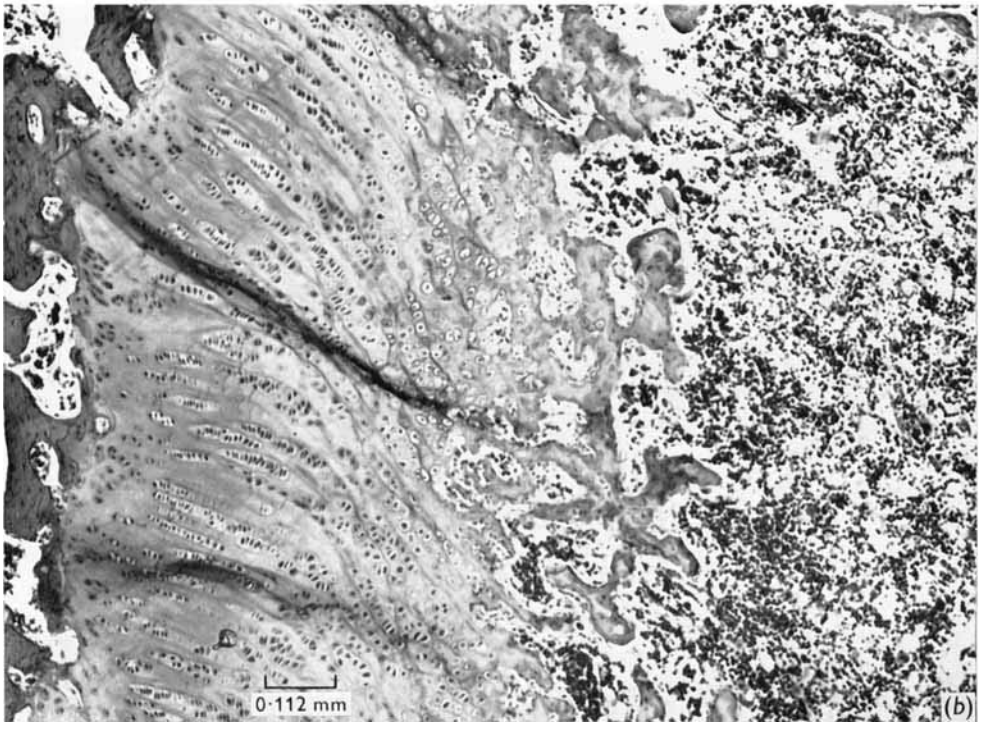
Skeletal defects resulting from Cu deficiency have been reported in several species but not in the rabbit. In our studies lesions were observed in the bones of animals which received supplemental ascorbic acid in the diet. Gross evidence of skeletal changes became apparent after 5 weeks in animals fed the Cu-deficient diet supplemented with ascorbic acid. There was abnormal curvature of the radius and ulna and the joints were enlarged. The gross appearance of the forelimbs was much like that of the Cu-deficient dogs described by Baxter & Van Wyk (1953). The over-all histopathological picture in bones of Cu-deficient rabbits fed ascorbic acid was one of continued cartilaginous proliferation at the epiphyseal ossification centre accompanied by a decreased formation or increased resorption, or both, of bone trabeculae. Reduced numbers of trabeculae and widened epiphyses were also characteristic of bones from Cu-deficient dogs (Baxter & Van Wyk, 1953; Baxter, Van Wyk & Follis, 1953). Bone lesions in Cu-deficient pigs (Follis, Bush, Cartwright & Wintrobe, 1955) are similar to those described in the dog. In both species there was diffuse osteoporosis resulting from an impairment of osteoblastic activity. Our findings of normal ash, Ca, and P contents of femurs from rabbits are in agreement with results obtained on bones of Cu-deficient dogs (Baxter *et al.* 1953).

In summary, rabbits on a Cu-deficient diet supplemented with ascorbic acid undergo changes in bone which appear similar to those seen in Cu-deficient dogs and pigs. Chondroblastic activity in the epiphyseal plate is not decreased; it appears to be stimulated. (Osteoblastic activity seems to be impaired because there is a paucity of bone formation on the cartilaginous matrix.) The absence of chemical changes in bone ash suggests that there is no serious abnormality of mineralization of the organic matrix.

The alterations we observed are probably the result of an interaction between ascorbic acid and Cu deficiency. This interaction increased the severity of the signs and lesions that are usually associated with experimental Cudeficiency. We have not seen aortic ruptures in Cu-deficient rabbits, and bone lesions were evident only when ascorbic acid was added to the diet. Perhaps bone lesions and aneurysms in major vessels could be produced in rabbits kindled from dams whose tissue Cu stores are low. However, our attempts to raise young from Cu-deficient dams have not been successful.

This investigation was supported by grant no. HE10112 from the National Institutes of Health, Bethesda, Maryland. This manuscript is contribution no. 1407 from





C. E. HUNT, W. W. CARLTON AND P. M. NEWBERNE

(*Facing p.* 69)

the Department of Nutrition and Food Science, Massachusetts Institute of Technology.

The present address of Dr C. E. Hunt is Schick Pharmaceutical Co., Seattle, Washington. Dr W. W. Carlton's address is Department of Veterinary Physiology, School of Veterinary Science of Medicine, Purdue University, Lafayette, Indiana.

REFERENCES

Baxter, J. H. & Van Wyk, J. J. (1953). *Bull. Johns Hopkins Hosp.* **93**, 1.
Baxter, J. H., Van Wyk, J. J. & Follis, R. H. Jr. (1953). *Bull. Johns Hopkins Hosp.* **93**, 25.
Bennetts, H. W., Beck, A. B. & Harley, R. (1948). *Aust. vet. J.* **24**, 237.
Bird, D. W., Savage, J. E. & O'Dell, B. L. (1966). *Proc. Soc. exp. Biol. Med.* **123**, 250.
Bush, J. A., Jensen, W. N., Athens, J. W., Ashenbrucker, H., Cartwright, G. E. & Wintrobe, M. M. (1956). *J. exp. Med.* **103**, 701.
Carlton, W. W. & Henderson, W. (1964). *Avian Dis.* **8**, 48.
Carlton, W. W. & Henderson, W. (1965). *J. Nutr.* **85**, 67.
Chase, M. S., Gubler, C. J., Cartwright, G. E. & Wintrobe, M. M. (1952). *J. biol. Chem.* **199**, 757.
Elvehjem, C. A. (1935). *Physiol. Rev.* **15**, 471.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Follis, R. H. Jr, Bush, J. A., Cartwright, G. E. & Wintrobe, M. M. (1955). *Bull. Johns Hopkins Hosp.* **97**, 405.
Gallagher, C. H. (1964). *Nutritional Factors and Enzymological Disturbances in Animals.* Philadelphia: J. B. Lippincott Co.
Gallagher, C. H., Judah, J. D. & Rees, K. R. (1956). *Proc. R. Soc.* B **145**, 134.
Griffiths, D. E. & Wharton, D. C. (1961). *J. biol. Chem.* **236**, 1850.
Gubler, C. J., Cartwright, G. E. & Wintrobe, M. M. (1957). *J. biol. Chem.* **224**, 533.
Hill, C. H. & Matrone, G. (1961). *J. Nutr.* **73**, 425.
Hill, C. H. & Starcher, B. (1965). *J. Nutr.* **85**, 271.
Howell, J. McC. & Davison, A. N. (1959). *Biochem. J.* **72**, 365.
Hunt, C. E. & Carlton, W. W. (1965). *J. Nutr.* **87**, 385.
Hunt, C. E., Landesman, J. M. & Newberne, P. M. (1967). *Fedn Proc. Fedn Am. Socs exp. Biol.* **26**, 633 (abstract no. 2110).
Lahey, M. E., Gubler, C. J., Chase, M. S., Cartwright, G. E. & Wintrobe, M. M. (1952). *Blood* **7**, 1053.
Lemberg, R., Newton, N. & Clarke, L. (1962). *Aust. J. exp. Biol. med. Sci.* **40**, 367.
MacLennan, D. H. & Tzagoloff, A. (1965). *Biochim. biophys. Acta* **96**, 166.
Morrison, M., Horie, S. & Mason, H. S. (1963). *J. biol. Chem.* **238**, 2220.
Nacht, S., Lee, G. R., Cartwright, G. E. & Wintrobe, M. M. (1967). *Fedn Proc. Fedn Am. Socs exp. Biol.* **26**, 634 (abstract no. 2112).
Neufeld, H. A., Levay, A. N., Lucas, F. V., Martin, A. P. & Stotz, E. (1958). *J. biol. Chem.* **233**, 209.
Orr, C. W. M. (1966). *Biochem. biophys. Res. Commun.* **23**, 854.
Pearl, W., Cascarano, J. & Zweifach, B. W. (1963). *J. Histochem. Cytochem.* **11**, 102.
Scales, F. M. & Harrison, A. P. (1920). *J. ind. Engng Chem.* **12**, 350.
Schultze, M. O. (1939). *J. biol. Chem.* **129**, 729.
Schultze, M. O. (1941). *J. biol. Chem.* **138**, 219.
Smith, L. (1955). *Meth. Biochem. Analysis* **2**, 427.

EXPLANATION OF PLATE

PLATE 1

(*a*) Photomicrograph of the distal end of the radius from a control rabbit illustrates the normal thickness and organization of the epiphyseal cartilage and numerous bone trabeculae in the metaphysis.

(*b*) Radius from a rabbit given the Cu-deficient diet plus ascorbic acid. Note the increased thickness of the epiphyseal cartilage, crooked columns of chondrocytes, and reduced number of bone trabeculae. The dark lines running through the epiphyseal cartilage are artifacts of processing and bear no relation to the deficiency.

Printed in Great Britain