

The histological effects of copper and zinc on chick embryo skeletal tissues in organ culture

By JOAN R. REST*

*Department of Animal Pathology, University of Cambridge,
Cambridge CB3 0ES*

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1. The effects of copper and zinc on organ cultures of chick embryo cartilage and bone maintained in low-trace-metal, chemically defined media for up to 8 d were studied macroscopically, histologically and histochemically. Length and wet-weight measurement of explants were assessed statistically.

2. No effects were found with Cu concentrations of 0.5-1.5 $\mu\text{g/ml}$ medium. Between concentrations of 5 and 40 $\mu\text{g Cu/ml}$ medium, lengths and wet-weights of cartilage cultures decreased significantly ($P < 0.001$) compared with controls. The decrease was directly proportional to increasing Cu concentration, and that of the length was greater with increasing period of culture ($P < 0.001$).

3. With 5-20 $\mu\text{g Cu/ml}$ medium cartilage and bone became yellow in colour, and chondrocytes were swollen, rounded and basophilic. They were detached from their lacunae and the quantity of matrix was reduced. Loss of alkaline phosphatase (*EC* 3.1.3.1) activity and disappearance of glycogen accompanied the degeneration. Osteogenesis ceased, cells failed to divide and mature, lost their enzymes and died. Cu did not accumulate in the bone matrix.

4. The direct toxic effects of Cu for cartilage and bone may underlie some of the skeletal changes in hepatolenticular degeneration (Wilson's disease).

5. As Zn concentrations were increased from 2.5 to 7.5 $\mu\text{g/ml}$ medium, lengths and wet-weights of cartilaginous cultures were significantly increased ($P < 0.001$). As Zn concentrations were further increased (from 10 to 40 $\mu\text{g/ml}$ medium), lengths and wet-weights were significantly decreased ($P < 0.001$).

6. Zn stimulated chondrocyte division and vacuolation of cytoplasm. With higher Zn concentrations toxic changes of granular basophilia, lacunar detachment and necrosis were seen. Differentiation and functioning of osteoblasts, osteoclasts and chondroclasts were stimulated by Zn.

7. Zn was found in bone matrix, osteoblasts, osteocytes and hypertrophied chondrocytes.

Deficiency of copper causes leg weakness (Bennetts, 1932; Teague & Carpenter, 1951) with reduced, brittle bone formation (Cunningham, 1950; Lahey, Gubler, Chase, Cartwright & Wintrobe, 1952; Follis, Bush, Cartwright & Wintrobe, 1955; Suttle, Angus, Nisbet & Field, 1972) and increased bone resorption (Baxter, Van Wyk & Follis, 1953; Hartley, Kater & Mackay, 1963; Carlton & Henderson, 1964). Cytochrome oxidase (*EC* 1.9.3.1) levels and collagen cross-linkage levels are reduced (Gallagher, 1957; Rucker, Parker & Rogler, 1969). The increased width of growth cartilage (Baxter *et al.* 1953; Carlton & Henderson, 1964) is not always recorded (Suttle *et al.* 1972) and is secondary to poor osteogenesis (Follis *et al.* 1955). Macroscopic skeletal abnormalities have also been reported in Cu-deficient rat embryos although this occurs less frequently than a soft tissue haemorrhagic syndrome (O'Dell, Hardwick & Reynolds, 1961).

* Present address: Department of Pathology, South Laboratory and Pathology Block, Southampton General Hospital, Tremona Road, Southampton SO9 4XY.

Numerous reports of skeletal lesions in hepatolenticular degeneration (Wilson's disease) (Finby & Bearn, 1958; Rosenoer & Michell, 1959; Boudin & Pepin, 1961; Morgan, Stewart, Lowe, Stowers & Johnstone, 1962) indicate that Cu may be directly toxic to these tissues.

Zinc deficiency reduces chondrocyte division and development and osteoblast activity (Follis, Day & McCollum, 1941; O'Dell, Newberne & Savage, 1958; Macapinlac, Pearson & Darby, 1966). Alkaline phosphatase (*EC* 3.1.3.1) levels are reduced (Starcher & Kratzer, 1963; Prasad, Oberleas, Wolf & Horwitz, 1967) remote from blood vessels (Westmoreland & Hoekstra, 1969*b*). Skeletal abnormalities have been produced in Zn-deficient chick embryos (Blamberg, Blackwood, Supplee & Combs, 1960; Kienholz, Turk, Sunde & Hoekstra, 1961; Savage, 1968) and the offspring of Zn-deficient rats have teratogenic congenital abnormalities (Hurley & Swenerton, 1966). Histologically there is a reduction in bone formation caused by a decrease in cell number rather than cell size (Diamond & Hurley, 1970). Excess Zn has been associated with joint lesions (Grimmett, McIntosh, Wall & Hopkirk, 1937; Sampson, Graham & Hestin, 1942; Brink, Becker, Terrill & Jenson, 1959) but no histological studies have been reported. Zn accumulates in the bones of pigs and rats (Grimmett *et al.* 1937; Stewart & Magee, 1964; Huxley & Leaver, 1966) and possibly lambs (James, Lazar & Binns, 1966).

Zn has been claimed to stimulate fracture healing yet its importance in this process and its relationship with vitamins A and D still needs further clarification (Calhoun, Smith & Becker, 1974). Zn deficiency *in vivo* has been confused with changes due to inanition (Williams & Chesters, 1970).

Organ culture of embryonic skeletal tissues in low-trace-metal, chemically defined media was chosen as a suitable model for morphological study of the direct action of metals in deficiency and excess.

MATERIALS AND METHODS

Reagents and media

Reagents for culture and analytical procedures were of the highest purity obtainable and stored in metal-free containers. Glass, polyethylene and PTFE apparatus was cleaned by soaking in cold water, washing with detergent and rinsing. It was made metal-free by overnight soaking in 6 M-hydrochloric acid (technical grade). Stainless-steel grid platforms (FDP Quality, pore size $1.5 \times 0.010 \times 0.005$ mm; Expanded Metal Co., Hartlepool, Cleveland) for explants were soaked overnight in fuming nitric acid, and PTFE-coated grids (Plastic Coatings Ltd, Guildford, Surrey) for cartilaginous experiments were washed in 6 M-HCl for 2 h. After these treatments the grids were rinsed in numerous changes of distilled water, blotted on tissues and dried in a hot-air oven. Sterile stainless-steel scalpels (No. 11; Swan-Morton, Sheffield, Yorks.) were used in pairs for dissections, as obtained from the manufacturers. Other instruments were washed in running water, boiled in Calgon (Albright and Wilson Mfg Co. Ltd, Birmingham, West Midlands) (1 g/l) and metasilicate detergent for 20 min and rinsed as described previously. Where appropriate, apparatus was sterilized in a hot-air oven.

Table 1. Composition (g/l) ($\times 10^{-2}$) of low-trace-metal, chemically defined 'BGJ' medium* used to culture chick embryo cartilage and bone

L-lysine HCl	24.0	Pteroylmonoglutamic acid	0.02
L-histidine HCl. H ₂ O	15.0	Biotin	0.02
L-arginine HCl	7.5	<i>p</i> -Aminobenzoic acid	0.2
L-threonine	7.5	α -Tocopheryl phosphate	0.1
L-valine	6.5	Choline chloride	5.0
L-leucine	5.0	<i>myo</i> -Inositol	0.02
L-isoleucine	3.0	Cyanocobalamin	0.004
L-methionine	5.0	L-ascorbic acid†	15.0
L-phenylalanine	5.0	Calcium lactate	55.5
L-tryptophan	4.0	KCl	40.0
L-tyrosine	4.0	KH ₂ PO ₄	16.0
L-cysteine HCl. H ₂ O	9.0	NaCl	530.0
L-glutamine†	20.0	MgSO ₄ . 7H ₂ O	20.0
Nicotinamide	2.0	NaHCO ₃ †	350.0
Thiamin HCl	0.4	Sodium acetate	5.0
Calcium pantothenate	0.02	Glucose†	500.0
Riboflavin	0.02	Phenol red†	2.0
Pyridoxal phosphate	0.02	Streptomycin†	5.0
		Penicillin†	0.60

Maximum concentration (μ g/ml) in 'BGJ' medium: copper 0.4, zinc 0.7, manganese 0.02.

* Biggers, Gwatkin & Heyner (1961) as modified by H. Fell (personal communication).

† Not included in BGJ powder supplied by Strangeways Research Laboratories, Cambridge.

Chemically defined medium in powder form (given by Strangeways Research Laboratory, Cambridge) was used to prepare the 'BGJ' culture medium (Biggers, Gwatkin & Heyner, 1961; as modified by H. Fell, personal communication) (Table 1). The 'BGJ' medium was divided into 50 ml portions to which stock aqueous copper sulphate solution (1 mg Cu/ml) was added to give final Cu concentrations of 0.5, 1.0, 1.5, 5, 10, 20 and 40 μ g/ml. Stock zinc sulphate (1 mg Zn/ml) in 0.1 M-HCl was added to give final Zn concentrations of 2.5, 5.0, 7.5, 10, 20 and 40 μ g/ml medium. Media were sterilized by positive-pressure filtration through 0.3 μ m diameter Millipore filters (Millipore (U.K.) Ltd, Millipore House, Abbey Road, London NW10 7SP), and stored at -20° . All media were analysed for Cu and Zn contents before culture and at each medium change using an atomic absorption spectrophotometer (Model No. 303; Perkin-Elmer, Beaconsfield, Berks.). Immediately before analysis for Zn, media containing more than 10 μ g Zn/ml were diluted with deionized water because of the greater sensitivity of the spectrophotometer for this metal. The manganese content of the 'BGJ' medium was also measured.

Organ culture: measurements and statistical analysis

The organ culture technique used was the Fell technique (see Paul, 1965). Cartilaginous explants of tibias, femurs and humeri were dissected from 7.5-d-old chick embryos. Paired groups were placed on PTFE-coated grids with gauze wicks and were cultured in 'BGJ' medium alone or with different amounts of added Cu or Zn, in an atmosphere of carbon dioxide-air (5:95, v/v). Media were changed every 2 d and stored at -20° for Cu and Zn analysis.

Explants were drawn in outline on graph paper using a reticule in the eye-piece

of a dissecting microscope. The longitudinal axis of each drawing was used to calculate the length ratio $(L_E - L_C):L_C$, where L_E is the length of the explant incubated in the presence of Cu or Zn and L_C is the length of the paired control explant. The values obtained for the ratio at different Cu and Zn concentrations were analysed by covariance, and regression coefficients were calculated. Separate analyses for tibias, femurs and humeri were subsequently pooled. Slopes of regression lines were calculated. The slopes for different sections of the plots of $(L_E - L_C):L_C$ v. Zn concentration of the culture medium were compared.

Paired groups were fixed after 2, 4, 8, 12 and 24 h with Cu, and 2, 4, 6 and 8 d of culture with Cu and Zn. When cultured for 2 d or longer, explants were weighed before fixation. They were washed in saline (9 g sodium chloride/l), blotted once and weighed in screw-cap bottles. Weights of individual explants were used to calculate the weight ratio $(W_E - W_C):W_C$, where W_E is the weight of the explant incubated in the presence of Cu or Zn, and W_C is the weight of the paired control explant. Statistical analysis was similar to that described for length measurements.

Bony explants of tibias and femurs from 11 to 12-d-old chick embryos were similarly cultured in paired groups on stainless-steel grids. The cartilaginous epiphyses were removed before culture, therefore length and wet-weight measurements were not made. Paired groups were fixed after 2, 4, 6 and 8 d of culture. Cu concentrations used were 1, 5 and 20 $\mu\text{g/ml}$ medium and the atmosphere for culture was CO_2 -air (5:95, v/v). Zn concentrations used were 5, 10, 20 and 40 $\mu\text{g Zn/ml}$ medium and the atmosphere was oxygen- CO_2 -nitrogen (50:5:45, by vol). A few experiments were done in the less favourable atmosphere of CO_2 -air (5:95, v/v). Results from these experiments are not included except where mentioned specifically. Some bones were sliced to study healing processes.

Histological and histochemical methods

Cartilaginous tibias and femurs, and bony tibias were fixed in formol-saline (pH 7.0) overnight and then transferred to aqueous ethanol (50 ml/l). After histological processing they were sectioned and stained with Mayer's haematoxylin and eosin, alcian blue-van Gieson, Hale's colloidal iron, toluidine blue (Pearse, 1960), periodic acid-Schiff reagent, Best's carmine, rubeanic acid, by Herovici's method for collagens (Herovici, 1963), Von Kossa's method for calcium salts (Rest, 1970), and the dithizone technique (Gomori, 1952) for Zn after pretreatment of sections with the fumes of 12 M-HCl for 4 min. Cartilaginous humeri and bony femurs were washed in saline, quenched, and embedded in dextran solution on cork blocks and sectioned. They were stained with Best's carmine, oil-red-O, rubeanic acid, by the azo-dye coupling method for alkaline phosphatase and Burstone's method for cytochrome oxidase (Pearse, 1960). Staining methods are described in Carleton (1967) except where otherwise stated.

RESULTS

Cartilaginous explants from 7.5-d-old embryos

Cu added at concentrations 0.5-1.5 $\mu\text{g/ml}$ medium had no detectable effect on cartilaginous explants. Explants cultured in media containing 5-40 $\mu\text{g Cu/ml}$ had

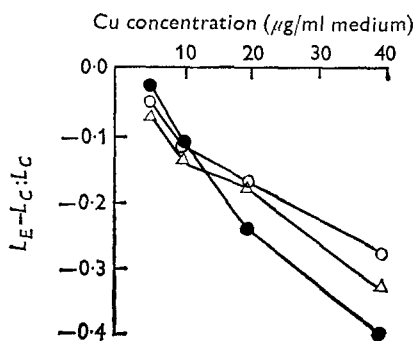


Fig. 1

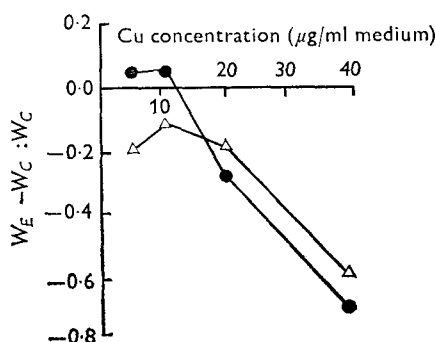


Fig. 2

Fig. 1. The relationship between length ratios and Cu concentrations of culture medium for cartilaginous explants from 7.5-d-old chick embryos. The length ratio was calculated as $(L_E - L_C) : L_C$, where L_E is the length of the explant incubated in the presence of Cu, and L_C is the length of the paired control explant. Values for ratios for tibias, femurs and humeri were combined and are shown as mean values: ○, 4 d culture; △, 6 d culture; ●, 8 d culture.

Fig. 2. The relationship between wet-weight ratios and Cu concentrations of the culture medium for cartilaginous explants from 7.5-d-old chick embryos. The wet-weight ratio was calculated as $(W_E - W_C) : W_C$, where W_E is the weight of the explant incubated in the presence of Cu, and W_C is the weight of the paired control explant. Values for ratios for tibias, femurs and humeri were combined and are shown as mean values: △, 6 d culture; ●, 8 d culture.

decreased length and wet-weight ratios compared to their paired controls (Figs. 1, 2). The negative regression coefficients for length ratio, and for weight ratio *v.* Cu concentration were significantly different from zero ($P < 0.001$).

With Cu concentrations of 5–20 μg/ml medium, cartilage became opaque and yellow in colour. Histologically chondrocytes were swollen, rounded and basophilic with granular cytoplasm and nuclear chromatin aggregation and margination (Plate 1*a*). Some necrosed completely. Glycogen content and alkaline phosphatase activity decreased, and the quantity of matrix (Plate 1*a*) and its mucopolysaccharide content decreased. Neither cytochrome oxidase nor Cu were found in these or any other cartilaginous cultures. In the medium containing 40 μg Cu/ml explants were brown and chondrocytes rapidly necrosed.

Length and wet-weight ratios increased directly with Zn concentrations between 2.5 and 7.5 μg/ml medium and decreased with Zn concentration higher than 10 μg/ml medium. The positive and negative regression coefficients were significantly different ($P < 0.001$) from zero and from each other (Figs. 3, 4).

Macroscopically, cartilage cultured in media containing 10–40 μg Zn/ml became white and opaque. Histologically, chondrocytes responded to Zn in all media (Plate 1*b–d*). Chondrocyte size was frequently increased. With lower Zn concentrations, this was mainly due to vacuolation of cytoplasm. Irregular cell division was stimulated with all Zn concentrations. Incomplete amitosis resulted in numerous binuclear chondrocytes. These and most other cells had basophilic, granular cytoplasm. With Zn concentrations of 20–40 μg Zn/ml, there was some loss of nuclear structure and rounding of cell outlines (Plate 1*d*). Many cells became eosinophilic with pyknotic nuclei.

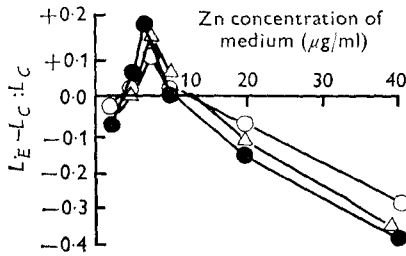


Fig. 3

Fig. 3. The relationship between length ratios and Zn concentrations of culture medium for cartilaginous explants from 7.5-d-old chick embryos. The length ratio was calculated as $(L_E - L_C) : L_C$, where L_E is the length of the explant incubated in the presence of Zn, and L_C is the length of the paired control explant. Values for ratios for tibiae, femurs and humeri were combined and are shown as mean values: ○, 4 d culture; △, 6 d culture; ●, 8 d culture.

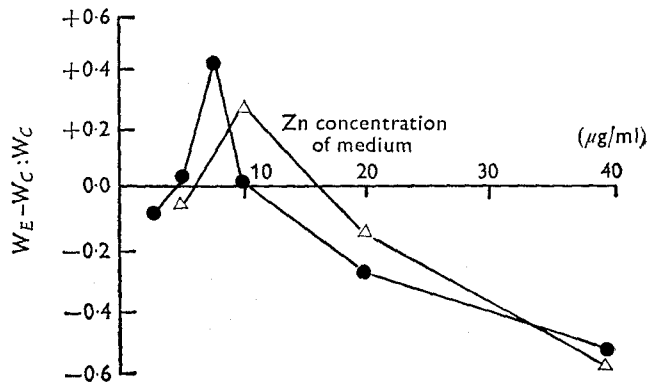


Fig. 4

Fig. 4. The relationship between wet-weight ratios and Zn concentration of the culture medium for cartilaginous explants from 7.5-d-old chick embryos. The wet-weight ratio was calculated as $(W_E - W_C) : W_C$, where W_E is the weight of the explant incubated in the presence of Zn, and W_C is the weight of the paired control explant. Values for ratios for tibiae, femurs and humeri were combined and are shown as mean values: △, 6 d cultures; ●, 8 d cultures.

The cytoplasmic glycogen content of chondrocytes was decreased when cultured in media with 20–40 μg Zn/ml but there was no change in alkaline phosphatase activity. Zn was found in hypertrophied chondrocytes and matrix adjacent to the perichondrium of explants cultured in media containing 10–40 μg Zn/ml. This distribution paralleled that of alkaline phosphatase activity. Matrix decreased in quantity but mucopolysaccharide staining was increased in intensity with low Zn levels. With 40 μg Zn/ml medium, matrix was decreased in quantity and in mucopolysaccharide stain affinity.

Bony explants from 11- to 12-d-old embryos

Explants cultured in media containing 5 or 20 μg Cu/ml were smaller than their paired controls, and yellow in colour. Histologically, cells were swollen and basophilic. There was no mitotic division, osteoblast differentiation or increased resorption. Some cytochrome oxidase activity was lost after 8 d in a medium containing 20 μg Cu/ml. Osteoid formation was inhibited but pre-formed bone matrix was not affected by Cu within the culture period studied.

Explants cultured with 5–20 μg Zn/ml medium were yellower in colour than their controls. Those with 40 μg Zn/ml were shorter, thinner and dull in appearance. Histologically more osteoblasts were seen initially than in controls but as the period of culture increased, the number decreased. In culture media containing 5–20 μg Zn/ml, they formed irregular groups with vacuolated cytoplasm and pale nuclei with enlarged nucleoli. Some formed syncytia which differentiated into osteoclasts. In culture media with 40 μg Zn/ml some necrosis was seen after 4 d, but after 8 d in

media with 10–20 μg Zn/ml cells became swollen, hyperchromatic and basophilic (Plate 2a). Osteoblasts stained strongly and osteocytes weakly for Zn. Cytochrome oxidase staining was lost in explants cultured for 8 d in the medium with 40 μg Zn/ml. Alkaline phosphatase activity was greater than that of controls. Osteoid was formed in trabeculae interwoven with pre-formed bone not as a subperiosteal sheet as in controls (cf. Plate 2b and c). There appeared to be some resorption by the osteoclasts formed during culture with Zn. Osteoid stained weakly and trabecular bone, particularly the edges, strongly for Zn. Hypertrophied chondrocytes also stained for Zn and multinucleate chondroclasts differentiated (Plate 2d).

In an atmosphere of CO_2 -air (5:95, v/v) maintenance of bone was less satisfactory than with O_2 - CO_2 - N_2 (50:5:45, by vol). Fibroblasts and foamy osteoblasts were more plentiful and the periosteum was thicker in explants cultured with 5–10 μg Zn/ml medium than in controls. There was some loss of cytochrome oxidase activity but less than in controls. More osteoid was laid down when cultured with added Zn. No osteoclasts were found but some chondroclasts differentiated when cultured with added Zn.

No conclusive results were obtained in fracture experiments.

DISCUSSION AND CONCLUSIONS

No requirement for Cu was indicated in these cultures. With inherently slow development in culture and a medium deficient in Fe, Mn and Zn, Cu was not the growth-limiting nutrient and was not depleted within the culture period studied. Cytochrome oxidase activity was found in bone but not cartilage, confirming the findings of Follis & Berthrong (1949).

It is unlikely that the Cu was unavailable. Metals are normally added to tissue culture media as sulphates (Ham, 1963, 1965; Thomas & Johnson, 1967) with Cu binding to amino acids. This binding is similar to that of albumin-Cu in blood, in which form it is available to cells (Bearn & Kunkel, 1954; Walshe, 1968).

Cu was toxic in concentrations of 5–40 $\mu\text{g}/\text{ml}$ medium. This was indicated by the decreased length and wet-weights compared to controls, and a Cu-specific deep yellow coloration. Explants exposed to concentrations of 10–20 μg Cu/ml medium showed cell rounding and basophilia similar to that seen when treated with toxic concentrations of Zn. At these Cu concentrations explants had less matrix than controls. A dramatic reduction in cartilage matrix in culture is caused by lysosomal damage (Fell & Mellanby, 1952; Fell & Thomas, 1960; Sledge & Dingle, 1965; Fell Coombes & Dingle, 1966), therefore a similar mechanism may be involved in these cultures.

Destruction of cartilage matrix and cessation of its production would lead to osteoarthritis and cessation of osteogenesis, to osteoporosis and fractures. The present *in vitro* findings are therefore supportive evidence for the hypothesis that these lesions of hepatolenticular degeneration are due to the direct action of Cu (Barka, Scheuer, Schaffner & Popper, 1964; Goldfischer & Sternlieb, 1968).

The rubenic acid histochemical method did not indicate toxic levels of tissue Cu

but this could be due to diffuse distribution of the metal as in early hepatolenticular degeneration (Goldfischer & Sternlieb, 1968). In organic bone, Cu is firmly bound (Rucker *et al.* 1969) and the staining may not have released this binding.

Atomic absorption spectrophotometry of medium levels was too insensitive to indicate Cu uptake, therefore, within the limits of experimental error, the Cu levels measured in the culture media corresponded to the Cu added.

Zn-deficient birds have shortened bones (Morrison & Sarett, 1958; Zeigler, Scott, McEvoy, Greenlaw, Heugin & Strain, 1962) and decreased chondrogenesis (Young, Edwards & Gillis, 1958). Longer explants with increased chondrogenesis when Zn is added at 5 and 7.5 $\mu\text{g/ml}$ medium, therefore indicate control Zn deficiency. Narrower flattened-cell zones are normally associated with Zn deficiency because of the lack of chondrogenesis (Follis *et al.* 1941; O'Dell *et al.* 1958; Macapinlac *et al.* 1966). In explants cultured with added Zn, narrower flattened-cell zones were due to an increased differentiation rate into hypertrophied cells because chondrogenesis was greater than in controls. Additional evidence of differentiation was the increased vacuolation of cytoplasm. The vacuoles did not stain for glycogen or neutral fat and may have been the hydration of hypertrophy (Lacroix, 1961). The vacuoles did not indicate pathological water imbibition as the concentration of ascorbic acid in this medium was greater than that found by Reynolds (1967) to prevent this phenomenon.

Explants cultured in a medium containing 2.5 $\mu\text{g Zn/ml}$ tended to be shorter in length than controls (Fig. 3), whereas with 5.0 and 7.5 $\mu\text{g Zn/ml}$ explants were significantly ($P < 0.001$) longer than paired controls. Increased chondrogenesis in culture will initially decrease matrix production because DNA and chondroitin sulphate syntheses are mutually exclusive (Abbott & Holtzer, 1966; Nameroff & Holtzer, 1967) so that non-matrix-producing cells continue dividing (Bryan, 1968). Chondrogenesis of explants with 2.5 $\mu\text{g Zn/ml}$ medium would not compensate for continuing matrix production of non-chondrogenetic controls whereas the greater stimulus to chondrogenesis more than compensated in explants cultured with media containing 5.0 and 7.5 $\mu\text{g Zn/ml}$ particularly with increasing period of culture. After 6 and 8 d culture in the latter media, explant epiphyseal inter-chondrocyte distances were less than those of controls although mucopolysaccharide stain intensity was greater. These findings are consistent with control Zn deficiency as Zn-deficient birds have increased quantities of matrix (Young *et al.* 1958; Westmoreland & Hoekstra, 1969*a*).

Addition of Zn to bone cultures initially increased osteoblast numbers compared to controls. A relative deficiency of these cells, as in controls, is seen in Zn-deficient chicks (Follis *et al.* 1941; O'Dell *et al.* 1958). Syncytia and fully differentiated osteoclasts were stimulated by Zn and bone remodelling occurred. Lack of remodelling would account for the thickened bones associated with Zn deficiency (O'Dell *et al.* 1958; Zeigler *et al.* 1962). Increased alkaline phosphatase levels were found in bone, although not in cartilage. The absence of this enzyme in control cultures indicates Zn deficiency as in vivo (Starcher & Kratzer, 1963; Prasad *et al.* 1967; Westmoreland & Hoekstra, 1969*b*; Prasad & Oberleas, 1971).

Uptake of Zn was indicated by dithizone staining, Pretreatment with acid to release the Zn from its binding was essential in this study but not in those of other workers

using samples from older animals (Haumont, 1961; Vincent, 1963) although the amounts found by them were minimal compared with the amounts present (Asling & Hurley, 1963). Zn concentrations in the culture medium, measured by atomic absorption spectrophotometry, were two-thirds those added. This disappearance of Zn cannot be explained completely by explant uptake. Interference by sodium (Prasad, Oberleas & Halsted, 1965), precipitation as phosphate and adsorption on containers also reduced Zn concentrations.

Fracture-healing experiments were inconclusive, probably because the media contained 5 mg glucose/ml, a concentration found unfavourable for healing by Prasad & Reynolds (1968). In the unfavourable atmosphere of CO₂-air (5:95 v/v), however, Zn did improve bone cellular activity.

Zn concentrations of 10–40 µg/ml medium significantly reduced lengths and wet-weights compared to control cultures of cartilage. Some histological features were also seen in Cu toxicity but abnormal mitoses resembled those of cobalt-treated cell lines (Daniel, Dingle, Webb & Heath, 1963). The relative increase in cell size was unlike that found in thallium chondrodystrophy of chick embryos by Ford, Eyring & Anderson (1968). Bone was morphologically normal with Zn concentrations at which cartilage showed severe cellular changes because of the ability of mineralized bone to accumulate Zn. There was differentiation of both chondroclasts and osteoclasts. Both alkaline phosphatase and cytochrome oxidase activities were reduced at high Zn concentrations.

It was frequently difficult to determine whether changes in culture due to Zn were beneficial or toxic. Zn increased chondrogenesis which was frequently incomplete or abnormal. Some binucleate cells and numbers of mitoses are normal in hypertrophied cartilage (Fell, 1925) although more are seen in cultures of unhealthy chondrocytes (T. S. P. Strangeways (unpublished results) cited by Fell, 1925). Amitosis has been seen in senescent mouse and human Purkinje cells (Andrew, 1955) and articular cartilage of the old mouse (Silberberg, Silberberg, Vogel & Wettstein, 1961). Zn stimulated cell differentiation but decreased the size of the ratio, nucleus: cytoplasm, which is a feature of senescent cells (Andrew, 1971). If Zn-treated chondrocytes have prematurely aged by increased cell differentiation this would be indicated conclusively if chondrocytes no longer synthesized keratan sulphate characteristic of embryonic cartilage (Shulman & Meyer, 1968). Zn stimulated bone resorption, deficiency of which may cause the thickened bones of Zn deficiency (Zeigler *et al.* 1962) and excess of which can cause bone rarefaction and collapse as found in Zn toxicosis (Grimmett *et al.* 1937).

The effects of Zn deficiency on DNA and RNA syntheses have been altered by both experimental animal inanition and different experimental diets (Hoekstra, 1969; Mills, Quarterman, Chesters, Williams & Dalgarno, 1969; Williams & Chesters, 1970; Leucke, Baltzer & Whitenack, 1974). However, in systems uncomplicated by inanition, there is good evidence that Zn is necessary for RNA synthesis (Wegner & Romano, 1963; Chesters, 1974; Falchuk, Fawcett & Vallee, 1975) and there is a net synthesis of RNA when osteoprogenitor cells are changed into osteoblasts or osteoclasts (Young, 1963). Stimulation of: (1) chondrogenesis and chondrocyte hypertrophy; (2) osteo-

blast differentiation and function; (3) osteoclast differentiation and bone resorption by Zn in these experiments is further evidence for a role for Zn in RNA synthesis. The organ culture technique is an inexpensive model for the study of nutritional requirements, interactions (e.g. Cu-Zn) and toxicity at the cellular level.

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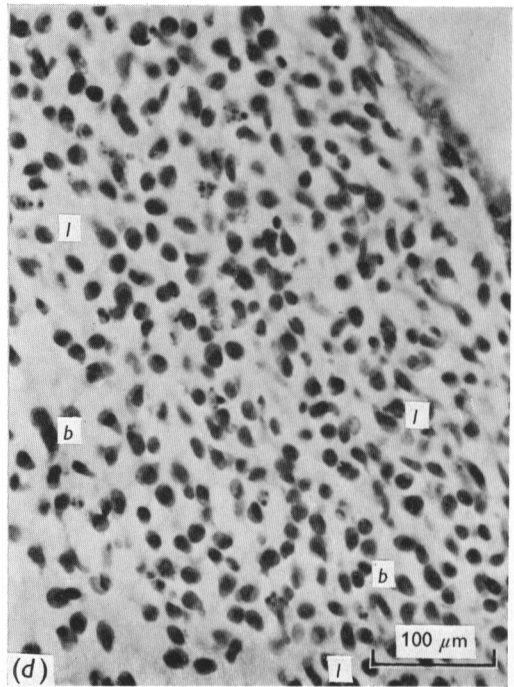
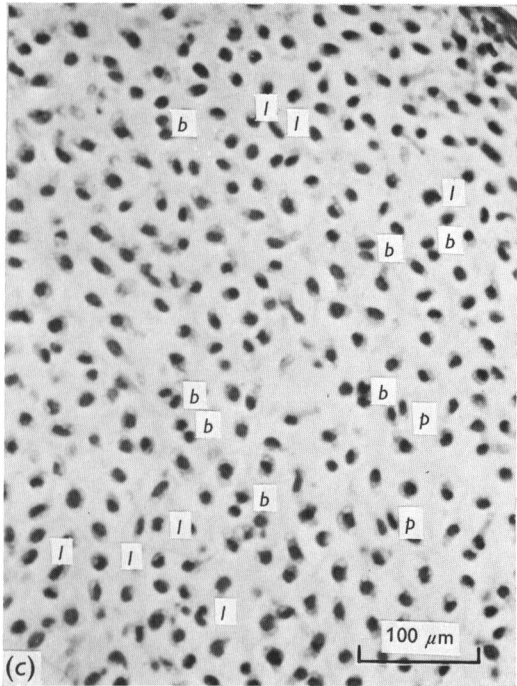
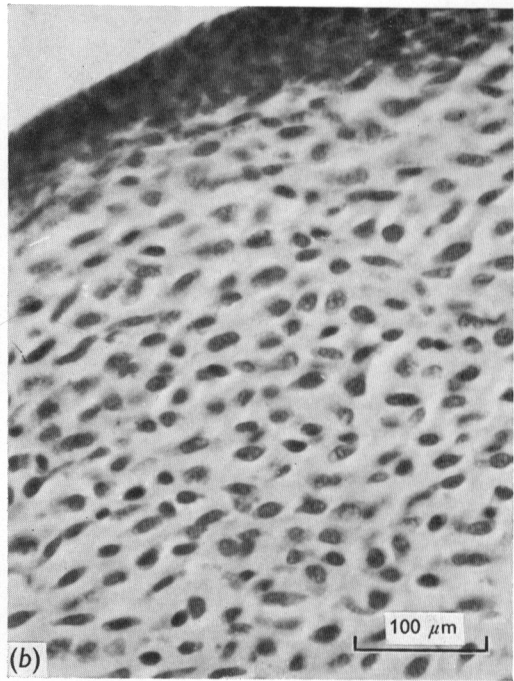
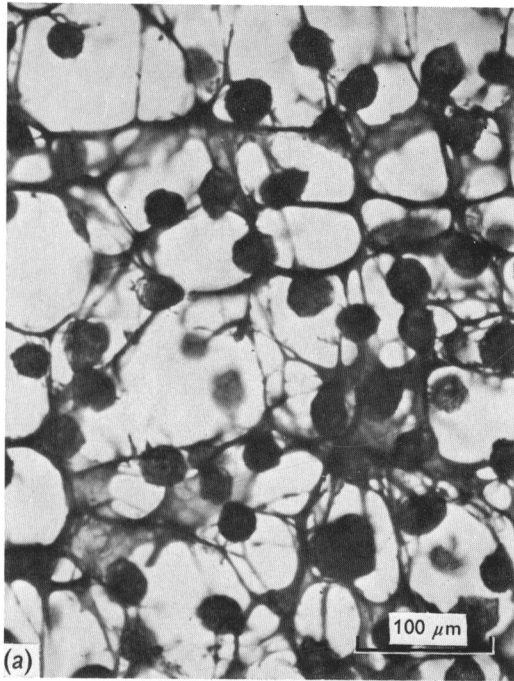
EXPLANATION OF PLATES

PLATE 1

(a) Hypertrophied cartilage of an explant from a 7.5-d-old chick embryo cultured for 8 d in 'BGJ' medium containing 20 μg Cu/ml (for details, see p. 000 and Table 1). Chondrocytes are swollen, rounded and basophilic. The cells are detached from the matrix, which is reduced in quantity (stain toluidine blue; $\times 500$); (b), (c), (d), epiphyseal cartilage and perichondrium of an explant from a 7.5-d-old chick embryo cultured: (b) for 8 d in 'BGJ' medium. Chondrocytes vary in shape from rounded to elongated and have stippled chromatin (stain haematoxylin and eosin; $\times 500$); (c) for 8 d in 'BGJ' medium containing 5 μg Zn/ml. Some chondrocytes are vacuolated, others are completely divided and therefore have lobed nuclei (l) or are binucleate (b). Recently divided cells are present as flattened cell pairs (p) (stain haematoxylin and eosin; $\times 450$); (d) for 4 d in 'BGJ' medium containing 20 μg Zn/ml. Chondrocyte morphology is varied. Some are rounded and hyperchromatic, others have nuclei showing karyorhexis. There are several lobed (l) or binucleate (b) chondrocytes (stain haematoxylin and eosin; $\times 450$).

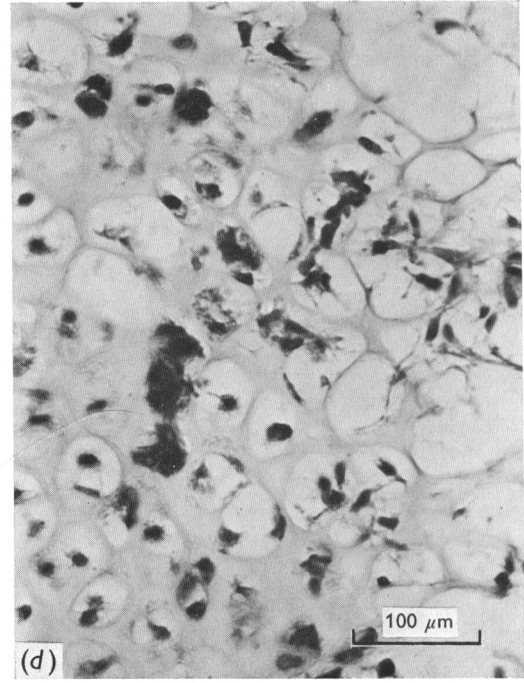
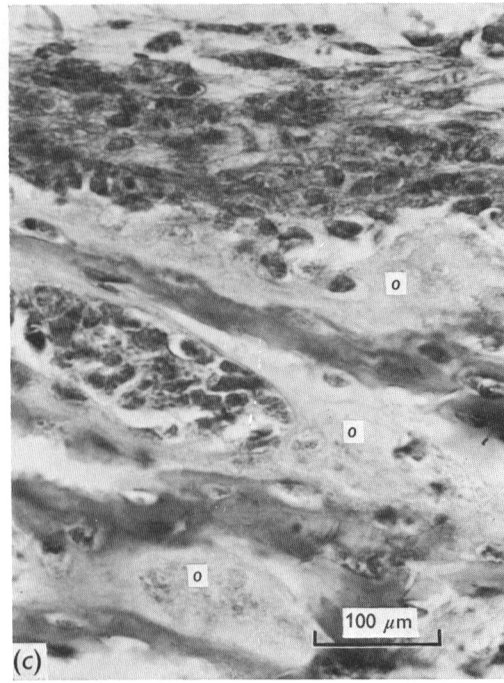
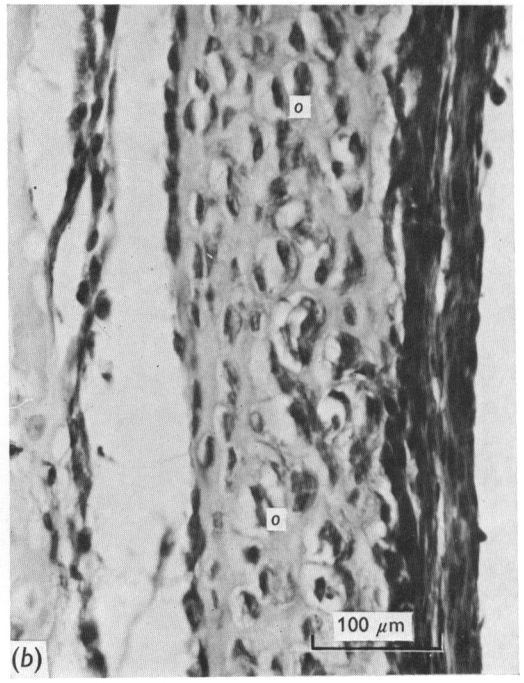
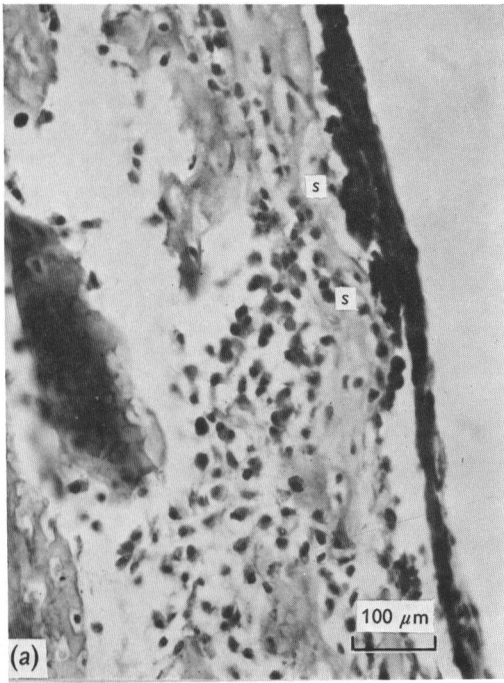
PLATE 2

Periosteum, osteoid and bone of an explant from an 11-12-d-old chick embryo cultured: (a) for 6 d in 'BGJ' medium containing 40 μg Zn/ml (for details, see p. 000 and Table 1). Hyperchromatic, rounded osteoblasts, have formed syncytia (s) (stain haematoxylin and eosin; $\times 300$); (b) for 6 d in 'BGJ' medium alone. There is a single thick layer of osteoid formed during culture (o) (stain haematoxylin and eosin; $\times 480$); (c) for 8 d in 'BGJ' medium containing 10 μg Zn/ml. Calcified trabecular bone is in the form of diagonal, deep-staining strands. Between these strands is lighter-coloured osteoid (o) formed during culture (stain haematoxylin and eosin; $\times 480$); (d) resorption edge of hypertrophied cartilage from an explant cultured for 8 d in medium containing 10 μg Zn/ml showing multinucleate osteoclasts (stain haematoxylin and eosin; $\times 480$).



JOAN R. REST

(Facing p. 254)



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