

Lesions produced by copper deficiency in neonate and older rats

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1. Three groups of rats of different mean weights were given a diet of milk treated with hydrogen sulphide and supplemented with copper-free minerals and vitamins. Also divided into three groups of similar weights were rats given the same diet supplemented with 500 μg Cu/week and stock rats which were given a commercial diet.
2. In a second experiment eight adult female rats were given the Cu-deficient diet for 2 weeks before mating and during pregnancy, and in a third experiment were mated and immediately after mating were given the Cu-deficient diet or the Cu-supplemented diet.
3. Cu deficiency reduced the growth rate of younger rats but had a much less marked effect on the weight of more mature rats. Achromotrichia appeared at 5 weeks; diarrhoea, with the faeces frequently containing undigested blood, and subcutaneous oedema were terminal changes. Deaths occurred in the youngest group of rats after 9 weeks and later in heavier, older rats.
4. Histological changes were seen in livers, spleens, testes and epididymes but not in blood vessels or bones.
5. Pups were not born to mothers given the deficient diet before mating, and the pups from mothers given the deficient diet only during pregnancy were born dead or showed congenital abnormalities.
6. Foetal and liver Cu concentrations for the various groups of animals are given.
7. Maintenance of the foetus appears to be the biological process most susceptible to Cu deficiency in the rat.

Several changes have been associated with experimentally produced copper deficiency in the rat including anaemia (Hart, Steenbock, Waddell & Elvehjem, 1928), poor growth (Gallagher, 1955), lesions in the central nervous system (Carlton & Kelly, 1969), achromotrichia (Keil & Nelson, 1931), reproductive failure (Dutt & Mills, 1960; Hall & Howell, 1969) and congenital abnormalities (O'Dell, Hardwick & Reynolds, 1961; O'Dell, 1968). Few of these changes have been extensively investigated. This paper describes the lesions and gives the Cu concentrations in neonatal rats and in rats made Cu-deficient at a variety of ages.

EXPERIMENTAL

Animals and diets

Hooded rats were used and they were divided into three dietary groups. The deficient groups were given a diet of milk treated with hydrogen sulphide and supplemented with Cu-free minerals and vitamins (Hall & Howell, 1969). The tocopheryl acetate supplement was given by mouth in two doses of 10 mg each week. The control groups received the same diet supplemented with 100 μg Cu, given as 0.2 ml of an aqueous copper sulphate solution and mixed with the minerals and vitamins on

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5 d of the week. These two groups of rats were housed in Perspex and Pyrex-glass cages modified from the design of McCosker (1967). The stock groups were housed in conventional rat cages and given a diet of Chardex GR₄ rat cubes (Wyatt, Chard, Somerset) and tap-water *ad lib*.

In Expt 1 in which the changes that occurred during the development of fatal Cu deficiency were to be studied, a total of fifty-five specific pathogen free (SPF) rats were divided into three groups, A, B and C, according to age and sex. Within each of these groups, rats were maintained under deficient, control and stock conditions. Group A contained twenty-eight males, 5 weeks old at the start of the experiment, with a mean weight of 113 g (twelve deficient, eight control, and eight stock). Group B contained thirteen females, 6 weeks old at the start of the experiment, with a mean weight of 143 g (five deficient, four control and four stock). Group C contained fourteen females 11 weeks old at the start of the experiment with a mean weight of 174 g (seven deficient, four control and three stock).

Rats given the Cu-deficient diet were either killed during the course of the experiment, allowed to die or killed in a moribund state. Some control and stock rats were killed during the course of the experiment and the remainder were killed at the end of the experiment.

In Expt 2, eight conventionally reared, adult, female rats, weighing approximately 200 g, were given a Cu-deficient diet for 2 weeks before being mated.

In Expt 3, twelve conventionally reared, adult, female rats, weighing approximately 200 g, were mated and immediately after mating six were given the Cu-deficient diet and six were given the control diet. A detailed examination was made of the tissues of the pups.

Investigations made during and at the end of the experiments

In Expt 1 all rats were weighed at the start of the experiment and weekly afterwards. Student's *t* test was used to analyse the weight changes. The animals were killed by diethyl ether inhalation and were then exsanguinated. Liver samples for Cu determination were taken with stainless-steel instruments which had been washed in deionized water and the samples were kept at -20° in Cu-free Polystyrene pots. Cu was determined with an atomic absorption spectrophotometer. A post-mortem examination was made of each rat, and lungs, liver, kidneys, heart, spleen, adrenals, brain, femur, ovaries, testes, epididymes, seminal vesicles, uterus, caecum, small intestines, bladder and aorta were examined histologically. These organs were fixed in 10% neutral formalin except for testes, epididymes and seminal vesicles, which were fixed in Bouin's fluid.

In Expts 2 and 3 vaginal smears were taken daily from each rat. Stock males were left overnight in the cage of the females on heat and mating was considered to have taken place if a copulation plug was found or if numbers of sperm were seen in the vaginal smear. Adult rats were killed and samples taken as described previously. Rat pups were removed from the mother after birth and killed with coal-gas. Two pups from each litter were washed in deionized water, stored and subsequently analysed for total Cu content as described previously. Two pups were stained for

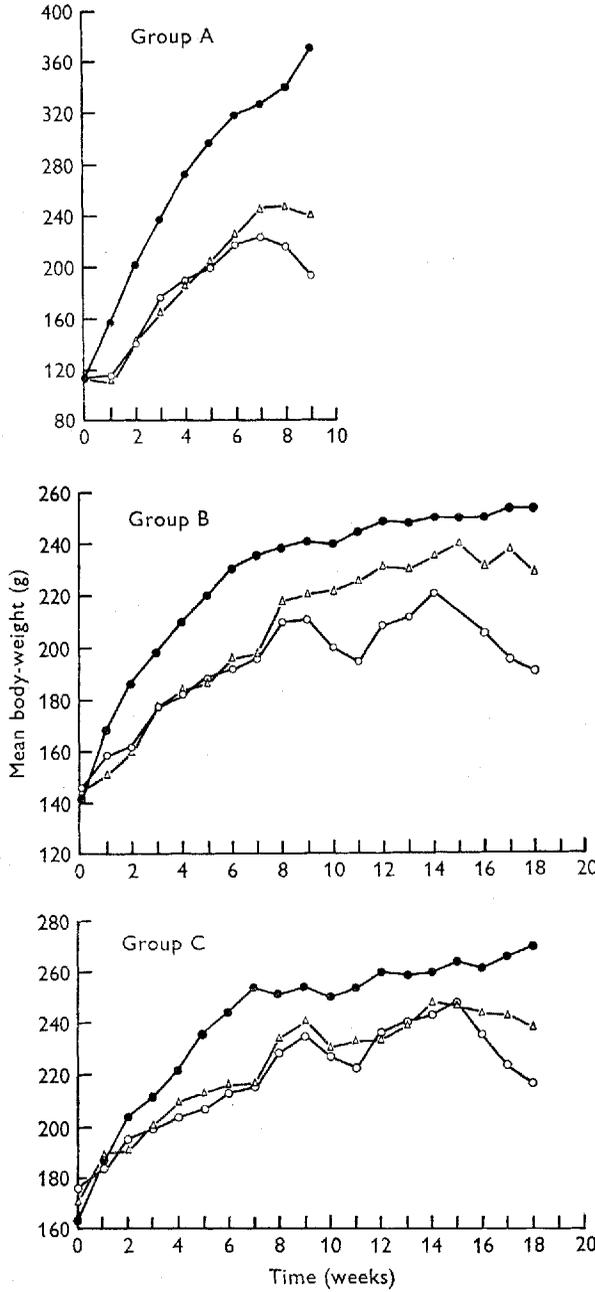


Fig. 1. Expt 1. Graph showing the weight of stock ●—●, control △—△ and copper-deficient rats ○—○ in groups A, B and C. (See p. 96 for details.)

Table 1. *Expt 1. Liver copper concentration (parts/10⁶ dry weight) in rats*

(Mean values with their standard errors)

Group*	Deficient		Control		Stock	
	Mean	SE	Mean	SE	Mean	SE
A	1.74	0.35	10.29	0.53	12.99	0.44
B	1.18	0.02	11.07	0.65	14.76	0.79
C	1.77	0.28	10.97	0.42	14.51	0.77

* For details see p. 96.

skeletal examination using alizarin red (Staples & Schnell, 1964, as modified by Cook & Moore, 1967). The remainder were fixed in Bouin's fluid and histological examination was made of transverse and longitudinal sections through complete foetuses.

RESULTS

Expt 1

The weight changes in Expt 1 are shown in Fig. 1. Milk-fed groups weighed significantly less ($P < 0.05$) than the stock group after 1 week in groups A and B; and after 2 weeks for the deficient group; and 5 weeks for the control group in group C. The deficient and control groups had similar weights for some time but the deficient animals eventually became lighter. The difference was first statistically significant after 7 weeks for group A and after 10 weeks for group B. In group C it had not reached significance (P was greater than 0.10) after 17 weeks.

The results of liver Cu estimations in Expt 1 are given in Table 1; values from all groups of deficient rats were significantly lower than those from corresponding control groups ($P < 0.001$).

Gross pathological changes. In all groups of rats in Expt 1 achromotrichia was first evident at the end of the 5th week of the experiment and visible in all rats given the Cu-deficient diet by the end of the 7th week. Achromotrichia and reduced body size were the only macroscopically visible changes seen in the rats killed during the course of the experiment, except for those which were killed when moribund. Six male rats (group A) and six female rats (groups B and C) died, or were killed when moribund, and diarrhoea had developed, before death, in six out of six male rats and five out of six female rats. Undigested blood was seen in the faeces of four male rats and two female rats, and at post-mortem examination these rats showed haemorrhage into the caecum. Subcutaneous oedema of thorax, abdomen and hind legs was seen in one male rat and two female rats which died.

Microscopical changes. There were no significant histological differences between the dietary subgroups in the lungs, hearts, aortas, bladders, uteri, ovaries, small intestines, central nervous systems, adrenals and femurs. In all groups differences were not seen between stock and control livers. Glycogen was absent from all the livers of deficient animals except in two rats in group B which were killed after 18 weeks of the experiment. In deficient rats the cytoplasm of hepatic parenchymal cells

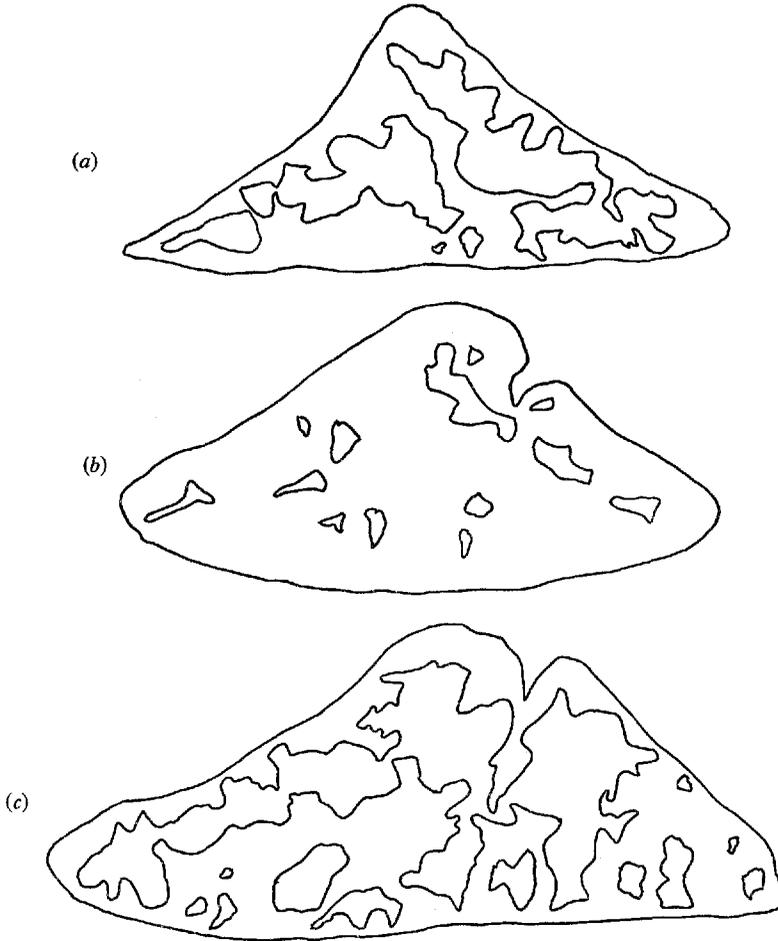


Fig. 2. An outline of the spleen and areas of white pulp within it from (a) a control rat, (b) a copper-deficient rat and (c) a stock rat. The image of the spleen, stained by Mayers haemalum and picro-orange, was projected on to fine-grain tracing paper.

had a homogeneous eosinophilic appearance compared with the foamy particulate appearance seen in control and stock livers. This change was more marked in periportal cells, and these cells were larger than centrilobular cells of the deficient rats. Significant differences between kidneys of Cu-deficient and control rats were not seen but there were differences between rats given the milk diet and those given the stock diet. Calcified material was present in the renal pelvis of twenty-seven out of forty milk-fed rats (Pl. 1a) and in none of the sixteen stock rats. Hydronephrosis was seen in fourteen out of forty milk-fed rats and in two out of fifteen stock rats. These animals had dilation of the renal pelvis and collecting tubules.

In the spleen of deficient rats there was depletion of lymphocytes, which was visible as a reduction in the area of the white pulp. This change was measured in transverse sections of spleen, using a projection system and a planimeter. The total area of white pulp was expressed as a percentage of the total cross-sectional area of the spleen. In all

Table 2. *Expt 3. Details of the litters from adult female rats given the copper-deficient diet from the day of mating, together with individual whole-foetal and maternal-liver Cu concentrations*

Group	Litter size	Pups born living/ dead	Comments	Cu concentration	
				Whole-foetus (parts/10 ⁶ wet wt)	Maternal liver (parts/10 ⁸ dry wt)
Deficient	7	3/4	All with SC haemorrhage and oedema	0.056 0.055	3.92
	13	12/1	All with SC haemorrhage	0.264 0.014	4.81
	6	0/6	All with SC haemorrhage	0.060 0.064	4.04
	10	0/10	All with SC haemorrhage	0.050 0.085	5.42
	8	0/8	A few with SC haemorrhage	0.125 0.055	4.99
Mean ± SE	8.8	3.0/5.8	—	0.083 ± 0.020	4.64 ± 0.3
Control	9	9/0	Normal	0.529 0.650	6.67
	13	13/0	1 pup showed SC haemorrhage	0.411 0.384	5.83
	13	13/0	Normal	0.461 0.060	5.93
	10	6/4	Normal	0.313 0.317	8.09
	14	13/1	Normal	0.419 0.240	9.48
	7	3/4	Normal	0.534 0.334	8.67
	Mean ± SE	11	9.5/1.5	—	0.388 ± 0.044

SC, subcutaneous.

the Cu-deficient male rats the area of white pulp was significantly reduced compared with stock rats but was less than that of control rats only in those rats which died or were killed when moribund (Fig. 2).

At post-mortem examination, haemorrhage was seen in the caecum and was found to originate from the mucous membrane, and erythrocytes were seen in and on its surface. The mucous membrane was intact but there was no inflammatory response.

Abnormalities were not detected in the testes, epididymes and seminal vesicles from any of the deficient male rats which were killed when active, but in the deficient male rats which died, or were killed when moribund, there was an increase of sloughed cells in the epididymes which was most marked in the tail (Pl. 1*b*). In two of these rats degenerative changes were seen in the testes; these consisted of sloughing of germinal cells into the lumen of some of the seminiferous tubules (Pl. 1*c*). Abnormalities were not detected in the Leydig cells in any of the groups.

Expts 2 and 3

The eight rats in Expt 2, which had been given a Cu-deficient diet for 2 weeks, were all mated but none produced litters. Foetal resorption, as described previously in Cu-deficient rats (Hall & Howell, 1969; Howell & Hall, 1969) was seen to have occurred.

One of the six rats given the Cu-deficient diet immediately after mating in Expt 3 did not produce a litter but all others given the deficient and control diets produced litters. Details of litter sizes, together with whole-foetal Cu concentrations are given in Table 2. In the deficient group five litters were produced, that is a total of forty-four pups of which twenty-nine were born dead. In the control group six litters were produced, a total of fifty-seven pups, of which nine were born dead. All litters from rats given the Cu-deficient diet contained pups which showed either subcutaneous haemorrhages or subcutaneous oedema or both. In the deficient pups subcutaneous haemorrhages were most common on the snout and over the shoulders. In the control litters one pup only had one small area of haemorrhage over one shoulder. The presence of subcutaneous haemorrhages was confirmed microscopically but other abnormalities were not detected. Histological examination of the blood vessels, including the aorta, and the central nervous system of the two groups did not reveal any differences. Examination of skeletons of cleared specimens stained with alizarin red did not reveal any abnormalities.

The mean liver Cu concentration (parts/10⁶ dry weight) of the group of eight rats which were given the Cu-deficient diet for 2 weeks before being mated (Expt 2) was 4.88 ± 0.45 . The results of whole-foetal and maternal liver Cu estimations in Expt 3 are given in Table 2. Total Cu concentrations of the foetuses born to dams given the Cu-deficient diet were significantly lower than those of the foetuses born to control dams ($P < 0.001$). The liver Cu concentrations of the two groups of dams, measured after parturition, were also significantly different ($0.005 > P > 0.001$), but the differences in whole-foetal Cu concentrations were larger.

DISCUSSION

A reduction in growth rate, due to Cu deficiency, has been reported by many workers and in several species, although most reports are the result of clinical observations rather than of controlled experiments. Poor growth in young Cu-deficient rats has been reported by Elvehjem & Kemmerer (1931), Gallagher (1955) and Warren (1962). The results of Expt 1 are in agreement with the findings of these authors, but our results additionally demonstrate a far more limited effect of Cu deficiency on the growth rate of more mature rats. When 5-week-old male rats were given a Cu-deficient diet, a significant depression of growth rate was seen after 7 weeks on the diet. When 6-week-old females were used, a significant depression of growth rate was not apparent until after 10 weeks. However, when rats were used which were 11 weeks old at the start of the experiment, the weight of the deficient group was never significantly less than that of the control group, even at the stage when rats

were apparently dying of Cu deficiency. Deaths occurred in the group of younger, lighter, rats at a much earlier stage of the experiment than in the group of older, heavier animals. Gallagher (1955) had recorded emaciation and diarrhoea in adult rats, but only after prolonged Cu depletion. Dreosti & Quicke (1966) reported that when adult rats were fed on a Cu-deficient diet throughout pregnancy and lactation they 'suffered from loss in body weight and acute diarrhoea' after only 5-6 weeks on the experimental diets, but they did not give details. Gallagher (1955) suggested that reduced growth rate in growing Cu-deficient rats was the result of impairment of oxidative processes, owing to reduced levels of cytochrome oxidase (*EC* 1.9.3.1). It seems likely that impairment of oxidative processes would reduce anabolic metabolism and, as the level of anabolic metabolism would be much higher in growing rats than in adults, Cu deficiency could be expected to produce a greater effect on the growth rate of growing rats.

The post-mortem findings in Expt 1 agree with those of Gallagher (1955), who noted achromotrichia as an early effect of Cu deficiency and generalized oedema and serous effusion as a late and inconstant finding. Histological changes in liver, spleen and testes have not been previously reported. The depletion of liver glycogen could be expected in a deficiency state in which cytochrome oxidase activity and, therefore, oxidative phosphorylation are impaired. The changes in the lymphoid tissue of the spleen were most marked in the terminal stages of deficiency but were present some time before. It is unfortunate that lymph nodes were not examined, for if a generalized depletion of lymphocytes were found it could be of the greatest significance in the terminal stages of deficiency. A defect in antibody-forming and transport mechanisms may account for such phenomena as the terminal diarrhoea which is seen in a variety of species. The testicular changes were not marked, were probably of recent origin, and clearly represent a feature of prolonged and severe Cu deficiency.

Changes in collagen (Rucker, Parker & Rogler, 1969; Waisman, Cancilla & Coulson, 1969) and elastin (Hill, Starcher & Kim, 1968) have been reported in a wide variety of species (Underwood, 1971). However, such changes have not been recorded in the bones or cardiovascular system of adult Cu-deficient rats, and this absence of change was confirmed in the study reported here.

The lesions seen in Cu-deficient rat pups were subcutaneous haemorrhage and oedema, and these lesions have been reported previously (O'Dell *et al.* 1961). These authors also reported skeletal abnormality, abdominal hernias and a reduction in the number of hair follicles, but we have not confirmed these observations. O'Dell (1968) has reported changes in the aortic elastin of such pups, but these were not present in our pups born in Expt 3.

The results of these investigations into Cu deficiency suggest that in the rat various processes have a different sensitivity to Cu deficiency. Reproduction in the female rat is apparently the process most sensitive to Cu deficiency. Rats given a Cu-deficient diet immediately after mating produce abnormal pups with markedly reduced Cu contents (Expt 3) but, in slightly more advanced Cu deficiency, reproduction fails (Expt 2). Reproductive failure is evident before any other manifestation of Cu deficiency. The initial lesion is in the foetus (Howell & Hall, 1969) and one reason for the

extreme sensitivity of reproduction in the female rat may be that the availability of Cu is reduced to a level below that required to support the intense cellular activity in the foetus. In Expt 3 the mean maternal liver Cu concentration of deficient rats at the end of the experiment was 4.64 parts/10⁶ and in the controls 7.45 parts/10⁶, but the mean whole-body Cu content of the deficient pups was 0.08 and of the control pups, 0.39 parts/10⁶. Thus a Cu-deficient diet had a much greater effect on foetal than on maternal Cu levels. The value for the Cu content of the liver in Cu-deficient adult animals is usually much lower than 4.64 parts/10⁶ (see Table 1) and the result not only indicates that foetal resorption occurs early in Cu deficiency but that the unknown mechanism for the transfer of Cu from mother to foetus may not be efficient when the diets contain a suboptimal level of Cu. Another factor may be that the developing foetus, which has a very high growth rate and an intense cellular activity, may have a very high requirement for Cu. A combination of high Cu requirement by the foetus and a poor supply, even in a mild Cu deficiency, may lead to death of the foetus.

The second detectable change was achromotrichia. Histological changes in liver and spleen developed when the deficiency was advanced and changes, including diarrhoea, dysentery, subcutaneous oedema, effusions in serous cavities and degenerative changes in spermatic tubules, developed in the terminal stages of the deficiency. It is well established that anaemia is produced by Cu deficiency in rats and, although we did not investigate this change, it is clear from previous reports that it develops only in extreme Cu deficiency (Gallagher, 1957; Underwood, 1971).

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EXPLANATION OF PLATE

Histological changes in tissues of rats. (*a*) Kidney from a milk-fed, copper-supplemented rat. Calcified material (arrows) is present within cystic spaces in papilliform proliferations of the epithelium of the renal pyramid. Haematoxylin and eosin. Both (*b*) and (*c*) are sections from Cu-deficient rats. Haematoxylin and eosin. (*b*) Epididymis containing many sloughed cells and few spermatozoa. (*c*) Testicular tubules with sloughed spermatids in the lumen.

