The effect of a copper-deficient diet on the concentration of copper in the nervous system and other tissues of the rat

By P. J. WARREN* (Bromiley Research Fellow)
Department of Chemical Pathology, Guy's Hospital Medical School, London, S.E. 1

(*Received* 18 *May* 1961—*Revised* 5 *February* 1962)

It has been clearly established that the experimental production of a copper deficiency in sheep, pigs and rats gives rise to severe anaemia, hypocupraemia, and a marked reduction in the concentration of Cu in the liver (Bennetts & Chapman, 1937; Underwood, 1956). Bennetts & Chapman (1937) have also shown that a severe Cu deficiency in pregnant ewes can produce a neonatal ataxia in their lambs. This original observation has been verified by many workers in different parts of the world, with animals under natural or experimental conditions.

More recently, Howell & Davison (1959) have shown that the Cu content of the brain and the liver of lambs with swayback was significantly lower than that in normal animals.

To my knowledge there have been no reports of the appearance of demyelination or abnormality of the brain or spinal cord in the rat after Cu depletion. Frick & Lampl (1953) attempted to produce such lesions in young and adult rats by deficient diets, but without success. The results of Mandelbrote, Stanier, Thompson & Thruston (1948) have suggested the possibility of reducing the Cu content of the rat's brain by giving repeated daily injections of British Anti-Lewisite (BAL) for a period of 10 weeks. Unfortunately, disadvantages have been found in the use of BAL for the removal of Cu from the tissues of human subjects and animals. Its toxicity and the painful abscesses sometimes produced at the site of the injection during treatment have restricted its use for experimental studies on Cu depletion. Some workers, notably Walshe (1956), have used D-penicillamine hydrochloride, a compound which has been found very effective in removing Cu from the body without any of these adverse effects. He found a considerable increase in the urinary Cu excretion of human patients with hepatolenticular degeneration, a disease in which Cu accumulates in the liver and the brain.

Because of the general interest in the role of Cu in the metabolism of the nervous system it seemed desirable to study two main problems. First, whether giving a Cu-deficient diet to young growing rats reduces the concentration of Cu in the central nervous system as well as in other tissues. Secondly, whether the concentration of Cu in the nervous system can be further reduced by the administration of D-penicillamine, by mouth, to the animals during the period on the deficient diet.

* Present address: Department of Biochemistry, London Hospital Medical College, Turner Street, London, E. 1.

EXPERIMENTAL

Experimental design. The rats used in the experiments were divided into four groups, A, B, C and D, and treated as follows:

Group A. Four rats were given a H_2S-treated milk diet for 71 days.

Group B. Four rats were given a H_2S-treated milk diet for 71 days with a daily supplement of 50 μg Cu/rat as $CuSO_4$ in 0·1 ml water.

Group C. Eight rats were given the H_2S-treated milk diet for 71 days together with a daily supplement per rat of 3 mg D-penicillamine hydrochloride (Distillers Company (Biochemicals) Ltd) in de-ionized water.

Group D. Six rats were given a standard rat-cake diet for 71 days.

Animals. All were male albino rats (Wistar strain), originally supplied by the Chester Beatty Research Institute, and bred at Guy's Hospital Medical School.

Housing of the animals. All the rats used in the experiments were born in clean well-galvanized metal cages, in which they spent 3 weeks up to weaning and 3 weeks thereafter. At 6 weeks of age they were divided at random into the four groups, A, B, C and D. Those to be maintained on the Cu-deficient diet were placed in Perspex cages fitted with glass-rod floors, the rods being spaced so as to allow faeces to pass easily between them. Each rat was kept in a separate isolated compartment, and the diet was offered in individual glass dishes securely fixed to the inside of each cage by a glass hook. The Perspex cages were designed to fit into a special wooden rack that provided a warm, well-ventilated environment for the animals, and were covered with a Polythene sheet arranged so as to reduce atmospheric dust contamination to the minimum.

Special cleaning of the cages. The galvanized metal cages were cleaned by scrubbing in hot water containing a detergent solution, and drying. The Perspex cages were carefully cleaned by washing the inside walls with 5 N-hydrochloric acid (AR) and with copious washings of de-ionized water and then allowing the cages to dry. This procedure was carried out at the beginning of the experiment and once a week during it.

Handling of animals and food containers. Rats fed on the low-Cu diet were handled only with rubber gloves, which were carefully washed to remove metal contamination and kept in an acid-cleaned Pyrex tray. The animals were weighed once a week in washed Polythene bags. The food containers were of glass, cleaned by washing with *aqua regia* and then repeatedly in de-ionized water. After cleaning, the food dishes were handled with clean rubber gloves and placed on a large clean Pyrex tray. The milk diet and supplements were added to each food dish, and were then offered individually to each rat.

Diets. The six rats in group D were given a standard rat-cake diet and tap water *ad lib.* The rat cake was supplied by the North Eastern Agricultural Society, Aberdeen, and had the percentage composition: fine bran 17·4, ground wheat 17·4, Sussex-ground oats 17·4, ground maize 8·7, ground barley 8·7, white-fish meal 4·8, meat-and-bone meal 9·6, dried skim milk 14·0, dried yeast 1·2, common salt 0·4, Adisco (a highly

stable vitamin supplement containing 1000 i.u. vitamin A and 200 i.u. vitamin D_3/g, Isaac Spencer and Co. Ltd, Aberdeen) 0·4.

The sixteen rats in groups A, B and C were given *ad lib.* a Cu-deficient diet of cow's milk treated with H_2S, together with purified mineral and vitamin supplements, prepared by the method of Gallagher, Judah & Rees (1956*a*, *b*), which was a modification of the original procedure of Mills (1955). The supplements, given to each animal daily, provided: 0·4 mg Fe^{3+} as ferric chloride, 0·05 mg Mn^{2+} as manganous sulphate, 12·5 μg thiamine hydrochloride, 25 μg riboflavin, 10 μg pyridoxine, 50 μg nicotinic acid, 100 μg calcium pantothenate, 25 μg *p*-aminobenzoic acid, 10 μg folic acid, 1 mg inositol, 2·5 mg choline chloride. In addition, each animal received 1·2 mg α-tocopheryl acetate and 37·4 μg vitamin A twice weekly. The mineral supplements were made up for convenience as aqueous solutions with de-ionized water such that the daily allowance was present in 0·1 ml of the solution. The water-soluble vitamins were similarly made up into one stock solution such that 0·2 ml of it contained the daily allowances. The supplements of minerals and water-soluble vitamins were given to each rat daily in a small quantity of H_2S-treated milk. The two fat-soluble vitamins were dissolved in arachis oil and made up to a solution such that 0·4 ml contained the weekly allowance.

Preparation of tissues. After 71 days on the experiment, the animals were killed with chloroform, and the tissues were dissected out with clean stainless-steel instruments on to glass Petri dishes. Portions of these tissues were then placed in clean weighed 50 ml Erlenmeyer flasks, dried to constant weight at 110° and analysed for Cu.

Preparation of apparatus and reagents. All glassware used in the experiments was made of borosilicate glass, and was cleaned in *aqua regia* overnight and washed four times in de-ionized water before use. Flasks used for the analysis of the tissues were boiled out with de-ionized water after the treatment with *aqua regia*. All reagents used were of AR quality and were tested for the presence of Cu. Those found to contain traces were purified by the methods described by Gallagher *et al.* (1956*a*, *b*). De-ionized water was prepared from glass-distilled water which was passed through a mixed-bed ion-exchange resin column consisting of equal parts of Zeo-Karb 225 in the H form and DeAcidite FF in the OH form (Permutit Co. Ltd). Water treated in this way contained no detectable Cu.

Determination of Cu. The procedure for the wet oxidation of the tissues and for the subsequent determination of Cu was that of Eden & Green (1940). The yellow colour formed in the presence of Cu with sodium diethyldithiocarbamate was extracted into redistilled n-pentanol and the absorption at 435 mμ was measured in a spectrophotometer.

RESULTS

General effect of the diet. The rats in groups A and C maintained for a period of 71 days on the low-Cu milk diet all showed signs of severe Cu deficiency.

The animals receiving the standard rat-cake diet (group D), and those fed on the milk diet containing a daily supplement of 50 μg Cu/rat (group B) appeared normal, and showed no signs of Cu deficiency. The mean gain in weight of the rats in groups

A, B and C during the experiment is shown in Fig. 1. Of the sixteen rats on the milk diet, two died during the experiment, one in group A after 35 days and the other in group C after 65 days.

In the first 40 days of the experiment the rats in groups A, B and C consumed daily about 75 ml milk/rat. During the remainder of the experiment, the milk consumption of the rats in group B given the Cu supplement increased to 90 ml/rat daily, whereas the intake of the animals in groups A and C, fed on the low-Cu diet, fell gradually to about 40 ml/rat daily.

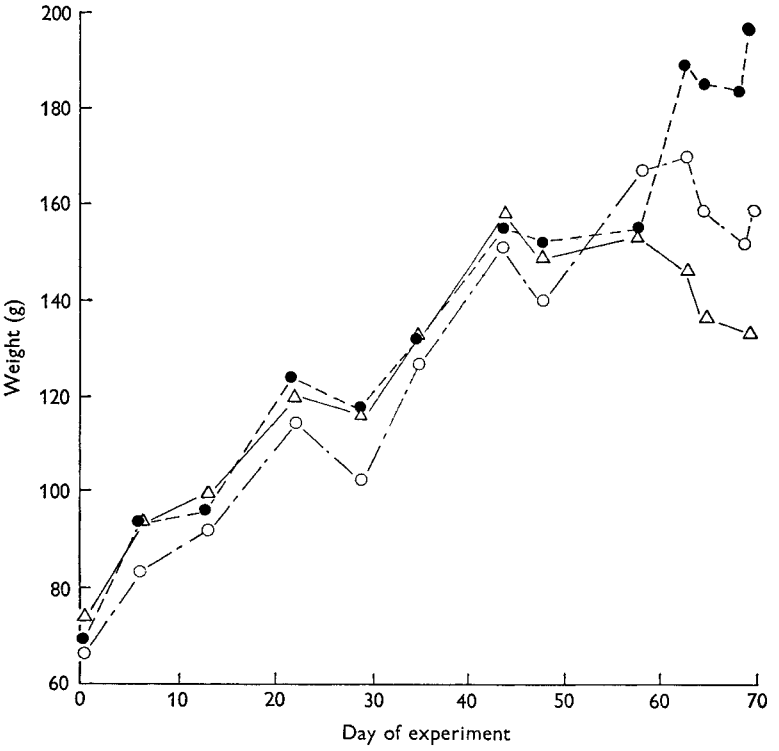


Fig. 1. Mean weights of rats fed for 71 days on diets of milk treated with H_2S. ○——○, group A (milk diet only); ●——●, group B (milk diet + 50 μg Cu/rat daily); △——△, group C (milk diet + 3 mg D-penicillamine hydrochloride/rat daily).

The first external signs of anaemia appeared after 44 days in the rats given D-penicillamine by mouth in addition to the low-Cu diet (group C). Later, after 60 days, the rats fed on the milk diet alone (group A) showed signs of anaemia. At this stage, the conjunctivae and the skin of the paws appeared extremely pale, the colour of the pupil, normally possessing a red tinge, was yellower in appearance. Textural changes of the hair, as illustrated in Pl. 1, were a common feature in these rats. No visible signs of neurological changes appeared in any of them.

Concentration of Cu in the tissues. The results for groups A, B, C and D are shown in Table 1. The mean values obtained for the rats fed on the normal rat-cake diet (group D) showed that the highest concentration of Cu was in the kidney. The con-

centrations in the liver and brain were similar, and smaller concentrations were found in the spinal cord and spleen. The concentrations of Cu in the tissues of rats in group B were very similar to those in group D, both groups having had a diet adequate in Cu. The concentrations in the tissues of rats in groups A and C were very similar, both groups having had a Cu-deficient diet.

Comparison of the concentration of Cu found in tissues of rats on a diet containing adequate amounts of Cu (groups B and D) with that for animals fed on a Cu-deficient diet (groups A and C) showed a significant reduction in the latter. For liver, brain and kidney $P < 0{\cdot}001$ and for spinal cord and spleen $P < 0{\cdot}01$.

Table 1. *Mean values with standard deviations and ranges for concentration of copper (μg/g dry tissue) in various tissues of four groups of rats after 71 days on the diets*

		Liver		Brain		Spinal cord	
Group	Diet	Value and SD	Range	Value and SD	Range	Value and SD	Range
A	H_2S-treated milk*	1·8 ± 0·64 (3)	1·3–2·5	9·5 ± 1·74 (3)	8·0–11·4	2·9 ± 1·67 (3)	1·1–4·4
B	H_2S-treated milk* with 50 μg Cu/day	12·7 ± 2·83 (4)	8·5–14·8	10·9 ± 2·38 (4)	7·5–12·6	6·2 ± 1·18 (4)	5·2–7·8
C†	H_2S-treated milk* with 3 mg D-penicillamine hydrochloride/day	3·4 ± 2·05 (8)	1·7–8·0	7·8 ± 1·31 (8)	5·3–9·6	4·0 ± 2·0 (7)	0·9–6·4
D	Rat cake	12·5 ± 2·55 (6)	9·9–16·6	11·4 ± 1·57 (6)	10·1–14·2	6·7 ± 1·64 (6)	5·0–8·9
B+D	Adequate in Cu	12·5 ± 2·51 (10)	8·5–16·6	11·2 ± 1·82 (10)	7·5–14·2	6·6 ± 1·42 (10)	5·0–8·9
A+C	Deficient in Cu	2·9 ± 1·9 (11)	1·3–8·8	8·3 ± 1·55 (11)	5·3–11·4	3·7 ± 1·89 (10)	0·9–6·4

		Kidney		Spleen	
Group	Diet	Value and SD	Range	Value and SD	Range
A	H_2S-treated milk*	8·9 ± 0·86 (3)	8·0–9·7	4·2 ± 2·99 (3)	2·1–7·6
B	H_2S-treated milk* with 50 μg Cu/day	18·6 ± 3·51 (4)	14·4–22·4	9·3 ± 6·16 (4)	5·0–18·4
C†	H_2S-treated milk* with 3 mg D-penicillamine hydrochloride/day	10·1 ± 2·12 (8)	7·3–13·7	2·8 ± 1·89 (5)	0·2–5·5
D	Rat cake	25·5 ± 3·22 (6)	20·4–29·7	6·7 ± 0·87 (6)	5·8–7·8
B+D	Adequate in Cu	22·7 ± 4·72 (10)	14·4–29·7	7·8 ± 3·83 (10)	5·0–18·4
A+C	Deficient in Cu	9·8 ± 1·9 (11)	7·3–13·7	3·3 ± 2·26 (8)	0·2–7·6

Figures in parentheses are the numbers of observations.
* Supplemented with minerals and vitamins, see p. 169.
† One rat in this group died after 65 days on the experiment.

DISCUSSION

The results show that giving young growing rats a diet of milk treated with H_2S for a period of 71 days produced a severe Cu deficiency. This deficiency could be prevented by the daily addition of 50 μg Cu to the diet of each rat. Comparison of the concentration of Cu in tissues of the animals given an adequate amount of Cu (groups B and D)

showed that there was no significant difference in the Cu concentrations in liver, brain, spinal cord or spleen of the two groups. This evidence strongly suggests that the addition of 50 μg Cu/day to the Cu-deficient milk diet permitted the rats to develop normal Cu levels in the tissues, and that the Cu deficiency induced by the diet without the Cu supplement was not complicated by other factors. These findings are in full agreement with those of Gallagher *et al.* (1956*a*, *b*). However, a small but significant lowering of the Cu level in the kidney was found in the rats of group B when compared with that of the animals in group D ($P < 0{\cdot}05$). This lowering had no effect on the statistical significance of the reduction in Cu concentration of the kidney produced by the Cu-deficient diet.

In rats with an adequate intake of Cu from their diet (groups B and D), the kidney contained the highest concentration of Cu, 23 μg/g dry tissue. The concentrations in liver and brain were similar, and of the order of 12 μg Cu/g dry tissue. The spinal cord and the spleen had the lowest concentrations, 7 μg Cu/g dry tissue.

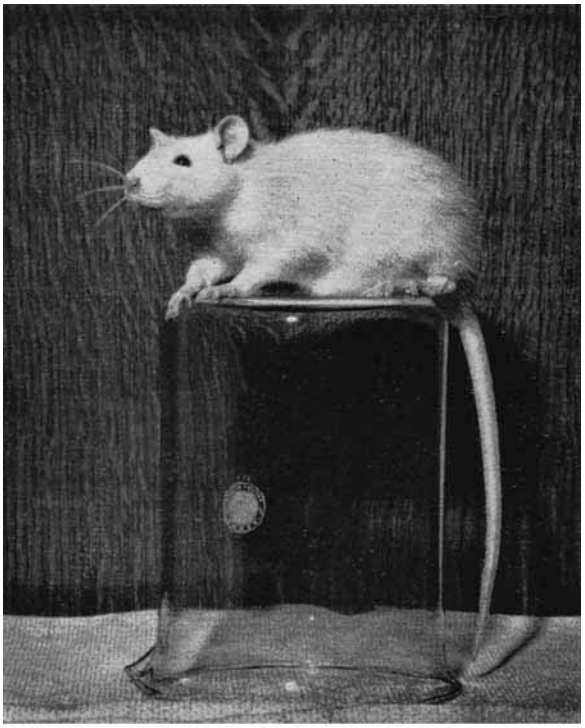
These values were considerably different from those for the animals fed on a Cu-deficient diet, for whom the concentration of Cu in the kidney was 10 μg/g dry tissue, which is 57 % less than the normal value for this organ. The levels in the spinal cord and the spleen were reduced by 44 and 57 % of the value for the animals given adequate amounts of Cu to 4 μg Cu/g dry tissue. In the Cu-deficient groups, A and C, the liver showed the lowest level, 3 μg Cu/g dry tissue, representing a reduction by 77 % of the concentration. Statistical comparison of the results from groups B and D with those from groups A and C showed that there was a highly significant difference in the concentration of Cu in the liver, brain and kidney ($P < 0{\cdot}001$) and a significant difference in the spinal cord and the spleen ($P < 0{\cdot}01$).

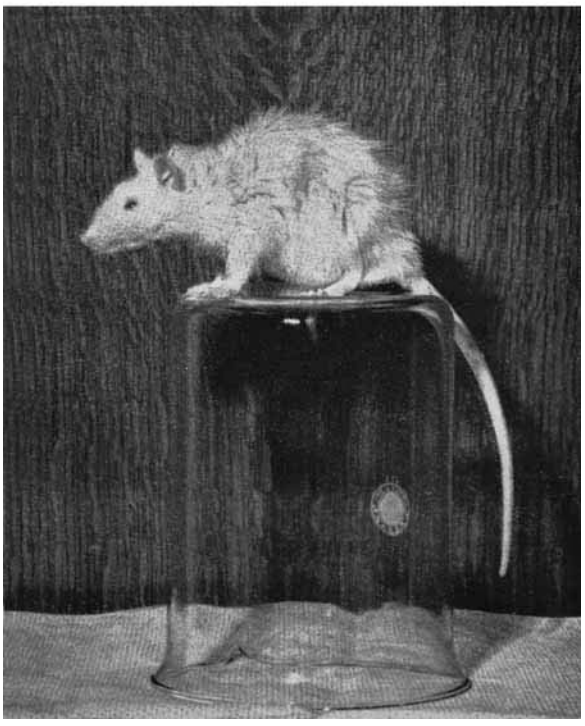
It had been hoped that giving 3 mg D-penicillamine hydrochloride daily to some of the Cu-deficient rats would produce the deficiency condition more readily, and that the Cu content of the brain and the spinal cord might have been substantially reduced. There was, however, no statistical difference between the results for the group of rats given penicillamine with the Cu-deficient diet and those for the animals receiving the deficient diet alone.

SUMMARY

1. A comparative study has been made of the concentration of copper in the liver, brain, spinal cord, kidney and spleen of four groups of albino rats. Three groups of rats were fed for 71 days on a Cu-deficient diet prepared from cow's milk treated with hydrogen sulphide to which purified mineral and vitamin supplements had been added. The first group of four rats was given this diet alone, the second group of eight rats received the milk diet together with 3 mg D-penicillamine hydrochloride/rat daily, and the third group of four rats was given a daily supplement of 50 μg Cu/rat with the milk diet. These rats were fed and housed in Cu-free Perspex and glass cages. The fourth group of six rats was fed for the same period on a standard rat-cake diet in galvanized metal cages.

2. The rats given the milk diet without Cu supplement developed external signs of anaemia and later became very deficient in Cu. The first signs of anaemia appeared





P. J. WARREN

(*Facing p.* 173)

in the group given the penicillamine supplement after 44 days, and after 60 days in the animals fed on the milk diet alone.

3. In the animals fed on the standard rat-cake diet, the kidney had the highest concentration of Cu (26 μg/g dry tissue). The concentrations of Cu in the liver and the brain were similar and of the order of 12 μg/g dry tissue. The spinal cord and the spleen had the lowest concentrations, 7 μg/g dry tissue.

4. The concentrations of Cu in the tissues of the rats fed on the Cu-supplemented milk diet were very similar to those of the rats fed on the standard rat-cake diet.

5. The tissues obtained from rats fed on the Cu-deficient milk diet, with or without penicillamine, had significantly lower concentrations of Cu than the tissues of animals having adequate amounts of Cu. The addition of D-penicillamine hydrochloride to the Cu-deficient diet did not have any significant effect on the concentrations.

I acknowledge the advice and helpful criticism given to me by Professor R. H. S. Thompson in whose department this work was carried out. I am also grateful to Dr C. H. Gallagher for details of the Cu-deficient diet. Dr E. V. B. Morton and Mr R. Cobb, of Boots Pure Drug Co. Ltd, kindly supplied me with vitamin samples of low Cu content. I thank Dr W. L. Magee for producing the photographs shown in Pl. 1.

The entire work was made possible by a generous grant from the Multiple Sclerosis Society.

REFERENCES

Bennetts, H. W. & Chapman, F. E. (1937). *Aust. vet. J.* **13**, 138.
Eden, A. & Green, H. H. (1940). *Biochem. J.* **34**, 1202.
Frick, E. & Lampl, F. (1953). *Klin. Wschr.* **31**, 912.
Gallagher, C. H., Judah, J. D. & Rees, K. R. (1956*a*). *Proc. roy. Soc.* B, **145**, 134.
Gallagher, C. H., Judah, J. D. & Rees, K. R. (1956*b*). *Proc. roy. Soc.* B, **145**, 195.
Howell, J. McC. & Davison, A. N. (1959). *Biochem. J.* **72**, 365.
Mandelbrote, B. M., Stanier, M. W., Thompson, R. H. S. & Thruston, M. N. (1948). *Brain*, **71**, 212.
Mills, C. F. (1955). *Brit. J. Nutr.* **9**, 398.
Underwood, E. J. (1956). *Trace Elements in Human and Animal Nutrition.* New York: Academic Press Inc.
Walshe, J. M. (1956). *Amer. J. Med.* **21**, 487.

EXPLANATION OF PLATE

Photographs of a rat fed on a copper-deficient milk diet for 71 days, but given 50 μg Cu/day during this period (upper) and of a rat fed on a copper-deficient milk diet alone for the same period (lower). The posture and the appearance of the hair of the latter animal were characteristics found in all animals of this group.

Printed in Great Britain