The effect of copper deficiency on reproduction in the female rat

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1. Eighty-seven rats were fed a diet of milk treated with hydrogen sulphide together with copper-free mineral and vitamin supplements. Forty-three of the eighty-seven rats were used as controls and given Cu supplements varying from 50 to 500 μg/week. In addition to the eighty-seven rats, twenty-eight rats were fed a commercial diet.

2. Rats which did not receive Cu supplements developed signs of Cu deficiency and their liver Cu levels were significantly lower than those of control and stock animals. Oestrous cycles were normal and the majority of the rats were successfully mated.

3. Litters developed to full term in stock rats and in those given Cu supplements but the Cu-deficient rats did not produce litters.

Copper deficiency has been associated with lowered fertility in cattle but the nature of the reproductive disturbance has not been determined (Underwood, 1962). Keil & Nelson (1931) reported that female rats fed a diet of milk supplemented with ferric chloride only reproduced if copper sulphate was added. Dutt & Mills (1960) recorded reproductive failure in female rats fed a Cu-deficient diet. These rats had normal oestrous cycles and conceived but resorbed their foetuses. The experiments reported here confirmed and extended these findings.

EXPERIMENTAL

Animals and diet. Hooded, specific pathogen-free rats were used and at the start of the experiments they were from 9 to 12 weeks old. Stock rats were kept in conventional rat cages and fed Chardex GR4 (Wyatt, Chard, Somerset) commercial rat diet with tap water *ad lib.* All other rats were kept in Perspex cages with Pyrex glass-rod bottoms modified from the design of McCosker (1967). These rats were given, from polythene bottles through Pyrex glass tubing, a diet of milk collected directly from the farm in polythene aspirators, and treated with hydrogen sulphide (Gallagher, Judah & Rees, 1956). They also received a vitamin and mineral supplement (Table 1). Rats fed on this diet alone will be called deficient rats. Control rats were given Cu as copper sulphate on 5 days of the week (Table 2). Rats receiving 100 μg Cu were given their supplement from the feeding bottle before the milk was added; the other controls received 10, 20 or 50 μg Cu *per os* from a plastic syringe.

Experimental procedures. Vaginal smears were taken daily from each rat. Stock males were left overnight in the cage of 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. Twenty-eight of the deficient rats, all the rats receiving 10, 20 and 50 μg Cu and sixteen of the rats receiving 100 μg Cu were fed the diet for 4 weeks before

the commencement of mating. Eight, eight and sixteen rats were fed the deficient diet for 2·5, 6 and 8 weeks respectively before mating, and twelve of the rats receiving 100 μg Cu were also fed the diet for 8 weeks before males were introduced.

Table 1. *Composition of vitamin and mineral supplements*

(A) Water-soluble vitamin and mineral supplement

	Daily dose, given to each rat 5 days a week
Thiamine	0·050 mg
Pyridoxine	0·050 mg
Riboflavine	0·10 mg
p-aminobenzoic acid	0·10 mg
Nicotinic acid	0·20 mg
Calcium pantothenate	0·80 mg
Folic acid	0·050 mg
Biotin	0·0050 mg
Inositol	4·0 mg
Vitamin B_{12}	0·250 μg
$MnSO_4.5H_2O$*	2·1 mg
$FeSO_4.7H_2O$*	1·3 mg
Choline chloride	10 mg

(B) Oil-soluble vitamin supplement

	Dose given to each rat twice weekly in 0·5 ml arachis oil
Vitamin A acetate	0·03740 mg
DL-α-tocopheryl acetate	Either 1·2 mg or 10·0 mg†

* Specpure (Johnson Matthey Chemicals Ltd, 73–83 Hatton Garden, London, EC1).

† The lower level of tocopheryl acetate was given to twenty of the twenty-eight deficient rats mated at 4 weeks, to sixteen of the deficient rats mated at 8 weeks, and to all the rats receiving the 100 μg Cu supplement. All the remaining rats received the 10 mg level.

Precautions taken to avoid contamination by extraneous Cu. The cages, feeding pots and other utensils were washed in a detergent, left standing for at least 12 h in 0·1 M-disodium ethylenediamine tetra-acetic acid, rinsed in a tank of deionized water and washed in running deionized water before they were reassembled. Rats fed the milk diet were handled only with rubber gloves washed in deionized water. They were weighed in polythene bags which had been washed in deionized water and were transferred to clean cages once a week. Wood wool which had been washed in deionized water and dried was provided as nesting material for rats with young. All storage vessels were of Pyrex glass (see Butler & Newman, 1956).

Examinations at the end of the experiments. Rats were killed by ether inhalation and exsanguinated. The animals were examined for changes in the colour of coat and teeth. Tissues for Cu analysis were removed using stainless steel instruments washed in deionized water and were kept at $-20°$ in chemically clean polystyrene pots. The Cu levels were determined by a modification of the method of Eden & Green (1940).

RESULTS

Reproduction. Details of the rats used and the results of the matings are given in Tables 2 and 3. Oestrous cycles were normal until mating and in the deficient group many rats recommenced oestrous cycles after an interrupted pregnancy (Table 4)

Table 2. *Summary of breeding records of copper-deficient, Cu-supplemented and stock rats*

Type of diet	Cu supplement as μg Cu given on 5 days of the week	No. of rats	No. not mated	No. of matings	No. not producing full-term pups	No. producing full-term pups
Milk treated	0	44	8	40	40	0
with	10	8	2	7	6	1
hydrogen	20	8	1	8	4	4
sulphide	50	7	0	8	1	7
	100	20	3	20	5	15
Stock	0	28	4	32	8	24

Table 3. *Details of the successful matings of control rats shown in the last line of Table 2*

Cu supplement as μg Cu given on 5 days of the week	No. of matings	No. of fertile matings	Remarks
10	7	1	Died at term with 3 full-grown pups and 6 resorbing sites *in utero*
20	8	4	(1) 11 born. All died within 24 h (2) 6 born. All died within 24 h (3) Blood and a half-eaten pup found (4) Killed on 20th day of pregnancy. One full-grown foetus and 3 resorbing foetuses *in utero*
50	8	7	(1) 10 born, 2 died, 2 removed at 2 days. Of the 6 remaining 5 lived to be weaned (2) 10 born, 4 removed at birth. Remaining 6 died before weaning (3) 9 born, 3 removed at 13 days. Of the 6 remaining 5 lived to be weaned (4) 8 born, 1 was dead, 1 removed at 2 days. 6 lived to be weaned (5) Died during parturition, 2 born dead, 6 dead *in utero* (6) Killed after 1 dead pup found. 3 dead *in utero* (7) Killed on the 17th day of pregnancy. 10 normal pups
100	20	15	Fifteen litters were born, a small number of pups died and were eaten but 15 litters were weaned. Unfortunately, the number of rats born and weaned is not available

and continued until they were killed at the end of the experiments. All groups mated well including the deficient group and the group supplemented with 100 μg Cu that were fed the diet for 8 weeks before males were introduced.

There were thirty-two matings in the stock rats and twenty-four produced full-term pups. A total of eighty-three matings occurred in the milk-fed rats and twenty-seven produced full-term pups, all of these were in the Cu-supplemented groups (Table 2) and some of their pups were successfully weaned and used in other experiments which will be reported elsewhere. The results of the productive matings in the Cu-supplemented groups are given in Table 3.

Table 4. *Details of vaginal bleeding and recurrence of oestrous cycles in rats that did not produce pups in the deficient group, and in those supplemented with* 10 μg *and* 20 μg *Cu*

Day on which bleeding first seen	Bleeding for 3 days or less			Bleeding for more than 3 days		
	No. bleeding	No. in which oestrous cycles re-commenced	Day after mating of recommence-ment of oestrus	No. bleeding	No. in which oestrous cycles re-commenced	Day after mating of recommencement of oestrus
9	4	1	9	0	—	—
10	5	2	12, 13	0	—	—
11	6	3	14, 15, 20	2	0	0
12	5	1	27	0	—	—
13	3	2	14, 15	1	1	21
14	1	1	16	10	6	20, 21, 21, 21, 21, 23
15	0	—	—	4	3	21, 22, 23
16	0	—	—	2	2	20, 22

Litters were not born to rats fed the diet without a Cu supplement (Table 2). A total of fifty-six rats fed the milk diet did not produce full-term pups (Table 2). Subsequent to mating, forty-three of these bled from the vagina and in a number of them oestrous cycles recommenced. These rats were in the deficient, 10 and 20 μg Cu-supplemented groups and could be divided into two types. In one vaginal bleeding occurred early, was of short duration and oestrous cycles usually recommenced within a short time. In the other, vaginal bleeding was prolonged and oestrous cycles did not recur for several days. The details are given in Table 4. Four of the deficient rats that bled early for a short time and immediately recommenced oestrous cycles were killed and their uteri were found to be non-gravid.

Evidence of Cu deficiency. The deficient rats which had been fed the diet for 12 or more weeks had grey-coloured head markings and their incisor teeth were depigmented. The control rats did not show these changes. The Cu levels in the liver of unmated female stock, Cu-deficient and control rats fed at the 100 μg level for 12 weeks were, respectively, $14{\cdot}43 \pm 0{\cdot}397$, $1{\cdot}34 \pm 0{\cdot}523$ and $10{\cdot}84 \pm 0{\cdot}858$ ppm dry weight.

DISCUSSION

These results clearly indicate that Cu is essential for the maintenance of pregnancy in the rat (Tables 2 and 3) and confirm the results of Keil & Nelson (1931) and Dutt & Mills (1960). Pups were borne by rats which had received five daily doses of 20 μg

Cu/week but the pups died within a short time of birth. Pups were born from and weaned by rats which had received five daily doses of 50 μg Cu/week. This level of Cu supplementation supported pregnancy and lactation.

Forty-three of the rats which did not produce full-term pups bled from the vagina after mating (Table 4). The rats which showed vaginal bleeding for several days and were slow to recommence cycling, or which never cycled again, were considered to be resorbing their foetuses in the manner described by Howell & Hall (1969). This is supported by the fact that over half of them were first seen to bleed on the 14th day of pregnancy, which is a critical time in the resorptive process (Howell & Hall, 1969). In many of the rats which bled for a short time and never recycled, or which did so after several days, resorption of the foetuses may have been taking place.

A group of eight rats bled for a short time and oestrous cycles commenced almost immediately. It is most unlikely that resorption of the foetuses was taking place in these rats. Four were killed and their uteri were non-gravid. This phenomenon was not seen in the rats in other groups nor is it recorded as an event in pseudopregnancy (Long & Evans, 1922). To test this we vasectomized six male rats and mated them to twelve females fed a stock diet. The post-mating vaginal smears of these rats were typical of pseudopregnancy (Long & Evans, 1922), and they did not show vaginal bleeding. The mechanisms involved in this phenomenon are not understood.

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