CXLI. SOME BIOCHEMICAL AND PHYSIOLOGICAL ASPECTS OF COPPER IN ANIMAL NUTRITION.

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WITHIN recent years our conception of the mineral nutrition of animals has had to be broadened to take account of certain "trace" elements which have been found to be widely distributed in the vegetable and animal kingdoms. These elements, *e.g.* manganese, iodine, zinc, copper, *etc.*, occur only in minute traces but their possible physiological significance cannot be neglected.

The claim that copper is essential for the utilisation of iron in haemoglobin building [Waddell *et al.*, 1928] arouses particular interest in this element. The work outlined below was undertaken with a view to obtaining further information on this and cognate subjects.

I. THE OCCURRENCE OF COPPER IN NATURE.

(a) The vegetable kingdom.

The occurrence of copper in vegetable materials has been investigated mainly with the object of discovering the amounts of this element in materials used as foodstuffs. Such investigations date as far back as 1816 [Meissner, 1816] but the greater number have been carried out within the last eleven years [Maquenne and Demoussy, 1920; Fleurent and Levi, 1920; Guérithault, 1920; Quartaroli, 1928; Elvehjem and Hart, 1929; Lindow, Elvehjem and Peterson, 1929, *etc.*]. The data available show that copper occurs very widely in foodstuffs, the amounts varying somewhere between 4 and 20 mg. per kg. dry matter, but the copper content may be affected by soil conditions [Webster and Jansma, 1929] and increased by manurial treatment of the crop [Elvehjem and Hart, 1929].

Maquenne and Demoussy [1920] have shown that, while copper occurs throughout all parts of the same plant, the concentration is greatest in buds, young leaves and cell sap. This has been confirmed to some extent by McHargue [1926], whilst Javillier and Guérithault [1927] found that the concentration of copper in the germs of seeds was much greater than in the endocarp.

Apart from these observations on the occurrence of copper in foodstuffs and on the concentration in young parts of plants, no evidence has been found of a definite function of copper in plant life.

In view of the connection, in animal life, between copper and the porphyrin pigment haemoglobin, it was decided to determine whether the copper content of different parts of plants bore any relation to the chlorophyll content. Comparison was therefore made of the copper contents of the root, the stem and the leaf of plants such as turnips and between the outer and the inner leaves of cabbages and lettuces. Results are shown in Table I. The second column refers to a second series of analyses.

The analytical method for copper used throughout the work reported in this paper was that of Biazzo [1926] as modified by Elvehjem and Hart [1929]. The only further modification employed was the addition of sodium phosphate to the solutions of the ashed materials in order to avoid interference by traces of iron.

Table I. *Comparison of the copper contents of aerial and root portions of plants.*

Plant	Part analysed	Mg. Cu/kg. dry matter	
Leek	Leaves	9.8	—
	White edible portion	5.8	—
Lettuce	Outer leaves	18.7	15.8
	Inner leaves	25.8	25.4
	Centre stalk	12.6	—
Cabbage	Outer green leaves	4.7	4.8
	Inner white leaves	4.0	5.8
	Centre vein of inner leaves	2.1	2.9
	Centre vein of outer leaves	3.1	
	Centre stalk of plant	3.4	—
Carrot	Leafy part (excluding lower leaves)	10.7	10.1
	Stalks of leaves	—	5.0
	Red part of root	4.5	5.4
	Yellow part of root	—	5.5
Beetroot	Leaves	10.2	12.6
	Stalks	6.8	8.4
	Roots	6.8	6.6
Potato	Stalk	11.2	—
	Tuber	5.4	—
Turnip	Leaves	7.1	—
	Stalks	3.0	—
	Roots	4.4	—
Garden pea	Mature old seed	7.3	—
	Young green seed	8.6	—
	Pods of young seed	5.4	—
	Leaves	10.6	—
		Mg. Cu/kg. dry matter	
Average (excluding cabbage and lettuce):		Leaves	9.9
		Stalks	6.7
		Roots	5.5

It is seen that there is more copper in the aerial parts of plants than in the roots. This is probably not connected with chlorophyll content as the white inner leaves of cabbages and lettuces contained as much (or more) copper as the outer chlorophyll-rich leaves. It is notable that in stems and pods, where a large amount of fibrous material is to be found, the copper content is lower.

(b) *The animal kingdom.*

The wide distribution of copper in vegetable foodstuffs suggests its occurrence in animal organisms, and data are available showing the presence of this element in insects, marine animals and land animals.

(i) *Insects and marine animals.* Information concerning the copper content of insects is not very definite. Muttkowski [1921] obtained a positive qualitative test in samples representing 34 different species and Haber [1926] showed qualitatively that copper occurs in the blood of *Blatella germanica*. Quantitative figures are available only for three species [Giunti, 1879; Aronsohn, 1911].

In the case of marine animals, on the other hand, a considerable number of copper determinations have been published by Rose and Bodansky [1920] and by Severy [1922]. It was shown that of 35 species the copper contents varied from about 60 mg./kg. dry matter in the oyster to traces in the whale, the amounts diminishing progressively in ascending from the comparatively simple to the more complex organisms.

The presence of copper in marine animals has been explained only in the case of those animals whose blood contains haemocyanin.

Analytical data obtained by the writer for the copper contents of insects and of marine animals are given in Table II.

Table II. *Copper contents of some insects and marine animals.*

		Mg. Cu/kg. dry matter	Mg. Cu in whole organ or tissue of one animal
<i>Insect</i>	Cockroach (<i>Blatta orientalis</i>)	32.4	—
	House fly (<i>Musca domestica</i>)	77.6	—
	Honey bee (<i>Apis mellifica</i>)		
	Whole worker	25.0	—
	Fully developed queen bee grubs	24.5	—
	Young queen bee grubs	22.7	—
	Royal jelly	17.8	—
<i>Marine animals</i> Octopus*			
	Skin	108.3	—
	Muscle	10.9	—
	Ink sac	914	8.8
	Ink (small sample)	No copper detectable	
Tunicate	Mantle	10.9	0.020
Lobster*	Liver	25.1	0.20
	Eggs	83.2	—
	Gonad	21.0	—
Herring	Liver	14.2	0.008
	Skin	2.3	0.004
	Flesh	2.3	0.039
	Female reproductive organs	2.1	0.024
	Male „ „	2.3	0.022

* Samples washed with many changes of saline and finally with pure distilled water to free from traces of haemocyanin.

The figures for insects call for small comment except that it is of interest that the copper percentage of the queen bee grub is higher than that of royal jelly, its sole source of food.

Referring to marine animals, it is seen that there is a very high proportion and amount of copper in the ink sac of the octopus. Such accumulation of copper in the ink sac is worthy of note particularly as the ink contains no appreciable quantity of this element. Reference will be made later to observations of a catalytic influence of copper on the oxidation of *l*-3:4-dihydroxy-phenylalanine to melanin. Since the ink of such sea animals contains melanin it is possible that, in the ink sac, copper has a function in the formation of the ink.

(ii) *Copper in land animals.* The occurrence of copper in land animals has been reported, but only in the case of the rat is there reliable information regarding the distribution of copper throughout the various organs of the body [Lindow, Peterson and Steenbock, 1929]. Some figures are available for different organs of man, the ox and the horse, but the data are scanty and the practice of taking different organs from separate animals has not permitted of definite conclusions being drawn regarding the distribution of copper throughout the body. The livers only, of a number of other species, have also been investigated for copper content.

In the present work, copper was determined in the organs from 12 different species of animals and the results are reported in Table III. The figures in a

Table III. *Copper in organs of different species expressed as mg./kg. dry matter.*

Species	Description	Liver	Heart	Lungs	Spleen	Kidney	Pancreas	Brain	Flesh	Skin	Hair
Human	Adult*	24.9	—	—	5.2	17.5	4.3	17.5	—	—	—
Bovine	Adult†	77.0	15.6	5.3	2.9	19.7	3.8	—	—	—	—
Bovine	Calf newly born	470.0	14.8	4.9	4.8	15.7	5.5	—	4.8	—	—
Bovine	Foetus	262.8 (16.3)	10.4 (0.086)	3.6 (0.115)	5.4 (0.060)	8.5 (0.087)	—	—	2.9	2.1 (0.458)	—
Sheep	Adult	236.6 (60.0)	17.9 (0.68)	9.6 (0.75)	5.0 (0.08)	17.8 (0.36)	7.7 (0.04)	—	—	—	—
Horse	Adult†	14.8 (30.5)	17.6 (11.9)	6.8 (10.0)	3.2 (1.7)	28.9 (8.2)	—	—	—	—	—
Pig	Adult†	41.3 (19.9)	14.9 (0.86)	5.3 (0.55)	6.0 (0.13)	21.1 (1.35)	—	—	—	—	—
Pig	Few days old	232.8 (2.0)	12.8 (0.020)	3.4 (0.010)	6.7 (0.0013)	14.7 (0.050)	—	—	—	—	—
Dog	9 days old	98.2 (0.320)	17.4 (0.006)	6.2 (0.005)	—	14.2 (0.009)	—	8.5 (0.012)	—	9.9 (0.076)	22.7 (0.056)
Cat	Adult†	25.3 (0.534)	14.4 (0.039)	3.8 (0.033)	5.2 (0.016)	10.1 (0.075)	—	14.6 (0.079)	2.3	4.2 (0.433)	11.9 (0.790)
Guinea-pig	Adult	17.0 (0.102)	21.2 (0.005)	9.5 (0.006)	—	19.9 (0.016)	—	—	—	—	—
Rabbit	Adult	9.2 (0.17)	22.3 (0.023)	8.1 (0.018)	—	13.7 (0.040)	—	—	—	—	—
Rat	About 90g. wt.†	10.0 (0.016)	27.8 (0.0023)	9.5 (0.0015)	8.1 (0.0016)	22.6 (0.005)	—	10.2 (0.003)	3.8	7.3 (0.025)	14.8 (0.014)
Badger	Adult†	21.7 (2.09)	12.8 (0.112)	5.6 (0.100)	3.0 (0.019)	9.4 (0.094)	—	10.8 (0.089)	—	3.2	24.7
Domestic fowl	Adult†	12.4 (0.081)	14.9 (0.014)	2.4 (0.003)	—	11.7 (0.018)	—	—	—	Feathers	4.9
Average: ...		...	16.8	6.0	5.0	16.4	—	—	—	—	—
Range of variation:		...	10.4-27.8	2.4-9.5	2.9-8.1	8.5-28.9	—	—	—	—	—

* Average of 3 series of analyses.

† Average of 2 series of analyses.

horizontal line refer to the organs from one and the same animal or group of animals and the total amount of copper, expressed in mg. in the entire organ from one animal, is given in brackets. These figures may be used in discussion of the distribution of copper throughout the various organs of animals from any one species without encountering any effect due to individuality or diet.

The averages and ranges of variation of the copper content of heart, lungs, spleen and kidneys for all species are also given in Table III.

Although the ranges of variation for heart, lungs, spleen and kidneys seem rather wide it should be noted that the extreme figures occur in only a few cases, most of the figures falling within 50 % of the average. The differences in copper content between these different organs therefore appear to be real differences and not due to chance variation.

Further investigation was undertaken to find the variation of copper in any one organ due to individuality, under controlled conditions of feeding. In this case, copper was determined in the livers of normal newly born piglings, whose mothers had all been receiving the same diet. The averages and range of variation for 15 livers are given in Table IV and figures for iron content are also included.

Table IV. *Copper and iron content of infant pig livers.*

	Mg. per kg. dry matter		Mg. in whole liver	
	Copper	Iron	Copper	Iron
Average	241.4	1390	1.15	6.14
Range	50.8-465.7	580-1910	0.11-2.00	2.63-12.12

Again, although the ranges of variation are rather large, only in 4 of the 15 cases did the total copper in the liver vary more than 50 % from the average. The proportion of copper in the dry matter varied in a similar manner. There was no correlation between the iron and copper contents, but the variations in respect of iron were as great as those in respect of copper.

In all the organs so far dealt with, then, the variation of copper content was too great to assign a definite value for it in a given organ. At the same time the differences between the different organs were significant, and the distribution of copper throughout the organs was substantially the same for all species examined. Classification of organs according to descending value for either proportion of copper or total weight of copper in the organ gave liver, kidneys, heart, lungs, pancreas, spleen.

Analyses of organs not referred to in this list or in Table III are given in Table V. It is seen that copper is present in all the parts of animals (and of plants) examined, and it is possible that this element may fill a small corner in the composition of protoplasm. Fox and Ramage [1931] have made a similar suggestion from spectrographic examination of animal tissues.

A general division of the parts of animals may be made, giving two classes

- (a) those containing a high proportion of copper;
- (b) those containing a low proportion of copper.

Table V.

Species	Sample	Mg. Cu/kg. dry matter	
Human	Hair	Av. 2	15.1
Bovine	Stomach wall	Av. 3	6.5
	Parotid salivary gland	Av. 2	6.6
	Horn		3.9
	Hoof		2.1
	Fatty bone marrow		0.16
	Red bone marrow, calf	Av. 2	4.4
	Thymus, calf		3.5
Sheep	Thymus		6.5
	Lymph glands		10.8
	Digestive tract		9.0
Horse	Adrenal		18.0
Dog	Melanotic tumour	Av. 2	8.0
	Bile		71.0
Cat	Bile	Av. 2	22.7
Rat	Tongues	Av. 2	16.6
Domestic Fowl	Skins and scales of leg		4.7
	Proventriculus		12.8
	Gizzard		3.2
	Gizzard lining	Av. 2	27.0

In (a) are included liver, kidneys, heart, brain, hair, while (b) includes skin, lungs, pancreas, spleen and flesh.

It was thought possible that some function might be associated with the presence of copper in certain organs and tissues, and further investigations were made of the skin and hair and the liver.

Skin and hair. Appreciably high amounts of copper were found in the skin and hair of all animals examined. Some figures were given in Table III and further figures are given in Table VI.

Table VI. *Copper in skin and hair of animals. Mg. Cu/kg. dry matter.*

Species	Skin	Hair or Wool
Bovine {Epidermis Dermis	6.0 2.3 (av. of 2)	
Sheep	2.8 (av. of 3)	8.3 (av. of 10)
Rabbit	6.6 (av. of 6)	15.4 (av. of 4)
Rat	9.7 (av. of 2)	12.2 (av. of 2)
Rats from same litters	At birth	18.6 (av. of 4)
		(0.0020)*
	1 week old	14.8 (av. of 4)
		(0.0090)
	2 weeks old	9.3 (av. of 4)
Guinea-pigs from same litter		(0.0105)
	3 weeks old	13.7 (av. of 2)
		(0.036)
	At birth	21.4
		(0.070)
Guinea-pigs from same litter	1 week old	10.1
		(0.035)
	2 weeks old	11.0
		(0.036)
		12.5
		(0.029)
		13.7
		(0.031)
		11.8
		(0.034)

* Figures in brackets represent the weight in mg. of copper in the whole tissue or part from one animal.

The figures quoted indicate that, in rats, the amount of copper in the skin increased rapidly from birth and reached a fairly steady value at about three

weeks of age, while in guinea-pigs the amount of copper decreased from the value at birth and became constant at about one week old. Several other series of analyses were made of both these species in which the age limits were narrowed or lengthened, and the findings from these data, which for the sake of brevity are not cited here, were in complete accord with the typical results quoted in Table VI.

The presence of copper in rat skins in somewhat different amounts has previously been reported by Lindow, Peterson and Steenbock [1929]. These workers found that the average amount of copper in the skin and hair of an adult rat was 0.161 mg. and the proportionate amount was 3.57 mg. copper/kg. dry matter. According to them, feeding a high level of copper to rats for 7 weeks did not increase the copper content of the skins above this value.

The author has investigated the effect of the amount of copper in the diet on the copper content of rat skins with the following results.

Table VII. *Copper content of rat skins (average values).*

No. of rats	Copper in diet mg. daily	Time fed diet months	Skin		Hair		Skin and Hair	
			mg. Cu per skin	mg. Cu/kg. dry matter	mg. Cu per animal	mg. Cu/kg. dry matter	mg. Cu per animal	mg. Cu/kg. dry matter
4	0.011	9	0.054	6.9	0.022	9.5	0.076	8.1
3	0.050	9	0.048	6.9	0.021	13.8	0.069	8.2
3	0.075	7	—	—	—	—	0.064	4.4
1	7.5	1	—	—	—	—	0.078	—
3	7.5	7	—	—	—	—	0.113	6.8

A slight increase in the total copper content of the skin and hair occurs after prolonged feeding of high doses of copper, but the effect is not very marked. At the same time almost identical amounts of copper were found in the skins of rats fed such different levels of copper as 0.011 and 0.050 mg. daily for long periods. It is therefore unlikely that the skin is a path for excretion of copper.

The occurrence of somewhat uniform amounts of copper in the skin and hair of many species merited some investigation. No explanation can be offered in the case of the hair, though it is of interest to note (1) that in bovines the concentration of copper is greater in the epidermis than in the dermis (Table VI), and (2) that in the sheep, an animal which produces large amounts of wool, the copper content of the liver is much higher than in any other species. On the other hand, while Angora rabbits produce large amounts of hair, the copper content of the livers is of the same order as that of short-haired rabbits.

Some instances were noticed amongst rats and rabbits in which the copper content of the skins of black animals was higher than for white animals. While this was not always so, the relation of copper to pigmentary processes was considered and the effect of this element on the oxidation of "dopa" (*l*-3:4-dihydroxyphenylalanine) was tried. "Dopa" is a chemical precursor of melanin into which it is converted on oxidation. It has been used by Bloch [1927] and by Stewart and Percival [1930] as a histological stain for detecting "dopa"

oxidase in skin, and by Koller [1930] in studying the genetics of pigmentation in rabbits. The technique adopted was largely based on the work of Koller.

It was found that the oxidation of a solution of "dopa" by means of an extract of the skin of newly born rats, rabbits or guinea-pigs only proceeded slowly, maximum coloration at room temperature occurring after about 60 hours. The addition of copper to the solution materially accelerated the oxidation process, always in proportion to the amount of copper added, and the addition of 0.05 mg. of copper in 2 cc. of solution sufficed to give maximum coloration in 18 hours.

It is difficult to assess the significance of this catalytic action of copper, but it is of interest to consider the possibility already alluded to that the copper in the octopus ink sac may possibly be definitely related to the formation of the melanin pigment present in the ink. It is possible also, that in land animals the copper in the skin may play some similar part in the formation of melanin pigments.

A further connection between copper and pigmentation is to be found in the practices of bird fanciers for obtaining a deep yellow colour in canaries, in which copper sulphate, or other substances such as cayenne pepper, are fed to moulting birds. The new feathers are said to have uniformly deep yellow coloured edges and this was confirmed experimentally.

This effect of copper feeding is probably not due to an increased formation of a copper pigment such as turacin, the pigment of Turaco feathers [Church, 1892], since the copper content of canary feathers was found to be low compared with the content of turacin-bearing feathers. Analytical results are given in Table VIII.

Table VIII. *Copper content of feathers.*

	Mg. Cu/kg. dry matter
Canary (average of 3)	40
<i>Turacus Hartlaubi medius</i>	
Crimson, turacin-bearing, secondary wing feathers	2900
Black wing feathers (no turacin)	14

Thus, although there is a possibility that the copper in the skin may exercise some influence on pigmentary processes, evidence bearing on the subject is too scanty for any conclusions to be drawn.

Copper in liver. In every species of land animal studied the liver was found to contain a much greater amount of copper than any other organ. The ratio, weight of copper in liver to weight of copper in kidneys, was found to be, for the sheep 160, the horse 4, the cat 6, the rabbit 40, and of similar order for the other species. This observation also applies to such figures as have been published by other workers.

A notable feature is the high proportion of copper in sheep's liver; the average copper content of 9 livers was 323 mg. copper per kg. dry matter, the range being 212.5-497.5. Since there is in this country no well-established practice of dosing sheep with copper sulphate for internal parasites, this high

copper content cannot be due to such administration of copper. Figures of the same order have been reported by Aston [1911] for livers of normal New Zealand sheep.

The high proportion of copper in livers generally might be due to filtration, from the blood, of copper absorbed in the digestion of foodstuffs. In this connection, figures published by Lindow, Peterson and Steenbock [1929] showed that feeding rats 5 mg. copper (as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) per rat daily for 7 weeks increased the copper content of the livers of the rats nearly 20 times. The author has fed different amounts of copper to rats with the results given in Table IX.

Table IX. *Copper content of rat livers. (Average values.)*

Diet	Copper in diet mg. daily	Time fed diet months	No. of rats	Mg. Cu in whole liver	Mg. Cu/kg. dry matter
Stock	about 0.050	Extended period	21	0.020	12.8
Cow's milk only	—	„	10	0.010	14.1
Synthetic	0.011	9	1	0.015	10.0
„	0.050	9	3	0.023	13.9
„	7.5	4	1	0.036	41.7
„	7.5	5	8	0.049	19.6

The copper content of rat livers, then, depended on the amount of copper in the food but the storage observed when high proportions of copper were fed was markedly less than was observed by Lindow, Peterson and Steenbock [1929].

Storage of copper in livers of foetal animals. That a considerable storage of copper occurs in intra-uterine life has previously been shown in the case of the human [Morrison and Nash, 1930], bovine [McHargue, 1925, 1, 2] and porcine species [Powick and Hoagland, 1924; Hart *et al.*, 1930]. Similar observations have been made by the writer for a number of species and the results are collected in Table X.

Table X. *Copper in livers of adult, foetal and newly born animals. (Average values.)*

Figures in brackets give the number of analyses.

Species	Mg. Cu/kg. dry matter		
	Adult	Foetal	Newly born
Human	25 (3)	281 (1)	—
Bovine	70 (3)	171 (4)	550 (2)
Sheep	323 (9)	—	117 (3)
Pig	35 (7)	—	241 (15)
Guinea-pig	17 (3)	122 (3)	203 (4)
Rabbit	10 (7)	29 (3)	55 (4)
Rat	12.5 (23)	—	70 (3)
Dog	88 (3)	—	89 (2)

In the bovine, rabbit and guinea-pig species, where comparisons of the copper contents of livers of foetal and newly born animals were made, it was found that there was more copper in the liver of the newly born animal than in that of the foetal animal.

With the exception of sheep, and possibly dogs, the figures for the different species show a much higher percentage of copper in the liver of the newly born or foetal than in that of the adult animal. In all cases, even with lambs and puppies, it was found that there was a steady drop in the percentage of copper in the liver from birth onwards, throughout early infancy (Table XI).

Table XI. *Copper content of infant livers at different ages.*

Figures in brackets give the mg. Cu in the whole liver.

Species	Age	Mg. Cu/kg. dry matter						
		At birth	3 days	1 week	2 weeks	3 weeks	4 weeks	8 weeks
Sheep		117.0	67.2	—	—	—	14.5	—
Dogs (same litter)		—	—	69.2 (0.209)	—	—	—	45.3 (0.534)
Rats (same litter)		87.8 (0.0045)	—	23.5 (0.0050)	14.5 (0.0063)	17.8 (0.022)	—	—
Guinea-pigs (same litter)		149.4 (0.171)	—	59.6 (0.102)	30.8 (0.042)	—	—	—

The observations recorded show that, of all organs the adult liver contains the highest proportion and absolute amount of copper, that the copper content of the diet influences the amount of copper in the adult liver, but not to a marked degree, and that the livers of newly born animals are provided with a large store of copper. This storage is possibly to provide the young animal with a supply of copper over the suckling period when only insignificant amounts of this element would be available in the diet of milk.

II. COPPER AND IRON METABOLISM.

Copper and haemoglobin building.

As has been mentioned, one of the chief interests of copper arises from the claim by Hart *et al.* [1928] that, in the presence of this element inorganic iron may be used by rats for haemoglobin building.

Bunge [1902] stated that only "food" iron, *i.e.* iron in firm combination with organic compounds, as in haematogen, could be used by animals for haemoglobin building, and that the iron of iron salts or "inorganic" iron could not be used for this purpose. Even at the present day opinion is divided on this point and experimental evidence has been conflicting. However, the value of iron therapy in all types of human anaemias is well known and the success of this practice in curing Bush Sickness in New Zealand [Aston, 1921] and anaemia in young pigs in Scotland [McGowan, 1924] renewed interest in iron metabolism and particularly in haemoglobin production.

Waddell *et al.* [1928] have shown that newly weaned rats became anaemic in 4–6 weeks when their diets were restricted to cow's whole milk, and that the anaemia was not cured by supplementary feeding of purified "inorganic" iron. When copper was fed along with the "inorganic" iron, however, a rapid cure was effected. McHargue, Healy and Hill [1928], McCollum and Becker [1930],

Weichselbaum [1930] and Krauss [1930] have confirmed this effect of copper, but, on the other hand, Beard and Myers [1929] and Keil and Nelson [1931] found that purified "inorganic" iron alone would slowly cure the anaemia produced in rats by milk feeding. It has further been claimed by Titus and Hughes [1929] that manganese is as effective as copper in bringing about a cure of such an anaemia, and Beard and Myers [1929] also found that nickel, cobalt and germanium were as good supplements as copper. Mitchell and Miller [1929] obtained a maximal regeneration of haemoglobin when the ash of spinach was fed with a milk-iron diet and, since spinach ash contains many "trace" elements, these authors postulated that a complex of "trace" elements must be present in the diet along with iron for optimal utilisation of the last named element in haemoglobin building. Nevertheless, Weichselbaum [1930] obtained no haemoglobin regeneration when iron *plus* manganese or iron *plus* cobalt was fed to rats, and Krauss [1931] found no better effect with iron *plus* copper *plus* manganese than with iron *plus* copper. Again Waddell, Steenbock and Hart [1929] reported no effect with the twelve "trace" elements zinc, chromium, germanium, nickel, cobalt, lead, antimony, cadmium, tin, mercury, arsenic and manganese, fed singly or all together as supplements to a milk-iron diet.

All the work cited above has been concerned with the supply to, and utilisation of iron by, rats fed a basal diet of milk. Drabkin and Waggoner [1930] studied the effect of protein on haemoglobin regeneration. They found that rats made anaemic with milk diets were brought to normal with great rapidity when the diet was changed to a solid one containing iron but practically devoid of copper. Drabkin and Miller [1931] observed relief from anaemia, when arginine or glutamic acid was used as a supplement to the milk diet, while no success was recorded from feeding histidine. These authors believed that the anaemia produced by milk feeding was not due to a deficiency of iron but to deficiency of the pyrrole nuclei necessary in formation of the porphyrin moiety of haemoglobin.

From this brief survey it is seen that, while the effect of copper in promoting the utilisation of iron in haemoglobin regeneration has received considerable support, it has not been thoroughly established that copper is essential. A study has therefore been made of haemoglobin building from "inorganic" iron, alone or with various supplements.

Rats were used throughout the investigations and the method first employed, due to Waddell *et al.* [1928] was as follows. Anaemia was produced by feeding newly-weaned animals a diet consisting solely of cow's whole milk, and supplements were then fed individually to the rats and their effects measured by response in haemoglobin content of the blood.

Haemoglobin was determined with the Haldane haemoglobinometer, and on this scale the normal haemoglobin content of rat's blood is 80–90 %. Blood samples were obtained by puncturing a coccygeal artery or vein on the ventral aspect of the tail. The advantage of this procedure over the usual one of

snipping a small piece from the end of the tail is that the flow of blood can be regulated according to the condition of the animal by making a puncture near the base or near the tip of the tail.

The conditions of experimentation demanded rigid exclusion of all possible sources of metallic contamination, and special cages were therefore constructed from glass battery jars 13" $\times$ 12" $\times$ 9" high. A false bottom of perforated celluloid allowed drainage of urine, whilst sufficient ventilation was provided by perforating the celluloid plate which closed the front of the cage. The milk used in feeding was drawn directly from the cow into an earthenware jar and the rats were fed from glass vessels.

It was found that deaths occurred very early when newly weaned rats were fed on the diet of cow's milk. In a number of experiments, 130 rats were used and of these 64 died after 2-5 weeks' milk feeding, the average time of survival being 3.2 weeks. After about 5 weeks' feeding the blood of the remaining animals contained low amounts of haemoglobin and these were given supplements. The iron used was purified by treatment in acid solution with H_2S , followed by several precipitations from acid solution by ammonia. For feeding purposes it was nearly neutralised with pure NaOH and for injection purposes the osmotic pressure was adjusted with pure NaCl . The response in haemoglobin content of the blood is shown in Fig. 1.

Fig. 1. Haemoglobin curves of rats fed milk diets from 0 weeks with various supplements from ↑.

The effect of copper feeding is at once obvious, for the rate of haemoglobin regeneration in rats fed this element is much more rapid than that observed when any other supplements were used. Curve *F* is of special interest. The animals to which it refers had previously been cured of anaemia by prolonged feeding of iron supplements and it was to be expected that considerable

quantities of iron would be stored in the liver. This was shown by comparing the iron contents of the livers of rats treated similarly up to the point marked ↑ on the curve, with those of rats fed milk alone. The following average figures were obtained.

	Mg. Fe in whole liver	% Fe in dry matter
Rats fed milk <i>plus</i> iron (long period)	2.493	0.158
Rats fed milk alone	0.917	0.078

The rats became anaemic when iron supplements were stopped notwithstanding this store of iron. When copper alone was fed, a rapid regeneration of haemoglobin followed, and it is therefore probable that the copper feeding caused the utilisation of the stored iron. After some time the animals were killed, and the iron content of the livers was determined. An average figure of 1.183 mg. Fe per liver or 0.127 % was found. The reduced quantity of iron supports the suggestion that copper feeding caused utilisation of the stored iron.

In view of these results it would appear that:

1. Newly weaned rats when fed a diet consisting solely of cow's whole milk developed a condition of anaemia very rapidly.
2. While a large proportion of the rats died if the diet was continued, slow recovery occurred in a small number of cases.
3. Additions of inorganic iron and copper to the diet prevented death and markedly hastened the recovery from anaemia.
4. Injection of iron, protoporphyrin or haematoporphyrin prevented death but had very little effect on the time of recovery.
5. Supplementary feeding of iron, manganese, cobalt or haematoporphyrin prevented death but had very little effect on the time of recovery.

These conclusions must, however, be considered in the light of certain complicating factors. Thus, the death rate was very high and, to investigate this further, 34 rats were fed a diet of cow's milk, and haemoglobin estimations made weekly from the commencement of feeding, for a period of 27 weeks. The results obtained may be arranged in three groups.

(a) Those rats which died soon after the milk diet was commenced and the haemoglobin of which was low. 8 deaths of this type occurred.

(b) Those rats which died after a longer period of milk feeding, and in which no marked lowering of haemoglobin occurred. 14 deaths of this type occurred.

(c) 12 rats which lived throughout the experiment exhibiting no dangerous lowering of haemoglobin.

In Fig. 2 are plotted haemoglobin curves: rat 628 typical of (a), rat 624 typical of (b) and the average of the 12 rats in (c).

The results indicate that there is some doubt as to whether a true nutritional anaemia was developed in rats fed on a diet of cow's milk. On the one hand the long period of survival of the 12 rats in (c) and, on the other hand, the large percentage of early deaths both in (a) and in earlier experiments suggest the possibility that some type of epidemic caused these deaths rather

than that they were due to an uncomplicated condition of iron starvation. Pathological examination of some of the anaemic rats from the earlier experiments was kindly made by Dr J. P. McGowan. The blood contained low

Fig. 2.

percentages of haemoglobin, but the bone marrow was hyperplastic, and marked reticulocytosis occurred in all cases. These conditions do not correspond with those of aplastic anaemias such as occur in iron deficiency and rather suggest an anaemia having as its characteristic feature a hyperplastic condition of the bone marrow. My attention was then drawn by Dr McGowan to the prevalence in rats of infections with Gärtner's bacillus and *Bartonella muris*.

Many of the animals which died in an acutely anaemic condition had *post mortem* symptoms corresponding to those in cases of death from *B. Gärtner* [Boycott, 1911] and this organism was isolated¹ from the intestine of one of the animals.

Infection by *Bartonella muris* is thought to be latent in most rats, though the organism does not usually develop until the spleen is extirpated [Mayer, Borchardt and Kikuth, 1927]. Acute anaemia then results in a large proportion of the animals. Intact rats fed diets deficient in vitamins A and C may develop the disease [Wells and Mehta, 1930], and Meyer [1929] has shown that rats 3–4 weeks old are at the most susceptible stage and may be infected even when the diet is complete. He also found that the presence of *B. Gärtner* sensitised some animals in a manner similar to splenectomy.

Newly-weaned rats (3–4 weeks old), restricted to a diet of cow's milk, consume only about 30 cc. daily, and thus receive only about 4 g. solid material per day compared with 10–15 g. consumed by rats on solid diets. The inadequate caloric value of the food coupled with the susceptible age of the rats [Meyer, 1929] give a set of conditions favourable to development of *Bartonella muris*.

It is likely, then, that the anaemia developed by milk feeding was partly due to *Bartonella* infection and such a possibility must be considered in other work, where milk diets have been employed for the production of anaemia. While it has been clearly demonstrated that copper feeding caused a more rapid regeneration of haemoglobin than did the feeding of iron alone, it was

¹ By kindness of Bacteriology Dept., Edinburgh University.

important to find if copper could exert any effect on haemoglobin production when the possibility of infection by *Bartonella muris* or *B. gärtner* was excluded.

A solid diet low in iron and copper was required, but the difficulty of compounding such a diet is illustrated by the results of analyses of foodstuffs (Table XII) likely to be free from these elements.

Table XII. *Iron and copper contents of some foodstuffs.*

Copper reported as mg. per kg. sample as purchased.			Iron reported as mg. per 100 g. sample as purchased.		
Material	Copper	Iron	Material	Copper	Iron
Maize starch	0.8-1.9	3.18	"Casein" B.D.H. fat and vit.-free	3.9	—
Rice starch	0.8	2.02	Cheese (Scottish)	2.0	—
Potato starch	1.8	1.10	Cheese (Kraft)	0.45	1.23
Wheaten starch	2.4	0.7	Gelatin	9.6	—
Dextrin	2.2	—	Egg-albumin B.D.H.	1.4	1.00
Sago	Traces (0.1)	0.52	Egg-albumin (Chinese)	1.0	0.72
Castor sugar (sucrose)	Traces (0.1)	0.13	Butter (Scottish)	0.6	0.14
Arrowroot biscuits	1.5	1.03	Milk (Rowett Institute)	—	0.065

mg./100 cc.

By a selection from these materials it was, however, possible to devise a complete diet sufficiently low in iron and copper for use in an attempt to produce anaemia in growing rats.

Three groups of 6 rats were used in an experiment and the basal diet was as follows:

Weight for 6 rats daily	
Kraft cheese	3.0 g.
Egg-albumin	9.5 g.
Butter	9.5 g.
Sago	16.0 g.
Castor sugar	16.0 g.
Salt mixture	2.5 g.
Milk	60 cc.
Radiostol	1 drop

The salt mixture was McCollum's No. 185 modified by exclusion of ferric citrate and made up commercially from A.R. chemicals. Slight contamination had apparently occurred in commercial mixing since the salt mixture contained 2.2 mg. Fe per 100 g. Two further salt mixtures were prepared in the laboratory by the addition of purified "inorganic" iron to one and purified iron and copper sulphate to the other. These salt mixtures were used in the diets of two further groups of rats. The following amounts of iron and copper were supplied to the rats of the different groups.

Group	Intake per rat daily	
	Mg. iron	Mg. copper
I	0.058	0.006
II	0.35	0.006
III	0.35	0.046

The experiment lasted 18 weeks, haemoglobin determinations and reticulocyte counts being made at weekly intervals. The average haemoglobin values for the different groups are plotted in Fig. 3, while in all cases the percentage of reticulocytes was within the normal value of 2 % for the rat [Krumbhaar,

1930]. This normal value is in distinct contrast to the marked reticulocytosis of rats made anaemic by milk feeding and it is therefore unlikely that there was any complication from the infections referred to earlier.

Fig. 3.

The results (Fig. 3) show that rats receiving no additions of iron or copper developed a mild anaemia which tended to disappear towards the end of the experiment. Supplementary feeding of inorganic iron had no effect in preventing the onset of this anaemia, but when copper was fed as well as iron the haemoglobin content remained normal throughout the entire period of the experiment.

Consideration of the weight increases shows that the effect of the copper is even more marked than is evident from the haemoglobin curves, for average growth in Group III was 76 g. while that in Group II was 57 g. and in Group I 55 g.

The additional copper, therefore, brought about the utilisation of iron for haemoglobin building, and inorganic iron was not used for this purpose unless copper also was available.

Other relations between copper and iron metabolism.

In the first experiment in this connection, 4 rats which had been fed for 3 weeks after weaning on a diet of starch and cow's whole milk were housed individually in metabolism cages. Faeces and urine were collected separately and 5 days' excretion from each rat was made into one sample and analysed for copper. After two initial periods on a basal diet low in iron and copper, inorganic iron was fed daily to each rat. The effect of this iron feeding on copper excretion was determined.

To avoid metallic contamination, metabolism cages were constructed from 8-inch Büchner funnels. The animals were kept on the perforated plate and, to provide sufficient height, the sides were lengthened by perforated sheet zinc

wrapped round the outside where it was out of contact with the rat or with excreta. A glass plate covered the top of the cage. Faeces remained on the perforated plate of the Büchner funnel and were removed daily, urine passed through and was collected in a beaker placed beneath. Cages were washed out at end of each five-day period. The analytical results are given in Table XIII.

Table XIII.

Average of 4 rats.

Date	Diet	Mg. Cu in		Cu in faeces Cu in urine
		Faeces	Urine	
10-15. ii. 30}	Basal	0.028	0.007	4.0
16-20. ii. 30}		0.021	0.010	2.1
21-25. ii. 30	Basal + 0.5 mg. Fe per rat daily	0.018	0.004	4.5
26. ii. 30-2. iii. 30 }		0.017	0.007	2.4
3-7. iii. 30 }		0.018	0.007	2.6
8-12. iii. 30 }		0.017	0.006	2.8
13-17. iii. 30 }	Basal + 2.0 mg. Fe per rat daily	0.016	0.007	2.3
18-22. iii. 30 }		0.016	0.006	2.6
23-27. iii. 30 }	Basal	0.018	0.006	3.0
29. iii. 30-1. iv. 30 }		0.017	0.006	2.8

Before being used for experiment the rats were kept for three weeks from weaning time on a diet low in copper. This precaution was adopted so that stored copper obtained from sharing the mother's food would be eliminated. The results for the first period are probably affected by incomplete preparation of the rats. Omitting the first period from consideration it can be concluded that feeding inorganic iron did not alter the total amount of copper excreted or the proportion appearing in faeces and urine.

The average ratio $\frac{\text{copper in faeces}}{\text{copper in urine}}$ is 2.8 for a diet low in copper.

In another experiment two groups of rats were housed in glass cages similar to those already described and fed a basal ration of cow's whole milk *ad lib.* plus 3 g. sucrose per rat daily. One group was fed in addition 0.2 mg. purified inorganic iron per rat daily. At the end of 6 weeks, the animals were killed and copper determined in liver, heart, lungs, spleen, kidneys, brain, flesh, skin, tongue and testes. The data for this experiment are averaged in Table XIV.

Table XIV.

	Basal diet 11 rats	Basal diet + iron 10 rats
Average gain in weight during experiment	36 g.	37 g.
Total copper in parts analysed (calculated for one rat)	0.037 mg.	0.038 mg.

No significant differences between animals of the two groups were found in any of the organs or parts analysed. The results therefore indicate that addition of inorganic iron to a diet of milk *plus* sucrose has no effect on the copper content of the rat.

An experiment was designed to study the effect of feeding a diet almost free from copper on storage of iron in the foetus. Work which is discussed below (see Table XIX) showed that the iron content of rat pups from mothers

receiving a low ration of copper was slightly, though not significantly, less than that of pups from mothers receiving a normal ration of copper. That complete removal of copper from the diet cannot be effected by selection of foodstuffs is illustrated in Table XII. In the case of the rat, storage in the liver takes place after continued ingestion of even small traces of copper (Table IX), and between pregnancies the adult maternal animal is probably able to store sufficient copper to supply the needs of the foetal and suckling young. On the other hand, the hen produces an egg at regular short intervals and is therefore constantly depleting iron and copper reserves. It was decided, therefore, to use the hen for this experiment.

Four groups of 5 birds were used, the feeding being as follows:

Group I	Basal ration + iron	Capons
II	Basal ration + iron	Pullets
III	Basal ration + iron + copper	Capons
IV	Basal ration + iron + copper	Pullets

The basal ration was as follows, the daily amounts for 5 hens being given:

Kraft cheese	$\frac{1}{4}$ lb.
Purified maize starch	$\frac{1}{4}$ lb.
Castor sugar	$\frac{1}{4}$ lb.
Salt mixture	10 g.
Radiostol	5 drops

thoroughly mixed and fed without further treatment.

Also

Whole milk	1 pint
Granite grit	<i>ad lib.</i> (purest form of grit available)
Glass-distilled water	<i>ad lib.</i>
Granulated pure CaCO_3	<i>ad lib.</i>

Precipitated CaCO_3 was granulated by mixing with paste prepared from starch and drying at 100° .

Salt mixture I	Groups I and II
	McCollum's salt mixture 185 21 parts
	Pure CaCO_3 9 parts
	Purified iron 0.03 part

Salt mixture II	Groups III and IV
	0.003 part copper (as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) were added.

In a preliminary experiment the diet for Groups I and II was shown to maintain weight and laying in a group of 5 hens over a period of 1 month.

The birds were confined inside on wooden floors covered with sawdust. Food was placed in wooden troughs and liquids in glass vessels. Trap nests were provided to obtain individual laying records. Feeding was started about one month before laying was expected to begin in order that first formation of eggs in the ovary should take place while the experimental diet was being consumed.

In all except two cases the experiment had been in progress for about 3 months before laying commenced.

Since reproduction makes a strong call on all reserves of the mother it was thought that the iron store in the liver might be depleted to fulfil the iron requirements of the egg. Two groups of capons were therefore included to act as controls for iron content of liver at the end of the experiment. These capons were procured from the same hatching of chickens as the pullets. The use of capons as non-laying controls for hens receives some justification from the facts of sex reversion in the fowl.

Results. Growth was similar in all groups and was rather slower than for normally fed hens. No significant differences in haemoglobin content of the blood occurred between any of the groups. When the experiment was concluded, 71 eggs had been laid by Group II hens and 69 by Group IV hens.

Iron estimations were made on the contents of all the eggs, composite samples being made from each hen with 2-4 eggs per sample. No significant differences were recorded and the averages of all eggs from each group are therefore given. Average analytical figures for twelve eggs from hens hatched at the same time as the experimental ones but fed a practical ration are included for comparison. Copper analyses are also included. These results appear in Table XV. In Table XVI are given the group averages of the results of analyses for iron and copper in the livers of all the birds at the end of the experiment.

Table XV. *Iron and copper content of eggs (averages).*

	% dry matter	Mg. Fe per egg	Mg. Cu per egg	% Fe on dry matter	Mg. Cu/kg. dry matter
Group II. Low copper	25.03	0.897	0.021	0.0087	2.0
Group IV. Fed copper	25.06	0.940	0.021	0.0085	1.7
Normal stock feeding	25.28	0.990	0.030	0.0090	2.2

Table XVI. *Iron and copper content of livers (averages).*

	Mg. Fe per liver	Mg. Cu per liver	% Fe on dry matter	Mg. Cu/kg. dry matter
Group I	18.64	0.061	0.206	6.3
	Range 7.00-38.50			
Group II	11.60	0.057	0.110	5.7
	Range 8.50-16.58			
Group III	9.87	0.085	0.088	7.5
fed copper	Range 5.40-12.83			
Group IV	14.05	0.057	0.118	5.0
fed copper	Range 9.40-23.75			

No significant differences were found between the iron and copper contents of eggs from Group II hens (each receiving about 0.07 mg. copper per day) and Group IV hens (each receiving 0.27 mg. copper per day). The amounts of iron in the livers of capons and of hens fed the experimental ration varied too much for any conclusions to be drawn.

Further experiments were conducted with rats to determine the effect of

copper feeding on the iron content of the livers and bodies of rats. The basal diet was cow's whole milk *ad lib.* plus 3 g. sucrose per rat daily, and the groups were treated as follows:

- Group I Killed at weaning and analysed.
- II Fed basal ration from weaning.
- III Fed basal ration from weaning plus 0.2 mg. iron/rat daily.
- IV Fed basal ration from weaning plus 0.2 mg. iron plus 0.05 mg. copper/rat daily.
- V Fed basal ration from weaning plus 0.05 mg. copper per rat daily.

Feeding was continued for one month, after which the rats were killed and the iron determined in the liver, spleen, and rest of the rat (intestinal tract excluded). These conditions apply to the first two experiments; in the third experiment Group I was omitted and, in analysis, the spleen was included with the rest of the rat.

The average analytical results for each experiment are given in Table XVII.

Table XVII.

	Killed at weaning Group I		Basal diet Group II		Basal diet + Fe Group III		Basal + Fe + Cu Group IV		Basal + Cu Group V	
	mg. Fe in organ	% Fe on dry matter	mg. Fe in organ	% Fe on dry matter	mg. Fe in organ	% Fe on dry matter	mg. Fe in organ	% Fe on dry matter	mg. Fe in organ	% Fe on dry matter
Exp. 1.	4 rats		2 rats		3 rats		2 rats		2 rats	
Liver	0.308	0.038	0.498	0.047	1.149	0.115	0.764	0.069	0.351	0.032
Spleen	0.066	0.090	0.119	0.090	0.251	0.166	0.272	0.195	0.185	0.109
Rest of rat	1.10	0.012	1.31	0.011	2.24	0.014	2.49	0.015	2.02	0.011
Exp. 2	4 rats		3 rats		4 rats		2 rats		3 rats	
Liver	0.249	0.053	0.302	0.037	0.716	0.079	0.845	0.086	0.320	0.038
Spleen	0.024	0.101	0.092	0.056	0.117	0.108	0.157	0.110	0.100	0.073
Rest of rat	0.89	0.015	1.01	0.007	1.65	0.010	1.79	0.017	1.30	0.011
Exp. 3			7 rats		4 rats		5 rats		3 rats	
Liver	—	—	0.615	0.056	0.663	0.046	0.650	0.055	0.597	0.043
Rest of rat	—	—	1.46	0.012	1.91	0.011	2.05	0.015	1.81	0.009

The results can be discussed from two aspects.

(a) *Effect of iron feeding.*

In Group II rats, which were fed a ration low in iron, the liver, spleen and body contain little more iron than do these organs from similar rats of Group I killed before commencement of feeding. Inclusion of iron in the ration of Group III has much increased the iron content, particularly in the case of liver. There is therefore some evidence to show that inorganic iron is stored in rats fed this element for a period of one month.

These remarks do not apply to the results of the third experiment, which provides no evidence of iron storage.

This disagreement regarding iron storage cannot be explained. A period of one month, however, is short and comparison of the iron content of liver from a

rat (single result) injected with 0.5 mg. iron daily for 6 months, with the content of liver from a normally fed stock rat illustrates the effect of continued dosage.

	Mg. Fe in liver	% Fe on dry matter
Rat injected	9.5	0.441
Rat normal	1.0	0.049

(b) *Effect of copper feeding.*

Comparison is made between Groups II and V and between Groups III and IV. In each case the only difference in the ration is the presence of copper. There is no evidence to show that copper increases the absorption or storage of iron. On the other hand, the results, while not perfectly clear cut, provide a balance of evidence to show that inclusion of copper in the diet has the effect of lowering the iron content of the liver and increasing that of the body. A possible interpretation of this is that copper promotes the utilisation of inorganic iron after it has been stored in the liver, and such an interpretation would agree with the results shown in Fig. 1, *F*.

The results just discussed together with those given in Fig. 1, *F*, showed that copper feeding probably caused utilisation of stored inorganic iron for haemoglobin building, and it was of interest to investigate this effect further. A method [Hill, 1930] became available for the determination in biological materials of "inorganic" iron as distinct from "organic" iron and some work was conducted to test the effect of copper feeding on the proportion of inorganic iron in the liver (Table XVIII). Hill has shown that $\alpha\alpha'$ -dipyridyl, the reagent used in this method, does not react with reduced haematin, and the writer failed to obtain a test for iron with this reagent and the washed corpuscles of cat's blood. There are then grounds for the claim that the method will differentiate between "inorganic" and "organic" iron.

Table XVIII. "*Inorganic*" and "*organic*" iron in livers of rats.
(Average values.)

No. of rats, adults	Diet	"Inorganic" Fe		Total Fe		% Fe in "inorganic" state	% Fe in "organic" state (by difference)
		mg. Fe per liver	% on fresh matter	mg. Fe per liver	% on fresh matter		
10	Milk only	0.666	0.011	1.372	0.021	44.5	55.5
3	Milk + iron + copper	0.501	0.010	0.819	0.017	63.9	36.1
7	Milk + iron	0.650	0.011	1.403	0.023	45.2	54.8
2	Milk + manganese	0.222	0.004	0.476	0.008	46.6	53.4
2	Milk + cobalt	0.443	0.011	0.716	0.017	61.8	38.2
6	Milk + copper	0.204	0.004	0.614	0.012	28.6	71.4
3	Copper fed for 1 month						
	Milk + copper	0.054	0.0008	0.361	0.005	15.2	84.8
Newly born rats	Mothers' diet						
8	Normal copper content	0.067	0.019	0.116	0.033	57.3	42.7
22	100 times normal copper content	0.034	0.013	0.077	0.030	42.0	58.0

These results indicate that the highest percentage of "organic" iron in terms of total iron was obtained in the cases where copper alone was the supplement to a milk diet: this percentage was increased by prolonged feeding

and was accompanied by a definite diminution in the total iron content of the liver. Where "inorganic" iron was added as a supplement along with the copper, there was no increase in the proportion of "organic" to total iron, probably owing to a continual addition to the stores of "inorganic" iron in the liver. Further it was observed that a high proportion of copper in the mothers' diets had a slight tendency to increase the proportion of "organic" iron in the livers of newly born rats.

No similar effects were observed when cobalt or manganese were fed as supplements without iron.

The mechanism of the action of copper in promoting haemoglobin building.

The effect of copper in promoting the utilisation of inorganic iron in haemoglobin building has been confirmed and it is of importance to consider the mechanism of this action.

The chief activity of copper is probably located in the liver, since, at all stages of growth of the animal, this organ is by far the richest in that element, and since, also, it has been shown that copper feeding causes the utilisation of iron stored in the liver for haemoglobin production (Fig. 1, *F*, and Table XVII).

Further, it has been shown, in the case of rats, that copper feeding definitely increases the proportion of "organic" to total iron in the liver, and this change from "inorganic" to "organic" iron is probably a step in haemoglobin building dependent on the presence of copper. Any modification of the state of the iron would be expected to be in the direction towards the condition of iron in haemoglobin, and it is therefore possible that copper may promote the change from inorganic iron to an iron porphyrin. Elvehjem [1931] found that the addition of copper to the nutrient medium of baker's yeast caused the formation of cytochrome α from inorganic iron, and if a similar process goes on in the rat some indication would be given that haemoglobin is derived from a haemochromogen.

It is of interest to enquire into the process whereby copper may bring about the formation of an iron porphyrin, though the suggestion to be made is, of course, entirely speculative.

(a) Turacin, the pigment of certain South African birds, is a naturally occurring copper porphyrin, and by chemical manipulation the copper can be displaced by iron giving a compound practically indistinguishable from haematin [Laidlaw, 1904].

(b) It was observed by Warburg and Krebs [1927] that after bleeding, *i.e.* when blood regeneration is rapid, the percentage of loosely bound copper in blood-serum is increased, while the percentage of loosely bound iron is decreased.

It is possible therefore, that the effect of copper in increasing the proportion of "organic" iron is achieved by a preliminary formation of a copper porphyrin and subsequent replacement of copper by iron. This would fit in with the observations outlined above and with the general chemical nature of the elements. The fact that injection of haematoporphyrin or protoporphyrin did

not increase blood regeneration (Fig. 1, *I*) indicates that the formation of the porphyrin unit itself is not the function of copper.

III. TOXICITY OF COPPER TO ANIMALS.

It is now fairly generally agreed by writers on toxicology that acute poisoning by copper, resulting in death, is rare, since toxic doses are so large that they are immediately rejected by the stomach.

The position in respect to chronic copper poisoning is not so clear and whilst Pecholier and St Pierre [1856] stated that slow absorption of copper was advantageous, Mallory [1925] has expressed the view that continued exposure to copper, such as is experienced by workers in copper foundries, will cause haematochromatosis. In support of Mallory, Funk and St Clair [1930] have reported a case of haematochromatosis in man where the proportion of copper in the liver was extremely high.

Feeding experiments with animals have been conducted chiefly with rabbits, the procedure being to feed copper acetate for a period and to observe the extent of pigmentation in the livers. No detailed observations regarding growth, fecundity *etc.* of animals receiving an abnormally high ration of copper have yet appeared, and experiments on this aspect have therefore been conducted with rats.

The experiments included, in all, six groups—the same basal ration being used throughout but with differing amounts of copper salts included in the salt mixture for Groups II–VI.

Details of the groups were as follows:

Group I. 14 newly weaned rats, 9 females and 5 males. Basal ration 0.011 mg. copper per rat daily.

Group II. 14 newly weaned rats, 9 females and 5 males. Copper sulphate added to give 0.050 mg. copper per rat daily.

Group III. 8 newly weaned rats, 5 females and 3 males. Copper sulphate added to give 7.5 mg. copper per rat daily.

Group IV. 5 adults, 3 females, 2 males. Copper sulphate added to give 7.5 mg. copper per rat daily.

Group V. 5 adults, 3 females, 2 males. Copper acetate added to give 7.5 mg. copper per rat daily.

Group VI. 5 adults, 3 females, 2 males. Basic copper acetate (verdigris) added to give 7.5 mg. copper per rat daily.

The basal ration low in copper was as follows:

Kraft cheese	...	...	...	45 g.
Butter	...	...	...	7.5 g.
Maize starch	...	...	...	65 g.
Castor sugar	...	...	...	35 g.
Salt mixture I	...	...	...	5 g.
Radiostol	...	...	...	3 drops
Milk	...	...	...	150 cc.

This weight of food was sufficient for fifteen rats.

Fig. 4. Average gain in weight of rats.

Fig. 5. Average weight of rats.

The rats were weighed weekly. For Groups I–III the average gains for one rat at weekly intervals are plotted in Fig. 4, although the growth curve for Group III is not strictly comparable with curves for Groups I and II since the former group was started at a different time of the year. In Fig. 5 are plotted the average weights per rat for each of groups IV–VI.

Haemoglobin determinations were made at irregular intervals on all rats and normal values were found without exception.

Table XIX contains the reproduction records of rats from each group. Some of the rat pups were killed at birth and analysed for copper and iron; the results are also included. The remaining young were reared successfully in all cases.

Table XIX.

Group ...	I	II	III	IV	V	VI	Stock diet; 1 litter for comparison.
Copper in diet per rat daily:	0.011 mg. CuSO ₄	0.050 mg. CuSO ₄	7.5 mg. CuSO ₄	7.5 mg. CuSO ₄	7.5 mg. Cu acetate	7.5 mg. verdigris	About 0.05 mg. Cu
No. of females in group	8	8	5	3	3	3	—
No. of litters	8	9	4	3	3	Nil	—
No. of pups per litter (av.)	6.9	7.9	6.3	8.0	6.7	—	8
Av. wt. of one pup at birth (g.)	4.8	6.6	6.0	5.7	4.8	—	5.0
Total number analysed	24	21	Nil	13	7	—	8
Mg. Fe/rat pup (av.)	0.268	0.312	—	0.352	0.316	—	0.275
Mg. Cu/rat pup (av.)	0.0045	0.0073	—	0.0145	0.0147	—	0.0046
% Fe on dry matter (av.)	0.0374	0.0320	—	0.0412	0.0467	—	0.0354
Mg. Cu/kg. dry matter (av.)	6.3	8.0	—	16.2	20.9	—	5.9

The following conclusions may be drawn from this experiment.

(1) The first generation of rats thrive on a diet providing as little as 0.011 mg. copper to each rat daily. In comparison with rats receiving a normal amount of 0.05 mg. copper daily the low-copper rats grew at a slightly slower rate, the number of pups produced in one litter was slightly less and the average weight of individual rat pups was somewhat smaller. Differences were not marked and sufficient data are not available to decide whether these are significant. At the same time, since the ration proved to be a good one, the low-copper rats grew rather faster than is normally the case for the stock colony, and their haemoglobin was maintained at a normal level.

(2) Analyses of the young produced by these two groups of rats showed on the average no marked difference in iron content but the copper content was significantly lower in those pups whose mothers were fed a low ration of copper. It will be seen that there is a further increase in the copper content of young rats from breeding mothers receiving 7.5 mg. copper per day. There are then,

some indications that the copper content of the diet of rats will affect the storage of copper in the foetus. Hart *et al.* [1930] have shown that the copper content of the diet of sows has no effect on the copper transmitted to the young pigs. These differences between the two species perhaps have some bearing on the facts that while copper promotes the utilisation of inorganic iron for haemoglobin building in the rat it has been claimed that in the pig [Hart *et al.*, 1930] it is not necessary for this purpose.

(3) The presence in the diet of over 100 times the normal amount of copper (as copper sulphate) has no detrimental effect on the growth of rats. Normal young may also be produced on such a ration.

(4) The presence in the diet of over 100 times the normal amount of copper as copper acetate or as verdigris was not toxic to adult rats. Again, histological examination of livers after 5 months' feeding did not reveal any deposition of pigment. Breeding was normal in those rats fed copper acetate but no litters were produced in the same time by the animals receiving verdigris.

SUMMARY.

1. Determinations of copper have been made in plants, foodstuffs, insects and marine animals. A systematic survey has also been made of the occurrence of copper in the organs of twelve species of land animals (foetal, newly born and adult).

2. The copper content of the skin and of the livers of rats has been shown to be increased in relatively small degree compared with the additional copper fed in the diet. Feeding additional copper to the mother caused increased storage of copper in the newly born rat.

3. A catalytic effect of copper on the oxidation of "dopa" to melanin has been observed. A suggestion has also been made that the copper in the ink sac of the octopus plays some part in the formation of the ink.

4. Copper feeding has been shown to have little or no effect on the storage of iron in the foetal rat or in the hen's egg.

5. Iron feeding has been shown to have no effect on the storage or excretion of copper by the rat.

6. The use of milk diets for production of a nutritional anaemia in rats has been criticised. At the same time the stimulating effect of copper on the formation of haemoglobin in rats has been confirmed and a possible mechanism suggested for its action.

7. It has been shown that feeding high amounts of copper sulphate or copper acetate has no effect on the growth and reproduction of rats, while high amounts of verdigris were observed to stop reproduction. It has also been shown that rats receiving one-fifth of the normal amount of copper in the diet will grow and reproduce normally.

The work reported above was carried out during a visit of the writer from New Zealand. This visit put into effect a scheme for the interchange of workers

in Agricultural Science, proposed at the Imperial Agricultural Conference in 1927. I desire to express my appreciation of the opportunity thus afforded, of carrying out research work in this country.

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