Studies in iron metabolism

1. The experimental production of iron deficiency in the growing rat

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Experimental iron deficiency has frequently been produced by the giving of semi-synthetic diets with low Fe content to animals whose Fe requirements are high, either because they are growing (cf. Waddell, Steenbock, Elvehjem & Hart, 1928) or because of haemorrhage (cf. Beutler, 1957). Methods of Fe depletion such as induction of blood loss have the disadvantage that substances other than Fe are removed, and it is difficult to know whether they are adequately replaced. The aim of the work described here was to study the effect of a deficiency of Fe, by observing differences between animals fed on a diet containing sufficient Fe to meet their requirements and animals fed on a diet similar in every respect except for a lower Fe content. Dietary deprivation of Fe was chosen as the method most likely to ensure an uncomplicated deficiency, and in this instance was accentuated by the high Fe requirements of the animal in its growth phase. Only a synthetic or semi-synthetic diet was likely to be sufficiently free of Fe for our purpose, and such a diet was planned after a study of the literature about the dietary requirements of the rat (McCall, 1961). Since Fe sufficiency has been claimed with levels of from 50 mg Fe/kg diet (Cuthbertson, 1957) to 600 mg Fe/kg diet (Albritton, 1954), experiments were undertaken to ascertain the quantity of Fe required by the rat, as well as the overall nutritional adequacy of the diet when supplemented with Fe.

EXPERIMENTAL AND RESULTS

Materials and methods

Animals. Weanling albino rats derived from Wistar strain stock were obtained from the War Department, Allington Farm, Porton Down, Salisbury. Each animal was dusted with Lorexane (γ-benzenehexachloride) flea powder (Imperial Chemical Industries Ltd) and marked by ear clipping. Animals were housed in cages constructed of Perspex with aluminium-mesh floors and aluminium-mesh tops to permit free circulation of air. The living space in each cage was $45 \times 35 \times 25$ cm and the maximum number of weanling rats allotted to each was twelve. The cages were cleaned daily and washed thoroughly in dilute Lysol once a week. Glass-distilled

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water for drinking was provided in glass reservoirs, and the food was contained in straight-sided porcelain bowls fitted with aluminium-mesh grids to reduce spillage. The temperature of the animal house was maintained between 16 and 20°. It was noticed that the rats tended to chew the aluminium-mesh floors of the cages from time to time. So far, no injurious effects have been observed from this behaviour; it is assumed that the ingestion of aluminium has not affected the results described.

Animals were weighed on a direct-reading Mettler balance (Type K4); duplicate weighings were within 2 g.

Table 1. *Vitamin, essential amino acid and mineral contents of the basic iron-deficient diet*

Substance	Contribution of supplement to concentration in diet (mg/kg)	Final concentration in diet (mg/kg)	Substance	Contribution of supplement to concentration in diet (mg/kg)	Final concentration in diet (mg/kg)
Vitamin A	8 000*	8 200*	L-lysine	—	16 200
α-Tocopherol	300	300	D- or L-methionine	—	5 700
Thiamine	10	12	L-cystine	—	2 700
Riboflavin	5	14	D- or L-phenylalanine	—	10 400
Pyridoxine	5	7·5	L-threonine	—	9 100
Pantothenic acid	12	15	L-tryptophan	—	2 700
Cyanocobalamin	0·015	0·02	L-valine	—	15 600
Choline	2 000	2 000–3 000	L-histidine	—	7 150
Nicotinic acid	10	15	Sodium	2 360	5 260
Vitamin K (menaphthone sodium bisulphite)	1	1	Iodine	0·56	0·76–1·06
			Manganese	80	80–81
Folic acid	1	1	Copper	20·7	21–23
p-Aminobenzoic acid	10	10	Iron	—	1–3
Biotin	0·2	0·2	Calcium	—	8 400
Inositol	200	200	Phosphorus	—	6 800
Vitamin D	1 000*	1 000*	Potassium	—	7 800
Vitamin C	—	35	Magnesium	—	780
L-arginine	—	7 800	Zinc	—	20–33
L-leucine	—	20 800	Molybdenum	—	0·26–0·32
L-isoleucine	—	14 300	Cobalt	—	0·04

The amino acid content of the diet was calculated from an analysis of the spray-dried skim-milk powder provided by Glaxo Laboratories Ltd, Greenford, Middlesex.

* i.u.

Diet. The diet was prepared by mixing 650 g spray-dried skim milk with 205 g sucrose, 30 g salt mixture, 5 g choline dihydrogen citrate, 99 g fat mixture (lard and arachis oil, 3:1 by weight) 10 g of a mixture of water-soluble vitamins and 1 ml of a mixture of fat-soluble vitamins (prepared by mixing 0·4 g vitamin A palmitate and 0·05 g ergocalciferol with 50 ml of a 35% (w/v) solution of α-tocopherol in refined soya-bean oil). Each kg of the mixture of water-soluble vitamins contained 0·5 g pyridoxine, 1·2 g calcium pantothenate, 0·5 g riboflavin, 1 g thiamine hydrochloride, 1 g nicotinic acid, 0·1 g vitamin K (menaphthone sodium bisulphite), 0·1 g folic acid, 1 g *p*-aminobenzoic acid, 0·02 g biotin, 20 g inositol, 0·0015 g cyanocobalamin and 974·5 g D-glucose. Each kg salt mixture contained 200 g NaCl, 0·022 g $NaIO_3.H_2O$,

10·812 g $MnSO_4.4H_2O$, 2·722 g $CuSO_4.5H_2O$ and 786·5 g D-glucose. The general composition of the diet was 23% protein, 56% carbohydrate, 11% fat, and the calculated calorific value was 4·1 kcal/g. Essential fatty acids provided by the arachis oil and lard were calculated to be 7·5 g and 4·5–10 g/kg diet respectively. The various components of the diet are shown in Table 1. Values for the constituents in the dried milk were obtained from Underwood (1956), McCance & Widdowson (1960) and Geigy (1956).

Hereafter the basic Fe-deficient diet is referred to as diet 1.

Fe-supplemented diets were prepared by adding suitable quantities of $(NH_4)_2SO_4.FeSO_4.6H_2O$ to the salt mixture in place of an equal weight of glucose. The salt mixture used for the final Fe-supplemented diet, diet 2, contained 54·08 g $(NH_4)_2SO_4.FeSO_4.6H_2O$/kg. Diet 2 was thus diet 1 supplemented with 240 mg Fe/kg.

Table 2. *Nitrogen, iron, phosphorus and cobalt contents of semi-synthetic diets 1 and 2*

Substance	Method of analysis	No. of duplicate analyses	Content/kg diet	
			Diet 1	Diet 2
N	Micro-Kjeldahl	3	35·2–40·0 g	35·2–40·0 g
Fe	Thiocyanate	3	1–3 mg	232–280 mg
P	Berenblum & Chain (1938)	3	6·5–7·2 g	6·5–7·2 g
Co	Activation analysis	1	0·04 mg	0·18 mg

Vitamin mixtures were prepared at intervals of 8–10 weeks and sealed in dark-glass bottles at −20°. The fat mixture was stored at 4° for up to 2 weeks and the complete diets were made up at intervals of 7 days and stored in covered Polythene containers at 4°. A ball mill (Pascall Engineering Co. Ltd, Crawley, Sussex) was used to reduce the materials to fine powder form and to facilitate their subsequent mixing. Suitable portions of the various components of the diet were thoroughly mixed in the order: sucrose, choline, salt mixture, water-soluble vitamins, fats and fat-soluble vitamins. The milk powder was added last. Vitamins were obtained from Vitamins Ltd, London, and the dried milk from Glaxo Laboratories Ltd, Greenford, Middlesex. Inorganic salts, sucrose and glucose were of AR grade.

Analyses were undertaken to determine the nitrogen, iron, phosphorus and cobalt contents of the fully mixed diets, and the results are shown in Table 2. It is believed, as a result of later studies (McCall, Newman, O'Brien & Witts, 1962*b*), that the difference between the Co concentration of diets 1 and 2 was not sufficient to have had any haematological effect.

Analysis of diet. The Fe content was determined, after wet ashing with HNO_3 and $HClO_4$, by a thiocyanate method (cf. Sandell, 1959) described in detail in the next paper (McCall *et al.* 1962*a*).

The Co content was determined by neutron activation analysis by Dr D. Gibbons, Isotope Research Division, U.K.A.E.A. Research Group, Wantage Research Laboratory, A.E.R.E., Berks.

The N content was determined by the micro-Kjeldahl procedure.

The P content was determined, after wet ashing with H_2SO_4 and HNO_3, by the method of Berenblum & Chain (1938).

Measurement of haemoglobin. Blood was either taken with a syringe from the dorsal aorta of the ether-anaesthetized rat after slaughter and expelled into a clean dry glass tube containing 2 mg (200 i.u.) heparin (Evans Medical Supplies Ltd) as an anticoagulant for each 10 ml blood, or was obtained from the live rat's tail. The tail was held in warm water for about 15 sec and then wiped clean and dry; a small nick was made into a superficial vein with a sharp scalpel blade, and the blood was drawn directly into a haemoglobin pipette.

Haemoglobin was determined as oxyhaemoglobin. Blood was drawn into a haemoglobin pipette and 0·02 ml washed into 6 ml 0·007 N-NH_4OH. The optical density of the resulting solution was determined in a Hilger H 810 Biochem Absorptiometer with filter no. 55 (peak transmission at 545 mμ). The haemoglobin content of the solution was calculated from a standard curve prepared for the absorptiometer by Dr G. H. Spray (Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford) with a solution of human haemoglobin of known oxygen capacity and Fe content. Although all rat haemoglobin determinations were consequently based on a human haemoglobin standard, it was shown that standard dilutions of rat haemoglobin gave a straight-line relationship of haemoglobin concentration to optical density over the range studied. All haemoglobin pipettes used were calibrated with mercury; those having an error greater than $\pm 2\%$ were discarded. The precision of the method was such that the standard deviation of replicated observations was $\pm 0{\cdot}38$ for haemoglobin values less than 8 g/100 ml blood and $\pm 0{\cdot}05$ for values greater than 15 g/100 ml blood.

Experiments with the rats

Establishment of iron requirement for normal performance. Experiments were undertaken to establish the dietary Fe level required by rats fed on the semi-synthetic diet to maintain a rate of weight gain and haemoglobin concentration equivalent to that of rats receiving a standard mixed diet of rat cake (modified diet 41 B, based on diet 41 of Bruce & Parkes, 1949 and Bruce, 1950) supplied by Oxo Ltd, Thames House, London, E.C. 4. This diet was found to contain 95 mg Fe and 30 g N/kg. Seventy-two rats were divided into six groups of twelve and fed *ad lib.* on the rat cake or the basic semi-synthetic diet supplemented with various quantities of Fe (Table 3). At the beginning of the experiment, the mean weight of the rats was 63 ± 12 g. Results are shown in Table 3. Rats receiving the semi-synthetic diet with Fe supplements of 50 mg/kg diet, or more, gained weight as rapidly as animals fed on the rat cake and maintained a similar haemoglobin concentration throughout the growth phase. Animals receiving less Fe gained weight more slowly and developed anaemia, whose severity depended on the level of Fe in the diet.

Reproduction. A rapid growth rate does not necessarily indicate well-being (McCay, Crowell & Maynard, 1935). A satisfactory diet should be adequate to support the animal at any stage in its life cycle, and the ability of diet 2 to support reproduction was tested. Significant inadequacies in the diet may not be revealed by studying

reproduction through one generation (Evans & Philips, 1939; Greenstein, Birnbaum, Winitz & Otey, 1957), and an experiment was therefore planned to study growth and reproduction through three generations. A group of six male and six female weanling rats from parents fed on the rat cake was transferred to diet 2: this generation is referred to as F. At maturity the males and females were paired, and each pair was placed in a small 'breeding' cage. By this means the potency of each male and the fertility of each female could be tested. The progeny were counted as soon as possible after birth and were weighed at weaning, 21 days later. At maturity the blood haemoglobin concentration of each animal was measured, and one male and one female were arbitrarily selected from each litter to become generation F_1. The process of feeding, breeding and selection of a new generation was continued to produce generations F_2 and F_3. The results are shown in Table 4.

Table 3. *Comparison of the rate of weight gain and haemoglobin concentration in rats fed on iron-supplemented semi-synthetic diets or diet 41B rat cake*

(Mean values with standard deviations)

Diet	Fe content (mg/kg)	No. of rats	Days required for weight increase from 50 to 150 g	Haemoglobin concentration (g/100 ml blood) at body-weight of 150 g
Semi-synthetic	2	12	70 ± 19	7·2 ± 0·6
	18	12	59 ± 14	11·9 ± 1·0
	34	12	47 ± 14	14·6 ± 1·1
	50	12	46 ± 10	16·0 ± 1·0
	240	12	46 ± 11	16·8 ± 0·8
Rat cake	95	12	49 ± 10	16·6 ± 0·9

Table 4. *Adequacy of diet 2 (240 mg iron/kg) to support the growth and reproduction of rats*

Generation	F–F_1	F_1–F_2	F_2–F_3
Mean blood haemoglobin level (g/100 ml) in adult males	16·4	15·7	16·8
Mean blood haemoglobin level (g/100 ml) in adult females	16·3	15·6	16·6
No. of potent males	5/6	6/6	6/6
No. of fertile females	6/6	6/6	6/6
Total no. born in first litters	51	44	41*
Mean no./litter	8·5	7·3	7·0
No. weaned from first litter	44	41	34
Mean no. of weanlings/litter	7·3	6·9	6·9
Mean weight (g) at weaning	32·4	34·0	38·2
Days taken for males to grow from 50 to 150 g live weight	28	27	28
Days taken for females to grow from 50 to 150 g live weight	42	52	50

* One female was disturbed during delivery and ate her young.

In the three generations of animals studied the blood haemoglobin concentration at maturity was similar; both the potency of male animals and the fertility of female animals were high. Although there was a small decline in the number of rats per litter, there was a rise in the mean weight at weaning. There was a decline in the rate of weight gain of female animals, which was most pronounced in the second-generation animals.

Development of, and recovery from, anaemia. The production of anaemia in rats fed on diet 1 and the recovery from this anaemia on diet 2 were tested. Four litters, each of four weanling rats, were fed on diet 1. After 50 days three animals were chosen from each litter. One of these rats was retained on diet 1, and the other two were fed on diet 2. The results are shown in Tables 5 and 6. Anaemia developed progressively in animals confined to diet 1. After 50 days, at the time of transfer to diet 2, haemoglobin values were of the order of 6 g/100 ml blood. Animals remaining on diet 1 became still more anaemic, whereas animals transferred to diet 2 regained the haemoglobin levels associated with Fe sufficiency in 12 days or less (Table 5). At the same time there was a marked increase in the live weight of the rats fed on diet 2, compared with those on diet 1.

Table 5. *Development of anaemia in sixteen weanling rats fed on diet* 1 (2 *mg iron/kg*)

Days on diet ...	0	14	20	28	41	50
Mean haemoglobin level (g/100 ml)	13·7	9·3	7·7	7·0	6·3	5·5
Mean weight (g)	44	74	92	102	110	115

Table 6. *Recovery from anaemia in rats fed on diet* 2 (240 *mg iron/kg*)

Days after beginning of experiment ...	0	2	4	6	8	12
Mean haemoglobin level (g/100 ml) of four rats kept on diet 1	6·0	5·8	6·0	5·6	5·4	5·1
Mean haemoglobin level (g/100 ml) of eight rats changed to diet 2	5·7	8·7	11·7	13·5	14·5	15·4
Mean weight (g) of four rats kept on diet 1	116	117	116	117	121	123
Mean weight (g) of eight rats changed to diet 2	116	112	125	131	132	138

DISCUSSION

These experimental studies have established that growing rats fed on the unsupplemented diet, which was almost free of Fe, developed an anaemia which was quickly responsive to Fe supplementation. They also strongly suggest the overall nutritional adequacy of the semi-synthetic diet when supplemented with 240 mg Fe/kg (diet 2). A supplement of at least 50 mg Fe/kg semi-synthetic diet was necessary for a maximal rate of weight gain and for haemoglobin maintenance during this phase. The Fe supplement was increased to 240 mg/kg (diet 2) in order to meet the higher Fe requirements of pregnancy and lactation (Griffith & Farris, 1949). No evidence of Fe deficiency was noted in the reproduction experiments with diet 2.

The experiments in these and the subsequent three papers (McCall *et al.* 1962*a*, *b*; McCall, Newman & Valberg, 1962) are open to the criticism that the animals on the various treatments were fed as single groups and thus there was no true replication for testing treatment differences. The differences between the groups were nevertheless so large and consistent that there can be little doubt that they were significant and that the conclusions drawn from them are valid.

Though 240 mg Fe/kg is in excess of the 95 mg/kg present in the rat cake, it is less than the 600 mg/kg suggested by Albritton (1954), and is similar to levels used by

Jones & Foster (1942) and by Greenstein *et al.* (1957). Cuthbertson (1957) reported that 50 mg Fe/kg diet was adequate for the complete life cycle of the rat. This figure was based on studies of the Fe level that stimulated a maximal rate of haemoglobin regeneration in animals with established Fe-deficiency anaemia (Waddell, Elvehjem, Steenbock & Hart, 1928). However, Waddell and his co-workers were not attempting to determine the optimal amount of iron in a diet which was otherwise adequate but were comparing the effect of different preparations of iron and other materials on the anaemia produced in rats by a diet of cow's whole milk. The rate of regeneration of haemoglobin in their experiments did not exceed 10 g/100 ml in 10 weeks. Since a similar recovery took only 12 days on our diet 2, it is suggested that the Fe supplement of Waddell *et al.* was inadequate or that there were other constituents lacking from the diet.

The general nutritional adequacy of diet 2 was established to some degree by its ability to support growth, in comparison with a mixed diet such as the rat cake, and also by its ability to support reproduction through three generations. It is not suggested, however, that it represents an optimum diet. Indeed, the relative proportions of metallic constituents may need re-adjustment as further details become available, which may be equally true of the amounts and nature of other components, including the vitamins.

Numerous experiments with diet 1 have confirmed that the characteristic haematological picture of Fe deficiency develops in a standard time, which depends on the initial weight of the animal (McCall *et al.* 1962*a*). In view of results indicating that the difference in the Co levels in the two diets is not sufficient to have haematological effects (McCall *et al.* 1962*b*), it seems justifiable to regard this anaemia as the result of uncomplicated Fe deficiency, because the overall nutritional adequacy of a diet (diet 2) similar in every respect except its Fe content has been established.

SUMMARY

1. A semi-synthetic diet suitable for experimental studies of iron metabolism is described in detail.

2. Weanling rats fed on the basic diet (diet 1), without an Fe supplement, developed an anaemia and gained weight less rapidly than their litter-mates given an Fe supplement.

3. The nutritional adequacy of the Fe-supplemented diet was established. A supplement of at least 50 mg Fe/kg was necessary for maximal rate of weight gain and for haemoglobin maintenance during the growth phase. Growth and reproduction were supported through three generations of rats fed on the diet supplemented with 240 mg Fe/kg. Anaemic animals recovered to an Fe-sufficient blood-haemoglobin level and increased their body-weight within 12 days of transfer to the Fe-supplemented diet (diet 2).

4. It seems justifiable to regard the changes in animals fed on diet 1 as the result of uncomplicated Fe deficiency, since the overall nutritional adequacy of diet 2, which was similar except for its Fe content, has been established.

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