# Studies in iron metabolism

## 2.* The effects of experimental iron deficiency in the growing rat

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(*Received* 11 *October* 1961—*Revised* 6 *February* 1962)

Iron deficiency is recognized clinically as a hypochromic and often microcytic anaemia which responds to Fe therapy. There may be associated epithelial and other tissue changes. Fe deficiency may involve one or several of the haemoproteins and metallo-proteins constituting the active agents in Fe metabolism; the form manifested by the deficiency may be determined by the nature of the compound affected. The quantitative relationship of these compounds one with another is not known exactly, particularly in the presence of Fe deficiency. The recognition of the onset of body Fe deficiency by a study of a single Fe compound such as haemoglobin can only be justified if that compound is the first affected in every circumstance and if it has been established that the change in concentration of the compound is related to the degree of Fe deficiency. This problem has yet to be solved by either clinical or experimental studies.

Two major view-points have emerged over the years. Hahn & Whipple (1936) suggested that in Fe deficiency the concentration of Fe-containing compounds in cells other than erythrocytes is maintained at the expense of the haemoglobin Fe. On the other hand, Waldenström (1938), Beutler (1957), Beutler & Blaisdell (1958) and Beutler, Larsh & Gurney (1960) have described tissue changes responding to Fe therapy and therefore attributable to Fe deficiency, in the absence of anaemia. The definition of body Fe deficiency, and of Fe deficiency in tissues other than the red cells, will remain confused until the quantitative relationships between the various Fe compounds of the body have been established in health and in deficiency. In the work described here, the course of Fe deficiency produced experimentally in the growing rat by dietary means was studied with particular reference to the relation between haematological changes, the concentration of Fe in the blood plasma and carcass and the cytochrome *c* content of the liver and kidneys.

The results described previously by McCall, Newman, O'Brien, Valberg & Witts (1962) indicate that their diet 1 (2 mg Fe/kg), when given to weanling rats, provides a satisfactory means of producing Fe deficiency without the complications introduced by methods of Fe depletion such as haemorrhage. The results also show that weanling rats fed on diet 2 (i.e. diet 1 supplemented with 240 mg Fe/kg) maintain a rate of weight gain and a haematological picture comparing favourably with that of similar

* Paper no. 1: *Brit. J. Nutr.* (1962), **16**, 297.

rats fed on 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. In order to measure some of the effects of Fe deficiency upon blood and tissue compounds, an experiment was planned in which at intervals during 3 months rats fed on diet 1 were compared directly with litter-mates fed on diet 2.

## EXPERIMENTAL

### *Animals*

Rats were housed and weighed as described previously (McCall *et al.* 1962). The body lengths of the animals were determined by the method of Acheson, Macintyre & Oldham (1959). The weights of the internal organs were determined on a direct-reading torsion balance (Model O, White Instrument Co., London); the volume of the caecum was measured by displacement of water in a graduated cylinder.

### *Haematological procedures*

Methods for taking blood and determining the haemoglobin concentration were as described previously (McCall *et al.* 1962). Haematocrit values were determined on a 'macro' scale by transferring blood to a Wintrobe tube with a Pasteur pipette and centrifuging at 1500 ***g*** for 30 min. The level of packed cells was expressed as a percentage of the total volume of blood, and the precision of the method was such that the standard deviation of replicated observations was $\pm 0{\cdot}25$ over the range encountered. For small quantities of blood the haematocrit values were determined with a micro-haematocrit centrifuge (Hawksley and Sons Ltd, London). Values obtained with this procedure were consistently 1–2 % lower than those obtained with Wintrobe tubes.

Red-cell counts, reticulocyte counts and bone-marrow smears were made by standard procedures. The precision of the method used for counting red cells was such that the standard deviation of replicated observations was $\pm 0{\cdot}3$ (cells $\times 10^{-6}/mm^3$ blood) for the range of values estimated.

### *Determination of Fe in tissues, diet and plasma*

Fe was determined as its red thiocyanate complex essentially in accordance with long-established procedures (cf. Sandell, 1959). As the colour intensity is affected to only a small extent by acidity, provided enough acid is present to prevent appreciable hydrolysis of the Fe salt and provided the acid does not form a complex with ferric ions (Sandell, 1959), the method is useful for the analysis of samples in strong acid, for example after wet ashing. In this study, samples of tissues and diets were prepared for analysis by wet ashing, and plasma by treatment with acid, and the Fe contents of the resulting aqueous solutions were determined by measuring the light absorption at 495 m$\mu$ of the ferric-thiocyanate complex extracted into an immiscible solvent of low polarity (Marriott & Wolf, 1905–6), in this instance, 1-pentanol.

Only Pyrex or hard borosilicate glassware was used; when ground-glass stoppers were not available, Polythene stoppers or Parafilm were employed. Precautions were

taken to prevent contamination of reagents and specimens during collection, storage and analysis (Butler & Newman, 1956). Blood was taken with stainless steel needles (Vann Bros. Ltd, London) attached to ungreased all-glass syringes (Chance Bros. Ltd, Birmingham). Needles were rinsed with warm dilute detergent immediately after use, then with metal-free water and were finally dried with pure acetone. They were used three times, then discarded. AR grade chemicals were further purified by distillation or crystallization, and metal-free water was used for making up all solutions. The primary Fe standard was a solution of $FeCl_3$ in 2 N-HCl, containing 0·01 g Fe/ml, prepared from spectrographically standardized $Fe_2O_3$ (Johnson, Matthey and Co. Ltd, London). This solution was compared with one containing 500 $\mu$g Fe/ml, prepared by dissolving $(NH_4)_2SO_4.FeSO_4.6H_2O$ (3·5107 g/l.) in a solution of 0·02 M-$K_2S_2O_8$ in 0·1 N-$H_2SO_4$. Working standards were prepared immediately before use each time by diluting portions of the standards with water. No significant difference between appropriate dilutions of the two standards was observed.

Portions of minced carcass and diet were prepared for analysis by wet ashing with $HNO_3$ and $HClO_4$. Carcasses were stored individually in sealed Polythene bags at $-15°$. While cold, the bag containing the carcass was placed between blocks of wood, and the large bones were crushed by pressure. In this way rupture of the bag and contamination of the carcass were avoided. The carcass was then easily cut into strips with stainless steel shears and the material passed twice through a stainless steel mincer of the shearing type. Keeping the carcass cold during this process caused the mince to retain a 'dry' texture with little loss of material. The mince was covered with Parafilm and allowed to attain room temperature before being thoroughly mixed with a glass spatula, weighed and sampled. A weighed sample of mince (usually about 15 g) was transferred to a beaker of known weight, covered with a watch glass and dried to constant weight in a hot-air oven at 105°. In this way the water content of the material was obtained by weight; it was not thought to be unduly affected by storage and preparation of the tissues for analysis. The dried material was wet ashed in the beaker by heating with 30 ml conc. $HNO_3$ and 5 ml 6·5 N-$HClO_4$. Portions of conc. $HNO_3$ (10 ml) and 8 M-$H_2O_2$ (1 ml) were added from time to time to maintain a volume greater than 10 ml, until the solutions were colourless. Heating was continued until white fumes of $HClO_4$ were given off. The digest was transferred to a volumetric flask. Any deposit was dissolved in 6 N-HCl and the solution, together with six washings of water, was added to the flask. Enough HCl was added to adjust the solution to 0·4 N when diluted to the mark, and portions (usually 5 or 10 ml) were taken for analysis. Reagent blanks and Fe standards were included with each batch analysed. Samples of diet (usually 1–10 g) were wet ashed similarly. Smaller quantities of material were wet ashed in digestion centrifuge tubes used with a micro digestion heater similar to the type described by King (1951). In this way the Fe content could often be determined directly in the same tube.

Plasma samples often contained traces of haemoglobin, and wet ashing them would have yielded spuriously high results. The observation that dissociation of the Fe-binding protein isolated from serum by Schade & Caroline (1946) was complete at pH 4 and that treatment of serum with hydrochloric and trichloroacetic acids did not

liberate iron from haemoglobin (Heilmeyer & Plötner, 1937) has been used by many authors as a basis for methods of determining the Fe content of serum or plasma. In this study, plasma was obtained from whole blood within 60 min of collection, and its Fe content determined the same day. Heparinized blood was centrifuged at 2000 ***g*** for 15 min and the plasma separated with a Pasteur pipette. A measured volume (usually 0·1–2 ml) of plasma was transferred to a centrifuge tube and diluted to 1 or 2 ml with water. A volume of N-HCl equal to that of the plasma and water was added, and the contents of the tube were well mixed. After 5–60 min the mixture was adjusted to 0·6 N with respect to trichloroacetic acid to precipitate the proteins, and after a further 15 min the mixture was centrifuged at 2500 ***g*** for 10 min. A measured portion (usually 2 ml) of the supernatant liquid was taken for analysis. Alternatively the supernatant liquid was removed, the protein residue was washed with 1 ml 0·6 N-trichloroacetic acid, and the supernatant liquids were combined for analysis. Reagent blanks and Fe standards were included with each batch analysed. No interference by haemoglobin in amounts equivalent to 0·5 g/100 ml plasma was observed. Under similar conditions Bothwell & Mallett (1955) obtained good recovery of Fe from plasma labelled with $^{59}Fe$.

The ferric-thiocyanate reaction was carried out in centrifuge tubes of 15 or 50 ml capacity. The final concentration of the solutions for analysis was adjusted to 0·2 N with respect to HCl, to 0·01 M with respect to $K_2S_2O_8$ (to ensure complete oxidation of Fe) and to 0·5 M with respect to KSCN. The colour was extracted with measured quantities of 1-pentanol, separation and clarification of the organic phase being effected by centrifuging at 2000 ***g*** for 5 min. The light absorption of the organic phase was measured against 1-pentanol in glass cells of 1 or 4 cm path length at 495 mμ ($\lambda_{max.}$) in a Unicam SP. 500 spectrophotometer fitted with an SP. 580 micro-cell attachment. Division of the extinction value, corrected for the reagent blank, by the calibration factor obtained from a standard curve gave the amount of Fe present. There was no interference in determining 5 μg Fe by 10 μg Cu and 10 μg Co or by other substances in the relative concentrations found in the organs and blood of the rat, as judged by recovery experiments, or by the presence of up to 0·6 N-trichloroacetic acid. Beer's law was obeyed with 0–5 μg Fe/ml 1-pentanol, no significant change being observed in the extinction for 60 min. The colour intensity depended to some extent on the particular sample of 1-pentanol used, and a quantity of the alcohol (b.p. = 138°) sufficient for the work was therefore distilled. Under these conditions the precision of the method was such that the mean $E^{1\%}_{1\,cm}$ (495 mμ) for Fe was 2250 with a standard deviation of ±36.

The precision of the whole procedure calculated from duplicate analyses on fifteen different samples of plasma (nine rats fed on diet 2, with plasma Fe levels of 144–334 μg/100 ml, and six rats fed on diet 1, with plasma Fe levels of 5–40 μg/100 ml) was such that the standard deviation was ±9. The percentage recovery of 1 μg Fe added/ml plasma was 98·5 ± 5·2 and of 25 μg Fe added/g dry weight carcass, diet 1 or rat cake diet was 97·0 ± 7·8.

### *Extraction of lipids*

Glassware was cleaned with dilute detergent and sulphuric acid–dichromate mixture, rinsed well with water and finally rinsed with ethanol and diethyl ether and air-dried. Extraction thimbles (Whatman) and filter-paper (Whatman no. 1) were washed with ether before use. Solvents were AR grade ethanol and 'anaesthetic' grade diethyl ether.

A weighed sample of carcass mince (about 25 g) was transferred to a wide-necked conical flask, 120 ml of a mixture of ethanol and diethyl ether (3:1, v/v) were added and the contents mixed as quickly as possible to prevent clumping (Entenman, 1957). It was done by stoppering the flask tightly with a cork covered with tinfoil and shaking violently. The flask was placed in a water-bath at 60° for 2 h, the contents were mixed thoroughly from time to time and the top of the flask was covered with a watch glass. While still warm the ethanol–ether phase was decanted through filter-paper into a distilling flask. The residue was extracted a second time in similar fashion with 120 ml ethanol–ether mixture, and the extract was filtered through the same paper into the distilling flask. The residue was then transferred to the filter-paper, ether being used to make the transfer complete, and allowed to drain. When dry, the filter-paper with the residue was transferred to a Soxhlet extraction thimble and extracted with 120 ml ether for 16 h. The volume of the combined extracts was reduced to a minimum by distilling off the ether and ethanol. The aqueous fat residue was extracted eight times by shaking with 50 ml portions of ether, and the ether phase was siphoned off each time into a thin-walled glass tube of known weight, standing in a water-bath at 37°. After the solution of fat in ether had been reduced to a minimum volume at 37°, the tube was heated at 60° for 30 min and then dried to constant weight in a desiccator under reduced pressure, the quantity of fat extracted being determined by weight.

### *Determination of cytochrome* c

The method for extracting cytochrome *c* from tissue and its assay by spectrophotometry was based on that described by Rosenthal & Drabkin (1943). Modifications were made in the homogenization and haemoglobin separation stages. Except for the sodium dithionite (BDH 'Laboratory Reagent' grade) used in the measurement stage, chemicals were of AR grade and glass-distilled water was used for making up all solutions. Hard borosilicate or Pyrex glassware was used, and was cleaned with dilute detergent and sulphuric acid–dichromate mixture and finally rinsed with glass-distilled water. The homogenizer was of the Potter–Elvehjem type with tubes (internal diam. = 16 mm) and pestle (external diam. = 15·8 mm) made of Pyrex glass and with inner surfaces ground with carborundum powder (320 mesh). The pestle was driven at 1200 rev/min. The light absorption of the final cytochrome *c* solution was measured in fused glass cells of 4 cm light path in a Unicam SP. 500 spectrophotometer fitted with the SP. 580 micro-cell attachment. A direct-reading pH meter (no. 23A, Electronic Instruments Ltd, London) and micro-sampling glass electrode system (SMS.23) were used to obtain pH values.

Before being killed, each rat was starved for 18–24 h to reduce the glycogen content of the liver. Under ether anaesthesia, as much blood as possible was withdrawn from the dorsal aorta. The liver and kidneys were removed, blotted, weighed and either homogenized immediately or stored at $-15°$. The final yield of cytochrome *c* is not significantly affected by storage of the organs for up to 4 h. As a convenient quantity of tissue required for analysis was 1–2 g, the kidneys were usually processed together, but the liver was divided into duplicate pieces of appropriate size. The tissue was minced with scissors and then water (0·5 ml/g tissue), N-$H_2SO_4$ (2·5 ml/g tissue) and 2 N-$NH_4OH$ (1 ml/g tissue) were added in that order, the mixture being homogenized for 50–60 sec after each addition. In preliminary experiments the efficiency of the homogenizing procedure was tested. Intact cells remaining in the homogenate were identified with the phase-contrast microscope (Barer, Joseph & Esnouf, 1956), and the number counted/100 nuclei. Immediately after homogenizing, there were high intact-cell counts, whereas after 2 h at room temperature few such cells were identified. Since an increase in the final yield of cytochrome *c* was observed, the homogenate was always allowed to stand for 2 h at room temperature before processing. The homogenate was centrifuged for 30 min at 2100 ***g***, a measured volume of the supernatant liquid (usually 4 ml/g tissue) was then mixed with an equal volume of $(NH_4)_2SO_4$ solution (0·5 g/ml), and the mixture was allowed to stand for 12–16 h at 4°. The pH of the supernatant liquid was always less than 3, and under these conditions the quantity of $(NH_4)_2SO_4$ added was sufficient to precipitate all the haemoglobin without loss of cytochrome *c*. Subsequent heating of the extract was unnecessary. The extract was filtered (Whatman no. 40 paper), and a measured volume of filtrate (usually 7 ml/g tissue) was transferred to a conical centrifuge tube containing 0·1 ml saturated $(NH_4)_2SO_4$ solution. To each ml filtrate were added 0·1 ml saturated $(NH_4)_2SO_4$ solution and 0·1 ml of a solution of trichloroacetic acid (0·9 g/ml $H_2O$), and the mixture was cooled to 4° . After 60 min the precipitated cytochrome *c* was separated by centrifuging at 4° for 45 min at 2960 ***g*** and carefully decanting the supernatant liquid. The precipitate was dissolved in 2 ml 0·2 N-NaOH and the solution centrifuged for 5 min at 2000 ***g***. The clear supernatant liquid was transferred to a cuvette, 0·1 mg sodium dithionite/ml solution was added, the cuvette was covered with an air-tight glass lid, and the contents were mixed by inversion. The sodium dithionite quickly dissolved, and the extinction, *E*, of the solution of reduced cytochrome *c* was measured against water at 550, 535 and 520 mμ. The final pH of this solution was about 12; under these conditions the reduced cytochrome *c* was stable for at least 8 h, and the extinction was at its maximum. The quantity of cytochrome *c* measured was calculated from the difference in extinction between 550 and 535 mμ. For cytochrome *c* in 0·2 N-NaOH $\epsilon = 28\,380$ and 7290 at 550 and 535 mμ respectively (Rosenthal & Drabkin, 1943), and $28\,380 - 7290 = 21\,090$. If the molecular weight of cytochrome $c = 13\,000$, the concentration (μg/ml) of an unknown solution can be calculated from the equation

$$\frac{E(550-535\ \text{m}\mu)\times 13\,000}{21{\cdot}09}.$$

In the method described, measured quantities were removed from the supernatant

liquid after centrifuging the homogenate, and from the filtrate after the separation of haemoglobin; the cytochrome *c* was dissolved in 2 ml 0·2 N-NaOH, and the extinction was measured in cells of 4 cm light path; hence

$$\mu\text{g cytochrome } c/\text{g fresh tissue} = \frac{E(550-535\ \text{m}\mu)\times 13\,000\times 2\times \text{ml homogenate}\times \text{ml before filtration}}{21{\cdot}09\times 4\times \text{g fresh tissue}\times \text{ml supernatant liquid used}\times \text{ml filtrate used}}.$$

The precision of the method was such that the standard deviation calculated from duplicate analyses on thirty-one different livers with cytochrome *c* contents in the range 136–235 $\mu$g/g liver (mean = 193·3) was $\pm 4{\cdot}4$, and the recovery of rat cytochrome *c* added to the homogenate supernatant stage was within this range. The addition and recovery of cytochrome *c* could not, however, establish quantitative extraction of cytochrome *c* from the tissue. Rosenthal & Drabkin (1943) assumed that the cytochrome *c* of the homogenate after centrifugation was evenly distributed in each unit volume of supernatant liquid and deposit. Beinert (1951), studying the extent of artificial redistribution of cytochrome *c* in rat liver homogenates, showed that successive tissue homogenization with distilled water and 0·154 M-NaCl resulted in an almost complete extraction of the pigment. These observations were substantially confirmed, and it was found that extraction of the tissue with 0·154 M-NaCl after the $H_2O/H_2SO_4/NH_4OH$ procedure left no further cytochrome *c* in the residue extractable by repetition of either procedure. The quantity of cytochrome *c* extracted by this double homogenization procedure was calculated by altering the numerator of the equation, substituting 'ml supernatant liquid' for 'ml homogenate'. A comparison of the cytochrome *c* content of twelve different livers and the kidneys of six different rats, calculated from results obtained after homogenizing with $H_2O/H_2SO_4/NH_4OH$, was made with the yield of that procedure after extraction with 0·154 M-NaCl, and was within experimental error.

### *Experiments with the rats*

Weanling female rats (cf. McCall *et al.* 1962) were obtained as litters, which had been selected to contain even numbers of animals. They were divided between two groups so that each litter was equally represented in each group, and each group contained thirty-six animals divided equally between three cages. The first group was fed on diet 1 (2 mg Fe/kg diet) and the second on diet 2 (240 mg Fe/kg diet) (McCall *et al.* 1962), both *ad lib.* The age of the rats ranged from about 21 to 36 days on the day they were first given the diets. Throughout the experiment the animals were weighed each week, their lengths being measured each fortnight and haemoglobin estimations carried out at fortnightly intervals. Measurements of body length were undertaken to obtain an additional index of rat growth, for it was considered that changes in body composition might occur in rats fed on the two diets, thus affecting the body-weight. In fact body-weight and length were found to be closely related indices of growth, and this relation was independent of the diet. The onset of Fe deficiency was followed by observing the haemoglobin concentration, haematocrit value, red-cell and reticulocyte counts and the concentrations of Fe in the plasma and carcass. The effects on tissue

Fe compounds were evaluated in terms of the cytochrome *c* concentrations of liver and kidneys. The weights of the heart, liver, kidneys and spleen and the volume of the caecum were measured. Since these determinations involved killing the animal, it was necessary to kill closely matched ones in regular succession to observe the progression of Fe deficiency. On the day the diets were first given, and thereafter at intervals of 2, 2 and 3 days in succession for 3 months, two litter-mates, one animal from each group, were killed, as described on p. 310. To avoid effects due to diurnal variations in the quantities of some compounds, the animals were killed at the same time of day throughout the period of the experiment.

The Fe, water and fat contents of the carcasses of the rats that had received the diets for 24, 42 and 63 days were determined. Carcasses were without livers and kidneys, and the intestinal tracts were also removed, to exclude the effect of contained food. The volume of blood removed at slaughter was recorded, its Fe content being calculated from its haemoglobin concentration.

## RESULTS

Compared with the rats receiving diet 2, the Fe-deficient animals were lethargic, had poor appetites and did not grow as big in any given time. They had rough coats with thin dull hair, their eyes were pale and their incisor teeth were white. Bleaching of teeth may have been due to a deficiency of an Fe-containing yellow pigment laid down by the enameloblasts. This bleaching also occurs in deficiencies of magnesium or vitamins A or E and in fluoride poisoning (Moore & Mitchell, 1955). The rate of increase in weight of rats fed on diet 2 was greater than that of rats fed on diet 1 (Table 1), and the time required for the mean live weight of rats fed on diet 2 to

Table 1. *Comparison of the increases in weight of two groups each of twelve female rats fed on diet* 1 (2 *mg iron/kg*) *or diet* 2 (240 *mg iron/kg*)

| Days on diet | Mean body-weight with standard deviation (g) | |
|---|---|---|
| | Diet 1 | Diet 2 |
| 0 | 50± 7 | 49± 7 |
| 7 | 56± 8 | 58± 8 |
| 14 | 84±10 | 83± 7 |
| 21 | 102±13 | 106± 8 |
| 28 | 111±16 | 125±10 |
| 35 | 118±19 | 139±11 |
| 42 | 128±16 | 150±15 |
| 49 | 134±15 | 155±16 |
| 56 | 138±16 | 164±14 |

increase from 50 to 150 g was 42 days. These results are based on figures obtained for the twelve rats in each group that, owing to the nature of the experiment, survived a period of 56 days.

When the weight/g body-weight for liver and kidney was calculated, no difference was found in the ratios, whether the animals had been fed on diet 2 or diet 1. Pro-

gressive changes occurred in the hearts and spleens of rats fed on diet 1. Both these organs were relatively much larger in the Fe-deficient animals. The correlation between heart size and haemoglobin level (Fig. 1) was not linear, since the heart size increased more rapidly as the haemoglobin level fell below 10 g/100 ml blood.

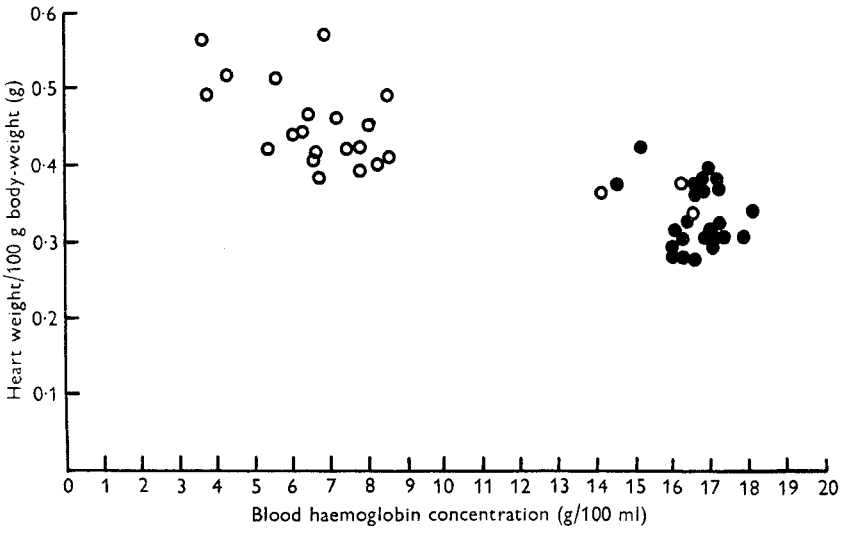


Fig. 1. Relationship of the blood haemoglobin concentration to the heart weight relative to body-weight in rats fed on diet 1 (2 mg Fe/kg) or diet 2 (240 mg Fe/kg). ○, rats fed on diet 1; ●, rats fed on diet 2.

Table 2. *Iron, water and fat contents of carcasses of six rats fed on diet 1 (2 mg Fe/kg) or diet 2 (240 mg Fe/kg)*

| Days on diet ... | 24 | | 42 | | 63 | |
|---|---|---|---|---|---|---|
| Diet | 1 | 2 | 1 | 2 | 1 | 2 |
| Live weight of rat (g) | 86 | 90 | 75 | 128 | 121 | 166 |
| Weight of carcass (g) | 61 | 63 | 52 | 106 | 92 | 138 |
| Concentration of Fe in carcass (mg/100 g) | 3·11 | 5·6 | 2·79 | 5·85 | 2·85 | 6·85 |
| Total Fe content of carcass (mg) | 1·89 | 3·53 | 1·46 | 6·19 | 2·61 | 9·45 |
| Haemoglobin concentration (g/100 ml blood) | 7·8 | 16·9 | 6·3 | 16·9 | 3·7 | 15·7 |
| Plasma Fe concentration (μg/100 ml plasma) | 31 | 204 | 22 | 198 | 58 | 295 |
| Concentration of Fe in carcass (mg/100 g) corrected to include Fe in the blood removed at slaughter | 3·82 | 7·45 | 3·39 | 7·33 | 3·30 | 8·14 |
| Total Fe content of carcass (mg) corrected to include Fe in the blood removed at slaughter | 2·50 | 5·20 | 1·91 | 8·09 | 3·16 | 11·60 |
| Calculated Fe content (mg) of the total haemoglobin of the rat | 1·21 | 2·74 | 0·86 | 3·90 | 0·81 | 4·70 |
| Calculated concentration of Fe in the carcass (mg/100 g) without Fe in blood haemoglobin | 2·41 | 4·40 | 2·25 | 4·29 | 2·63 | 5·31 |
| Water content of carcass (g/100 g) | 65·9 | 66·7 | 69·8 | 68·1 | 68·4 | 65·6 |
| Fat content of carcass (g/100 g) | 8·6 | 10·0 | 8·2 | 11·7 | 8·7 | 13·3 |

The volumes of the caecums of the rats fed on diet 1 became relatively much larger than in animals receiving diet 2. This increase in size became evident a few days after the diet was first given and continued throughout the period of the experiment.

The Fe, water and fat contents of the six carcasses investigated are summarized in Table 2. The concentration of Fe in the carcasses of animals fed on diet 1 showed a slight decline with time, whereas in the rats fed on diet 2 it rose rapidly in the first 24 days and thereafter more slowly. A suggestive increase occurred with time in the total Fe content of the rats fed on diet 1, and a rapid rise occurred in the animals fed on diet 2.

When the carcass Fe concentration was calculated to include haemoglobin Fe and was compared with the plasma Fe concentration, higher plasma Fe values coincided with higher carcass Fe levels. On re-calculating the carcass Fe concentration to

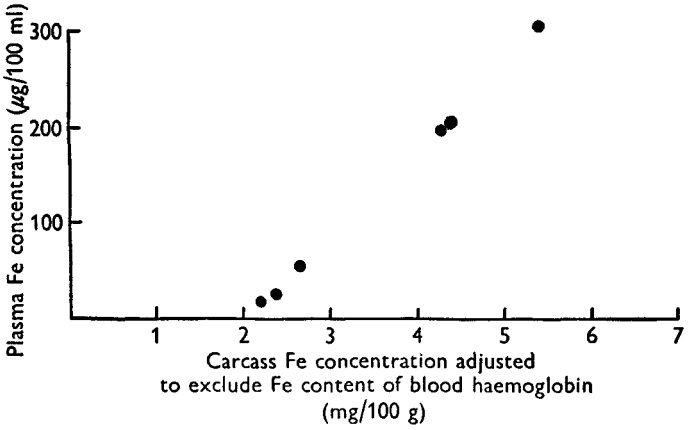


Fig. 2. Relationship between the plasma iron concentration (μg/100 ml) and the concentration of Fe in the carcasses (mg/100 g) adjusted to exclude the Fe content of the haemoglobin of the blood of rats fed on diet 1 (2 mg Fe/kg) or diet 2 (240 mg Fe/kg).

Table 3. *Haematological results obtained at slaughter on rats fed on diet 1 (2 mg Fe/kg) or diet 2 (240 mg Fe/kg)*

(Results are expressed as mean values, and the lower figures refer to the animals fed on diet 2)

| Days on diet ... | 3–7 | 10–17 | 19–35 | 38–52 | 54–68 |
|---|---|---|---|---|---|
| No. of rats | 4 | 3 | 7 | 7 | 7 |
| | 4 | 3 | 7 | 7 | 7 |
| Haemoglobin concentration (g/100 ml blood) | 15·5 | 9·7 | 8·7 | 6·0 | 5·3 |
| | 16·6 | 16·3 | 16·8 | 16·5 | 16·4 |
| Haematocrit value (%) | 42·4 | 32·3 | 30·0 | 21·4 | 19·8 |
| | 45·0 | 44·3 | 45·5 | 45·3 | 44·3 |
| Mean corpuscular haemoglobin concentration (%) | 35·8 | 30·0 | 29·0 | 28·4 | 26·5 |
| | 36·8 | 35·7 | 37·0 | 36·5 | 37·0 |
| Mean corpuscular volume ($\mu^3$) | 59 | 39 | 40 | 35 | 31 |
| | 57 | 64 | 64 | 61 | 61 |
| Red-cell count (cells $\times 10^{-6}$/mm$^3$ blood) | 7·2 | 5·8 | 6·5 | 6·1 | 6·5 |
| | 8·0 | 7·2 | 7·1 | 7·5 | 7·2 |
| Reticulocyte count (%) | 6 | 9 | 24 | 25 | 20 |
| | 10 | 10 | 11 | 5 | 4 |
| Colour index | 0·7 | 0·6 | 0·5 | 0·3 | 0·3 |
| | 0·7 | 0·8 | 0·8 | 0·8 | 0·8 |
| Plasma Fe concentration (μg/100 ml plasma) | 87 | 27 | 35 | 21 | 20 |
| | 135 | 171 | 201 | 220 | 268 |

exclude haemoglobin Fe (a calculation based on the blood-volume:body-weight relationship observed by Everett, Simmons & Lasher, 1956), the relationship between carcass Fe concentration and plasma Fe concentration was found to be linear (Fig. 2). During the period of the experiment there was no significant change in water content of the carcasses of rats fed on either diet, but the increase in the concentration of fat in the carcasses of rats fed on diet 2 was greater than that in rats fed on diet 1 (Table 2).

Table 4. *Comparison of the haematological results obtained on female rats fed on diet 2 (240 mg Fe/kg) or on rat cake 41B (95 mg Fe/kg)*

(Results are expressed as means with standard deviations)

| Diet ... ... ... | Diet 2 | Rat cake |
|---|---|---|
| No. of rats | 31 | 12 |
| Age of rats (days) | 60±15 | 285±100 |
| Haemoglobin concentration (g/100 ml blood) | 16·6±0·75 | 15·7±0·62 |
| Haematocrit values (%): | | |
| micro method | 45·2±1·9 | — |
| macro method | — | 44·5±2·0 |
| Mean corpuscular haemoglobin concentration (%) | 36·7±0·98 | 35·4±0·80 |
| Mean corpuscular volume ($\mu^3$) | 61·7±4·4 | — |
| Red-cell count (cells × $10^{-6}$/mm$^3$ blood) | 7·37±0·34 | — |
| Reticulocyte count (%) | 7·8±5 | 4·8±3 |
| Colour index | 0·8±0·03 | — |
| Plasma Fe concentration ($\mu$g/100 ml plasma) | 214±64 | 296±50 |

Haematocrit values determined by the micro method were consistently 1–2 % less than those obtained by the macro method. A mean corpuscular haemoglobin concentration of 36·7 % (micro-haematocrit) is therefore equivalent to 35·7 % (macro-haematocrit).

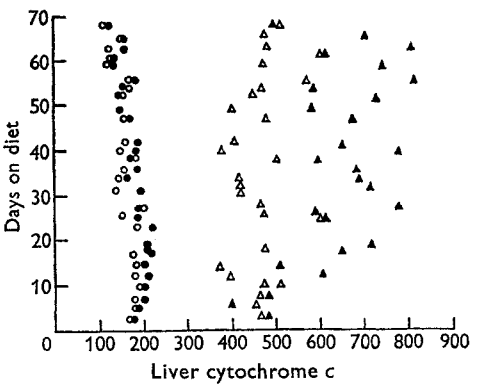


Fig. 3

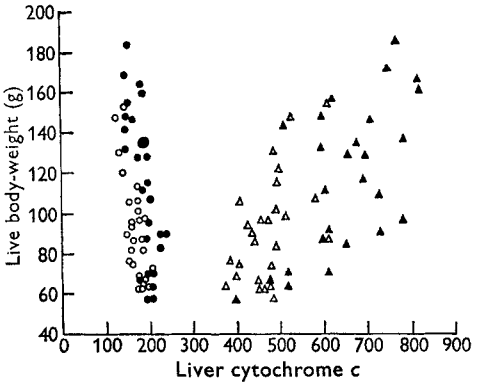


Fig. 4

Fig. 3. Change with time in the cytochrome *c* content of the livers of rats fed on diet 1 (2 mg Fe/kg) or diet 2 (240 mg Fe/kg). ○, concentration of cytochrome *c* ($\mu$g/g liver) of rats fed on diet 1; ●, concentration of cytochrome *c* ($\mu$g/g liver) of rats fed on diet 2; △, total cytochrome *c* content ($\mu$g) of the livers of rats fed on diet 1; ▲, total cytochrome *c* content ($\mu$g) of the livers of rats fed on diet 2.

Fig. 4. Relationship between body-weight and the cytochrome *c* content of the livers of rats fed on diet 1 (2 mg Fe/kg) or diet 2 (240 mg Fe/kg). ○, concentration of cytochrome *c* ($\mu$g/g liver) of rats fed on diet 1; ●, concentration of cytochrome *c* ($\mu$g/g liver) of rats fed on diet 2; △, total cytochrome *c* content ($\mu$g) of the livers of rats fed on diet 1; ▲, total cytochrome *c* content ($\mu$g) of the livers of rats fed on diet 2.

The haematological results obtained at slaughter are summarized in Table 3. Observations on the rats receiving diet 1 began while they were Fe-sufficient and make clear the progressive nature of the anaemia. A normoblastic hyperplasia of the bone marrow developed in the Fe-deficient animals.

The haematological indices established in thirty-one rats receiving diet 2 are compared in Table 4 with those obtained in mature rats fed on the rat cake.

The cytochrome *c* content of the livers of the rats is shown in Figs. 3 and 4. Throughout the period of the experiment, the concentration of cytochrome *c* in the livers of rats receiving diet 1 declined, but the total cytochrome *c* content of the livers of these rats increased significantly as the livers increased in size. The concentration of cytochrome *c* in the livers of rats receiving diet 2 rose at first, peak concentrations

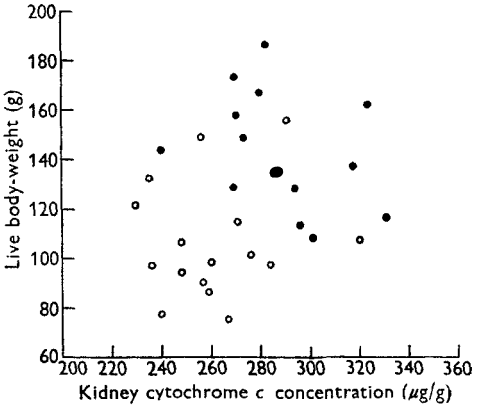


Fig. 5

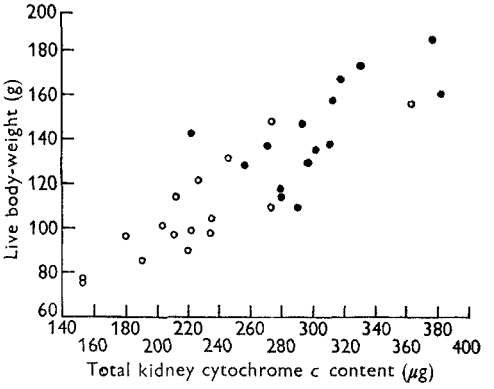


Fig. 6

Fig. 5. Relationship between the concentration of cytochrome *c* in the kidneys and the live weight of rats fed on diet 1 (2 mg Fe/kg) or diet 2 (240 mg Fe/kg). ○, concentration of cytochrome *c* (μg/g kidney) of rats fed on diet 1; ●, concentration of cytochrome *c* (μg/g kidney) of rats fed on diet 2.

Fig. 6. Relationship between the total kidney cytochrome *c* content and the live weight of rats fed on diet 1 (2 mg Fe/kg) or diet 2 (240 mg Fe/kg). ○, total cytochrome *c* (μg) of the kidneys of rats fed on diet 1; ●, total cytochrome *c* (μg) of the kidneys of rats fed on diet 2.

occurring between the 15th and 25th days. This period coincided with the phase of rapid growth. Thereafter, although the total amount of cytochrome *c* in the livers of these rats increased, the concentration declined.

Because of possible variations in the amounts of intrarenal fat between the two groups of rats, cytochrome *c* was determined in the renal cortex during the first part of the experiment. It soon became evident that the concentration of this cytochrome *c* of the animals receiving diet 2 was varying. When the values were compared with the ratio of the weight of cortex dissected to the total kidney weight, high cytochrome *c* values were related to low weight ratios, suggesting that some cortical component contained a higher concentration of cytochrome *c* than the medulla. In the kidneys of rats fed on diet 1, low values were found with animals that had been longest on the diet. In view of the difficulty of dissecting the cortex consistently, the cytochrome *c*

content of the whole kidneys was measured thereafter, and the results are shown in Figs. 5 and 6.

Between the 30th and 80th days of the experiment the concentrations of cytochrome *c* in the kidneys of rats fed on diet 1 and diet 2 were $260 \pm 24$ and $285 \pm 24$ $\mu$g/g kidney, respectively (mean values with standard deviations). During this time the total amounts of cytochrome *c* in the kidneys of the Fe-deficient rats and rats receiving diet 2 were $229 \pm 50$ and $299 \pm 40$ $\mu$g, respectively (mean values with standard deviations). There was no significant difference in the concentrations of cytochrome *c* in the kidneys as the rats increased in size (Fig. 5), but the total cytochrome *c* contents of the kidneys of both groups of rats increased significantly as the rats grew (Fig. 6).

## DISCUSSION

In the first part of the discussion the experimental findings are considered in relation to those of other workers and in the second, the application of these findings to the clinical recognition of Fe deficiency and to the current theories of body Fe distribution in Fe deficiency (Waldenström, 1938; Beutler, 1957; Beutler & Blaisdell, 1958; Beutler *et al.* 1960; Hahn, 1937, 1948).

### *Findings in experimental Fe deficiency*

The detailed haematological findings in growing rats fed on a semi-synthetic diet containing adequate Fe for growth and reproduction (diet 2) were established (Table 4), and the consistency of these haematological findings suggests a physiological saturation of the red cell with haemoglobin (Table 3). It is believed that these values represent a state of Fe sufficiency. The blood picture of growing rats receiving this diet was similar to that of mature animals fed on the rat cake. The haemoglobin values in these animals were similar to those reported by Griffith & Farris (1949) and Kaldor (1955) but the blood picture described by some other workers (e.g. Cameron & Watson, 1949) is by these standards erythraemic, hypochromic and microcytic. Since these latter workers also used female albino rats from Allington Farm, Salisbury, it is suggested, in the absence of available figures for the Fe content of their experimental diet, that their animals were Fe-deficient. The anaemia that developed in growing rats fed on diet 1 was hypochromic and microcytic, as judged by the fall in mean corpuscular haemoglobin concentration and colour index and the mean corpuscular volume (Table 3). The red-cell count did not fall significantly in these rats, but a reticulocytosis closely related to the degree of anaemia was observed.

A range of plasma Fe concentration greater than 100 $\mu$g/100 ml was observed in rats fed on diet 2, high values occurring most frequently in the animals that had been longest on the diet. High values were also associated with high tissue Fe concentrations (Fig. 2). As the amount of Fe available in the diet was greater than the minimum required by the growing animal, the rising plasma Fe values in animals fed on this diet seem to be related to increasing body Fe deposits. On the other hand, the concentration of Fe in the plasma of the Fe-deficient rats was less than 90 $\mu$g/100 ml (Table 3). It is suggested that, in the growing rat under the conditions of these experiments,

plasma Fe values below 90 $\mu$g/100 ml represent the exhaustion of available body Fe and in the absence of adequate dietary Fe this is the first stage in the anaemia of Fe deficiency. The onset of Fe deficiency in rats fed on diet 1 was indicated at about 1 week after the beginning of the experiment when the concentration of Fe in the plasma fell below 90 $\mu$g/100 ml. Subsequent to this fall anaemia developed. The relationship between the haemoglobin concentration and the concentration of Fe in the plasma is shown in Fig. 7. Blood haemoglobin concentrations and mean corpuscular haemoglobin concentrations were consistently below the ranges associated with Fe sufficiency when the concentration of Fe in the plasma was less than 90 $\mu$g/100 ml.

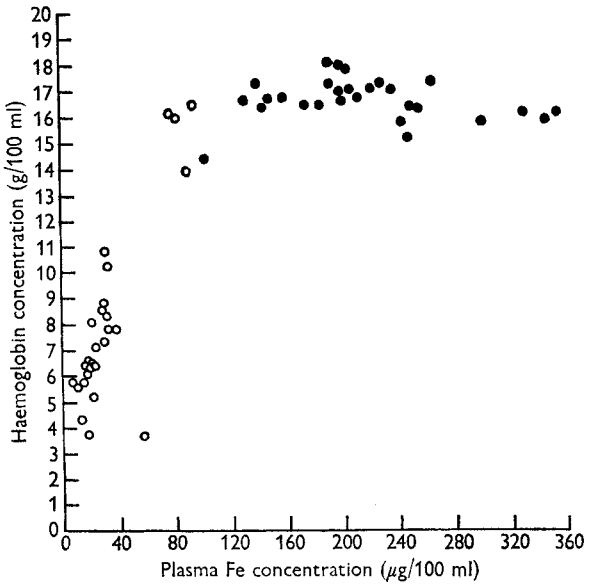


Fig. 7. Relationship between the blood haemoglobin concentration and the plasma Fe concentration of rats fed on diet 1 (2 mg Fe/kg) or diet 2 (240 mg Fe/kg). ○, rats fed on diet 1; ●, rats fed on diet 2.

Low concentrations of cytochrome *c* in the heart, liver and kidneys of Fe-deficient rats have been reported (Cohen & Elvehjem, 1934; Beutler, 1957; Beutler & Blaisdell, 1958). In this study the cytochrome *c* contents of the livers and kidneys of thirty growing rats were measured during the progressive development of an Fe-deficiency state. The values obtained were compared with those of a similar number of littermates fed on the same diet supplemented with a quantity of Fe shown to be nutritionally adequate. The concentrations of cytochrome *c* in the livers of rats fed on diet 1 decreased with time (Fig. 3) and increasing liver weight (Fig. 4), but the total quantity of the pigment in the livers of these animals increased significantly as the animals grew (Figs. 3 and 4). There was little change in the concentrations of cytochrome *c* with time or live weight in the kidneys of Fe-deficient rats during the period when whole kidneys were used for analysis (Fig. 5), but there was a clear increase with live weight of the total quantity of cytochrome *c* in the kidneys of these animals (Fig. 6).

Beutler (1957) and Beutler & Blaisdell (1958) ascribed the fall in concentration of

tissue cytochrome *c* to a depletion (i.e. removal) of the enzyme in the course of Fe deficiency; however, in view of the slow turnover rate of cytochrome *c* (Helwig & Greenberg, 1952), the rise in total tissue quantities reported here does not support their suggestion. It is difficult to compare these results directly with those of Beutler (1957) and Beutler & Blaisdell (1958) because of the differences due to the nutritional state, age and strain of the animals and the methods of inducing Fe deficiency. The fall in the tissue concentrations of cytochrome *c* in the rats receiving diet 1 appears to be a 'dilution with growth' rather than a depletion, the rise in total cytochrome *c* occurring as Fe became available either from absorption (the diet not being entirely Fe-free) or perhaps from the turnover of other Fe-containing compounds, such as haemoglobin. Increases in the total cytochrome *c* content of the livers and kidneys of these animals occurred while the haemoglobin levels were falling.

To some extent these findings seem to be in agreement with those of Josephs (1932) and Hahn & Whipple (1936), who suggested that tissue levels of functional Fe compounds are maintained at the expense of blood haemoglobin. However, after the first 10 days of the experiment, the total quantity of cytochrome *c* in the livers and kidneys of rats receiving diet 1 was lower in proportion to the weights of the animals than that of rats receiving diet 2. The functional effect of this difference is not known. Although depletion of cytochrome *c* in growing Fe-deficient rats was not demonstrated, the relative quantities of the pigment may depend to some extent upon the age or stage of the life cycle of the animal. For example, during the first part of the experiment, the concentration of cytochrome *c* in the livers of rats fed on diet 2 increased. A similar change in the concentration of catalase in the livers of growing animals has been reported (Beutler & Blaisdell, 1958). Highest cytochrome *c* values occurred between the 15th and 25th days (Fig. 3), and this period coincided with the phase of rapid weight gain. No such increase was observed in rats fed on diet 1.

When the concentration of Fe in the carcasses of the rats fed on diet 1 was recalculated to exclude the Fe content of haemoglobin, the total Fe content of the carcasses increased as the animals increased in weight. Josephs (1932) observed that though the total body Fe content of Fe-deficient rats increased, their total haemoglobin Fe remained unchanged. The effect of Fe deficiency on the distribution of Fe between the red-cell mass and the remainder of the carcass was measured in this study by Josephs's technique. The Fe content of the red-cell mass was calculated from the measured haemoglobin concentration and the blood volume calculated from the rat's body-weight, according to the relationship observed by Everett *et al.* (1956). There was a fall in the total Fe content of the red-cell mass in rats that had been fed for between 30 and 80 days on the Fe-deficient diet (Fig. 8). Over the same period the Fe content of the tissues, calculated from carcass analysis and body-weight, increased, suggesting that, with insufficient Fe available to meet all requirements, the relative competitive power of some body processes for Fe may increase at the expense of others, resulting in a redistribution of Fe within the body. This calculation presupposes that there is no alteration in the ratio of blood volume to body-weight in Fe-deficient rats (Scott & Barcroft, 1924). In spite of the gross anaemia developing in Fe-deficient animals, none died; in view of their retarded growth it is possible that an equilibrium

was reached. The concentration of Fe in the tissues does not appear to fall below a certain minimum level (Fig. 2), and the animal grows within the limiting amount of available Fe.

The cardiomegaly that developed in the Fe-deficient rats (Fig. 1) is unlikely to have been due to an increase in cardiac water content, for the mean water content of the hearts of three rats fed on diet 1 was 77·0% and of the hearts of two rats fed on diet 2 was 76·3%. The mechanism of enlargement of the heart at a time when general body growth is reduced invites speculation. It is generally considered to be a 'work hypertrophy' (Wintrobe, 1956). The increase in the relative size of the spleens of these animals may have been due to marrow hyperplasia, since the spleen is a site of

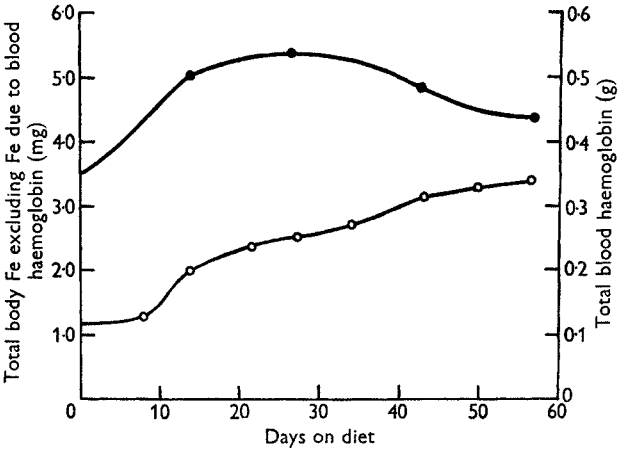


Fig. 8. Effect of iron deficiency, produced in the rat by diet 1 (2 mg Fe/kg), on the distribution of Fe between the red-cell mass and the other tissues. ○, total body Fe content; ●, total blood haemoglobin content.

erythropoiesis in the rat and there was hyperplasia of the bone marrow in the Fe-deficient animals. Histological examination of the spleens was not included in our study. There was no close correlation between the splenomegaly and the degree of reticulocytosis. The reason for the increase in the volume of the caecums of the Fe-deficient rats, which occurred about 10 days after being given diet 1, is obscure.

The effects of age and the nutritional state of the animal upon body composition may play a part in the interpretation and comparison of results. For example, the carcasses of rats fed on diet 2 contained a higher concentration of fat than those of rats fed on diet 1 (Table 3) although the diets differed only in Fe content. In this instance the quantity of fat present did not alter materially the relative concentrations of Fe in the tissues of the two groups of animals. There were also changes in the concentration of cytochrome *c* in the livers of control rats during the growing phase.

In the experimental animal, control or 'normal' values may well depend on the investigator's knowledge of dietary requirements; unless the 'normal range' has been demonstrated to represent a dietary sufficiency it is an inadequate standard for the assessment of deficiency, as, for example, in the work of Cameron & Watson (1949). The relationship of this view to clinical practice is important.

*Clinical recognition of Fe deficiency*

The conventional recognition of Fe deficiency assumes that the existing haematological standards permit diagnosis of anaemia and preclude the existence of unrecognized Fe deficiency. If the 'normal range' upon which the diagnosis of anaemia is based is not that associated with adequacy of Fe, it is possible that a tissue Fe deficiency might exist without apparent anaemia. The findings, that 'normal' haemoglobin levels in children (Mackay, 1931), in pregnant women (Benstead & Theobald, 1952; Fisher & Biggs, 1953; Whiteside, 1960; and others) and in menstruating women (Beutler *et al.* 1960; Yudkin, 1944) rise when Fe supplements are given orally, suggest that current criteria for the recognition of anaemia are based on populations of which a material proportion is Fe-deficient. The description of tissue Fe deficiency without anaemia, in women who subsequently showed a significant increase in blood haemoglobin concentration after treatment with Fe (Beutler *et al.* 1960), supports the contention that the diagnosis of tissue Fe deficiency in the absence of anaemia is an artifact resulting from the use of haematological 'normal' standards not derived from Fe-sufficient populations.

Even if this explanation of the clinical syndrome of 'tissue iron deficiency in the absence of anaemia' is correct, it does not explain the basic conflict between the theories of Hahn (1937, 1948), and Waldenström (1938), Beutler (1957), Beutler & Blaisdell (1958), Beutler *et al.* (1960). Clinically it is important to know whether Fe deficiency is manifest primarily by its effect on the red-cell mass (Hahn, 1937, 1948) or by its effects on other tissues (Waldenström, 1938; Beutler, 1957; Beutler & Blaisdell, 1958; Beutler *et al.* 1960), and in what particular circumstances a particular effect will predominate. There is little experimental evidence available on the distribution of Fe in Fe deficiency. It seems unlikely from our work reported here that the effect of Fe deficiency is ever borne by only one tissue or by a change in the production of a single pigment (haemoglobin). Our findings only partly support Hahn's (1937, 1948) hypothesis that metabolically active tissue Fe is inviolable, since a reduction in tissue Fe level, and in tissue cytochrome *c* content, was observed in Fe-deficient animals. Although this change was slight in proportion to the gross alteration in blood haemoglobin concentration, it is not possible to assess its significance without studies of tissue function. On the other hand, these findings are contrary to Beutler's (1957) suggestion that metabolically active tissue compounds are depleted of Fe for the maintenance of blood haemoglobin levels. However, it must be emphasized that these findings in experiments on the growing Fe-deficient rat may differ from those in adult Fe-deficient animals. The relative competitive power of metabolic processes for Fe may depend upon the overall nutritional state of the animal, the phase of the life cycle studied and the method of producing Fe deficiency.

SUMMARY

1. Growing rats on a diet deficient in iron but otherwise nutritionally adequate have been compared with rats maintained on the same diet supplemented with Fe. Particular attention has been paid to the relative effects of Fe deficiency on the haemoglobin Fe and the tissue Fe.

2. The overall picture of Fe sufficiency in the growing rat has been established. The following features of these animals were observed: the rate of weight gain; the size of the heart, liver, kidney, spleen and caecum; the carcass Fe, fat and water content; the liver and kidney cytochrome *c* content. In particular the haematological findings are described in detail.

3. Fe deficiency is recognized as a departure from the picture of Fe sufficiency, and its progressive development in growing rats fed on an Fe-deficient diet is described.

4. Fe-deficient rats gained weight more slowly, were lethargic and had rough coats and white incisor teeth; they developed cardiomegaly, splenomegaly and enlargement of the caecum in comparison with controls given Fe supplements.

5. Carcass Fe concentrations were lower in Fe-deficient animals, but did not appear to fall below a certain lower limit, despite an increasing degree of anaemia.

6. The water contents of the carcasses of Fe-deficient rats and of rats given Fe supplements were similar, but rats given Fe supplements had a higher proportion of body fat.

7. The cytochrome *c* concentration in the livers of rats given Fe supplements was found to increase during the animal's phase of most rapid growth and subsequently to decline. The total cytochrome *c* content of the livers increased throughout the period of study. In Fe-deficient rats there was a fall in the liver cytochrome *c* concentration, as the organ increased in weight, but there was a significant increase in the total liver cytochrome *c* during the same period.

8. The observation of kidney cytochrome *c* content in both groups of animals was complicated by the initial attempt to measure the concentration of cytochrome *c* in the renal cortex. The value of the cytochrome *c* concentration was found to depend on the degree of separation of cortex from medulla. Subsequent results, when the cytochrome *c* content of the whole kidneys was measured, again showed that in Fe deficiency the concentration of the pigment was lower, but that the total cytochrome *c* content of the kidney increased with growth.

9. A characteristic sequence of changes in the blood picture occurred in rats fed on the Fe-deficient diet. First, the plasma Fe concentration fell; when it was less than 90 $\mu$g/100 ml there was a subsequent decline in the blood haemoglobin concentration. This anaemia was due to a decrease in the red-cell saturation with haemoglobin, as measured both by the colour index and the mean corpuscular haemoglobin concentration. At the same time there was a fall in the mean corpuscular volume, but even in severe anaemia there was little change in the red-cell count. In severely anaemic animals a reticulocytosis was observed.

10. Higher plasma Fe levels were observed in the animals that had been longest on the Fe-supplemented diet. A straight-line relationship was found between the plasma

Fe concentration and the carcass Fe concentration, adjusted to exclude Fe due to blood haemoglobin.

11. The clinical concept of tissue Fe deficiency in the absence of anaemia is discussed, together with current theories on the effect of Fe deficiency on body Fe distribution.

The technical assistance of Mr J. Halfacree and his staff in the animal house, and of Miss E. Clinkard, is gratefully acknowledged. We thank Mr P. J. Tosh for making the homogenizing tubes and pestles.

This work was supported in part by a grant to L. J. W. from the Medical Research Council.

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*Printed in Great Britain*