Iron Deficiency: Impaired Liver Growth and DNA Synthesis in the Rat

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(Received 14 March 1974; accepted for publication 2 May 1974)

SUMMARY. This study deals with liver growth and DNA synthesis in young, rapidly growing rats weaned to an iron deficient diet. Shortly after the development of anaemia there was a depression in the rate of liver growth that was more profound than that of body growth. The rate of increase in total liver DNA was similarly depressed, in comparison with iron-supplemented animals. Paradoxically, in the deficient rats, two normally sensitive indices of DNA synthesis, incorporation of \(^{3}H\)thymidine into DNA and activity of thymidine kinase, were unaffected. This was also the case in animals acclimated to a 4 hr/day restricted feeding regimen in order to control for possible variations in feeding patterns. Although \(^{3}H\)thymidine incorporation was normal throughout a 24 hr period, incorporation of \(^{32}P\) into liver DNA was depressed in the iron deficient rats. The results indicate changes in liver DNA synthesis due to iron deficiency that are distinct from those seen in starvation and protein-calorie malnutrition. Probably DNA synthesis in iron deficiency is modified through a depression of de novo synthesis, in the presence of normal salvage pathway activity.

Severe iron deficiency is most common between 6 months and 3 years of age, an interval normally characterized by a more than doubling of body weight. Iron deficient children of this age, who consume large quantities of milk, may appear pale but otherwise well-nourished. Quantitative study, however, shows that this population as a whole is underweight (Judisch et al., 1966), an abnormality that is reversible with iron treatment.

Depression of growth is more readily detected in the rat. In the young rat, it is a relatively late consequence of nutritional iron deficiency (McCall et al., 1962b). Iron lack has no discernible effect on growth until the haemoglobin concentration has stabilized at one-quarter to one-third of the normal value; at this time, myoglobin and cytochrome c in skeletal muscle have reached a plateau at about half of the control concentration (Dallman & Schwartz, 1965; Dallman, 1969). After this, a gradual slowing of growth helps to maintain stable concentrations by preventing additional dilution of these iron compounds within a larger body mass. In a preliminary experiment in the rat, we detected a depression in the rate of liver growth that was more profound than that of body weight. This observation led to further investigation of the effects of iron deficiency on liver growth and DNA synthesis.

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MATERIALS AND METHODS

Animals and Diets

Nursing Sprague-Dawley rats were obtained from Simonsen Laboratories, Gilroy, California. Female pups were randomized to eight rats per litter at 10 days of age. The litters were divided into two groups; one was provided with an iron supplemented diet and the other with the same diet without iron. The composition of the diet (Dallman & Manies, 1973) and the experimental conditions (Dallman & Goodman, 1970) have been reported previously. The rats were kept in a windowless room with temperature regulation and an 8.30 a.m. to 8.30 p.m. light cycle and were weaned at 21 days of age.

Two experimental designs were used, one to study the sequential development of certain manifestations of iron deficiency (experiment 1), and the second to determine the effect of a controlled feeding regimen by study of the animals at frequent intervals during a 24 hr period (experiment 2).

Experiment 1. The two diets were provided ad libitum until the time of killing at 9.30 a.m., at ages 21, 27, 33, 39 and 48 days. Some of the iron deficient rats were injected intramuscularly with 12 mg of iron as iron dextran (Imferon) at 46 days of age and killed 2 days later, concurrently with iron deficient and control animals of the same age.

Experiment 2. The two diets were provided ad libitum only from 10 days of age to weaning at 21 days. After this, the feeding period was progressively restricted: food was available for 8 hr, between 8.30 a.m. and 4.30 p.m. from 23 to 25 days of age; for 6 hr, between 8.30 a.m. and 2.30 p.m. from 26 to 28 days; and for 4 hr, between 8.30 a.m. and 12.30 p.m. from 29 to 32 days. A total of 33 rats in each dietary group was killed at age 32 days, three rats from each group at intervals of 1.5-4 hr during the subsequent 24-hr time period.

Laboratory Measurements

Isotopes. 1 hr before killing rats were injected either intravenously with 100 μCi thymidine [3H]methyl (6 Ci/mnmole)/0.2 ml of saline/100 g body weight (experiment 1) or intraperitoneally with 100 μCi each of thymidine [3H]methyl and H32PO4/0.2 ml of saline/100 g body weight (experiment 2).

DNA concentration and thymidine kinase activity. The concentration of DNA and the activity of thymidine kinase (EC No. 2.7.1.21) were determined in liver as previously described (Siimes & Dallman, 1974). DNA in liver homogenates was extracted, precipitated, and hydrolysed by a modification of the Schmidt-Thannhauser method. Pentose was measured spectrophotometrically after reaction with diphenylamine. Thymidine kinase activity was assayed in the 100 000 g supernatant fluid of liver homogenate by measuring the rate of incorporation of [3H]thymidine into phosphorylated nucleotides. The activity was expressed as counts in phosphorylated nucleotides per microgram of protein in the 100 000 g supernatant fluid. The radioactivity of the samples was measured in a Beckman Liquid Scintillation Counter. In the channels used, the 32P window contained virtually no 3H counts, whereas the 3H window had approximately 5% of the 32P counts. Efficiency of the counting was 39% for 32P and 22% for 3H. Addition of internal standard revealed no significant differential quenching.

Liver iron. Non-haem iron was measured by the method of Weinfield (1964) after in situ perfusion of the liver with isotonic saline solution after ether anaesthesia.
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RESULTS

Experiment 1. The sequence of development of some manifestations of iron deficiency in rats fed ad libitum is summarized in Fig 1. The decrease in liver iron stores and PCV began early, before complete weaning, whereas retardation in liver and body growth occurred later in the course of iron deficiency, only after the PCV had already declined to its lowest value. At 21 days of age, when the rats were completely weaned, the non-haem iron in liver was 32% and the PCV was 70% of the control values. There was no difference in body or liver weight either at 21 or 27 days of age (Tables I and II). Subsequently, both were significantly below control values. Liver weight was disproportionately depressed compared to body weight, particularly during the early period of weight loss ($P<0.01$ at 33 days, $P<0.02$ at 39 days, and $P<0.2$ at 48 days). At 48 days of age the liver iron, PCV, liver weight and body weight were depressed to 89, 64, 36 and 23% below control values, respectively.

The mean total liver DNA became decreased after 33 days of age in the iron deficient rats (Table II). However, the standard error was large, and the significance of the decrease was variable ($P<0.01$ at 33 days, not significant at 39 days, and $P<0.05$ at 48 days). In contrast, DNA synthesis in liver, as estimated by the in-vivo incorporation of $[^3H]$thymidine into DNA or by the in-vitro activity of thymidine kinase, was unaffected at 33, 39 and 38 days of age. Only thymidine kinase activity was transiently depressed at 21 and 27 days.

Response to treatment with iron dextran. The response of the group of iron deficient rats that was injected with iron dextran 2 days before killing at 48 days of age is shown in Tables I and II. Non-haem iron in the liver rose to 601% of the iron-deficient values, almost equivalent to the control values at this age. The pretreatment PCV was 0.16 and rose to 0.23, an increase of 44%. The liver weights and body weights rose 22%, from 6.8 to 8.3 g; and 7%, from

![Graph showing effect of iron deficiency on body weight, liver and PCV](image-url)
TABLE I. Development of iron deficiency and response to iron treatment. Ten-day-old rats were started either on the iron deficient or iron supplemented diet and subsequently groups of five rats were killed at the ages indicated. An additional iron deficient group was injected with iron dextran at the age of 46 days and killed 2 days later.

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>48 days (2 days after iron treatment)</th>
<th>% Increase</th>
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<tbody>
<tr>
<td></td>
<td>21 days</td>
<td>27 days</td>
<td>33 days</td>
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<tr>
<td>Body wt (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>57 ± 1.2†</td>
<td>88 ± 2</td>
<td>129 ± 4</td>
</tr>
<tr>
<td>Deficient</td>
<td>56 ± 1.7</td>
<td>88 ± 3</td>
<td>103 ± 3***</td>
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<tr>
<td>PCV</td>
<td></td>
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<tr>
<td>Control</td>
<td>0.30 ± 0.01</td>
<td>0.16 ± 0.003</td>
<td>0.37 ± 0.007</td>
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<tr>
<td>Deficient</td>
<td>0.21 ± 0.004***</td>
<td>0.13 ± 0.006***</td>
<td>0.14 ± 0.008***</td>
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<tr>
<td>Liver non-haem iron (µg/liver)</td>
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<tr>
<td>Control</td>
<td>64 ± 5.25</td>
<td>—</td>
<td>642 ± 110</td>
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<tr>
<td>Deficient</td>
<td>54 ± 4.4**</td>
<td>68 ± 7.3***</td>
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† Means ± SEM. * P < 0.05; ** P < 0.01; *** P < 0.001.
<table>
<thead>
<tr>
<th>Age</th>
<th>21 days</th>
<th>27 days</th>
<th>33 days</th>
<th>39 days</th>
<th>48 days (2 days after iron treatment)</th>
<th>% Increase</th>
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<tr>
<td>Liver wt (g)</td>
<td></td>
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<tr>
<td>Control</td>
<td>2.5 ± 0.15†</td>
<td>4.3 ± 0.24</td>
<td>6.4 ± 0.29</td>
<td>7.3 ± 0.50</td>
<td>10.6 ± 1.03</td>
<td>8.3 ± 0.78 22%</td>
</tr>
<tr>
<td>Deficient</td>
<td>2.2 ± 0.06</td>
<td>4.2 ± 0.32</td>
<td>3.7 ± 0.22***</td>
<td>4.5 ± 0.22***</td>
<td>6.8 ± 0.56*</td>
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<tr>
<td>Liver DNA (mg/liver)</td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>5.4 ± 0.50</td>
<td>4.9 ± 0.21</td>
<td>10.8 ± 0.54</td>
<td>11.3 ± 1.1</td>
<td>13.9 ± 1.5</td>
<td>9.0 ± 0.97 0%</td>
</tr>
<tr>
<td>Deficient</td>
<td>4.9 ± 0.34</td>
<td>6.8 ± 0.62*</td>
<td>6.8 ± 0.62**</td>
<td>9.0 ± 1.4</td>
<td>9.0 ± 0.97*</td>
<td>9.0 ± 0.97 0%</td>
</tr>
<tr>
<td>[3H]Thymidine into liver DNA (cpm/μg DNA)</td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>82 ± 12</td>
<td>41 ± 8.2</td>
<td>37 ± 9.7</td>
<td>36 ± 5.6</td>
<td>21 ± 1.5</td>
<td>70 ± 8.9 106%/**</td>
</tr>
<tr>
<td>Deficient</td>
<td>70 ± 4.8</td>
<td>51 ± 5.6</td>
<td>65 ± 8.2</td>
<td>28 ± 3.0</td>
<td>34 ± 7.5</td>
<td></td>
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<tr>
<td>Liver thymidine kinase (cpm/μg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>24.3 ± 5.1</td>
<td>10.3 ± 1.5</td>
<td>11.3 ± 2.1</td>
<td>4.6 ± 0.59</td>
<td>2.5 ± 0.63</td>
<td>18.4 ± 2.9 309%/***</td>
</tr>
<tr>
<td>Deficient</td>
<td>8.6 ± 1.7*</td>
<td>4.7 ± 0.66**</td>
<td>9.4 ± 2.3</td>
<td>3.7 ± 1.0</td>
<td>4.3 ± 1.2</td>
<td></td>
</tr>
</tbody>
</table>

† Means ± SEM. * P < 0.05; ** P < 0.01; *** P < 0.001.
163 to 175 g, respectively. The incorporation of [3H]thymidine and thymidine kinase increased 106 and 309%, respectively (Table II); in both cases, these values were far above those in the control animals. However, no change in total liver DNA was evident at this time (Table II).

Experiment 2: Four-hour feeding regimen. The data from experiment 1 (Table II) showed a slower than normal accumulation of total liver DNA during the development of iron deficiency even though the rate of DNA synthesis seemed unaffected. These apparently discordant findings could be attributable to differences in feeding patterns between the two groups. Glover & Jacobs (1972) reported that iron-deficient rats were most active during the daytime, opposite to the normal nocturnal pattern of activity. It is probable that the feeding pattern could be similarly altered by iron deficiency. Since the timing of the diurnal fluctuation in liver DNA synthesis is dependent on the timing of food intake, we instituted a controlled feeding regimen in which the rats were gradually acclimated to a 4 hr feeding period of either an iron-deficient or iron-supplemented diet. They were then killed at 32 days, near the

![Graph](image-url)

Fig 2. Body and liver weight during a 24 hr period in 32-day-old rats fed either iron deficient (●) or iron supplemented (■) diet. Animals were acclimated to a 4 hr feeding regimen (8.30 a.m. to 12.30 p.m.) as described in the 'Methods'. Each of the 11 time points represents the mean of values from three rats. At the start of the experiment at 8 a.m., the mean body weights were 73 g and 86 g, and the mean liver weights were 2.4 and 2.6 g in the iron deficient and iron supplemented rats, respectively. After the feeding period, liver weights increased 30 ± 4% in the iron deficient group and 80 ± 4% in the iron supplemented group (the mean of the values between 2 p.m. and 2 a.m. are compared with the 8 a.m. values).
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age when body and liver weight were first found to deviate significantly from normal in experiment 1. In preliminary experiments with control rats, the 4 hr feeding period was followed by a sharp and distinct peak in incorporation of $[^3]$H]thymidine into DNA about 12-14 hr later, as also described by Barbiroli & Potter (1971).

The restricted food intake resulted in a slower weight gain than in animals fed ad libitum. Rats on the regimen of restricted feeding of iron supplemented diet weighed 94 g (mean weight at time of killing throughout the 24 hr period) at 32 days of age when compared to the usual 117 ± 5 g in rats allowed free access to the same diet. Restricted feeding of the iron deficient diet resulted in a weight of 78 g (24 hr mean).

![Fig 3. Incorporation of $[^3]$H]thymidine (above) and $^{32}$P (below) into liver DNA during a 24 hr period in iron deficient and iron supplemented rats. The isotopes were injected 1 hr before killing. The results are from the same experiment as in Fig 2.](image)

On the 4 hr feeding regimen both groups of rats had a similar diurnal fluctuation of their body weight of c. 15% (Fig 2). However, diurnal changes in liver weight were different in the two groups. In the control rats the liver weight for a 12 hr period after feeding (2.00 p.m. to 2.00 a.m.) was 80% over the prefeeding weight at 8.00 a.m. The corresponding increase was only 30% in the iron deficient rats (Fig 2). The difference in this response was highly significant ($P<0.001$). DNA content of the livers did not deviate significantly from a mean of 5.0 mg in the iron deficient and 5.2 mg in the iron supplemented groups.

The incorporation of $[^3]$H]thymidine into liver DNA reached a sharp peak 14 hr after the start of the feeding period in the control animals (Fig 3). In the iron deficient rats a less prominent, earlier peak of incorporation of $[^3]$H]thymidine into DNA occurred between 10
and 15 hr after the onset of feeding. Each peak value was significantly higher than the combination of all other values in the corresponding diet groups \((P<0.001)\). However, the mean incorporation during the entire 24 hr period was similar in the two groups, in accord with the results of experiment 1.

The incorporation of \(^{32}\text{P} \) into DNA was less variable during the 24 hr period than that of \([^{3}H]\text{thymidine}\). There was a significant \((P<0.05)\) elevation in the control group between 8 and 12 hr after the start of feeding, above the values at other time points. In the iron-deficient group there was an even less prominent, but still significant \((P<0.02)\) peak in the incorporation of \(^{32}\text{P} \) after 14–16 hr. The overall incorporation of \(^{32}\text{P} \) into DNA over the 24 hr period was depressed in the iron-deficient groups, \(P<0.001\), as it was at almost all the individual time points (Fig 3).

**DISCUSSION**

The early and disproportionate depression in the rate of liver growth and accumulation of total DNA in experiment 1 appeared to be a direct result of iron deficiency rather than a non-specific consequence of anaemia. Firstly, with the continuation of an iron-deficient diet, the slowing of liver growth became progressively more severe, whereas the degree of anaemia had reached a plateau. Secondly, the rapid hepatic response to iron therapy suggested that the altered rate of growth and production of DNA in the liver were related more to iron supply than to anaemia.

Recently it has been proposed that iron has a specific role in nucleic acid synthesis and cell division. The most convincing evidence is from the observations of Robbins & Pederson (1970) who showed that iron chelating agents could selectively inhibit incorporation of labelled thymidine into DNA and mitosis in HeLa cells while leaving RNA and protein synthesis intact. Furthermore, iron was localized in the nucleolus during interphase and became associated with the chromosomes during mitosis. Another study suggested that incorporation of labelled thymidine into DNA in bone marrow cell suspensions was decreased in iron deficiency (Herschko et al, 1970), but this result is likely to be a secondary consequence of diminished availability of the iron that is a requirement particularly for erythroid precursors. Cellular growth in other tissues was studied by Canale & Lanzkowsky (1970) in iron deficient rats. They found that liver DNA was decreased disproportionately to liver protein. However, their results showed an unusually low liver weight (4.1 g at 143 days of age) in the ad libitum fed control group, suggesting that these animals were also malnourished.

Although total liver DNA became depressed during the development of iron deficiency in experiment 1, it was surprising that there was not a corresponding depression in the incorporation of \([^{3}H]\text{thymidine} \) into DNA or in the activity of thymidine kinase between 33 and 48 days of age. We realized that this apparent discrepancy could be due to differences in diurnal patterns in the two diet groups, and such differences proved to be present under the controlled feeding conditions of experiment 2. However, the findings were similar, even with the 4 hr feeding regimen. The mean incorporation of \([^{3}H]\text{thymidine} \) into DNA over a 24 hr period was not depressed in the iron deficient group at a time when differences in the rate of accumulation of DNA should have become evident. In contrast, there was a depression of incorporation of \(^{32}\text{P} \) into DNA. The disagreement between these results could be due to
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The incorporation of thymidine into DNA is via the salvage pathway of DNA synthesis and should reflect primarily the activity of that pathway. Although $^{32}$P has many alternative pathways of metabolism, its incorporation into deoxypolynucleotides constitutes one of the last steps in DNA synthesis after the salvage and de novo pathways have joined. It is intriguing to postulate that liver DNA synthesis in iron deficiency may be decreased via de novo synthesis, but that partial compensation for this decrease is effected through continued activity of the salvage pathway. In accord with this hypothesis is the recent study of Hoffbrand et al (1974) which suggests that the activity of ribonucleotide reductase, an enzyme on the de novo pathway, may be decreased by iron deficiency.

The mechanism for depressing DNA production is likely to be different in starvation and protein-calorie malnutrition from that in iron deficiency. In starvation or protein-calorie malnutrition, no discrepancy between DNA content and measures of DNA synthesis was observed. The prompt and profound depression in the incorporation of $[^3H]$thymidine into DNA (Dallman & Manies, 1973) was consistent with depression in liver growth and accumulation of DNA (Winick & Noble, 1966).

The disproportionate depression of liver weight in comparison to body weight that is described in this study is not unique to iron deficiency. The liver also loses weight and protein more rapidly than other tissues of the body in other forms of malnutrition that are characterized by suppression of body growth, such as fasting or protein deficiency. After feeding a normal diet the liver recovers with similar rapidity. One probable basis for this lability in liver weight is the unusually rapid turnover of protein in liver, an average half life of less than 5 days (Schimke et al, 1969). Glycogen and fat are also subject to rapid changes in response to diet because of the central role that the liver plays in their storage and metabolism. Liver weight therefore anticipates changes that are slower to develop in other tissues. In addition to an early and disproportionate sensitivity of liver growth to iron deficiency, it seems possible that malabsorption may play a role in its etiology. Experiment 2 showed the restricted feeding regimen resulted in a similar post-feeding increase of 15% in body weights of both dietary groups, suggesting that the total food intake was of the same magnitude. In the control rats there was a diurnal doubling in liver weight, whereas total DNA remained stable. However, in the iron deficient animals the increase in liver weight after feeding was less than half of that in the control rats. It is possible that the diurnal increase in body weight represents a normal amount of food ingested, but the diminished weight response of the liver suggests a failure to assimilate the food in normal quantities. The caecal enlargement with engorgement by faeces that has been noted in iron deficient rats suggests the possibility of malabsorption (McCall et al, 1962a). Abnormalities in intestinal absorption are also present in some iron deficient children but have not been successfully reproduced in the rat (Dallman et al, 1967).

ACKNOWLEDGMENTS

We thank Mr Robert Spirito and Mrs Evelyn Manies for technical assistance. This investigation was supported by Grant No. AM 13897, National Institutes of Health, Bethesda, Maryland.
REFERENCES


