DIETARY VITAMIN A

AND

IRON METABOLISM IN THE RAT

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DIETARY VITAMIN A AND IRON METABOLISM IN THE RAT

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STELLINGEN

1. In observationele studies bij mensen is het gevonden verband tussen vitamine-A- en ijzerstatus betwistbaar omdat geen rekening is gehouden met infektie.

2. Door gevoeligheid voor een verstoorde waterbalans bij vitamine-A-tekort, is de rat een slecht diermodel voor vitamine-A-deficiëntie-geïnduceerde anemie.
(o.a. dit proefschrift)

3. Bij studies met jonge ratten naar vitamine-A- en/of ijzerstofwisseling is het belangrijk om de voeders van de moeders te standaardiseren.
(dit proefschrift)

4. Ferrokinetische studies kunnen uitsluitsel geven over de vraag hoe vitamine-A-tekort het metabolisme van rode bloedcellen beïnvloedt.
(Hoofdstuk 7, dit proefschrift)

5. Experimentele bevestiging van het veronderstelde "retinoic acid response element" in de promoter van het erythropoietine-gen, kan impliceren dat vitamine A de synthese van rode bloedcellen beïnvloedt op het nivo van DNA-transcriptie.
(Hoofdstuk 9, dit proefschrift)

6. Het schrijven van een algemene discussie voor het proefschrift bevordert het psychologisch verwerken van promotieonderzoek.

7. De voedingskundige van de toekomst zal zich meer moeten verdiepen in de genetica.

8. It is a capital mistake to theorise before one has data; insensibly one begins to twist facts to suit theories, instead of theories to suit facts.
(Sherlock Holmes in 'A scandal in Bohemia', Sir Arthur Conan Doyle)

9. Proeven waarin veel gemeten wordt, leveren doorgaans weinig op.

10. Onderzoek is als voetbal: gebrek aan techniek is slechts ten dele te compenseren door inzicht en snelheid.

11. Een werkende vader geniet minder sociale erkenning dan een werkende moeder: niemand zal hem vragen hoe hij het toch klaarspeelt, omdat ervan uitgegaan wordt dat zijn vrouw wel thuis zal zitten bij de kinderen.

12. Het argument dat deeltijd werken ten koste gaat van de bereikbaarheid van de werknemer, hoor je vaak uit de mond van voltijds werkende mensen die zelf moeilijk bereikbaar zijn.

13. Alleen de wapenindustrie is gebaat bij oorlog.

Stellingen behorend bij het proefschrift
Dietary vitamin A and iron metabolism in the rat by Annet J.C. Roodenburg,
Wageningen, 5 maart 1996.

Aan mijn ouders
Voor Bart, Tim en Koen

Abstract

Dietary vitamin A and iron metabolism in the rat

PhD thesis by Annet J.C. Roodenburg, Wageningen Agricultural University The Netherlands. March 5, 1996

The experiments presented in this thesis were carried out to simulate the interrelationship between vitamin A and iron metabolism, which has been illustrated in human studies in third world countries and to elucidate underlying mechanisms.

Effects of manipulation with dietary vitamin A on vitamin A and iron status were studied in rats. Recurrent effects on iron metabolism with moderate vitamin A deficiency were: iron accumulation in spleen and bone; reduction in total iron-binding capacity, a measure of transferrin and reduced total liver iron, caused by a reduction in liver weight. These effects were reversed by vitamin A supplementation for 10-12 days. Mild vitamin A deficiency produced anaemia followed by increased iron absorption. When vitamin A status further deteriorated, haemoglobin concentrations rose, due to haemoconcentration. Rats with chronic vitamin A deficiency may be less affected by haemoconcentration. But there was no anaemia in mature female rats with stable low plasma retinol levels.

As to the mechanism by which vitamin A might influence iron metabolism, we have speculated that with vitamin A deficiency, blood cell synthesis is impaired, leading to increased destruction of malformed red blood cells and increased iron stores in the macrophages of bone marrow and spleen. In addition synthesis of transferrin might be impaired, possibly at the level of transferrin glycosylation. These hypotheses could not be confirmed by measuring transferrin glycosylation and indicators of red blood cell synthesis and breakdown in rats fed diets deficient in vitamin A.

Alternatively, transferrin synthesis might be regulated by vitamin A at the level of gene transcription. With the use of a gene bank, candidate retinoic acid response elements for transferrin and erythropoietin were located. However, it was not possible to confirm a role for vitamin A in transferrin gene transcription by using cultured liver cells incubated with all-*trans* and 9-*cis* retinoic acid.

Finally, evaluating the rat as animal model it can be deducted from these studies that for optimal standardization of experiments with young growing rats involving nutrients such as vitamin A and iron, diets fed to their dams should also be defined and controlled.

Contents

CHAPTER 1

General introduction

BACKGROUND

Nutritional anaemia is the most prevalent nutritional disease in the world. Not only iron deficiency causes anaemia, but also deficiencies of vitamin B_{12}, folate and vitamin A. Early evidence that vitamin A can contribute to nutritional anaemia comes from studies in both man and animals.

Studies in man

Blackfan and Wolbach reviewed a collection of 13 case studies of children suspected of vitamin A deficiency. In these children anaemia and haemosiderin in spleen was observed [1]. Induction of experimental vitamin A deficiency in 10 human volunteers produced reduced blood haemoglobin levels after a period of 188 days. Simultaneously, red blood cells were observed that were abnormal in shape (poikilocytes) or size (anisocytes) [2]. In a similar experiment with 8 volunteers, vitamin A deficiency appeared after 357 to 771 days of consuming diets low in vitamin A. The reduction in plasma retinol was accompanied by a reduction in blood haemoglobin. The anaemia did not respond to iron treatment, but was reversed when ß-carotene (pro-vitamin A) was added to the diet [3].

More recent evidence has come from studies carried out in children and women in third world countries in areas where vitamin A deficiency is endemic. Observational studies [3-9] showed that low vitamin A status was associated with impaired iron status, whereas intervention studies indicated that supplementation with vitamin A improved iron status [4,6,7,10-13].

Studies in animals

The observed interrelation between vitamin A and iron metabolism has been studied intensively in animals in order to reveal the mechanisms underlying this interaction. In 1922, it was reported that bone marrow of vitamin A deficient rats commonly shows a replacement of haematopoietic tissue by patches of gelatinous degeneration [14]. Such marked changes were not observed by Wolbach and Howe although they did see a reduction in haematopoietic tissue [15]. Mild vitamin A deficiency produced anaemia in rats [3,16,17], but when vitamin A deficiency became more severe, haemoglobin levels rose [16,17]. Many studies failed to find anaemia with vitamin A deficiency in rats, but reported increased blood

haemoglobin levels or haematocrit [18-21]. This increased concentration of blood components was associated with a disruption of water balance in severe vitamin A deficient rats, probably related to growth retardation [20,22].

Other changes with vitamin A deficiency in rats are poikilocytosis [17,19], reduced plasma iron concentration [17,21] and iron accumulation in liver and spleen [17,23-25]. In some studies iron absorption was increased [19,26] and incorporation of radioactive iron into red cells was reduced with vitamin A deficiency [21].

Possible mechanisms

It has been suggested that vitamin A deficiency impacts on iron metabolism by:
- impairing erythropoiesis
- increasing red cell destruction
- impairing iron mobilization

The reduced haemoglobin levels or haematocrit measured with vitamin A deficiency [3-5,7-9,16,17] and the observed alterations in red blood cell morphology [2,17,19] in both man and animals, suggests that red blood cell synthesis might be impaired when insufficient vitamin A is available. This was supported in studies showing a reduced incorporation of radioactive iron into rat red blood cells [21].

Reduced haemoglobin levels and increased iron stores in organs measured in animals [17,23-25] could be caused by increased red blood cell destruction. However, this hypothesis is not supported by the finding that red blood cell destruction was not affected when estimated using radiolabelled iron and measuring red blood cell osmotic fragility in vitamin A deficient rats [27].

Iron mobilization might be impaired in vitamin A deficiency, as is suggested by the increased iron stores observed in vitamin A deficient animals. Reduced plasma iron levels in humans and rats with low vitamin A status [5,7,9,17,21] or increased levels with vitamin A supplementation [4,6,7,10,12] support the hypothesis that vitamin A influences iron mobilization. In addition, some studies have found a positive association between vitamin A status and total iron-binding capacity, the iron transport protein, transferrin, or transferrin saturation [6,7,10].

OUTLINE OF THIS THESIS

Using the rat as animal model, the effects of vitamin A deficiency on iron metabolism have been studied. Different experimental designs were used to optimally mimic the situation described in humans. For defining the rat model changes in iron metabolism over time as caused by the development of vitamin A deficiency are described. The effects of vitamin A deficiency on iron metabolism were compared with those of iron deficiency [**chapter 2**]. In the following two studies both iron and vitamin A intake were manipulated. In these experiments the effect of supplementation of vitamin A together with iron was studied in rats with reduced vitamin A and iron status [**chapters 3 and 4**]. Different degrees of vitamin A deficiency as caused by different levels of dietary vitamin A in a two-generation experimental design and the relation with iron metabolism, is evaluated in **chapter 5**. Iron metabolism after long term various low levels of vitamin A intake was studied in female rats and evaluated in **chapter 6**. From the changes in iron metabolism measured in **chapters 2 to 6** and on the basis of literature data the hypotheses were generated. The hypotheses were tested in rat experiments [**chapters 7 and 8**] and in an *in vitro* study with human liver cells [**chapter 9**]. In **chapter 10** the main findings are summarized; the rat model is evaluated with respect to the metabolisms of vitamin A and iron. Possible mechanisms are discussed, leading to conclusions and directions for further research.

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CHAPTER 2

Comparison between time-dependent changes in iron metabolism of rats as induced by marginal deficiency of either vitamin A or iron

ABSTRACT

To compare the changes in iron metabolism during the development of vitamin A and iron deficiency, rats were fed either a control diet with sufficient iron (35 mg added iron/kg feed) and retinol (1200 retinol equivalents/kg feed), a diet without added vitamin A or a diet with sufficient vitamin A but 3.5 mg added iron/kg feed. During a period of 10 weeks, indicators of vitamin A and iron status were monitored. Neither vitamin A nor iron deficiency produced clinical signs. Iron deficiency induced an immediate fall of blood haemoglobin concentrations. Vitamin A deficiency produced a mild anaemia as the first change in iron metabolism, pointing to impaired erythropoiesis. This effect was followed by a rise in iron absorption and increased amount of iron in spleen. By the end of the study, blood haemoglobin, haematocrit, plasma iron and iron content in kidney and femur had increased above control levels, while total iron-binding capacity had decreased. We speculate that the initial anaemia was masked later by haemoconcentration. The decrease in iron mobilization, shown by lower total iron-binding capacity, and the increase in iron absorption may have caused the observed continuous rise in tissue iron concentration in rats with vitamin A deficiency. In the rats with iron deficiency, low tissue iron levels coincided with high iron absorption and high total iron-binding capacity. Thus, changes in iron metabolism with vitamin A deficiency differed from those with iron deficiency.

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INTRODUCTION

Observational and experimental studies in humans have shown that lack of vitamin A can contribute to the development of anaemia. In 1940, Wagner reported that subjects maintained on a vitamin A deficient diet for 6 months developed low haemoglobin and haematocrit levels, and concluded that haematopoiesis was impaired [1]. In later studies, vitamin A deficiency led to moderate anaemia which was refractory to iron but responsive to vitamin A [2,3]. A series of correlation studies, mostly in developing countries, has shown a positive relationship between serum retinol and haemoglobin levels in non-pregnant and non-lactating women [3], pregnant women [4] and children [5-8]. In addition, intervention studies in children [5,9-11] and in adults [12] have shown that supplementation with vitamin A resulted in increased haemoglobin levels.

Work to elucidate the mechanisms underlying the development of anaemia as induced by vitamin A deficiency, will have to involve the use of laboratory animals. However, experiments with laboratory animals were at first confusing [13]. As reported in 1926 by Koessler et al., anaemia early in vitamin A deficiency in rats is followed by increased haemoglobin levels and haematocrit as the severity of vitamin A deficiency developed [14]. This can be explained by water loss as the vitamin A deficiency proceeds which leads to haemoconcentration [14-16]. At the present time, little is known about the effects of vitamin A deficiency on iron metabolism in rats although it would appear that in vitamin A deficiency, iron absorption is increased [17,18], liver iron is raised [18-20], and the amount of iron in bone is lowered [18]. It is not clear how these changes in iron metabolism are interrelated.

A time-course study on the changes in various aspects of iron metabolism which take place during the progression of vitamin A deficiency may allow us to distinguish between primary and secondary features of the altered iron metabolism. Further clues to the mechanisms underlying the development of altered iron metabolism as produced by vitamin A deficiency may be obtained by a comparison with the development of the anaemia as induced by iron deficiency. Thus, we have compared the time-dependent changes in iron metabolism of weanling rats as induced by deficiency of either vitamin A or iron. We chose to produce marginal deficiencies of vitamin A and iron because this would limit interference by non-specific influences on iron metabolism such as those caused by reduced feed intake and retarded growth. In addition, marginal deficiencies of vitamin A and iron may bear more resemblance to the situation of humans in developing countries than do severe deficiencies. Infections are known to interact with both vitamin A and iron

metabolism [21] while animals deficient in vitamin A or iron have increased sensitivity to infectious agents [22,23]. In the above-mentioned rat studies on vitamin A deficiency and iron metabolism, no information about the infectious status was provided. Thus, during the course of the present study we regularly checked the differential white blood cell counts and have shown that there were no signs of infection.

MATERIALS AND METHODS

This study was approved and supervised by the animal welfare officer of Wageningen Agricultural University.

Animals, housing and diets

Male Wistar rats (Cpb:WU), derived from a commercial breeder (Harlan CPB, Zeist, The Netherlands), were used. On arrival, the rats, aged 3 weeks, were housed in groups of six animals in wire-topped, polycarbonate cages ($34.5 \times 22.5 \times 16$ cm) with a layer of sawdust as bedding. During the pre-experimental period of 2 weeks, all rats were fed the control diet containing adequate amounts of vitamin A (1200 retinol equivalents/kg feed) and iron (35 mg added iron/kg feed). The diet (*Table 1*) was formulated according to the nutrient requirements of rats [24]. After the pre-experimental period (day 0), the rats were divided into three groups of 96 rats each. The rats were housed in groups of four animals in stainless steel cages with wire mesh bases ($30 \times 42 \times 19$ cm). Mean body weights of the rats in the three groups were similar, and so were mean body weights per cage. One group continued to receive the control diet and the other groups were transferred either to a diet without added vitamin A, or to an iron-deficient diet containing 3.5 mg added iron/kg feed (*Table 1*). Analysed iron concentrations of the diets were as follows (mg/kg feed): control diet, 38; vitamin A deficient diet, 38; iron deficient diet, 10. The purified diets were in powdered form and stored at 4°C until fed. The animals had free access to feed and demineralised water. Feed intakes, corrected for feed spillage, were measured per cage twice weekly, and individual body weights once a week. A regimen of controlled light/dark cycle (light on: 0600-1800 h), temperature (20-22°C) and relative humidity (50-60%) was maintained in the animal room.

Table 1 Composition of the diets

Ingredients	Control diet	Vitamin A deficient diet	Iron deficient diet
Casein (g)	151	151	151
Maize oil (g)	25	25	25
Coconut fat (g)	25	25	25
Glucose (g)	709.2	709.2	709.2
Cellulose (g)	30	30	30
$CaCO_3$ (g)	12.4	12.4	12.4
$NaH_2PO_4.2H_2O$ (g)	15.1	15.1	15.1
$MgCO_3$ (g)	1.4	1.4	1.4
KCl (g)	1.0	1.0	1.0
$KHCO_3$ (g)	7.7	7.7	7.7
$FeSO_4.7H_2O$ (mg)	174	174	17.4
Vitamin A preparation† (mg)	8	-	8
Mineral premix‡ (g)	10	10	10
Vitamin premix§ (g)	12	12	12

† Rovimix A 500®, 150 retinol equivalents/mg (F. Hoffmann-La Roche & Co. Ltd, Basle, Switzerland), consisting of retinyl acetate and retinyl palmitate; of this preparation 1200 retinol equivalents/kg feed was added as indicated.

‡ The mineral premix consisted of (mg): MnO_2, 79; $ZnSO_4.H_2O$, 33; $NiSO_4.6H_2O$, 13; NaF, 2; KI, 0.2; $CuSO_4.5H_2O$, 15.7; $Na_2SeO_3.5H_2O$, 0.3; $CrCl_3.6H_2O$, 1.5; $SnCl_2.2H_2O$, 1.9; NH_4VO_3, 0.2; maize meal, 9853.2.

§ The vitamin premix consisted of (mg): thiamin, 4; riboflavin, 3; niacin, 20; D,L-calcium pantothenate, 17.8; pyridoxine, 6; cyanocobalamin, 50; choline chloride, 2000; folic acid, 1; biotin, 2; menadione, 0.05; D,L-α tocopheryl acetate, 60; cholecalciferol, 2 (1000 IU); maize meal, 9834.15.

Collection of samples

On day 0, 12 animals were killed. After 1, 2, 4, 6, 8 and 10 weeks, 12 animals from three cages in each dietary group were killed. For a period of seven days prior to being killed, the animals were housed individually in metabolism cages ($314cm^2 \times 12$ cm). Over the last four days of this period, feed and water intake were measured. Faeces and urine of each rat were collected and stored at -20°C until analysis. Between 09.00 and 11.00 h, heparinized blood was obtained from the non-fasting rats by orbital puncture while they were under diethyl ether anaesthesia. The blood was stored at 0°C for haematological examination and differential white blood cell counting on the same day. The plasma collected was

stored at -20°C until analysis, except for 250 μl which was stored at -80°C prior to analysis of retinol. Immediately after bleeding, the anaesthetized rats were decapitated. The left kidney, liver, spleen and both hindlegs were removed and stored at -20°C until analysis. Organs were weighed before storage. After thawing and prior to ashing, spleen and liver had been washed with saline and the femur was rid of adhering tissue.

Blood volume determination

At 4 and 8 weeks of the experimental period, blood volume was determined in 9-12 animals of each dietary group using a modified, Evans Blue dye dilution method [25,26]. After the induction of anaesthesia with an intramuscular injection of 0.3 ml of Hypnorm (10 mg fluanison and 0.315 mg fentanylcitrate/ml, Janssen Pharmaceutics, Tilburg, The Netherlands), about 0.6 ml of Evans Blue solution (3 mg/ml of saline) was injected into the lateral tail vein of the animals. The exact volume of dye injected was determined by weighing the syringe before and after injection. Complete mixing of the dye in the circulation occurs within 5 min [25], and blood samples were taken without anticoagulant after 7, 10, 15 and 22 min by orbital puncture. The volume of the first three samples was 0.25 ml and that of the last sample was 0.7 ml, part of which was collected in a heparinized vial for the determination of haematocrit. Animals were killed by asphyxiation with carbon dioxide after blood samples had been taken. After allowing to stand for at least 10 min, the blood samples were centrifuged (1580 g, 10 min), and the serum obtained was diluted 50 fold, and extinction was measured at 620 nm. Standards with a known concentration of dye in 50-fold diluted rat serum were used for calibration. In order to obtain samples for determination of the background extinction of serum, a similar experiment was carried out with 2 rats in which saline without Evans Blue was injected. Blood volume was calculated from the zero time value, assuming exponential decay of the plasma dye concentration and correcting for the haematocrit. The method was checked by measuring blood volume in 7 animals after an average blood sample of 1.55 ml/100 g body weight had been taken. In these animals, blood volume was found to be 5.16 ml/100 g body weight (SE 0.18), whereas in 8 non-bled animals blood volume was 6.37 ml/100 g body weight (SE 0.15). Thus, the method may underestimate blood volume by about 20%. This may not distract from the value of the method in comparing blood volumes between different groups of rats.

Chemical analyses

Haemoglobin concentration, haematocrit, red blood cell count, mean corpuscular haemoglobin concentration and mean cell volume were analysed with a blood cell counter (Model K-1000, Sysmex, IJsselstein, The Netherlands). Differential counting of white blood cells was done in blood smears.

Plasma iron concentrations and total iron-binding capacity were determined spectrophotometrically using a test kit (Roche Nederland, Mijdrecht, The Netherlands). Iron was measured by flame atomic absorption spectrometry following wet ashing with nitric acid for feed and liver samples and dry ashing for faeces, spleen, the left kidney and femur. From each liver, standardized portions of the left lateral and median lobe were taken for iron determination. All analyses for each rat were carried out singularly, except for liver iron which was done in duplicate. Iron in femur was calculated as the mean of values for the left and right femur. Absorption of iron was calculated as iron ingested minus that excreted in faeces and expressed as percentage of intake. Iron intake and excretion were measured over 4-day periods. Negligible amounts of iron were found in urine, and the data are thus not given.

Plasma and liver retinol was measured by reversed phase high performance liquid chromatography (HPLC). Plasma (100 μl) was mixed vigorously with ethanol (90% v/v, 400 μL), and after centrifuging (1580 g, 10 min) retinol was determined directly in the supernatant and calibrated against solutions of retinol in ethanol (72%, v/v). Serum pools with retinol concentrations of 0.63 μmol/L and 2.16 μmol/L were used as external controls. Of the two control levels, the between-run variation was 9% (low level) and 4% (high level). The within-run variation was 2% (both levels). The combined variation of retinol determination of the two control levels was 6% and 4% (coefficient of variation), respectively. The particulars of the HPLC system used were as follows: injection volume, 50 μl; pre-column, 10x3 mm stainless steel packed with Chromguard reversed phase (Chrompack, Middelburg, The Netherlands); column, 100x3 mm stainless steel packed with Spherisorb-ODS (5μm) cartridge (Chrompack); isocratic pump (Spectra Physics, San Jose CA, USA); UV/vis detector (Perkin Elmer, Norwalk CO, USA) with wavelength of 325 nm; mobile phase, methanol:water (90:10, v/v); flow rate, 0.4 ml/min; run time, 5 min; retention time, 3.5 min.

Liver total retinol was determined after saponification and extraction. 200 μl liver homogenate (liver:demineralised water, 1:5, w/v) was digested by heating at 100°C for 15 min in 1500 μl of 0.7 mol/L KOH in 50% ethanol (containing 6 g/L pyrogallol) and after cooling, the mixture was extracted twice with 4 ml of

hexane. Standards with retinol acetate in ethanol absolute were processed identically and used for calibration. The upper layer of hexane extract was removed after centrifugation (1580 g, 6 min), and retinol was determined using the HPLC method described above, except for the following conditions: injection volume, 20 μl; mobile phase, methanol:water (95:5, v/v); flow rate, 0.4 ml/min; run time, 3 min; retention time, 1.6 min. Recovery was determined by adding known amounts of retinol acetate to homogenates prior to digestion; it was 90-95%. A pooled liver homogenate containing 98 μmol retinol/L was used as an external control. The between-run variation was 8% and the within-run variation was 2%. The combined variation of retinol determination was 6% (coefficient of variation).

Statistical analysis

Groups fed the vitamin A deficient or iron deficient diet were compared with the control group. A new variable was calculated, combining time and feed effects in a matrix, and used for one-way analysis of variance. Contrasts with pooled variances were used for comparison of group means, after checking for normality with Kolmogorov-Smirnov goodness of fit tests. All variables were distributed normally except for iron in spleen and retinol in liver, which were transformed logarithmically before statistical testing. A pre-set p value of 5% was used.

RESULTS

Feed intake and body weight

In rats given the vitamin A deficient diet as compared to control rats, feed intake (*Figure 1b*) started to fall after 8 weeks ($p = 0.016$), and was further reduced after 10 weeks ($p < 0.001$). Feed intake when expressed per 100 g body weight was not depressed (not shown). Water intake in vitamin A deficient rats was decreased at week 8 ($p = 0.032$) and week 10 ($p < 0.001$) (*Figure 1d*). Body weight (*Figure 1a*) was decreased in the vitamin A deficient animals after 10 weeks ($p = 0.005$). Liver weight was decreased by vitamin A deficiency (week 6, $p < 0.001$; week 8 and 10, $p < 0.001$) (*Figure 1c*). The iron deficient diet had no effect on body weight and feed intake (*Figures 1a, 1b*), but reduced liver weight from week 2 ($p = 0.013$) until the end of the study ($p < 0.001$) (*Figure 1c*).

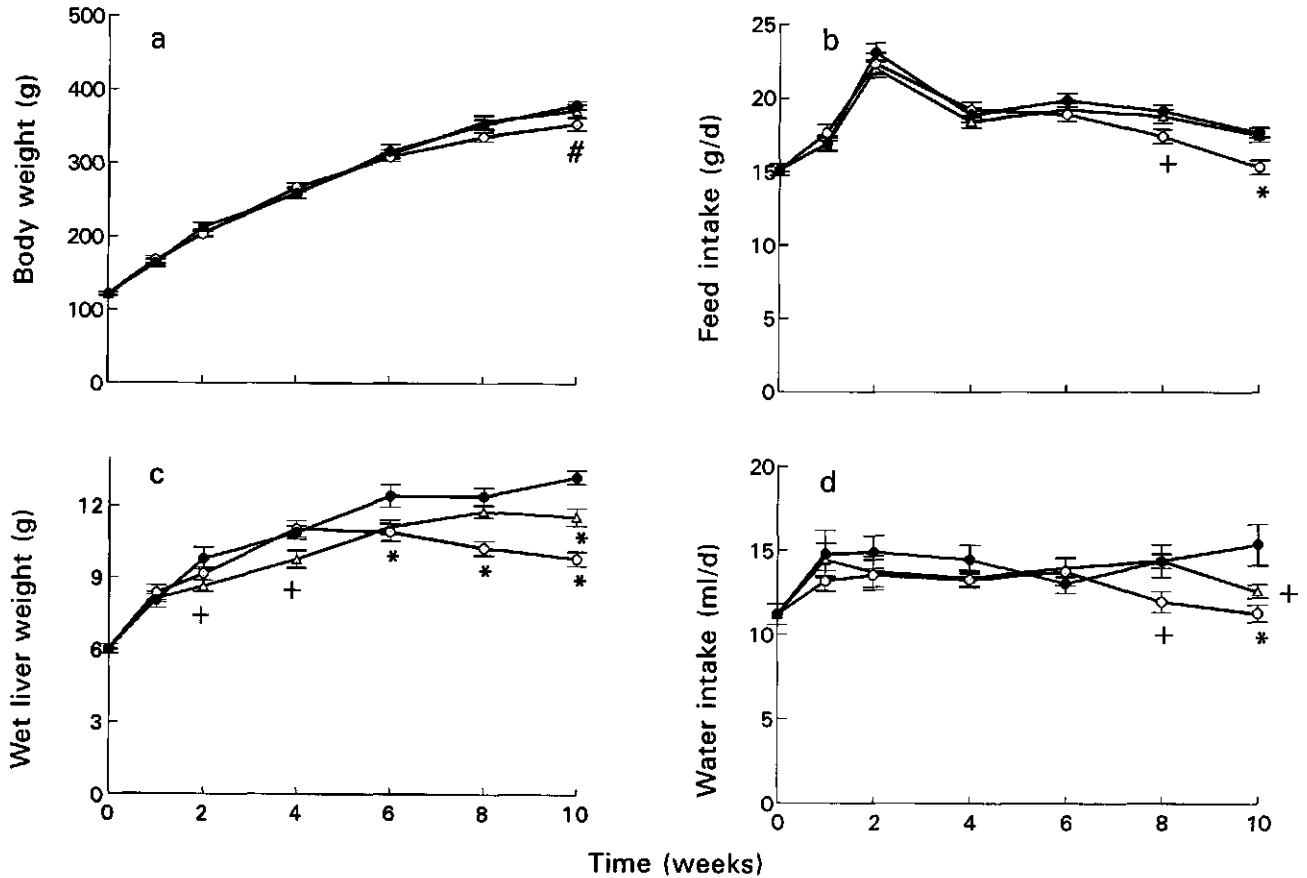

FIGURE 1 Time course of body weight (panel a), feed intake (panel b), wet liver weight (panel c) and water intake (panel d) in rats fed either the control diet (●), vitamin A deficient diet (○) or iron deficient diet (△). Values represent means for 12 rats, with standard errors (SE) indicated as error bars. Differences between vitamin A deficient or iron deficient and control groups: +, $p<0.05$; #, $p<0.01$; *, $p<0.001$.

Blood volume

Blood volume, expressed as ml/100 g body weight, measured at week 4 and 8 was neither affected by the vitamin A deficient diet nor by the iron deficient diet (*Table 2*). There was a decrease in blood volume with age, which has been shown earlier [25].

Table 2 Blood volume (ml/100 g body weight) in rats fed the experimental diets.

Period of experiment	Control diet		Vitamin A deficient diet		Iron deficient diet	
	Mean	SE	Mean	SE	Mean	SE
Week 4	8.84	0.27	8.60	0.18	8.24	0.24
Week 8	7.04	0.21	7.06	0.16	6.55	0.15

Values represent means and their standard errors for 9-12 rats per group.

White blood cell counts

In the course of the experiment, the relative numbers of white blood cells were as follows. Granulocytes (basophils, 0%; eosinophils, 0-6%; bandcells, 0-2% and polymorphonuclear cells, 1-48%) lymphocytes, 52-99% and monocytes, 0-1%. There were no systematic differences between the dietary groups. Differential counts of all animals were within the normal range [27]. Thus, no signs of infection were detected.

Vitamin A status

Total retinol in liver was decreased after 1 week of giving the vitamin A deficient diet ($p<0.001$) (*Figure 2b*). After 8 weeks, retinol in liver was not detectable. Plasma retinol concentration in vitamin A deficient rats declined steadily as from week 2 ($p<0.001$) (*Figure 2a*). In the iron-deficient group, plasma retinol was reduced from week 2 ($p<0.001$) and settled at a level which was about 10%

lower than that of the control group. Thus, it seems that iron deficiency affected vitamin A metabolism because it lowered plasma retinol concentrations as was also found by Amine et al. [17]. However at least after 8 weeks, this effect may be explained by dilution due to an increase in plasma volume at the expense of blood cell volume (see below).

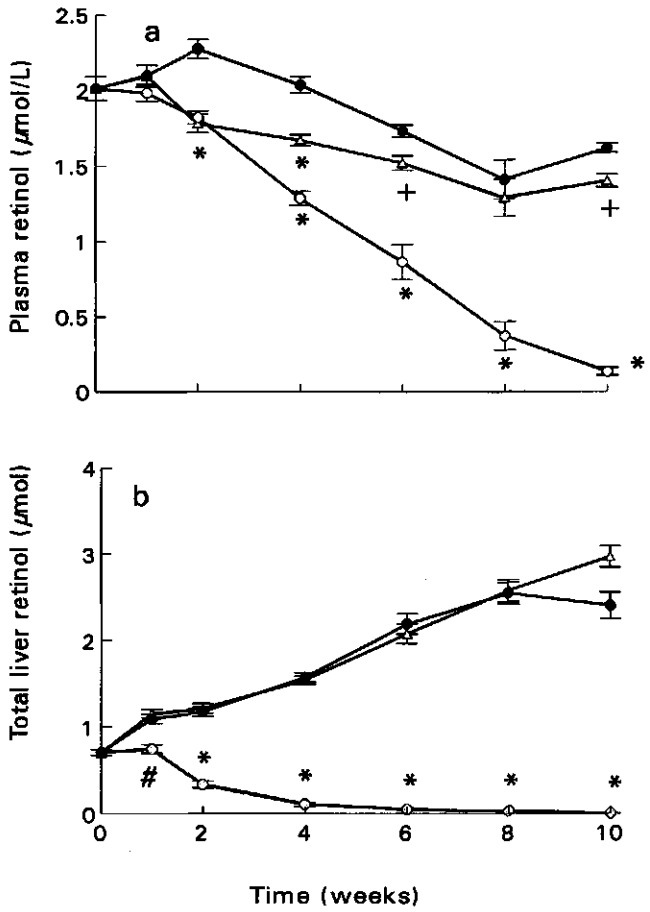

FIGURE 2 Time course of plasma retinol concentrations (panel a) and total liver retinol (panel b) in rats fed either the control diet (●), vitamin A deficient diet (○) or iron deficient diet (△). Values represent means for 12 rats, with SE indicated as error bars. Differences between vitamin A deficient or iron deficient and control groups: +, $p<0.05$; #, $p<0.01$; *, $p<0.001$.

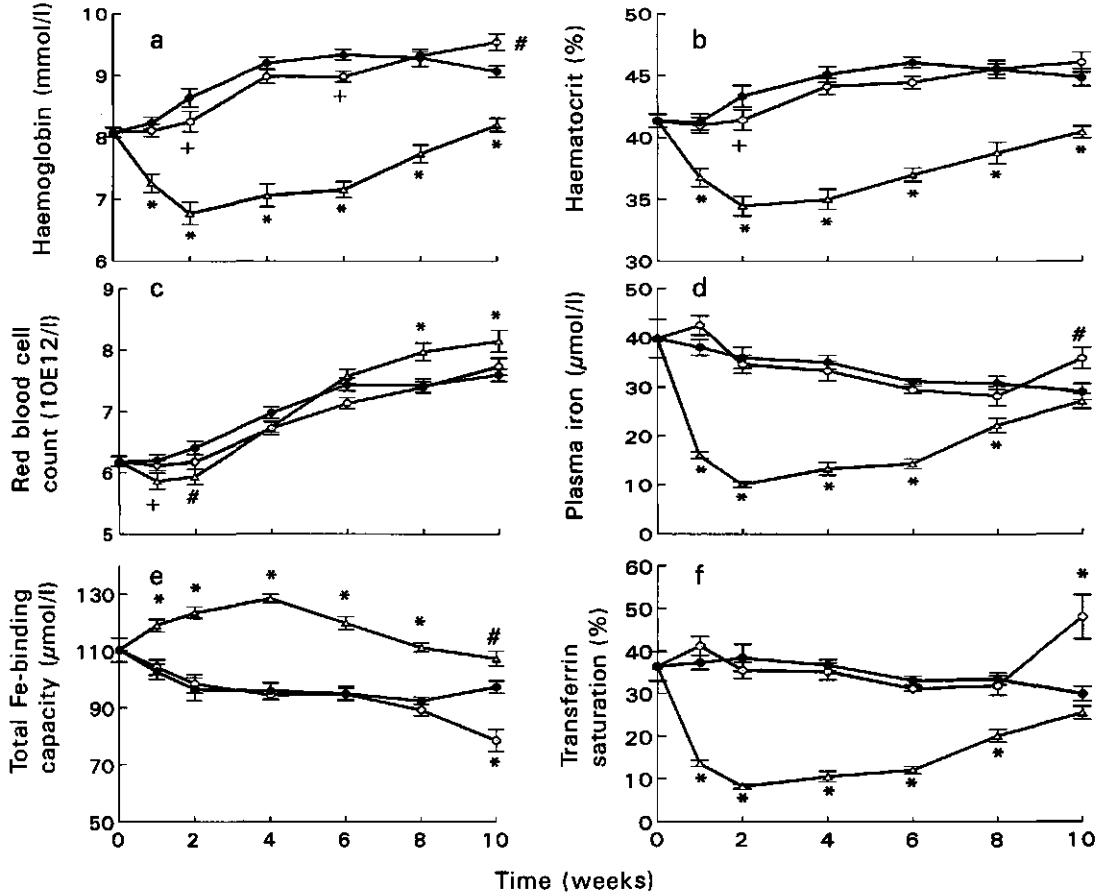

FIGURE 3 Time course of haemoglobin concentrations (panel a), haematocrit (panel b), red blood cell count (panel c), iron in plasma (panel d), total iron-binding capacity (panel e) and percentage transferrin saturation (panel f) in rats fed either the control diet (●), vitamin A deficient diet (○) or iron deficient diet (△). Values represent means for 12 rats, with SE indicated as error bars. Differences between vitamin A deficient or iron deficient and control groups: +, $p<0.05$; #, $p<0.01$; *, $p<0.001$.

Haematology

Effects of the vitamin A deficient diet on haematological parameters were time dependent. Haemoglobin concentrations and haematocrit (*Figures 3a,3b*) were below the control level from week 2 ($p = 0.030$ and $p = 0.046$) until week 6 ($p = 0.045$ and $p > 0.05$, not significant). Thus, there was a mild anaemia in vitamin A deficient rats. However after 6 weeks haemoglobin and haematocrit rose and reached a level above that of the control group at 10 weeks ($p = 0.008$ and $p > 0.05$, not significant). No clear effects of vitamin A deficiency on red blood cell count (*Figure 3c*), mean cell volume and mean corpuscular haemoglobin concentration were found (not shown).

Anaemia was induced by iron deficiency. After one week, haemoglobin and haematocrit were significantly reduced (*Figures 3a,3b*). Haemoglobin concentrations and haematocrit were lowest at week 2 and thereafter levels gradually increased, but remained lower than those of the control group ($p < 0.001$). Red blood cell count in iron-deficient rats was reduced during the first two weeks ($p = 0.032$ and $p = 0.003$), but was increased by the end of the study ($p < 0.001$) (*Figure 3c*). Mean cell volume was reduced during the whole experiment ($p < 0.001$), but mean corpuscular haemoglobin concentration was not affected (not shown).

Plasma iron and total iron-binding capacity

In the vitamin A deficient group, plasma iron levels were not affected until week 10 when there was a rise ($p = 0.006$). Likewise, transferrin saturation suddenly rose at week 10 ($p < 0.001$) (*Figures 3d,3f*). Total iron-binding capacity remained unchanged in vitamin A deficient rats until 10 weeks when it dropped ($p < 0.001$) (*Figure 3e*).

The iron deficient diet produced an immediate decrease in plasma iron and transferrin saturation and an increase of total iron-binding capacity within one week. Both plasma iron and transferrin saturation reached their lowest value after 2 weeks and then rose to control values at the end of the experimental period. Total iron-binding capacity in iron deficient rats peaked after 4 weeks and then dropped, but remained higher than control values until the end of the experiment ($p = 0.008$).

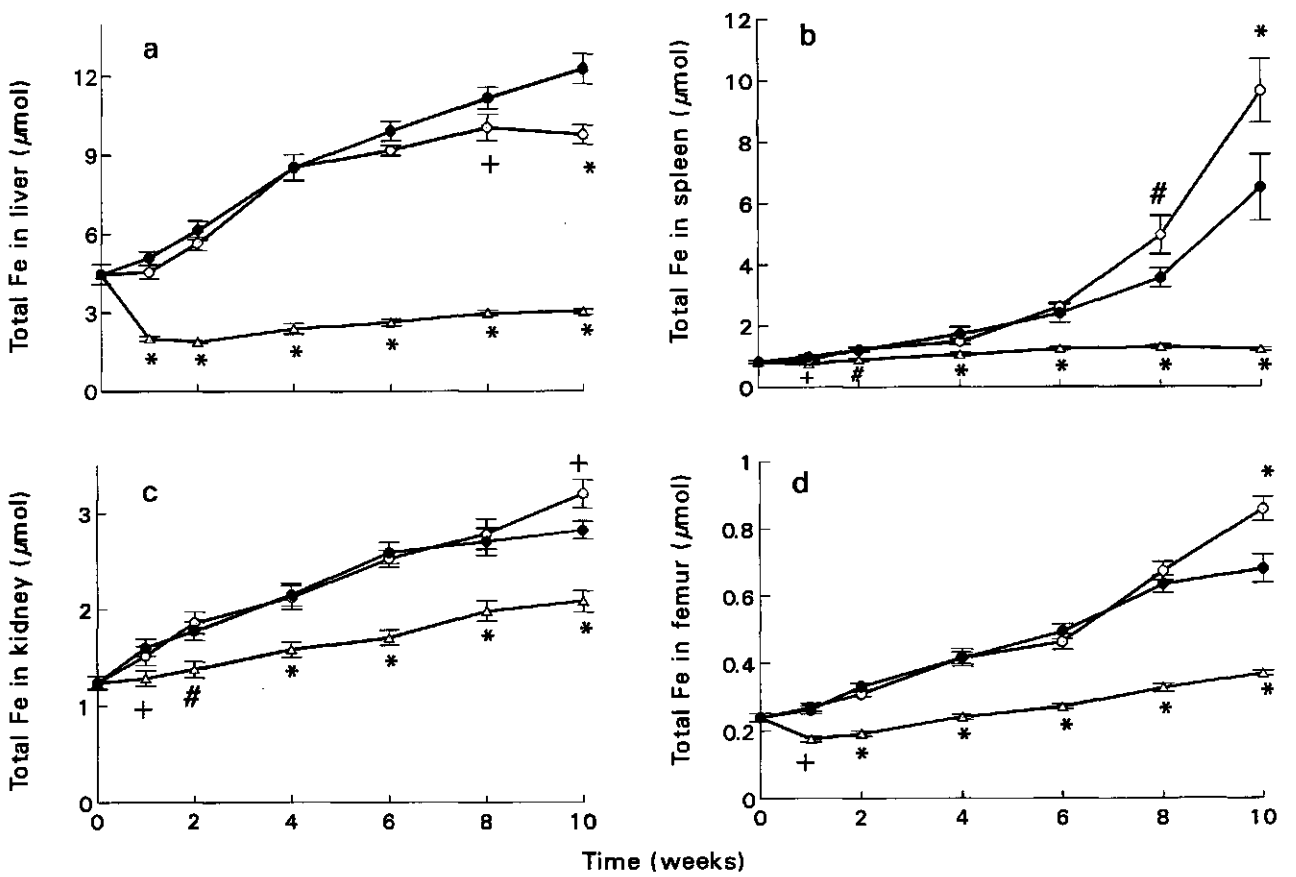

FIGURE 4 Time course of total iron in liver (panel a), spleen (panel b), kidney (panel c) and femur (panel d) in rats fed either the control diet (●), vitamin A deficient diet (○) or iron deficient diet (△). Values are expressed as μmol per organ and represent means for 12 rats, with SE indicated as error bars. Differences between vitamin A deficient or iron deficient and control groups: +, $p<0.05$; #, $p<0.01$; *, $p<0.001$.

Iron in organs

After feeding the vitamin A deficient diet, total iron in liver decreased below control levels at week 8 ($p = 0.020$) and dropped further after week 10 ($p < 0.001$) (*Figure 4a*). This effect can be explained mainly by a reduction of liver weight (*Figure 1c*) because hepatic iron concentrations after 10 weeks were not significantly affected by vitamin A deficiency (4.36 (SE 0.15) μmol/g dry weight) versus control treatment (4.05 (SE 0.20) μmol/g dry weight). The weights of spleen and kidney were not affected by the vitamin A deficient diet (not shown). Total amounts of iron in spleen ($p < 0.001$), kidney ($p = 0.012$) and femur ($p < 0.001$) in vitamin A deficient rats had increased above the control level after 10 weeks. Total iron content of spleen in vitamin A deficient animals was already significantly raised after 8 weeks ($p = 0.011$) (*Figures 4b-4d*). This can be explained by a rise in splenic iron concentration in the vitamin A deficient group (38.7 (SE 4.5) μmol/g dry weight) versus the control group (27.4 (SE 2.3) μmol/g dry weight) at week 8 ($p = 0.001$). After 10 weeks, iron concentration of both spleen and femur were higher in the vitamin A deficient group (72.8 (SE 8.41) and 1.78 (SE 0.09) μmol/g dry weight, respectively) versus the control group (46.7 (SE 7.27) and 1.41 (SE 0.09) μmol/g dry weight, respectively) ($p < 0.001$). In rats given the iron deficient diet, total iron content of liver, spleen, kidney and femur were significantly decreased below control levels after one week and remained low until the end of the experiment (*Figures 4a-4d*). This is also demonstrated by the lower iron concentrations in these organs after 10 weeks, when compared to the control group ($p < 0.001$) (μmol/g dry weight): liver 1.14 (SE 0.02), spleen 9.06 (SE 0.31), kidney 4.45 (SE 0.21) and femur 0.81 (SE 0.03). For comparison, kidney iron concentration in the control group, at week 10 was 5.94 (SE 0.21) μmol/g dry weight.

Iron absorption

From weeks 2 to 6 of the experiment, the percentage of apparent iron absorption fell in the control rats and then remained more or less stable. After 8 ($p = 0.003$) and 10 ($p < 0.001$) weeks, apparent iron absorption was raised in vitamin A deficient animals (*Figure 5*). In iron deficient rats, apparent iron absorption was essentially constant during the entire experimental period and was significantly higher ($p < 0.001$) than in control rats from 4 weeks (*Figure 5*).

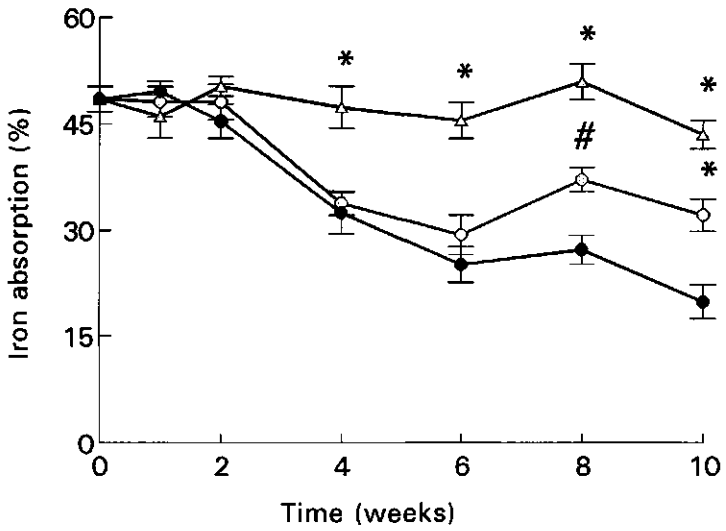

FIGURE 5 Time course of iron absorption, in rats fed either the control diet (●), vitamin A deficient diet (○) or iron deficient diet (△). Values represent means for 12 rats, with SE indicated as error bars. Differences between vitamin A deficient or iron deficient and control groups: #, $p<0.01$; *, $p<0.001$.

DISCUSSION

This study shows the time course of changes in iron metabolism during the development of vitamin A deficiency in rats. No clinical signs nor evidence of subclinical infection were observed. In the deficient rats, it was only after 6 to 8 weeks that body weight and liver weight, feed and water intake declined. Blood volume, expressed as ml/100 g body weight, was not affected by feeding the vitamin A deficient diet for at least 8 weeks. However, in the course of the experiment, plasma retinol concentrations fell steadily and liver retinol stores were depleted after 4 weeks. Thus, the rats given the diet without added vitamin A could be considered marginally deficient in vitamin A.

When making measurements on iron metabolism during the development of marginal vitamin A deficiency, it is possible to distinguish between primary and secondary features of the cascade of changes in iron metabolism. In the vitamin A deficient rats, haemoglobin concentrations and haematocrit were slightly, but consistently lower than control levels from week 2 to week 6 of the experiment. This effect of vitamin A deficiency has been reported earlier [3,18,19,28]. After

6 weeks, apparent iron absorption was somewhat higher in the vitamin A deficient than in the control rats; this effect became more pronounced later. The observed increase in iron absorption as induced by vitamin A deficiency agrees with earlier work [17,18]. After 8 weeks, vitamin A deficiency was associated with raised iron concentrations in liver and spleen. In other studies vitamin A deficiency has been shown to produce an increase in liver iron concentrations [19,20,28], but liver weights were not reported so that the effect on liver iron mass cannot be ascertained. As found in this study, Sijtsma et al. [29] also observed that vitamin A deficiency reduced the total iron content of liver. Unlike liver weight, vitamin A deficiency did not affect spleen weight. Thus, the increased splenic iron concentration, which was also found by Mejía et al. [19], resulted in raised total iron content of spleen.

When rats had been given the vitamin A deficient diet for 10 weeks, the amounts of iron in kidney and femur were higher than in control animals, total iron-binding capacity was reduced and transferrin saturation was elevated. As far as we know, these effects are reported here for the first time. After 10 weeks, haemoglobin concentration and plasma iron concentration were all increased above control levels. These effects, which have been reported previously [16,17,19,30] may be explained by haemoconcentration [15,16,30,31]. We did not measure blood volume after 10 weeks, but at that time point, water intake was significantly reduced in the vitamin A deficient rats, which could have caused dehydration [3].

Thus, four successive stages of changes in iron metabolism may be discerned during the development of vitamin A deficiency. The first stage is characterized by a slight fall in haemoglobin concentration and haematocrit. During the second stage, apparent iron absorption is enhanced. Then, iron concentrations in liver and spleen are raised. During the fourth stage, iron in kidney and femur is increased and total iron-binding capacity of plasma is decreased. As mentioned, many of these changes have been reported earlier, but this study has now identified the time course and the sequence of the changes.

The primary feature of altered iron metabolism in vitamin A deficiency probably is impaired erythropoiesis. Indeed, Mejía et al. [16] found in vitamin A deficient rats that the rate of incorporation of intravenously administered ^{59}Fe into red blood cells was depressed. The impaired erythropoiesis may stimulate, by some unknown mechanism, the absorption of iron. The resulting enhanced influx of dietary iron could have produced the higher iron concentrations in liver and spleen followed by those in kidney and femur. Since the greater efficiency of iron absorption may not overcome the depressed erythropoiesis as induced by vitamin A deficiency, iron absorption remains enhanced and iron amounts in spleen, and

possibly also in kidney and femur, continue to rise. The excessive iron status thus developed, may inhibit transferrin synthesis as reflected by the decrease in total iron-binding capacity. Iron loading, which also leads to excessive iron status, has been shown to reduce total iron-binding capacity [32]. The observed decrease in total iron-binding capacity in vitamin A deficient rats agrees with some studies with humans showing a positive relationship between plasma retinol concentration and either total iron-binding capacity or transferrin in plasma [5,11], but other studies did not find such relationships [4,9]. The reduced plasma concentration of transferrin in vitamin A deficient rats might contribute to iron accumulation in organs by reducing iron mobilization.

It is clear that the slight lowering of haemoglobin concentration in early vitamin A deficiency has a metabolic basis different from the marked fall in haemoglobin seen in the iron deficient rats. The iron deficiency progressed very rapidly and thus primary and secondary features are difficult to untangle. It appears however, that the initial reduction in haemoglobin concentration was followed by an increase in the efficiency of iron absorption. This partly corrected haemoglobin, plasma iron concentrations, transferrin saturation and haematocrit, during the course of the experiment, but was not sufficient to raise tissue iron concentrations to control levels. In vitamin A deficiency, in contrast to iron deficiency, the increase in apparent iron absorption was associated with increased tissue iron concentrations.

In summary, we have provided evidence that the anaemia often seen in marginal vitamin A deficiency in both man [3-8] and rats [3,16,18] is not caused by impaired intestinal absorption of dietary iron and thus has features different from those in iron deficiency. In fact, vitamin A deficiency is associated with adequate iron status as based on tissue iron concentrations, which is caused by enhanced iron absorption. This would suggest that the primary derailment of iron metabolism in marginal vitamin A deficiency probably is impaired erythropoiesis.

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CHAPTER 3

Vitamin A status affects the efficacy of iron repletion in rats with mild iron deficiency

ABSTRACT

In populations with vitamin A deficiency, vitamin A administration in addition to supplemental iron has been shown to further improve blood indicators of iron status. To obtain clues to associated changes at the level of organ indicators of iron status, we have attempted to mimic previous human studies in a rat model. The influence of vitamin A on selected indices of iron metabolism was studied during iron repletion of rats with mild iron deficiency combined with either vitamin A deficiency or normal vitamin A status. In the vitamin A deficient rats, but not in those with normal vitamin A status, the administration of vitamin A raised body weight and liver weight. Vitamin A administration also raised total iron-binding capacity and plasma iron concentrations but depressed iron concentrations in liver, kidney, spleen and bone in the vitamin A deficient rats. We conclude that vitamin A administration to rats with both vitamin A and iron deficiency enhances the utilization of iron.

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INTRODUCTION

Studies with children [1-4] and pregnant women [5] have shown that vitamin A deficiency is associated with impaired iron status, while vitamin A supplementation produced an increase in blood haemoglobin concentrations [2,6-10]. The mechanism by which vitamin A affects iron metabolism is poorly understood. Vitamin A deficiency in rats may impair erythropoiesis [11,12,**chapter 2**] but increased iron concentrations in liver [13-15] and spleen [12,13,**chapter 2**] may point to disturbed iron transport.

The administration of vitamin A in addition to supplemental iron has been found to enhance the response of serum iron concentration, transferrin saturation and blood haemoglobin concentration in children and pregnant women with low vitamin A and iron status [8,10]. This suggests that vitamin A improves the utilization of ingested iron and affects iron concentrations of organs. To test this hypothesis and to develop an animal model for studying the underlying mechanisms, we examined the influence of vitamin A administration on selected indices of iron metabolism during iron repletion in rats with mild iron deficiency. Because the effect of vitamin A administration may depend on the vitamin A status of the rats, we used rats with either normal or low vitamin A status.

In vitamin A supplementation studies with children, growth has been found to be enhanced [7,16] which in turn might influence the observed interrelationship between vitamin A and iron metabolism. In order to mimic the situation in children, we allowed our rats to have access to feed ad libitum so that not only feed composition but also feed intake could influence growth. Any confounding effects of growth on iron parameters were assessed by using body weight as a covariate in the statistical analysis.

METHODS

This experiment was approved and supervised by the animal welfare officer of Wageningen Agricultural University.

Animals, diets and housing

Seventy-two, three-week old male Wistar rats (Cpb:WU) were purchased from a commercial breeder (Harlan CPB, Zeist, The Netherlands). They were divided

into two groups of 36, matched for body weight. The experimental design is illustrated in *Figure 1*. During the first experimental phase of 25 days (*Figure 1*), one group was fed a diet with adequate vitamin A (1200 retinol equivalents/kg) and iron (35 mg/kg). The other group was fed a diet without vitamin A but with adequate iron. The diet adequate in vitamin A was formulated according to the nutrient requirements of rats (National Research Council) [17]. The diets were in the form of powder and stored at 4°C until used for feeding.

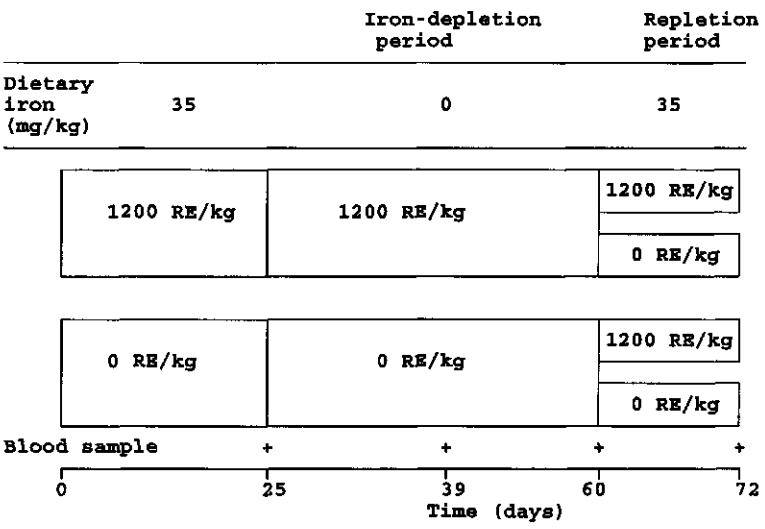

FIGURE 1 Experimental design: During the first experimental phase, two groups of 36 rats were fed different levels of dietary vitamin A. From day 25, when the rats were 45 days old until day 60, all rats were maintained on an iron-depletion diet, followed by an iron-repletion period (days 60 to 72). Simultaneously, vitamin A intake was varied so that during the repletion period there were four groups each of 18, differing in vitamin A intake during the periods. The rats were killed on day 72 for the removal of tissues. The diets used differed in vitamin A and iron content only. In the figure the amounts are indicated as retinol equivalents (RE) and mg iron/kg feed. The amount of vitamin A, added to the diets as Rovimix A 500^{R} (150 RE/mg, F. Hoffmann-La Roche & Co. Ltd, Basle, Switzerland, consisting of retinyl acetate and retinyl palmitate) was 1200 RE/kg. For the vitamin A deficient diets, no vitamin A was added. Per kg iron-sufficient diet, 174 mg $FeSO_4.7H_2O$ was added. No iron was added to the diet low in iron. All diets contained (g/kg feed): casein, 151; maize oil, 25; coconut fat, 25; glucose, 709.2; cellulose, 30; $CaCO_3$, 12.4; $NaH_2PO_4.2H_2O$, 15.1; $MgCO_3$, 1.4; KCl, 1.0; $KHCO_3$, 7.7; mineral premix (iron-free), 10; vitamin premix (vitamin A-free), 12. The mineral premix consisted of (mg): MnO_2, 79; $ZnSO_4.H_2O$, 33; $NiSO_4.6H_2O$, 13; NaF, 2; KI, 0.2; $CuSO_4.5H_2O$, 15.7; $Na_2SeO_3.5H_2O$, 0.3; $CrCl_3.6H_2O$, 1.5; $SnCl_2.2H_2O$, 1.9; NH_4VO_3, 0.2; maize meal, 9853.2. The vitamin premix consisted of (mg): thiamin, 4; riboflavin, 3; niacin, 20; D,L-calcium pantothenate, 17.8; pyridoxine, 6; cyanocobalamin, 50; choline chloride, 2000; folic acid, 1; biotin, 2; menadione, 0.05; D,L-α tocopheryl acetate, 60; cholecalciferol, 2 (1000 IU); maize meal, 9834.15.

On day 25 (*Figure 1*), when the rats were about 45 days old, a blood sample

was taken for haematological examination and analysis of plasma retinol. All animals entered the iron-depletion period and continued to receive their respective diets, except that iron was omitted (*Figure 1*). On days 39 and 60 (*Figure 1*), blood samples were taken again for haematological examination and analysis of plasma retinol.

On day 60 when the iron-repletion period started, both the vitamin A deficient and sufficient groups were divided into two groups each of 18 rats, which were matched for body weight and haemoglobin concentration. Within each group of rats with identical dietary history, half were fed an iron-sufficient diet with 1200 RE/kg and the other half were given the same diet but without added vitamin A (*Figure 1*). After a further 12 days (day 72) all animals were killed and blood and organs were collected for analysis.

All animals had free access to food and demineralized water. Body weight was measured once a week and feed intake twice weekly. The iron concentration of the diets as analyzed was 10 mg/kg for the low iron diets and 43 mg/kg for the diets with adequate iron. The rats were housed in groups of four in stainless steel cages with wire mesh bases (30x42x19 cm). A controlled 12 hours light/dark cycle (light on: 0600-1800 h), temperature (20-22°C) and relative humidity (50-60%) were maintained in the animal room.

Collection of samples

Blood was collected in heparinized vials by orbital puncture while the rats were under diethyl ether anaesthesia. The blood was stored at 0°C for hematological examination on the same day. Then, plasma was isolated by centrifugation (10 min, 1580 g) and stored at -20°C until analysis, except for a portion which was stored at -80°C prior to analysis of retinol. On day 72 (*Figure 1*), the anaesthetized rats were decapitated immediately after bleeding. The left kidney, liver, spleen and both hindlegs were removed and stored at -20°C until analysis. Organs were weighed before storage.

Chemical analyses

Haemoglobin concentration, haematocrit, red blood cell count and mean cell volume were analyzed with a blood cell counter (Model K-1000, Sysmex, IJsselstein, The Netherlands). Plasma iron concentrations and total iron-binding

capacity were determined spectrophotometrically using a test kit (Roche Nederland, Mijdrecht, The Netherlands). Iron in liver, spleen, kidney and tibia was measured by flame atomic absorption spectrometry after dry ashing of the samples. Prior to ashing, spleen and liver were washed with saline, and liver was homogenized as described elsewhere [12,**chapter 2**]. Tibia iron was calculated as the mean of left and right tibia. Plasma and liver retinol were measured by reversed phase high performance liquid chromatography (HPLC) as described previously [12,**chapter 2**].

Statistical analysis

The data for days 25 and 39 (*Figure 1*) were subjected to one-way analysis of variance with feed composition as independent variable. Contrasts with pooled variances were used for comparison of group means (Student's t-test). For comparison of group means of data for days 60 and 72, a multiple comparison test (Tukey-test) was used after demonstration of a significant diet effect using one-way analysis of variance. The data for day 72 were also subjected to a two-way analysis of variance with previous vitamin A intake (prior to day 60) and vitamin A supplementation during iron-repletion (day 60 to 72) as independent variables. The two-way analysis of variance for all iron parameters and organ weights was done without or with body weight at day 72 as a covariate. Prior to the analysis with the covariate, we checked for any interaction between the independent variables and the covariate. No significant interactions were found. When body weight as covariate contributed significantly to the explained variance, the adjusted means are also given. If the variances were not homogeneous (Cochran's-C test), the data were log-transformed prior to statistical testing. A pre-set p value of 5% was used throughout. Data were analyzed using the of SPSS/PC+ software package [18].

RESULTS

Body weight and feed intake

On days 25 and 39, there was no effect of the lack of dietary vitamin A on body weight (*Table 1*) and feed intake (not shown). On day 60, body weight (*Table 1*) was significantly reduced in the vitamin A deficient rats. Average feed intake per animal from days 53-60 was 13.9 g/d in vitamin A deficient and 17.3 g/d in the

vitamin A sufficient rats (Student's t test, $p < 0.001$; pooled SE = 0.3 g/d; n = 9 cages with 4 rats each).

Body weights were increased in the rats transferred from the vitamin A deficient diet to the diet containing vitamin A during the final 12 days of the experiment. The rats deprived of vitamin A throughout the experiment did not grow during the iron-repletion period (*Table 1*) and ate less than those in the other groups. In the vitamin A deprived rats feed intake per animal, as measured on days 65-72, was on average 12.3 g/d (n = 5 cages with 4 or 2 rats) whereas it was 18.3 g/d in the other groups (n = 15 cages with 4 or 2 rats) given vitamin A during any phase of the experiment (Student's t-test, $p < 0.001$; pooled SE = 0.2 g/d).

Vitamin A status

On days 25 and 39, plasma retinol concentration was reduced in the rats fed the diet without vitamin A (*Figure 2*). On day 60, plasma retinol concentrations were significantly reduced in the vitamin A deficient rats (*Figure 2*).

The rats transferred from the diet deficient in vitamin A to the diet containing vitamin A during the final 12 days of the experiment had increased concentrations of retinol in liver (*Table 2*) and almost normal plasma retinol concentrations (*Figure 2*). In the rats transferred from the diet sufficient in vitamin A to a deficient diet, hepatic retinol concentrations were reduced (*Table 2*). The group deprived of vitamin A throughout the experiment had, on day 72, low liver weights, vitamin A in liver less than the level of detection with the method used (*Table 2*), and very low plasma retinol concentrations (*Figure 2*).

Haematology

On days 25 and 39, there was no effect of the lack of vitamin A on haemoglobin concentration, red blood cell count and mean cell volume (*Table 1*). A small reduction in haematocrit was seen on day 25, but this had disappeared by day 39 (*Table 1*). On day 60, at the end of the iron-depletion period, haemoglobin concentration, haematocrit and red blood cell count were generally higher in the groups fed the vitamin A deficient diet before iron repletion (*Table 1*). Mean cell volume had decreased during the iron-depletion period, but there was no effect of vitamin A status (*Table 1*). After iron repletion (day 72), haemoglobin concentrations, haematocrit and red blood cell count were reduced by simultaneous

vitamin A administration in the vitamin A deficient but not in the vitamin A sufficient animals (*Table 1*). Mean cell volume was increased by vitamin A administration in the vitamin A deficient animals (*Table 1*). The effects on mean cell volume were slightly, but significantly influenced by body weight as shown by analysis of variance.

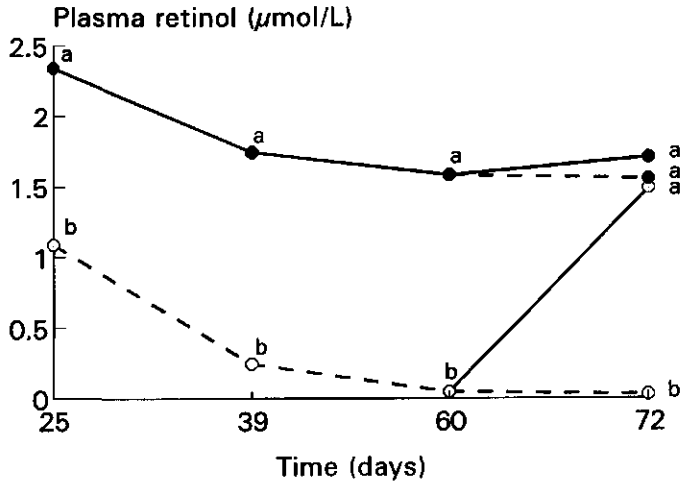

FIGURE 2 Plasma retinol concentrations throughout the experiment. The vitamin A sufficient group (●) was fed adequate vitamin A (1200 RE/kg feed) from day 0 to 60 (*Figure 1*). The vitamin A deficient group (○) received no vitamin A from day 0 to 60. From day 25 to 60 all animals were fed diets without added iron. The solid lines (——) represent groups that received adequate vitamin A (1200 RE/kg feed); the broken lines (----) correspond to the groups that received no dietary vitamin A. The number of rats per group from days 0 to 60 was 36. During the last 12 days of the experiment each of the four groups comprised 18 animals.
Statistically significant differences between groups at the same time point as based on contrasts (days 25, 39 and 60) or Tukey's multiple comparison test (day 72) are indicated by different letters ($p < 0.05$). Analysis of variance for the data from day 72 showed significant effects of body weight ($p < 0.001$) as a covariate, vitamin A intake prior to day 60 ($p < 0.001$), vitamin A administration during iron repletion ($p < 0.001$), their interaction ($p < 0.001$). The adjusted means for the levels at day 72 were 1.65, 1.50, 1.41 and 0.14 μmol/L. Values at days 60 and 72 were log-transformed prior to statistical testing.

Table 1 Body weight and haematological characteristics at the beginning of the iron-depletion period (d 25), after 14 days of iron depletion (d 39), and before (d 60) and after (d 72) iron repletion.

Dietary vitamin A, RE/kg		Body weight, g		Haemo-globin, mmol/L		Haemato-crit %		Red blood cell count, $x10^{-12}/L$		Mean cell volume, fL	
d 0-60		(d 25)	(d 39)	(d 25)	(d 39)	(d 25)	(d 39)	(d 25)	(d 39)	(d 25)	(d39)
1200		182.6[a]	243.6[a]	8.47[a]	9.07[a]	43.0[a]	46.0[a]	6.3[a]	7.2[a]	68[a]	64[a]
0		180.8[a]	234.5[a]	8.35[a]	9.21[a]	42.0[b]	46.3[a]	6.2[a]	7.4[a]	67[a]	63[a]
	Pooled SE	3.1	3.6	0.07	0.09	0.3	0.4	0.1	0.1	0.3	0.4
d 0-60	d 60-72	(d 60)	(d 72)	(d 60)	(d 72)	(d 60)	(d 72)	(d 60)	(d 72)	(d 60)	(d 72)†
1200	1200	326.4[a]	361.3[a]	8.54[a]	9.49[a]	44.2[ab]	48.6[a]	7.8[ab]	8.2[a]	57[a]	58[a] (58)
1200	0	320.8[a]	357.4[a]	8.53[a]	9.47[a]	42.9[a]	47.3[a]	7.6[a]	8.0[a]	56[a]	59[a] (59)
0	1200	275.1[b]	330.4[b]	9.09[b]	8.95[b]	45.9[b]	44.7[b]	8.1[bc]	7.6[b]	57[a]	59[a] (59)
0	0	274.1[b]	279.9[c]	9.19[b]	9.46[a]	46.2[b]	46.3[ab]	8.3[c]	8.3[a]	56[a]	56[b] (56)
	Pooled SE	6.3	7.2	0.14	0.09	0.7	0.5	0.1	0.1	0.7	0.6
Day 72: analysis of variance, p value‡											
Body weight at d 72					NS		NS		NS		0.004
Previous vitamin A feeding			<0.001		0.002		0.001		0.049		NS (NS)
Vitamin A feeding during iron-repletion			<0.001		0.006		NS		0.001		0.030 (NS)
Interaction			0.002		0.003		NS		<0.001		0.002(0.005)

Values represent means and pooled SE (n=36, d 25 and d 39; n=18, d 60 and d 72). Values in a column not sharing the same superscript letter are significantly different ($p<0.05$). † When the covariate "body weight at day 72" contributed significantly to the explained variance adjusted means are given in parentheses. ‡ Analysis of variance with previous vitamin A feeding and vitamin A feeding during iron repletion as independent variables without or with body weight at d 72 as covariate. When the covariate contributed significantly to the explained variance, the matching p values are given in parentheses. NS = not significant.

Table 2 Liver retinol levels and liver wet weight after iron repletion (d 72).

Dietary Vitamin A, RE/kg		Liver retinol concentration, μmol/g wet weight†	Liver wet weight, g	
d 0-60	d 60-72			
1200	1200	0.119[a]	12.0[a]	(11.1)‡
1200	0	0.081[b]	11.8[ab]	(11.0)
0	1200	0.015[c]	11.0[b]	(11.0)
0	0	ND [d]	7.4[c]	(9.0)
	Pooled SE	0.002	0.3	
Analysis of variance, p value*				
Body weight at d 72		NS	<0.001	
Previous vitamin A feeding		<0.001	<0.001	(<0.001)
Vitamin A feeding during iron-repletion		<0.001	<0.001	(<0.001)
Interaction		<0.001	<0.001	(<0.001)

Values represent means and pooled SEs (n = 18). Values in a column not sharing the same superscript letter are significantly different ($p < 0.05$). ND = not detectable. † Values are log-transformed prior to statistical testing, unadjusted means are given. ‡ When the covariate "body weight at d 72" contributed significantly to the explained variance adjusted means are given in parentheses. * Analysis of variance with previous vitamin A feeding and vitamin A feeding during iron repletion as independent variables without or with body weight at d 72 as covariate. When the covariate contributed significantly to the explained variance, the matching p values are given in parentheses. NS = not significant.

Total iron-binding capacity, plasma iron, transferrin saturation.

On day 72 (*Figure 1*), total iron-binding capacity and plasma iron concentration were reduced by about 20% in the animals that were deprived of vitamin A throughout the entire experiment (*Table 3*). Administration of vitamin A during iron repletion significantly raised total iron-binding capacity and plasma iron in the vitamin A deficient rats but had no effect in their vitamin A sufficient counterparts. However, total iron-binding capacity in the vitamin A deficient rats supplemented with vitamin A remained below the level seen in the vitamin A sufficient groups. After correction for body weight, the effect of vitamin A feeding on plasma iron concentrations disappeared but that on total iron-binding capacity remained statistically significant. Transferrin saturation was not affected by the dietary regimes (*Table 3*).

Table 3 Total iron-binding capacity, plasma iron and transferrin saturation after iron repletion (d 72).

Dietary Vitamin A, RE/kg		Total iron-binding capacity, μmol/L	Plasma iron, μmol/L	Transferrin saturation, %
d 0-60	d 60-72			
1200	1200	91.87[a] (90.81)†	38.11[a] (36.97)†	41.47[a]
1200	0	91.21[a] (90.29)	39.82[a] (38.84)	43.99[a]
0	1200	84.06[b] (84.13)	38.17[a] (38.25)	45.84[a]
0	0	72.39[c] (74.31)	31.35[b] (33.39)	43.27[a]
Pooled SE		1.78	1.59	2.09
Analysis of variance, p value*				
Body weight at d 72		<0.001	<0.001	NS
Previous vitamin A feeding		<0.001 (<0.001)	0.010 (NS)	NS
Vitamin A feeding during iron-repletion		0.001 (0.025)	NS (NS)	NS
Interaction		0.003 (0.017)	0.009 (NS)	NS

Values represent means and pooled SEs (n = 18). Values in a column not sharing the same superscript letter are significantly different ($p < 0.05$). † When the covariate "body weight at d 72" contributed significantly to the explained variance, adjusted means are given in parentheses. * Analysis of variance with previous vitamin A feeding and vitamin A feeding during iron repletion as independent variables without or with body weight at d 72 as covariate. When the covariate contributed significantly to the explained variance, the matching p values are given in parentheses. NS = not significant.

Iron in organs

In the vitamin A deficient animals that were supplemented with vitamin A during iron repletion, iron concentrations of liver, kidney, spleen and tibia were significantly reduced when compared with the vitamin A deficient rats not supplemented with vitamin A (*Table 4*). In the former animals, liver weight was markedly higher (*Table 2*), so that supplemental vitamin A produced an increase in total liver iron in the vitamin A deficient rats. In the vitamin A sufficient rats, the absence or presence of vitamin A in the diet during iron repletion had no effect on iron concentrations in organs (*Table 4*). Iron concentrations in spleen and tibia were highest in the group that was deprived of vitamin A throughout. Analysis of variance with body weight as covariate revealed that spleen and tibia iron concentrations were influenced by changes in body weight (*Table 2*).

Table 4 Iron concentrations in organs after iron repletion (d 72).

Dietary Vitamin A, RE/kg		Iron concentration in organs, μmol/g dry weight			
		Liver	Kidney	Spleen†	Tibia
d 0-60	d 60-72				
1200	1200	4.10^{ab}	4.03^{ab}	23.40^{a} (23.88)‡	1.07^{a} (1.08)‡
1200	0	4.35^{ab}	4.02^{ab}	24.51^{a} (24.92)	1.14^{ab} (1.14)
0	1200	3.81^{a}	3.70^{a}	25.02^{a} (24.99)	1.23^{b} (1.23)
0	0	4.49^{b}	4.39^{b}	44.17^{b} (43.30)	1.59^{c} (1.58)
	Pooled SE	0.16	0.14	1.99	0.04
Analysis of variance, p value*					
Body weight at d 72		NS	NS	<0.001	<0.001
Previous vitamin A feeding		NS	NS	<0.001 (0.007)	<0.001 (<0.001)
Vitamin A feeding during iron-repletion		0.007	0.031	<0.001 (<0.001)	<0.001 (0.001)
Interaction		NS	0.013	<0.001 (<0.001)	<0.001 (0.001)

Values represent means and pooled SEs (n=18). Values in a column not sharing the same superscript letter are significantly different ($p<0.05$). † Values are log-transformed prior to statistical testing, unadjusted means were given. ‡ When the covariate "body weight at d 72" contributed significantly to the explained variance, adjusted means are given in parentheses. * Analysis of variance with previous vitamin A feeding and vitamin A feeding during iron repletion as independent variables without or with body weight at d 72 as covariate. When the covariate contributed significantly to the explained variance, the matching p values are given in parentheses. NS = not significant.

DISCUSSION

The main objective of this study was to test whether vitamin A status affects the efficacy of iron repletion in rats with mild iron deficiency. The rats with mild iron deficiency and either a low or normal vitamin A status, were repleted with iron alone or with iron plus vitamin A.

At the end of the iron depletion period (day 60), vitamin A deficiency versus normal vitamin A status had produced an increase in haemoglobin concentrations, haematocrit and red blood cell count. This effect of vitamin A deficiency had been reported previously [12,13,19,20,**chapter 2**]. Both water imbalance and reduced growth might have caused this haemoconcentration [21,22]. In the rats with normal vitamin A status, there was no effect of vitamin A consumption during iron

repletion on blood haemoglobin, haematocrit and red blood cell count. In contrast, when vitamin A was administered to the vitamin A deficient rats during iron repletion, the values of the haematological parameters dropped significantly. This vitamin A effect does not necessarily reflect a specific influence on iron metabolism but could be secondary to overcoming the haemoconcentration.

In this study, the effect of vitamin A on feed intake and body weight may confound effects of vitamin A on iron metabolism. The use of restricted or pair-feeding would have ruled out any such influence of differences in feed intake and body weight. From studies with children it is known that vitamin A status affects growth [7,16,23] and thus body weight was considered a parameter of interest in our study which dictated the use of an ad libitum feeding regimen. To assess possible confounding by differences in growth, a two-way analysis of variance was also performed with body weight as covariate. This approach served to isolate the effects on iron metabolism of body weight from those of vitamin A feeding. Only the effects of vitamin A feeding on plasma iron concentrations were affected by body weight. The changes in other parameters of iron metabolism can be considered rather specific effects of vitamin A.

Despite possible haemoconcentration, total iron-binding capacity and also plasma iron concentrations were reduced in vitamin A deficiency, which corroborates earlier work in both humans [1,2,5,9] and rats [11,13]. The administration of vitamin A to the vitamin A deficient rats during iron repletion significantly raised total iron-binding capacity and plasma iron. A greater increase in plasma iron as a result of vitamin A in addition to iron administration also occurred in pregnant women [10]. Perhaps, vitamin A deficiency interferes with iron transport so that absorbed iron cannot be utilized optimally. This is supported by the finding that vitamin A is necessary for the synthesis of the glycoprotein transferrin [24]. However in the present study the increased plasma iron concentrations were also related to enhanced growth and possibly point to an indirect effect of vitamin A feeding.

This experiment with rats allowed us to study the effect of vitamin A on the distribution of iron between organs. The observed increase in iron concentrations in liver, kidney, spleen and tibia in vitamin A deficiency is consistent with earlier studies in rats [12,13,25,**chapter 2**]. The new observation is that in rats with vitamin A deficiency, but not in those with normal vitamin A status, vitamin A administration during iron repletion reduced the concentration of iron in liver, kidney, spleen and tibia.

We have hypothesized [12,**chapter 2**] that in vitamin A deficiency, red blood cell synthesis is impaired. However, reduced haemoglobin concentrations as seen in

humans [1-5] are masked in growing rats by haemoconcentration [12,13,**chapter 2**]. It may be that the extra iron in the spleen and tibia of the vitamin A deficient rats is located in macrophages due to increased destruction of abnormal erythrocytes. This is supported by the report by Mejía et al [11] that with vitamin A deficiency, the incorporation of radioactive iron was increased in spleen but decreased in red blood cells.

In conclusion, this experiment with rats shows that supplementation of vitamin A together with iron was more beneficial in restoring normal iron status than was iron repletion alone. This can be seen from the increase in total iron-binding capacity and decrease in spleen and tibia iron when animals were fed diets containing vitamin A. The effect of vitamin A only occurred when the vitamin A status of the rats was low. The present results are compatible with those from human studies [8,10] and indicate that vitamin A might interfere with plasma iron transport and the location of iron stores. The rat model described here will be useful in unravelling the underlying mechanisms.

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CHAPTER 4

Supplemental vitamin A enhances the recovery from iron deficiency in rats with chronic vitamin A deficiency

ABSTRACT

Studies with anaemic children and pregnant women from areas where vitamin A deficiency is endemic have shown a beneficial effect on iron status of supplemental vitamin A in addition to iron supplementation. This suggests a relationship between vitamin A and iron status, which we attempted to mimic in rats with anaemia and chronic vitamin A deficiency. Male rats were fed iron adequate diets (35 mg iron/kg) containing different levels of vitamin A (1200, 450, 150, 75 and 0 retinol equivalents (RE)/kg feed) until they were 5 weeks old. These diets were identical to the diets fed to their mothers. Then, the young male rats were transferred to diets containing the same levels of vitamin A but no added iron. After another two weeks, the rats were repleted with iron (35 mg/kg feed) without or with vitamin A to a level of 1200 RE/kg feed. Increased vitamin A intake by the groups previously fed on diets with either 0 or 75 RE/kg, produced a reduction in blood haemoglobin concentration, haematocrit and red blood cell count. In the group which had been fed the diet without vitamin A, supplemental vitamin A raised mean cell volume, plasma iron concentration and total iron-binding capacity. Vitamin A supplementation during the period of iron repletion produced a decrease in splenic and tibia iron concentration, the effect being greater with increasing severity of previous vitamin A deficiency. The paradoxal effect of supplemental vitamin A on haemoglobin, haematocrit and red blood cell count can be explained by a decrease in the degree of haemoconcentration. Thus, the positive effect of supplemental vitamin A seen in humans, is also observed with rats under controlled experimental conditions. We speculate that supplemental vitamin A during iron repletion contributes to optimum erythropoiesis and iron mobilization when baseline vitamin A status is impaired.

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British Journal of Nutrition 1996, in press

INTRODUCTION

In children [1-4] and pregnant women [5] from areas where vitamin A deficiency is endemic, low plasma retinol levels were associated with low concentrations of haemoglobin and serum iron and low degrees of transferrin saturation. When children with relatively low blood haemoglobin levels (<7.5 mmol/L) were supplemented with vitamin A the haemoglobin levels rose [2,6-9]. Interestingly, when vitamin A was given together with iron as compared to iron supplementation alone, not only serum iron concentration and transferrin saturation [8] but also blood haemoglobin levels [10] rose to higher values. Thus, there appears to be an additional effect of vitamin A on iron metabolism during the recovery from iron deficiency.

In order to investigate the mechanisms underlying the relationship between vitamin A and iron status, experiments with animals are required. In rats, vitamin A deficiency impairs erythropoiesis [11,12] and raises the concentrations of iron in liver [13-16] and spleen [13,17,**chapter 2**], but generally lowers the total amount of iron in liver [16,17,**chapter 2**]. The effect of vitamin A supplementation on dietary-iron-induced regeneration of iron status had not yet been studied in rats. It was hypothesized that this effect of vitamin A depends on the vitamin A status of the rats. Thus, we determined iron parameters in blood and organs of anaemic rats with various degrees of chronic vitamin A deficiency before and after supplementation with vitamin A together with iron. It was anticipated that this study would provide clues as to the underlying mechanisms of the additional effect of vitamin A on iron status, as found in iron supplementation studies with children and pregnant women.

METHODS

The experiment was approved and supervised by the animal welfare officer of Wageningen Agricultural University.

Animals, housing and diets

A controlled light/dark cycle (light on: 0600-1800 h), temperature (20-22°C) and relative humidity (50-60%) were maintained in the animal room. All animals had free access to feed and demineralised water throughout.

Fifty-two female and 28 male Wistar rats (Cpb:WU), aged 10 weeks, were used for breeding. The rats were housed in groups of 4 or 5 animals of the same sex in stainless steel cages with wire mesh bases (30 × 42 × 19 cm). During a period of 4 weeks, all animals received a control diet (*Table 1*) containing sufficient vitamin A (1200 retinol equivalents (RE)/kg feed). This diet was formulated according to the nutrient requirements of rats [18]. Then, two weeks prior to mating, the female rats were divided into 5 groups matched for body weight, and transferred to wire-topped, polycarbonate cages (34.5 × 22.5 × 16 cm) with a layer of sawdust as bedding. They were housed one or two per cage. The groups received diets with different levels of vitamin A (1200, 450, 150, 75 or 0 RE/kg feed).

Table 1 Composition of the diets‡.

Ingredients	
Casein (g)	151
Maize oil (g)	25
Coconut fat (g)	25
Glucose (g)	709.2
Cellulose (g)	30
$CaCO_3$ (g)	12.4
$NaH_2PO_4.2H_2O$ (g)	15.1
$MgCO_3$ (g)	1.4
KCl (g)	1.0
$KHCO_3$ (g)	7.7
$FeSO_4.7H_2O$ (mg)	174/0
Vitamin A preparation (RE)§	0/75/150/450/1200
Mineral premix (iron-free) (g)†	10
Vitamin premix (vitamin A-free)(g)¶	12

‡ The diets were in powdered form. The breeder rats were given the diets in pelleted form with the same composition, except that per kg feed 379.6 g glucose was replaced by 329.6 g maize starch plus 50 g molasses. The amount of iron added to the pelleted diet (124 mg $FeSO_4.7H_2O$) was corrected for the iron content of molasses. § Rovimix A 500®, 150 RE/mg (F. Hoffmann-La Roche & Co. Ltd, Basle, Switzerland), consisting of retinyl acetate and retinyl palmitate; of this preparation 1200, 450, 150, 75, 0 retinol equivalents (RE)/kg feed was added. † The mineral premix consisted of (mg): MnO_2, 79; $ZnSO_4.H_2O$, 33; $NiSO_4.6H_2O$, 13; NaF, 2; KI, 0.2; $CuSO_4.5H_2O$, 15.7; $Na_2SeO_3.5H_2O$, 0.3; $CrCl_3.6H_2O$, 1.5; $SnCl_2.2H_2O$, 1.9; NH_4VO_3, 0.2; maize meal, 9853.2. ¶ The vitamin premix consisted of (mg): thiamin, 4; riboflavin, 3; niacin, 20; D,L-calcium pantothenate, 17.8; pyridoxine, 6; cyanocobalamin, 50; choline chloride, 2000; folic acid, 1; biotin, 2; menadione, 0.05; D,L-*α* tocopheryl acetate, 60; cholecalciferol, 2 (1000 IU); maize meal, 9834.15.

The purified diets (*Table 1*) were pelleted (diameter 10 mm). Male rats continued to receive the control diet. For mating, one male rat was housed together with one or two females, aged 16 weeks, for a period of 10 days.

After this period, the female rats were housed individually in the polycarbonate cages until the pups were weaned. All female rats appeared healthy and body weights and litter size were similar for the five groups. From each group, 16 male pups entered the pre-experimental period at about 3 weeks of age. The pups were born 24-29 days after the start of temporary cohabiting of the males and females.

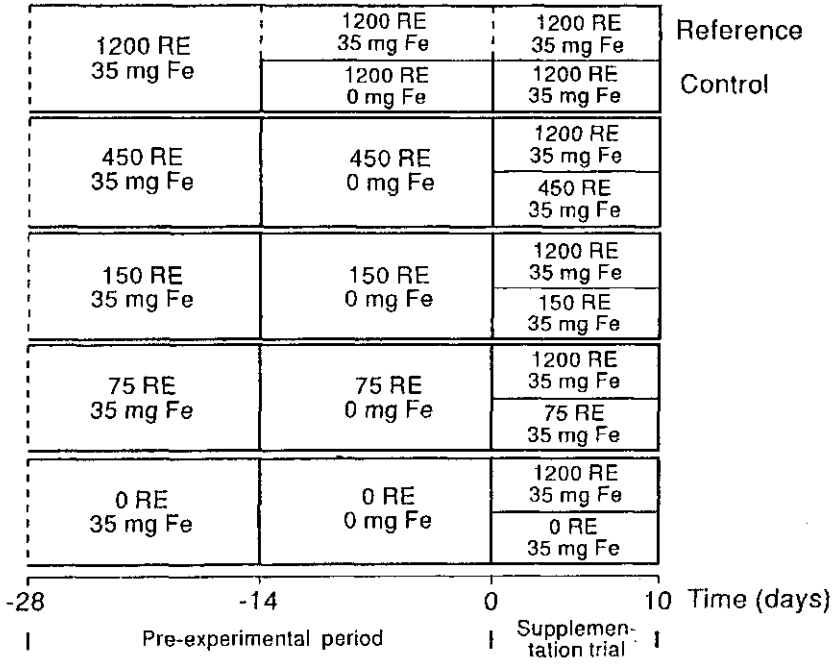

FIGURE 1 Experimental design: except for the reference group (top bar) the rats with different vitamin A status went through an iron-depletion period (days -14 to 0) followed by an iron-repletion period without or with vitamin A supplementation. At weaning on day -28, the rats were 3 weeks old. Before weaning, the pups were raised by dams that had been fed, since two weeks prior to mating, on the same diets the pups were to be given at weaning. The rats were killed on day 10 for the removal of tissues. The number of rats per group on days -28 and -14, was sixteen and on day 10 it was eight. The diets used differed only in vitamin A and iron content (*Table 1*); the amounts are indicated as retinol equivalents (RE) and mg iron (Fe) /kg feed.

Pre-experimental period

The pups were housed in groups of four animals in stainless steel cages with wire mesh bases (30 × 42 × 19 cm). The rats were randomly assigned to the cages which were distributed uniformly over the animal room. For a period of 2 weeks (days -28 to -14), the rats were fed the same diet as their

respective mothers (*Figure 1*), except that the diets were in powdered form. Then (day -14), when the rats were 5 weeks old, a blood sample was taken for haematological examination and analysis of plasma retinol concentration. Body weight, feed intake and haematological characteristics at day -14 (*Figure 1*) are shown in *Table 2*. Haemoglobin concentration, haematocrit and red blood cell count were increased in the groups fed either the diets without added vitamin A or with 75 RE/kg. This corroborates earlier work [13,19] and is explained by haemoconcentration due to chronic vitamin A deficiency [12,13,19,20]. On day -14 (*Figure 1*) the rats receiving the diet with 1200 RE/kg were divided into two groups of 8 animals each, which were matched for body weight and haemoglobin concentration. One group of 8 animals (reference group) continued to be fed on the control diet. The other group (control group) received the same diet but without added iron. All other groups were deprived of added dietary iron but continued to receive their respective levels of dietary vitamin A as illustrated in *Figure 1*.

Table 2 Body weight, feed intake, plasma retinol concentrations and haematological characteristics at day -14‡.

Dietary vitamin A (RE/kg)	Body weight (g)	Feed intake (g/day) §	Plasma retinol (μmol/L) ‖	Haemo-globin (mmol/L)	Haema-tocrit (%)	Red blood cell count (10E12/L)	Mean cell volume (fL)
1200¶	145.5	16.9	2.55 ± 0.05	7.9	39.8	5.75	69.3
450	141.7	16.8	1.92 ± 0.09	7.9	40.6	5.67	71.6*
150	133.5	16.3	1.08 ± 0.12*	8.1	41.7*	5.87	71.1
75	135.2	15.8	0.60 ± 0.12*	8.2*	41.9*	6.12*	68.6
0	141.6	15.8	0.23 ± 0.02*	8.5*	43.7*	6.29*	69.4
Pooled SE	3.4			0.1	0.4	0.10	0.6
P values for trend effects of vitamin A intake: †							
L term:	0.044		<0.001	<0.001	<0.001	<0.001	>0.05
Q term:	>0.05		<0.001	<0.001	0.004	<0.001	<0.001

‡ Means with pooled standard errors (SE) for 8 animals per group. § Feed intake was measured per cage with four animals from day -28 to -14 (*Figure 1*); the pooled SE was not calculated because of the low number of degrees of freedom. ‖ Prior to statistical analysis the data were log-transformed (unadjusted means and separate SE are given). ¶ Statistically significant difference (Student's-t test) versus group fed diet with 1200 RE/kg: *, $p<0.0125$. † Value for linear (L) and/or quadratic (Q) term in effect of vitamin A intake.

Table 3 Body weight, feed intake, plasma retinol concentrations and haematological characteristics after the iron depletion period (day 0)‡.

Dietary vitamin A (RE/kg)	Body weight (g)	Feed intake (g/day)§	Plasma retinol (μmol/L)#	Haemo-globin (mmol/L)	Haemato-crit (%)	Red blood cell count (10E12/L)	Mean cell volume (fL)
1200 (reference) ‖	227.2	17.1	2.28 ± 0.03	8.8*	44.0*	6.42*	68.6*
1200 (control)¶	224.9	17.1	2.16 ± 0.06	6.9	35.3	5.95	59.3
450	219.9	16.7	1.64 ± 0.05	6.7	34.4	5.75	59.7
150	198.7*	14.5	0.72 ± 0.05*	7.4	37.9	6.20	61.0
75	200.2*	14.4	0.36 ± 0.05*	7.4	37.3	6.33	58.9
0	173.4*	8.4	0.05 ± 0.01*	8.3*	42.3*	7.06*	59.9
Pooled SE	6.1			0.2	0.9	0.12	0.7
P values for trend effects†:							
Vitamin A intake,							
L term:	<0.001		<0.001	<0.001	<0.001	<0.001	>0.05
Q term:	<0.001		<0.001	<0.001	<0.001	<0.001	>0.05
Iron intake:	>0.05		>0.05	<0.001	<0.001	0.008	<0.001

‡ Means for 8 animals in the reference group and control group and 16 animals for all other groups. Pooled standard errors (SE) are calculated for 8 animals per group.

§ Feed intake was measured per cage with four animals from day -14 to 0 (*Figure 1*); the pooled SE was not calculated because of the low number of degrees of freedom.

Prior to statistical analysis the data were log-transformed (unadjusted means and separate SE are given).

‖ The reference group was the only one receiving iron with the diet (35 mg/kg) from days -14 to 0.

¶ Statistically significant difference (Student's t-test) versus the control group: *, $p<0.01$.

† Values for linear (L) and/or quadratic (Q) term in effect of vitamin A intake and iron intake.

After another 14 days (day 0), again a blood sample was taken for the same measurements as above. *Table 3* shows a similar pattern of results as does *Table 2*, except that body weights were lowered with decreasing vitamin A intakes in the iron depleted groups. When compared with the reference group, iron depletion for 14 days produced low haemoglobin concentrations in all groups except for the one given the diet without added vitamin A. Apparently, the progression of haemoconcentration caused by vitamin A deficiency had counteracted the decrease in blood haemoglobin concentration caused by iron deficiency.

Supplementation trial

The iron and/or vitamin A repletion was introduced on day 0 of the experiment (*Figure 1*). The iron deficient groups receiving the diets with either 450, 150, 75 or 0 RE/kg were each divided into two groups of 8 animals, which were stratified within dietary vitamin A level for haemoglobin concentration and body weight both measured on day 0 (*Figure 1*). All animals received iron again (35 mg/kg feed), but half of the animals continued to receive the reduced levels of vitamin A, whereas the other half was supplemented by 1200 RE/kg feed. The reference group remained on the control diet. The rats that had been fed the diet containing 1200 RE/kg feed but without added iron were transferred to the iron adequate control diet again. After another 10 days (day 10), all animals were killed. Throughout the supplementation trial, body weight and feed intake were monitored.

Collection of samples

Blood was collected, in heparinized vials by orbital puncture while the rats were under diethyl ether anaesthesia. In our hands, the orbital puncture technique itself does not affect the endocrine stress response and induces laesions that heal without detectable scars [21,22]. The blood was stored at 0°C for haematological examination on the same day. Then, plasma was isolated by centrifugation (10 min, 1580 g) and stored at -20°C until analysis, except for 250 μl which was stored at -80°C for subsequent analysis of retinol. Immediately after bleeding, the anaesthetized rats were decapitated. The left kidney, liver, spleen and both hindlegs were removed and stored at -20°C until analysis. Organs were weighed before storage.

Chemical analyses

Haemoglobin concentration, haematocrit, red blood cell count and mean cell volume were analyzed with a blood cell counter (Model K-1000, Sysmex, IJsselstein, The Netherlands). Plasma iron concentrations and total iron-binding capacity were determined spectrophotometrically using a commercial test kit (Roche Nederland, Mijdrecht, The Netherlands). Spleen and liver were washed with saline and liver was homogenized as described below. Liver homogenate,

spleen, kidney and tibia were dried (100°C, 12 hours) and ashed (500°C, 16 hours). The ash was dissolved in 1 ml 6N HCl and diluted with demineralized water. Iron was measured by flame atomic absorption spectrometry (Model AA-475, Varian, Springvale, Australia). All analyses were carried out singly. Iron in tibia was calculated as the mean of left and right tibia.

Plasma and liver retinol was measured by reversed phase high performance liquid chromatography (HPLC) as described in **chapter 2.**

Statistical analysis

Estimated values of the components of variance of cage and litter were calculated from the mean squares [23] and compared with the mean squared errors. They were all considerably smaller than the residual variance, except for six variables (body weight at days -14, 0 and 10, red blood cell count at day -14, iron in kidney and tibia at day 10) of which the cage component of variance was of similar size. We therefore ignored any litter or cage component of variance.

The data for the two pre-experimental time points (days -14 and day 0; *Figure 1*) and the time point at the end of the supplementation trial (day 10), were evaluated by one-way analysis of variance with vitamin A intake as factor. If variances were not homogeneous (Cochran's C test) data were log-transformed prior to statistical testing. Contrasts with standard errors calculated from pooled variances were used to identify statistically significant differences. A pre-set p value of 5% with Bonferroni's adaptation was used for controlling the experiment-wise error rate of any set of 8 comparisons with the control group (effect of differences in vitamin A intake in pre-experimental period) or of any set of 4 pair-wise comparisons relating to the effect of vitamin A supplementation during the experimental period. In addition, linear contrasts were used to test polynomial (linear and quadratic) regressions on measurement values at days -14 and 0 and day 10 against previous (prior to day 0) vitamin A intake. This analysis was also carried out for the day 10 values in the groups fed 450, 150, 75 or 0 RE/kg with supplemental vitamin A as extra independent variable. For the day 0 values in all the six groups dietary iron was added as independent variable in the trend analysis.

RESULTS

Body weight, feed intake and organ weights

For the reference group (n = 8) which received the diet with 1200 RE and 35 mg iron/kg throughout (*Figure 1*), the following values were obtained: body weight, 266.9 g; feed intake per day, 16.83 g; liver wet weight, 11.2 g; spleen wet weight, 0.43 g; kidney wet weight, 0.77 g; pooled SEs are given in *Table 4*. After iron repletion for 10 days, body and liver weight in the rats fed the diets with less than 450 RE/kg remained depressed when compared with the control group, irrespective of vitamin A supplementation (*Table 4*). Supplemental vitamin A only raised body and organ weights in the group that had been deprived of vitamin A until day 0. These effects were associated with an increase in feed intake (*Table 4*). In the rats fed diets without added vitamin A throughout, there was no body weight gain during the iron repletion period (*Tables 3 and 4*). In these animals liver, spleen and kidney weight were also markedly lower (*Table 4*). For the groups with dietary vitamin A concentrations lower than 1200 RE/kg feed prior to day 0, previous vitamin A intake and supplemental vitamin A significantly influenced body and organ weights as based on the trend analysis.

Vitamin A status

The differences in plasma retinol levels between the four groups deficient in vitamin A (*Table 3*) was maintained during iron repletion but not during vitamin A supplementation from days 0 to 10 (*Figure 2*) and was reflected in the liver retinol concentrations at day 10 (*Table 5*). In fact, no retinol was detectable in the livers of the groups fed diets with 150 RE/kg or less. The reference group fed on the diet with 1200 RE and 35 mg iron throughout had a mean liver retinol concentration of 54.3 ± 3.6 nmol/g wet weight. Vitamin A supplementation increased both liver and serum retinol levels significantly although the final values remained below those seen in the rats maintained on a diet adequate in vitamin A throughout. Plasma retinol concentrations were 2.15 (SE 0.04) and 2.08 (SE 0.09) μmol/l for the reference group (n = 8) and the control group (n = 8), respectively.

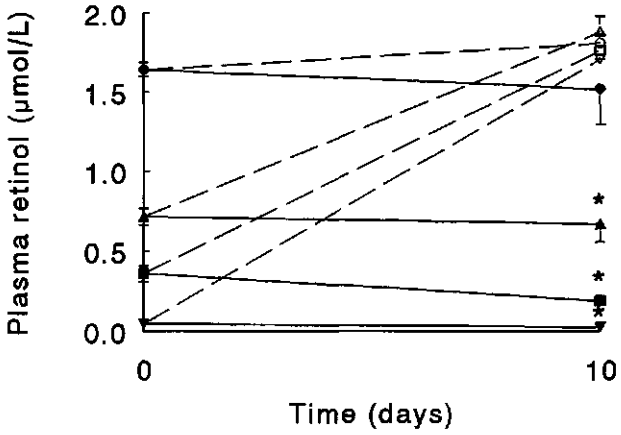

FIGURE 2 Plasma retinol concentrations at days 0 and 10. Explanation of symbols: ●, 450 RE/kg feed; ▲, 150 RE/kg feed; ■, 75 RE/kg feed and ▼, 0 RE/kg feed prior to supplementation. All diets were iron sufficient from day 0, but only half of the groups were supplemented with vitamin A to a dietary level of 1200 RE/kg feed. Dotted lines and open symbols at day 10 refer to vitamin A supplemented groups. Means and SEs (vertical bars) are given. Values were log-transformed before they were analysed statistically. Trend analysis revealed significant linear and quadratic terms of vitamin A intake prior to day 0 ($p<0.001$) for values at both day 0 and day 10, of vitamin A supplementation ($p<0.001$) and of interaction of these effects ($p<0.001$) for the values at day 10. Statistically significant difference (Student's t-test) versus the group fed the same diet prior to day 0: *, $p<0.001$.

Haematology

Iron repletion during days 0 to 10 produced an increase in haematological parameters (*Tables 3 and 6*). In the groups fed either the diet without vitamin A or with 75 RE/kg, haemoglobin, haematocrit and red blood cell count at day 10 (*Table 6*) were higher than in their counterparts repleted with vitamin A. Mean cell volume was lowest in the rats maintained on the diet without vitamin A but supplementation resulted in an increase (*Table 6*). Trend analysis for the groups with dietary vitamin A concentrations of 0 to 450 RE/kg until day 0, had the following outcome. Previous vitamin A intake significantly influenced red blood cell count and mean cell volume, and supplemental vitamin A intake significantly affected haemoglobin, haematocrit and red blood cell count. For the reference group ($n=8$) which received the diet with 1200 RE and 35 mg iron/kg throughout, the values at day 10 were: haemoglobin, 9.2 mmol/L; haematocrit, 44.6 %; red blood cell count, 6.68 10E12/L; mean cell volume, 66.9 fL.

Table 4 Body weight, feed intake and organ weights at day 10‡.

Dietary vitamin A (before day 0 - after day 0, RE/kg)	Body weight (g)	Feed intake§ (g/day)	Organ wet weight		
			Liver (g)	Spleen (g)	Kidney (g)
1200-1200 (control) ‖	272.3	18.59	11.0	0.46	0.82
450-1200	272.6	18.63	10.6	0.45	0.81
450-450	269.1	18.84	10.8	0.49	0.85
150-1200	243.5*	17.26	8.9*	0.41	0.77
150-150	235.7*	15.74	8.5*	0.38*	0.78
75-1200	248.8	17.01	9.0*	0.46	0.73
75-75	244.5*	17.31	8.2*	0.41	0.82
0-1200	222.9*†	15.76	8.4*†	0.45†	0.72
0-0	176.4*	9.39	4.9*	0.34*	0.62*
Pooled SE	6.9		0.3	0.02	0.03
P values for trend effects¶:					
Previous vitamin A intake,					
L term:	<0.001		<0.001	<0.001	<0.001
Q term:	<0.001		<0.001	>0.05	0.021
Supplemental vitamin A:	0.001		<0.001	0.007	>0.05
Interaction:	0.004		<0.001	0.002	0.016

‡ Means with pooled standard error (SE) for 8 animals per group; the calculation of the pooled SEs included the reference group. § Feed intake was measured per cage with four animals from day 0 to 10; the pooled SE was not calculated because of the low number of degrees of freedom.

‖ Statistically significant difference (Student's-t test) versus control group: *, $p<0.00625$, or versus the group which had been fed the same diet until day 0: †, $p<0.0125$.

¶ Values for linear (L) and/or quadratic (Q) term in effects of previous vitamin A intake (prior to day 0) and supplemental vitamin A. Only the groups with dietary vitamin A concentrations lower than 1200 RE/kg feed prior to day 0 are included.

Plasma iron and total iron-binding capacity

At the end of the supplementation trial (day 10), total iron-binding capacity was depressed in the groups fed either 0 or 75 RE/kg throughout the

experiment (*Table 7*). The group given the retinol-free diet was most seriously affected and this was associated with a low plasma iron concentration. Vitamin A repletion produced a significant rise in total iron-binding capacity. Transferrin saturation was not affected by vitamin A intake (*Table 7*). Previous vitamin A intake significantly affected plasma iron concentration and total iron-binding capacity. The reference group (n = 8) which received the adequate diet throughout showed the following values: plasma iron, 32.30 μmol/l; total iron-binding capacity, 88.05 μmol/l; transferrin saturation, 36.82 %.

Iron in organs

Liver iron concentrations at the end of the supplementation trial were similar for all groups (*Table 8*). In the groups given diets with either 75, 150 or 450 RE/kg there was a decrease in kidney iron concentrations when compared with the control group. Vitamin A supplementation raised kidney iron concentration in the group that had been fed on the diet with 75 RE/kg until day 0. Decreasing vitamin A intakes in the groups not supplemented with vitamin A were associated with increases in spleen and tibia iron concentrations. The concentrations of iron in spleen and tibia were generally reduced in response to vitamin A supplementation (*Table 8*). Thus in the trend analysis previous vitamin A intake and vitamin A supplementation were found to significantly reduce spleen and tibia iron concentrations. The reference group (n = 8) had the following organ iron concentrations (μmol/g dry weight): liver, 3.33; spleen, 14.22; kidney, 4.49; tibia, 1.39.

DISCUSSION

After iron depletion for 14 days, the rats with different vitamin A status were repleted with iron without or with supplemental vitamin A. This experimental design allowed to examine the effect of vitamin A supplementation on iron-induced regeneration of iron status as influenced by the degree of chronic vitamin A deficiency. The baseline of the experiment (day 0) may simulate the situation in humans with anaemia in areas where marginal vitamin A intake is a problem. The additional effect of supplemental vitamin A on iron status after iron supplementation, as has been shown in two independent studies with children and pregnant women [8,10] can now be described in more detail.

Table 5 Liver retinol concentrations at day 10‡.

Dietary vitamin A (before day 0 - after day 0, RE/kg)	Liver retinol (nmol/g liver wet-weight)
1200-1200 (control)	60.7 ± 2.9
450-1200	23.3 ± 3.2
450-450	6.1 ± 0.7
150-1200	15.0 ± 0.8
150-150	ND
75-1200	13.2 ± 2.6
75-75	ND
0-1200	11.9 ± 1.6
0-0	ND

‡ Means with standard errors for 8 animals per group.
ND = not detectable, the detection limit is about 3 nmol/g wet liver weight.

Iron repletion raised the selected haematological parameters as would be anticipated [24]. Vitamin A repletion of the vitamin A deficient rats clearly improved their vitamin A status as based on plasma and liver retinol concentrations. Supplemental vitamin A significantly influenced iron metabolism in the two groups with most severe vitamin A deficiency. At the end of the supplementation period, extra vitamin A in the diet had reduced blood haemoglobin concentrations, red blood cell count and haematocrit. These effects can probably be explained by a diminished haemoconcentration and thus attenuation of the vitamin A deficiency-induced haemoconcentration as described earlier [12,13,19,20], and cannot be interpreted as a specific effect of vitamin A on iron metabolism. However, supplemental vitamin A also raised mean red blood cell volume in the group previously given the diet without vitamin A. Perhaps this points to a stimulatory effect of vitamin A on erythropoiesis.

Although there was indirect evidence of haemoconcentration in the group fed the diet without vitamin A, plasma iron concentrations and iron-binding capacity were reduced and were increased after vitamin A supplementation. Low levels of plasma iron is a consistent feature of poor vitamin A status in

children [2,6] and pregnant women [5]. In some human studies [1,9] but not all [5,8] low total iron-binding capacity or transferrin concentration were associated with poor vitamin A status. Possibly vitamin A controls the synthesis of transferrin, which is the major iron transport protein carrying iron from the storage depots such as liver to the erythropoietic system in bone marrow. An impaired transferrin synthesis in vitamin A deficiency is compatible with the observation that vitamin A is involved in the synthesis of the glycosyl moieties of the transferrin molecule [25].

Table 6 Haematological characteristics at day 10[‡,§].

Dietary vitamin A (before day 0 - after day 0, RE/kg)	Haemoglobin (mmol/L)	Haematocrit (%)	Red blood cell count (10E12/L)	Mean cell volume (fL)
1200-1200 (control)	8.7	42.7	6.82	62.6
450-1200	8.8	43.7	6.81	64.3
450-450	8.8	44.0	6.73	65.4
150-1200	8.8	43.7	6.79	64.4
150-150	8.8	43.4	6.88	63.2
75-1200	8.4†	41.8	6.66	62.9
75-75	9.0	44.6	7.07	63.1
0-1200	8.5†	42.2†	6.86†	61.7†
0-0	9.4*	45.9*	7.83*	58.6*
Pooled SE	0.1	0.8	0.14	0.8
P values for trend effects§:				
Previous vitamin A intake,				
L term:	>0.05	>0.05	0.001	<0.001
Q term:	>0.05	>0.05	0.003	0.002
Supplemental vitamin A	<0.001	0.004	0.001	>0.05
Interaction:	0.001	0.025	0.001	>0.05

‡ Means are given with pooled standard error (SE) for 8 animals per group; the calculation of the pooled SEs included the reference group.
§ For indications of statistical significance, see legend to Table 4.

In any event, this study shows that in rats with iron deficiency with the highest degree of vitamin A deficiency, supplemental vitamin A had a raising effect on plasma iron and total iron-binding capacity that is superimposed on that of iron supplementation. This observation illustrates the involvement of vitamin A in iron metabolism.

Table 7 Plasma iron, total iron-binding capacity and percentage transferrin saturation at day 10[‡,§].

Dietary vitamin A (before day 0 - after day 0, RE/kg)	Plasma iron (μmol/L)	Total iron-binding capacity (μmol/L)	Transferrin saturation (%)
1200-1200 (control)	40.36	95.19	42.80
450-1200	41.55	89.01	46.71
450-450	43.39	90.42	48.19
150-1200	42.51	89.56	47.59
150-150	44.13	87.86	50.20
75-1200	37.77	83.71*	45.27
75-75	39.47	86.49*	45.97
0-1200	34.97	83.17*†	42.30
0-0	30.11*	71.86*	42.40
Pooled SE	3.63	2.82	4.91
P values for trend effects§			
Previous vitamin A intake,			
L term:	<0.001	0.004	>0.05
Q term:	<0.001	0.003	>0.05
Supplemental vitamin A:	>0.05	>0.05	>0.05
Interaction:	>0.05	0.002	>0.05

‡ Means are given with pooled standard error (SE) for 8 animals per group; the calculation of the pooled SEs included the reference group.
§ For indications of statistical significance, see legend to Table 4.

A major advantage of our study with rats is that it provides information on the effects of supplemental vitamin A on the distribution of iron between

organs. Liver iron concentrations were unchanged after feeding the vitamin A deficient diets but the reduction in liver weight resulted in a decreased total liver iron content. A decreased total amount of iron in the liver of vitamin A deficient rats has been found earlier in this laboratory [16,17,**chapter 2**]. Vitamin A supplementation of the group previously fed on the diet without vitamin A reduced liver iron concentration but significantly raised total liver iron content. In the vitamin A deficient animals, there was an increase of splenic and tibia iron as found earlier [12,13,17,**chapter 2**]. This study shows that, during iron repletion, supplemental vitamin A lowers iron contents of spleen and tibia. This effect of vitamin A was greater with increasing severity of the previous vitamin A deficiency.

Table 8 Iron concentrations in organs at day 10‡,§.

Dietary vitamin A (before day 0 - after day 0, RE/kg)	Liver	Spleen	Kidney	Tibia
		(μmol/g dry weight)		
1200-1200 (control)	2.55	12.74	4.30	1.30
450-1200	2.52	13.58	3.45*	1.40
450-450	2.52	14.56	3.37*	1.46
150-1200	2.73	14.56	3.57*	1.38
150-150	2.66	17.11*	3.37*	1.51
75-1200	3.00	15.41	4.20†	1.43†
75-75	2.57	17.24*	3.53*	1.69*
0-1200	2.40	14.46†	4.00	1.66*†
0-0	3.06	21.53*	4.13	2.12*
Pooled SE	0.29	1.27	0.22	0.08
P values for trend effects§:				
Previous vitamin A intake,				
L term:	>0.05	0.002	<0.001	<0.001
Q term:	>0.05	<0.001	0.013	<0.001
Supplemental vitamin A:	>0.05	<0.001	>0.05	<0.001
Interaction:	>0.05	0.014	>0.05	0.008

‡ Means are given with pooled standard error (SE) for 8 animals per group; the calculation of the pooled SEs included the reference group.
§ For indications of statistical significance, see legend to Table 4.

We speculate that in vitamin A deficiency blood cell synthesis is impaired leading to increased iron stores in the macrophages in tibia and spleen, this process possibly being enhanced by increased destruction of inferior red blood cells. In addition iron mobilization might be impaired because iron is trapped in the macrophages. The effects of vitamin A deficiency on iron metabolism can be reversed by supplemental vitamin A. This reasoning could explain why supplemental vitamin A contributes to recovery from iron deficiency associated with vitamin A deficiency.

In conclusion, the outcome of this experiment carried out with rats under strictly controlled conditions mimics the observation [8,10] in humans with marginal vitamin A intake that vitamin A together with iron is more effective in normalizing iron status than is iron supplementation alone. This study indicates that vitamin A supplementation under those conditions stimulates the utilization of iron stores in spleen and bone.

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CHAPTER 5

Evaluation of a two-generation rat model for vitamin A deficiency and the interrelationship with iron metabolism

ABSTRACT

In order to induce a range of vitamin A deficient states in young growing rats and to study the effect of vitamin A deficiency on iron status, we designed the following two-generation experiment. Dams were fed on diets with one of five vitamin A levels from two weeks before and throughout pregnancy and lactation. The pups received the same diets as their mothers both before and after weaning. The five dietary levels of vitamin A were 1200, 450, 150, 75 and 0 retinol equivalents/kg feed. Vitamin A intake did not affect reproduction outcome, nor was body and liver weight of the pups affected when they were 3.5 weeks old. Male pups with normal vitamin A status had higher plasma retinol levels than female pups. Vitamin A status of the offspring was affected from 3.5 weeks on. Body and liver weight was decreased in the male pups fed the lowest dietary vitamin A levels from week 6.5 onwards but not in the female pups. Iron status was marginally affected. Haemoglobin levels were increased and total iron-binding capacity decreased in the groups fed no dietary vitamin A at week 9.5. Splenic iron was increased only in the male pups fed the lowest levels of dietary vitamin A. However as a whole, iron status was only mildly affected and subject to considerable variation. We conclude that the two-generation rat model described here is not suitable for studying effects of vitamin A deficiency on iron metabolism.

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INTRODUCTION

Vitamin A deficiency is a major public health concern and affects not only child morbidity and mortality [1], but is also associated with impaired iron status [2-4]. Intervention trials, in areas where vitamin A deficiency is endemic, have shown that vitamin A supplementation produces increased blood haemoglobin levels [5-9]. However the mechanisms by which vitamin A influences iron metabolism are still unclear. To develop an animal model for studying these mechanisms, we have induced acute vitamin A deficiency in young growing male rats by feeding them no vitamin A from the age of 6 weeks onwards [10, **chapter 2**]. Vitamin A deficiency in the rats developed quickly, and consequently does not mirror the situation in humans with impaired vitamin A status. Therefore, we have attempted to design a rat model that would resemble the situation in children with chronic vitamin A deficiency. For this purpose we used a two-generation model with dams and their pups fed on vitamin A deficient diets. On the basis of studies in chickens by West et al. [11] it could be anticipated that the two-generation rat model would produce young rats with different, but more or less constant vitamin A status. In the male and female offspring, we assessed vitamin A status by determination of plasma and liver retinol concentrations. Iron status was assessed by measuring haemoglobin, plasma iron, total iron-binding capacity and iron in liver, spleen and bone.

MATERIALS AND METHODS

The experiment was approved and supervised by the animal welfare officer of Wageningen Agricultural University.

Animals, diets and housing

Fifty two female and 28 male Wistar rats (Cpb:WU), aged 10 weeks, were used for breeding. Before they entered the study the rats had been fed a commercial pelleted diet (RMH-B, Hope Farms, Woerden, The Netherlands). According to the manufacturer, minimum levels of vitamin A and iron in the natural-ingredient diet were 5940 retinol equivalents (RE)/kg and 165 mg/kg, respectively.

During the experiment, the rats were housed in groups of 4 or 5 animals of

the same sex in stainless steel cages with wire mesh bases (30 × 42 × 19 cm). All animals received a control diet (*Table 1*) containing sufficient vitamin A (1200 RE/kg feed), for a period of 4 weeks. This diet was formulated according to the nutrient requirements of rats [12]. Then, two weeks prior to mating, the female rats were divided into 5 groups matched for body weight, and transferred to wire-topped, polycarbonate cages (34.5 × 22.5 × 16 cm) with a layer of sawdust as bedding. They were housed one or two per cage. The 5 groups of 10 or 11 animals received diets with different levels of added vitamin A (1200, 450, 150, 75 or 0 RE/kg feed) until they were killed after weaning of the pups. The analyzed iron content of the diets was 60 mg iron/kg feed. The purified diets (*Table 1*) were pelleted (diameter 10 mm). Male rats continued to receive the control diet.

Table 1 Composition of the pelleted diets (g/kg).

Ingredients	
Casein	151
Corn oil	25
Coconut fat	25
Glucose	329.6
Maize starch	329.6
Cellulose	30
$CaCO_3$	12.4
$NaH_2PO_4.2H_2O$	15.1
$MgCO_3$	1.4
KCl	1.0
$KHCO_3$	7.7
$FeSO_4.7H_2O$ (mg)	124
Molasses	50
Vitamin A preparation (RE)†	1200/450/150/75/0
Mineral premix (iron-free)‡	10
Vitamin premix (vitamin A-free)[1]	12

† Rovimix A 500®, 150 RE/mg (F. Hoffmann-La Roche & Co. Ltd, Basle, Switzerland), consisting of retinyl acetate and retinyl palmitate; of this preparation 1200, 450, 150, 75, 0 retinol equivalents (RE)/kg feed was added.

‡ The mineral premix consisted of (mg): MnO_2, 79; $ZnSO_4.H_2O$, 33; $NiSO_4.6H_2O$, 13; NaF, 2; KI, 0.2; $CuSO_4.5H_2O$, 15.7; $Na_2SeO_3.5H_2O$, 0.3; $CrCl_3.6H_2O$, 1.5; $SnCl_2.2H_2O$, 1.9; NH_4VO_3, 0.2; maize meal, 9853.2.

[1] The vitamin premix consisted of (mg): thiamin, 4; riboflavin, 3; niacin, 20; D,L-calcium pantothenate, 17.8; pyridoxine, 6; cyanocobalamin, 50; choline chloride, 2000; folic acid, 1; biotin, 2; menadione, 0.05; D,L-α tocopheryl acetate, 60; cholecalciferol, 2 (1000 IU); maize meal, 9834.15.

Table 2 Reproductive outcome and litter size of the dams fed different amounts of vitamin A.

Dietary retinol RE/kg feed	Dams delivering number/total (%)	Litter size n (range)	Live births %
1200	7/10 (70)	9.8 (2-12)	97
450	7/10 (70)	11.6 (9-14)	96
150	9/10 (90)	11.3 (2-14)	97
75	7/11 (64)	10.7 (9-14)	97
0	9/11 (82)	11.1 (7-14)	97

For mating, one male rat was housed together with one or two females, aged 16 weeks, for a period of 10 days. After this period, the female rats were housed individually in the polycarbonate cages until the pups were weaned. All female rats appeared healthy. The proportion of dams delivering, litter size and the proportion live births were similar for the five groups (*Table 2*). Nest size was standardized to 10-12 pups. Due to this procedure three dams that delivered small nests did not lactate and were excluded from the analysis. All mother rats were killed on the same day, one to seven days after the end of the lactation and nursing period (21 days). Blood and organs were collected. Data from the rats that did not deliver (n = 13) or delivered but not lactated (n = 3) were left out of the analysis.

Of the total of 427 pups, 342 were born 24-26 days after the start of the temporary cohabitation of the males and females; 150 out of the 342 were used in this experiment. The other pups were used in two other experiments [13,14, **chapters 4 and 6**]. When the pups were 3.5 weeks (25-27 days) old, 5 male and 5 female pups from each group were selected at random and killed. Blood and organs were collected. From the other animals, 10 male and 10 female pups from each group were housed in groups of 5 animals of the same sex in stainless steel cages with wire mesh bases (30 × 42 × 19 cm) and continued to receive the same diet as their respective mothers. At both age 6.5 and 9.5 weeks (46-48 and 67-69 days), one cage containing 5 male and 5 female pups from each group was removed from the study. The rats were then killed and blood and organs were collected.

All animals had free access to feed and demineralised water throughout. During the experiment, body weight and feed intake per cage were recorded at

weekly intervals. Feed intake was corrected for spillage losses. A controlled light/dark cycle (light on: 0600-1800 h), temperature (20-22°C) and relative humidity (50-60%) were maintained in the animal room.

Collection of samples

Blood was collected in heparinized vials by orbital puncture while the rats were under diethyl ether anaesthesia. The blood was stored at 0°C for haematological examination on the same day. Then, plasma was isolated by centrifugation (10 min, 1580 g) and stored at -20°C until analysis, except for 250 μL which was stored at -80°C for subsequent analysis of retinol. Immediately after bleeding, the anaesthetized rats were decapitated. Liver, spleen and both hindlegs were removed and stored at -20°C until analysis. Organs were weighed before storage.

Chemical analyses

Haemoglobin concentration was analyzed with a blood cell counter (Model K-1000, Sysmex, IJsselstein, The Netherlands). Plasma iron concentrations and total iron-binding capacity were determined spectrophotometrically using a commercial test kit (Roche Nederland, Mijdrecht, The Netherlands). Spleen and liver were washed with saline and liver was homogenized as described below. Liver homogenate, spleen and tibia were dried (100°C, 12 hours) and ashed (500°C, 16 hours). The ash was dissolved in 1 mL 6N HCl and diluted with demineralized water. Iron was measured by flame atomic absorption spectrometry (Model AA-475, Varian, Springvale, Australia). Iron in tibia was calculated as the mean of left and right tibia.

Plasma and liver retinol was measured by reversed phase high performance liquid chromatography (HPLC). Plasma (100 μL) was mixed vigorously with ethanol (90% v/v, 400 μL), and after centrifuging (1580 g, 10 min), retinol was determined directly in the supernatant and calibrated against solutions of retinol in ethanol (72%, v/v). Serum pools with retinol concentrations of 0.63 or 2.16 μmol/L were used as external controls. The combined between- and within-run variation of retinol determination in the two pools was 6% and 4% (coefficient of variation). The particulars of the HPLC system used were as follows: injection volume, 50 μL; pre-column, 10 × 3 mm stainless steel packed with Chromguard reversed phase

(Chrompack, Middelburg, The Netherlands); column, 100 × 3 mm glass cartridge packed with Spherisorb-ODS (5μm) (Chrompack); isocratic pump (Spectra Physics, San Jose, CA, USA); UV/vis detector (Spectra Focus, forward optical scanning detector, Spectra Physics, San Jose, CA, USA) with wavelength of 325 nm; mobile phase, methanol:water (90:10, v/v); flow rate, 0.4 mL/min; run time, 5 min; retention time, 3.5 min.

Liver total retinol was determined after saponification and extraction. Liver homogenate (200 μL; liver:demineralised water, 1:5, w/v) was digested by heating at 100°C for 15 min in 1.5 mL of 0.7 mol/L KOH in 50% ethanol (containing 6 g/L pyrogallol), and after cooling the mixture was extracted twice with 4 mL of hexane. Standards with retinol acetate in absolute ethanol were processed identically and used for calibration. After centrifugation (1580 g, 6 min), the upper layer of the hexane extract was collected and retinol determined using the HPLC method described above, except for the following conditions: injection volume, 20 μL; mobile phase, methanol:water (95:5, v/v); flow rate, 0.4 mL/min; run time, 3 min; retention time, 1.6 min. Recovery as determined by adding known amounts of retinol acetate to homogenates prior to digestion was 90-95%. A pooled liver homogenate with target value of 98 μmol retinol/L was used as an external control. The combined between- and within-run variation of retinol determination was 6% (coefficient of variation). No impurities were detected when peak purity was tested using the scanning function of detector over a wavelength range of 220-600 nm.

Statistical analysis

The data from the offspring were subjected to two-way analysis of variance with vitamin A feeding and gender as independent variables. The data from the dams and from the male and female pups separately at the three experimental points, were subjected to one-way analysis of variance with vitamin A feeding as independent variable. Contrasts based on Student's t-test were used to test whether the groups fed 0 to 450 RE/kg feed were significantly different from the control group fed 1200 RE/kg feed. Bonferroni's adaptation for multiple comparisons was used.

RESULTS

Table 3 Vitamin A in plasma and liver, body weight and liver weight of the dams at 22-29 days after parturition.

Dietary retinol RE/kg feed	n	Plasma retinol μmol/L	Liver retinol μmol/g wet weight	Body weight g	Liver weight g
1200†	6	0.82	0.73	260.9	10.9
450	7	1.02	0.33*	272.9	12.8
150	8	0.94	0.28*	261.5	10.4
75	6	1.04	0.29*	258.0	11.1
0	9	1.01	0.22*	253.3	10.1
Pooled SE‡		0.31	0.03	44.7	4.8

† Statistical significant difference (Student's t-test) versus control group fed diet with 1200 RE/kg; *, $p<0.0125$.

‡ Pooled SE are calculated as $SE=\sqrt{MS_{within}}/\sqrt{n}$, with $n=6$, the smallest group and MS_{within}, as mean squares derived from one-way analysis of variance.

Table 4 Iron status of the dams.

Dietary retinol RE/kg feed	n	Iron in organs Liver	Spleen	Tibia	Haemo-globin	Total iron-binding capacity	Plasma iron
		μmol/g dry weight			mmol/L	μmol/L	μmol/L
1200	6	8.04	43.4	1.48	10.12	86.81	62.86
450	7	4.88	45.5	1.57	9.80	94.31	60.31
150	8	7.25	62.9	1.80	9.68	85.71	62.03
75	6	8.25	58.3	1.76	10.05	87.14	60.05
0	9	8.85	72.1	1.94	9.99	82.54	62.08
Pooled SE†		7.20	49.7	0.81	1.03	18.52	21.57

† Pooled SE are calculated as $SE=\sqrt{MS_{within}}/\sqrt{n}$, with $n=6$, the smallest group and MS_{within}, as mean squares derived from one-way analysis of variance.

Characteristics of dams

The dams did not differ in body weight, liver weight and plasma retinol levels (*Table 3*). Only liver retinol concentration was decreased in the groups fed the restricted levels of vitamin A when compared to the control group (*Table 3*). Iron status, as illustrated by iron levels in organs, blood haemoglobin, plasma iron and total iron-binding capacity, did not differ among the five groups of dams (*Table 4*).

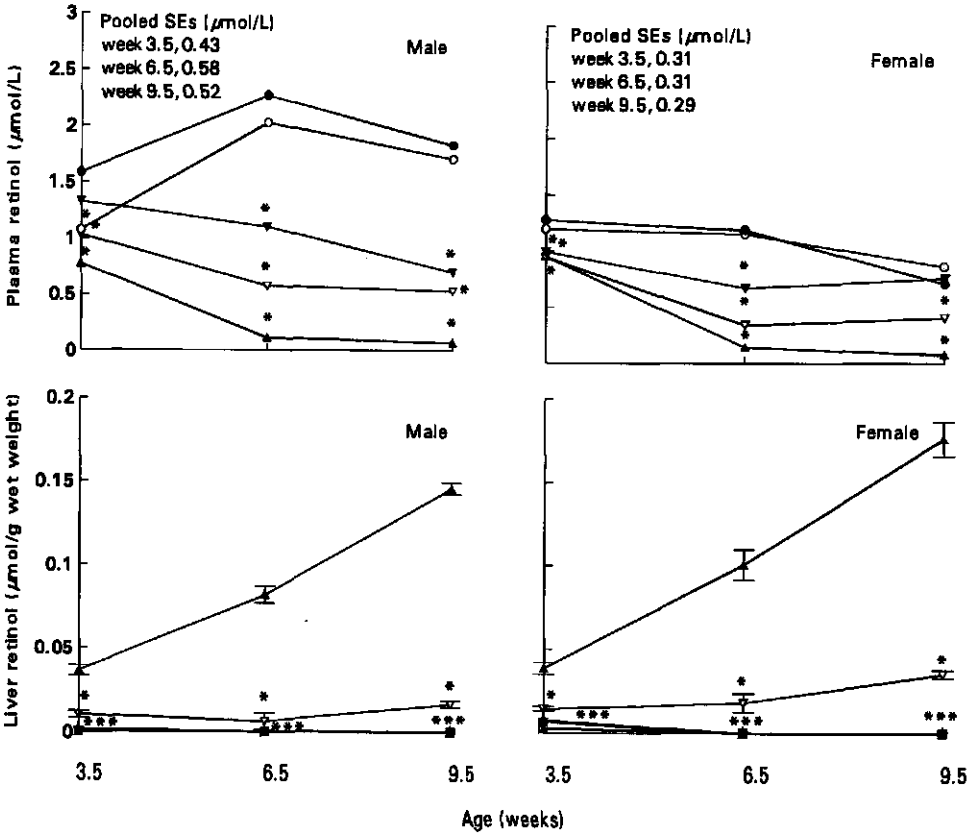

FIGURE 1 Plasma retinol and liver retinol concentrations in male and female pups fed diets containing the following amounts of vitamin A: • 1200 RE (control group), ○ 450 RE, ▼ 150 RE, ▽ 75 RE and ▲ 0 RE/kg feed. Means of 5 animals per group are given together with pooled standard errors (SE). Separate SEs for liver retinol are shown as error bars for the groups fed 1200 and 450 RE/kg. Differences between the groups fed diets with 0 to 450 RE/kg feed versus the control group fed 1200 RE/kg feed: *, $p < 0.0125$. Analysis of variance showed significant effects of gender ($p < 0.001$) and vitamin A feeding ($p < 0.001$) and their interaction ($p < 0.001$) for plasma retinol and of gender ($p = 0.031$) and vitamin A feeding ($p < 0.001$) for liver retinol.

Vitamin A status of pups

Plasma retinol was reduced in the groups fed 0, 75 and 150 RE/kg feed in both male and female pups when compared with their control group fed 1200 RE/kg feed. Levels of plasma retinol in the control groups were lower in female pups when compared with male pups (*Figure 1*). This was shown by a significant gender effect ($p < 0.001$) in the variance analysis. Liver retinol was low in the groups fed 450 RE/kg feed and undetectable in the groups fed lower levels of vitamin A both in female and in male pups. The control groups seemed to accumulate hepatic retinol (*Figure 1*). Analysis of variance revealed a small effect of gender ($p = 0.031$) on liver retinol concentrations.

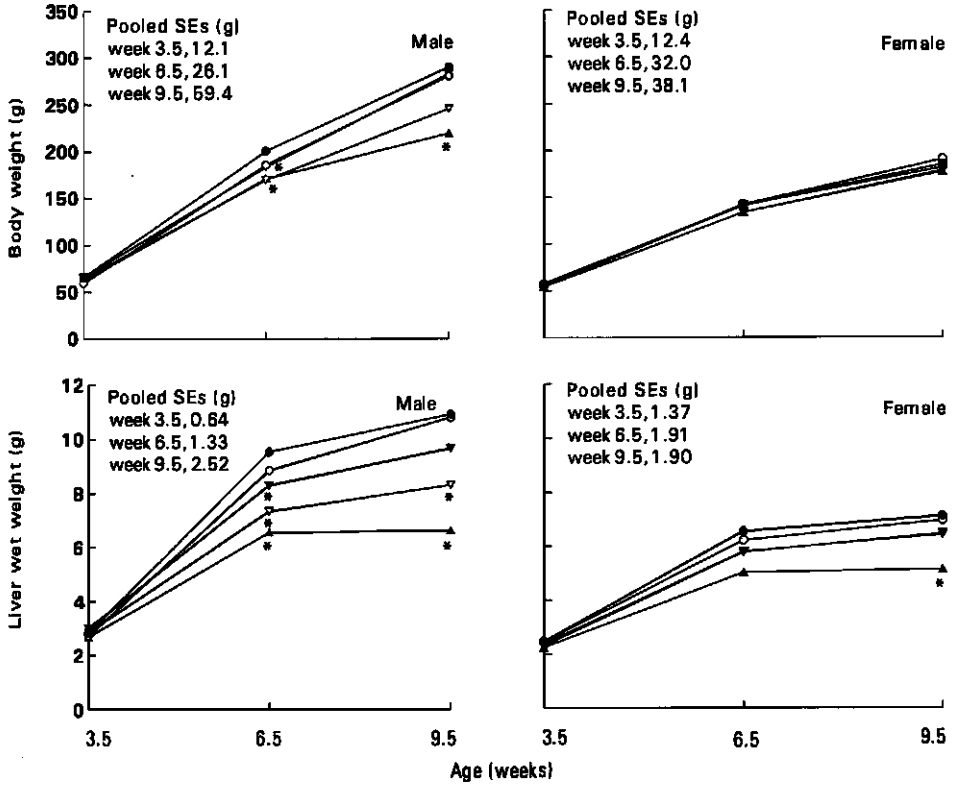

FIGURE 2 Body weight and liver weight in male and female pups fed diets containing the following amounts of vitamin A: ● 1200 RE (control group), ○ 450 RE, ▼ 150 RE, ▽ 75 RE and ▲ 0 RE/kg feed. Means of 5 animals per group are given together with pooled standard errors (SE). Differences between the groups fed diets with 0 to 450 RE/kg feed versus the control group fed 1200 RE/kg feed: *, $p < 0.0125$. Analysis of variance showed significant effects of gender ($p < 0.001$) for body weight and of gender ($p < 0.001$) and vitamin A feeding ($p = 0.007$) for liver weight.

Body weight and liver weight of pups

Dietary vitamin A reduced body weight and liver weight of the young rats from week 6.5 onwards in the groups of male pups fed the lowest levels of dietary vitamin A (0-150 RE/kg), compared with the control group, which received 1200 RE/kg feed (*Figure 2*). This could be explained in part by a reduced feed intake, which was (at week 9.5) 20.2, 19.2, 19.0 g/day in the groups fed 1200, 450 and 150 RE/kg respectively, 16.3 g/day in the group fed 75 RE/kg and 13.1 g/day in the male pups fed no vitamin A. The female pups that received no dietary vitamin A had lower liver weights at week 9.5 when compared to the control group (*Figure 2*). Body weight and liver weight were lower in the female then in the male pups ($p < 0.001$, analysis of variance). Also feed intake was lower in female pups, 12.9 g/day (overall mean) when compared to male pups, 17.6 g/day, at week 9.5.

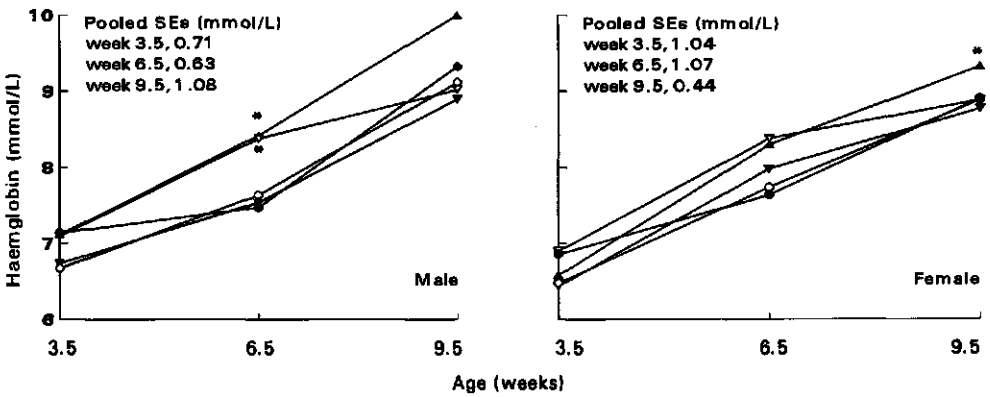

FIGURE 3 Haemoglobin concentration in male and female pups fed diets containing the following amounts of vitamin A: • 1200 RE (control group), ○ 450 RE, ▼ 150 RE, ▽ 75 RE and ▲ 0 RE/kg feed. *Means of 5 animals per group are given together with pooled standard errors (SE). Differences between* the groups fed diets with 0 to 450 RE/kg feed versus the control group fed 1200 RE/kg feed: *, $p < 0.0125$. Analysis of variance showed significant effects of gender ($p = 0.021$) and vitamin A feeding ($p = 0.004$).

Iron status of pups

Haemoglobin levels were increased in the groups fed 0 and 75 RE/kg dietary vitamin A at week 6.5 in the male pups and at week 9.5 in the female pups fed the diet without added vitamin A (*Figure 3*). Differences were relative to the control groups. Analysis of variance also showed that haemoglobin levels differed among sexes ($p = 0.021$).

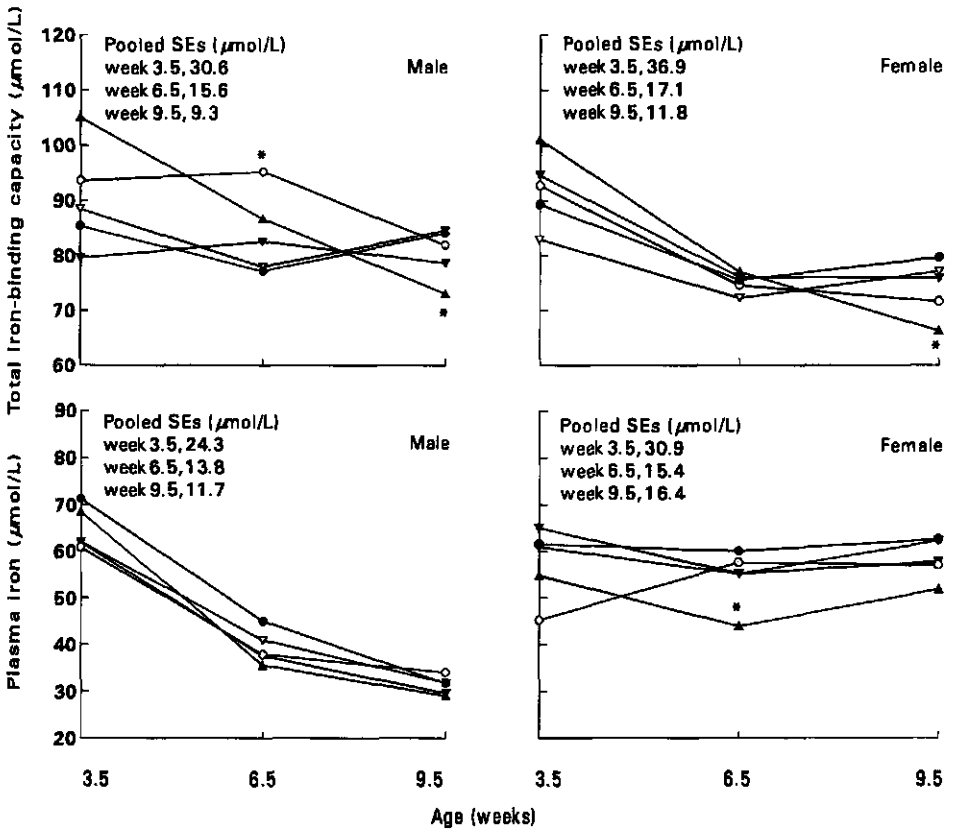

FIGURE 4 Total iron-binding capacity and plasma iron concentrations in male and female pups fed diets containing the following amounts of vitamin A: ● 1200 RE (control group), ○ 450 RE, ▼ 150 RE, ▽ 75 RE and ▲ 0 RE/kg feed. Means of 5 animals per group are given together with pooled standard errors (SE). Differences between the groups fed diets with 0 to 450 RE/kg feed versus the control group fed 1200 RE/kg feed: *, $p < 0.0125$. Analysis of variance showed significant effects of gender ($p = 0.024$) for total iron-binding capacity and of gender ($p < 0.001$) and vitamin A feeding ($p = 0.002$) for plasma iron.

Total iron-binding capacity was increased at week 6.5 in the male pups fed 450 RE/kg feed when compared to the control group. This effect disappeared at week 9.5, when total iron-binding capacity was reduced below control levels in both male and female offspring that received no vitamin A (*Figure 4*). Plasma iron levels were also below control levels in the female pups given no vitamin A at week 6.5, but this reduction failed to reach significance at week 9.5 (*Figure 4*). Analysis of variance revealed a significant effect of gender on total iron-binding capacity ($p = 0.024$) and plasma iron ($p < 0.001$).

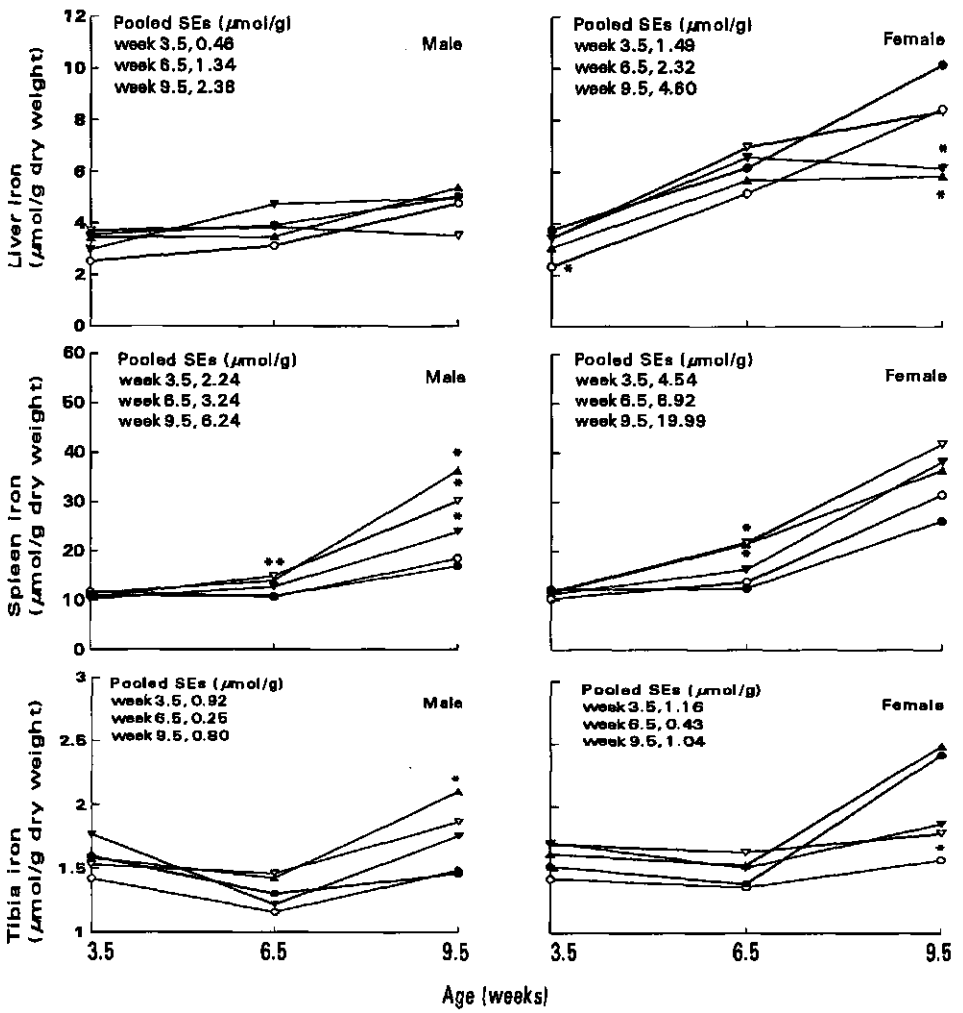

FIGURE 5 Iron concentrations of liver, spleen and tibia in male and female pups fed diets containing the following amounts of vitamin A: ● 1200 RE (control group), ○ 450 RE, ▼ 150 RE, ▽ 75 RE and ▲ 0 RE/kg feed. Means of 5 animals per group are given together with pooled standard errors (SE). Differences between the groups fed diets with 0 to 450 RE/kg feed versus the control group fed 1200 RE/kg feed: *, $p < 0.0125$. Analysis of variance showed significant effects of gender ($p < 0.001$) and vitamin A feeding ($p = 0.018$) for liver iron, of gender ($p = 0.001$), vitamin A feeding ($p < 0.001$) and their interaction ($p = 0.020$) for spleen iron and of gender ($p = 0.001$) and vitamin A feeding ($p < 0.001$) for tibia iron.

Hepatic iron concentrations were reduced below control levels in the female pups at week 3.5 in the group fed 450 RE/kg feed and at week 9.5 in the groups fed 150 and 0 RE/kg feed (*Figure 5*). Splenic iron was increased above control levels at week 6.5 in both male and female pups fed 75 and 0 RE/kg feed. At week 9.5, this increase was significant in the male rats fed 0, 75 or 150 RE/kg diet, in the female pups there were no significant differences due to large variation (*Figure 5*). Tibia iron was also increased above control level in the male but not in the female offspring fed diets without vitamin A (*Figure 5*). Iron in liver ($p < 0.001$), spleen and tibia ($p = 0.001$) differed between gender according to the variance analysis.

DISCUSSION

With the two-generation rat model described here we aimed at inducing chronic vitamin A deficiency with different degrees of severity so as to study the interrelationship between vitamin A and iron metabolism. Thus we attempted to influence vitamin A status of rat pups as early as at the time of conception by feeding diets with different vitamin A concentrations to the dams. Vitamin A status of the pups was not only influenced through the dams but also directly by consumption of the experimental diets. As from the age of about 12 days rat pups start to consume solid food in addition to the mother's milk. The first assessment of vitamin A status in the pups was at age 3.5 weeks. Vitamin A status of the dams was affected mildly by the amount of vitamin A in the diet. There was no effect on plasma retinol although liver retinol concentrations were reduced significantly (Table 3). Liver retinol concentration determines the amount of vitamin A transferred into the milk during the lactation period while plasma retinol levels remain constant [15]. Thus, the maternal vitamin A status contributed to at least part of the differences in vitamin A status of the pups as measured at the age of 3.5 weeks.

There was no effect of the dietary treatment on maternal plasma retinol levels. This could explain why there was no difference in reproduction outcome among the groups. Wallingford & Underwood [16] concluded from their studies in Sprague-Dawley rats, that dams require at least 0.011 μmol retinol/g liver to maintain systemic vitamin A metabolism during pregnancy. In this study liver retinol concentrations of the dams exceeded these levels substantially. This was almost certainly caused by the fact that the dams were fed a commercial diet until the age of 10 weeks, when they entered the study. The vitamin A concentrations

of the commercial diet used was almost five times higher than the recommended level and must have induced high vitamin A stores. The abundant vitamin A status of the dams probably caused that the initial plasma retinol concentrations of the pups were higher than those seen by Gardner & Ross [17] in a similar experiment.

In this study, male and female rats differed in plasma retinol levels. From the age of 6.5 weeks, male pups had higher plasma retinol levels than females when fed 450 and 1200 RE/kg feed. Females seem to accumulate more vitamin A in liver. This might partly be explained by a lower feed intake and growth rate and thus a lower body weight and liver weight in the female pups. Also the effects of restricted dietary vitamin A on body weight and liver weight are more pronounced in male than in female pups.

Vitamin A deficiency has been shown to affect iron status in humans [8,9] and rats [10,13,14, **chapters 2,4 and 6**, 18-20]. In the present study the increase in haemoglobin levels and reduction of plasma iron in vitamin A deficiency were very small and subject to large variation. Despite this, we found a significant reduction in total iron-binding capacity and a significant increase in splenic iron with vitamin A deficiency in both female and male rats. The increased haemoglobin levels with vitamin A deficiency had been found previously [10,13, **chapters 2 and 4**, 18,21,22]. This had been associated with haemoconcentration due to water imbalance and reduced growth [23,24].

Despite the well-known haemoconcentration in rats with vitamin A deficiency, we observed a decrease in total iron-binding capacity, which had been reported earlier in both humans [2-4,8] and rats with vitamin A deficiency [10,13,14, **chapters 2,4 and 6**, 18,19]. It has been suggested that vitamin A deficiency interferes with the synthesis of the glycoprotein, transferrin, resulting in low total iron-binding capacity [25]. This in turn might lead to impaired iron transport, explaining the slightly lowered plasma iron levels in the rats raised and fed on the diet without vitamin A. Impaired iron transport might lead to iron accumulation in organs. Indeed, we found an increase in splenic iron concentrations with vitamin A deficiency which has also been reported earlier by Mejía et al. [18] and Roodenburg et al. [10,13,14, **chapters 2,4 and 6**]. The extra splenic iron might be located in the macrophages.

In conclusion, this two-generation experiment resulted in a range of vitamin A deficient states in young growing rats, and revealed a difference between male and female rats concerning plasma retinol concentrations and the effects of vitamin A deficiency on growth, liver weight and iron status. We did find effects of vitamin A deficiency on total iron-binding capacity and splenic iron. However, effects of vitamin A deficiency on other iron parameters were unclear in this study. The large

variation in parameters such as iron concentrations in the different organs, total iron-binding capacity and plasma iron concentration might have confounded possible effects of vitamin A deficiency. We must state that the animal model described here is less suitable for studying effects of vitamin A deficiency on iron metabolism than rat models we have used before. The 10 weeks feeding of male weanling rats with a diet without vitamin A produced a progressive derailment of iron metabolism, including increases in splenic iron and decreases in total iron-binding capacity [10, **chapter 2**]. In young male rats with different degrees of vitamin A deficiency that were produced exactly as described in this paper, we could clearly demonstrate that impaired vitamin A status reduces the efficacy of iron repletion after the induction of iron deficiency [13, **chapter 4**].

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CHAPTER 6

Iron status in female rats with different, stable plasma retinol concentrations

ABSTRACT

To mimic the impaired iron status seen in children with chronic vitamin A deficiency, an attempt was made to produce rats with different, but more or less constant vitamin A status. Dams were fed different levels of vitamin A (1200, 450, 150 or 75 RE/kg feed) throughout pregnancy and lactation and their pups received the same diets. Blood parameters of vitamin A and iron status were measured throughout the study in the female pups until they were aged 41 weeks. At the end of the experiment blood and organs were collected for determination of vitamin A and iron status. In this rat model, different degrees of vitamin A deficiency were associated with almost constant plasma retinol concentrations. Vitamin A deficiency produced increases in spleen and tibia iron, and reductions in plasma iron, total iron-binding capacity and mean corpuscular haemoglobin concentration.

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INTRODUCTION

In children [1,2] and pregnant women [3,4] from areas where vitamin A deficiency is endemic, low plasma retinol levels are associated with impaired iron status. We have attempted to mimic the situation in children with chronic, but stabilized vitamin A deficiency in a rat model. Dams were fed diets with different vitamin A levels throughout pregnancy and lactation and their pups received the same diets as their mothers both before and after weaning [5,**chapter 5**]. It was expected on the basis of studies in chickens [6] that the young rats would have different, but more or less constant plasma retinol concentrations. However, in the female offspring aged 3.5-9.5 weeks there was no clear relationship between vitamin A intake and plasma retinol concentration, this concentration not being stable [5,**chapter 5**]. In this paper we report on female rats from the same study, but now describe their vitamin A and iron status for the age of 16-41 weeks. We had hypothesized that long-term, insufficient vitamin A intake would lead to a new steady state of plasma retinol concentration, pointing at a certain degree of chronic, marginal vitamin A deficiency. The rats in this study were given free access to the experimental diets with different vitamin A levels so as to optimally simulate the human target population. To enhance interpretation of the outcome by taking into account any group differences in growth performance, the data were subjected to analysis of variance with body weight as covariate.

METHODS

The experimental protocol was approved and its conduct supervised by the animal welfare officer of Wageningen Agricultural University.

Animals, housing and diets

The rats used in this study were derived from a breeding design described in detail earlier [5,**chapter 5**]. The female Wistar rats (Cpb:WU) were bred from dams that had been fed on diets with one of five vitamin A levels from two weeks before and throughout pregnancy and lactation. The diets were formulated according to the nutrient requirements of rats [7] and contained either the recommended vitamin A level (1200 retinol equivalents/kg feed) or

450, 150, 75 or 0 retinol equivalents per kg feed (*Table 1*).

Table 1 Composition of the pelleted diets.

Ingredients	
Casein (g)	151
Maize oil (g)	25
Coconut fat (g)	25
Glucose (g)	329.6
Corn starch (g)	329.6
Cellulose (g)	30
$CaCO_3$ (g)	12.4
$NaH_2PO_4.2H_2O$ (g)	15.1
$MgCO_3$ (g)	1.4
KCl (g)	1.0
$KHCO_3$ (g)	7.7
$FeSO_4.7H_2O$ (mg)	124
Molasses (g)¶	50
Vitamin A preparation (RE)†	1200/450/150/75/0
Mineral premix (iron-free) (g)‡	10
Vitamin premix (vitamin A-free) (g)*	12

¶ The molasses contained 0.2 mg iron/g.

† Rovimix A 500^R, 150 RE/mg (F. Hoffmann-La Roche & Co. Ltd, Basle, Switzerland), consisting of retinyl acetate and retinyl palmitate; of this preparation either 1200, 450, 150, 75 or 0 retinol equivalents (RE)/kg feed was added.

‡ The mineral premix consisted of (mg): MnO_2, 79; $ZnSO_4.H_2O$, 33; $NiSO_4.6H_2O$, 13; NaF, 2; KI, 0.2; $CuSO_4.5H_2O$, 15.7; $Na_2SeO_3.5H_2O$, 0.3; $CrCl_3.6H_2O$, 1.5; $SnCl_2.2H_2O$, 1.9; NH_4VO_3, 0.2; maize meal, 9853.2.

* The vitamin premix consisted of (mg): thiamin, 4; riboflavin, 3; niacin, 20; D,L-calcium pantothenate, 17.8; pyridoxine, 6; cyanocobalamin, 50; choline chloride, 2000; folic acid, 1; biotin, 2; menadione, 0.05; D,L-α tocopheryl acetate, 60; cholecalciferol, 2 (1000 IU); maize meal, 9834.15.

From each dietary group, 12 female pups were used for the present experiment. After weaning, the pups were housed in groups of four animals in stainless steel cages with wire mesh bases (30x42x19 cm). The rats were randomly assigned to cage position within the room which had a controlled light/dark cycle (light on: 0600-1800 h), temperature (20-22°C) and relative humidity (50-60%). The pups were fed the same diet as their mothers. All animals had free access to feed and demineralised water throughout. Feed was stored at -20°C until it was used. The animals were weighed weekly and feed intake per cage was monitored twice a week.

At the age of 16 weeks, the rats that had received the vitamin A free diet were killed, because their condition was deteriorating rapidly. Blood was collected before killing. From the other animals a blood sample also was taken at the age of 16 weeks for the assessment of vitamin A and iron status. A second and third blood sample was taken when the animals were 24 and 31 weeks old. At the age of 41 weeks, all animals were killed and blood and organs were collected.

Collection of samples

Blood was collected in heparinized vials by orbital puncture while the rats were under diethyl ether anaesthesia. The blood was stored at 0°C for haematological examination on the same day. Then, plasma was isolated by centrifugation (10 min, 1580 g) and stored at -20°C until analysis, except for 250 μl which was stored at -80°C for subsequent analysis of retinol. At the end of the experiment when the rats were aged 41 weeks, they were decapitated while still anaesthetized, immediately after bleeding. The left kidney, liver, spleen and both hindlegs were removed and stored at -20°C until analysis. Organs were weighed before storage.

Chemical analyses

Haemoglobin concentration, red blood cell count and mean corpuscular haemoglobin concentration were analyzed with a blood cell counter (Model K-1000, Sysmex, IJsselstein, The Netherlands). Plasma iron concentrations and total iron-binding capacity were determined spectrophotometrically using a commercial test kit (Roche Nederland, Mijdrecht, The Netherlands). Spleen and liver were washed with saline and liver was homogenized as described elsewhere [8,**chapter 2**]. Liver homogenate, spleen, kidney and tibia were dried (100°C, 12 hours) and ashed (500°C, 16 hours). The ash was dissolved in 1 ml 6N HCl and diluted with demineralized water. Iron was measured by flame atomic absorption spectrometry (Model AA-475, Varian, Springvale, Victoria, Australia). All analyses were carried out singly. Iron in tibia was calculated as the mean of left and right tibia. Plasma and liver retinol was measured by *reversed phase high performance liquid chromatography. Plasma retinol was* determined directly in the supernant fraction after extraction with ethanol [8,**chapter 2**]. Total liver retinol was measured after saponification and extraction [8,**chapter 2**].

Statistical analysis

For each time point (16, 24, 31 and 41 weeks) the data were evaluated by analysis of variance with body weight as covariate and vitamin A intake as independent variable. Contrasts based on Student's t-test and pooled variances

based on one-way analysis of variance were used to identify statistically significant differences between the control group fed on the diet sufficient in vitamin A and the four test groups given the vitamin A deficient diets. If variances were not homogeneous (Cochrans C-test) the data were log-transformed prior to statistical testing. A pre-set p value of 0.05 with Bonferroni's adaptation resulted in a level of significance of $p < 0.0125$ or < 0.017.

RESULTS

Vitamin A status

When compared with the control group that was given 1200 RE/kg feed, plasma retinol was reduced throughout in those groups that received 75 or 150 RE/kg diet (*Figure 1*). At the age of 16 weeks, the rats fed on the vitamin A-free diet had an extremely low level of plasma retinol. The rats given 450 RE/kg feed had plasma retinol levels similar to those of the control group (*Figure 1*). Liver retinol concentrations at the age of 41 weeks were markedly reduced in the group with 450 RE/kg, but even more so when the rats were given 150 or 75 RE/kg diet (*Table 2*). The group differences in plasma and liver retinol were associated with differences in body weight as can be concluded from the analysis of variance with body weight as covariate, but vitamin A intake remained a significant determinant after correction for body weight differences (*Tables 3 and 4*).

Table 2 Liver retinol concentrations in rats aged 41 weeks.

Dietary vitamin A (RE/kg feed)	Liver retinol concentration (nmol/g wet weight)†
1200	741.2 ± 191.4
450	23.2 ± 5.6*
150	1.3 ± 0.3*
75	0.2 ± 0.1*

Values represent means and SEs (n = 12).

† Data were log-transformed prior to statistical analysis.

Significant difference (Student's t-test) versus control group given 1200 RE/kg feed: *, $P < 0.017$.

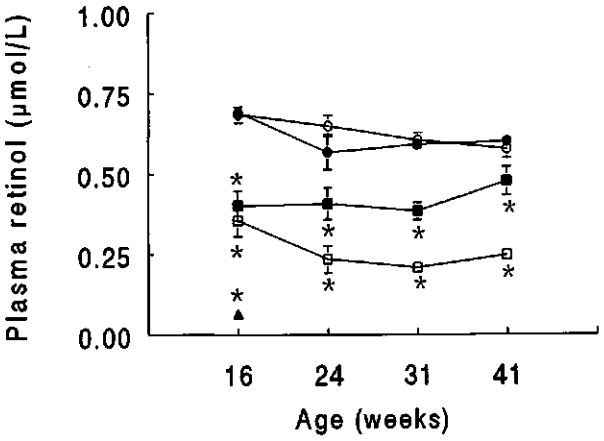

FIGURE 1 Plasma retinol concentration in female rats fed diets containing different amounts of vitamin A: ●, 1200 (control group); ○, 450; ■, 150; □, 75; and ▲, 0 RE/kg feed. Means of 12 animals per group and their standard errors are given. Differences versus the control group fed 1200 RE/kg feed: *, $p < 0.0125$ at the age of 16 weeks or $p < 0.017$ at the age of 24, 31 and 41 weeks.

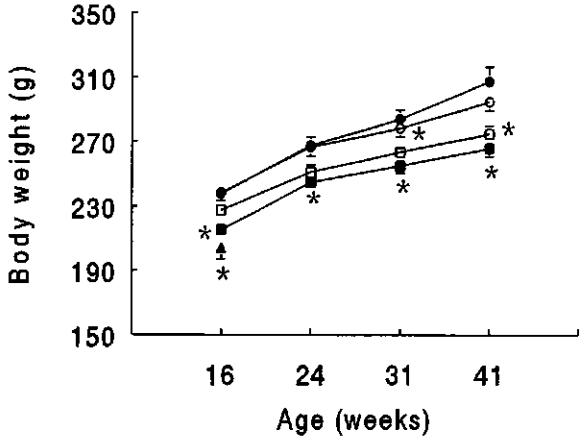

FIGURE 2 Body weight of female rats fed diets containing different amounts of vitamin A: ●, 1200 (control group); ○, 450; ■, 150; □, 75; and ▲, 0 RE/kg feed. Means of 12 animals per group and their standard errors are given. Differences versus the control group fed 1200 RE/kg feed: *, $p < 0.0125$ at the age of 16 weeks or $p < 0.017$ at the age of 24, 31 and 41 weeks.

Body weight and organ weights

Body weight at the age of 16 weeks was reduced in the rats fed either 0 or 150 RE/kg diet. From the age of 31 weeks, the animals that received either

75 or 150 RE/kg diet had a lower body weight compared with the control group fed on the diet with 1200 RE/kg, which was also reflected by a lower liver weight (*Figure 2, Table 4*). The weight of kidney and spleen were not affected by the dietary regimen (*Figure 2*). From the analysis of variance with body weight as covariate it followed that changes in body weight had influenced the vitamin A effects on liver weight (*Table 4*).

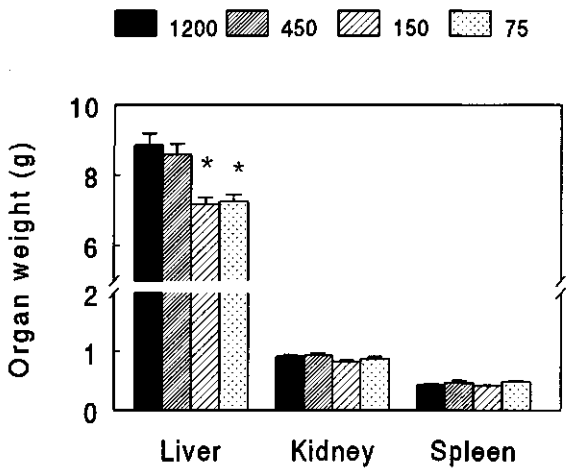

FIGURE 3 Organ weight of female rats aged 41 weeks fed diets containing different amounts of vitamin A: 1200 (control group), 450, 150 and 75 RE/kg feed. Means of 12 animals per group and their standard errors are given. Differences versus the control group fed 1200 RE/kg feed: *, $p < 0.017$.

Iron status

Haemoglobin concentration was not systematically affected by vitamin A intake (*Figure 4a*). Mean corpuscular haemoglobin concentration was consistently reduced in all groups fed less than the recommended amount of vitamin A (*Figure 4b*). Body weight had influenced mean corpuscular haemoglobin concentration but, except for the age of 41 weeks, vitamin A also was a significant determinant (*Table 3*). Red blood cell count and haematocrit did not differ between dietary groups (not shown).

Plasma iron concentrations were systematically reduced in the group fed on the diet with 75 RE/kg (*Figure 4c*). At the age of 24 and 31 weeks, the animals fed 150 RE/kg feed also had reduced plasma iron levels relative to the control group. At the age of 16 weeks total iron-binding capacity was reduced relative to the control group in the animals fed either 0, 75 or 150 RE/kg (*Figure*

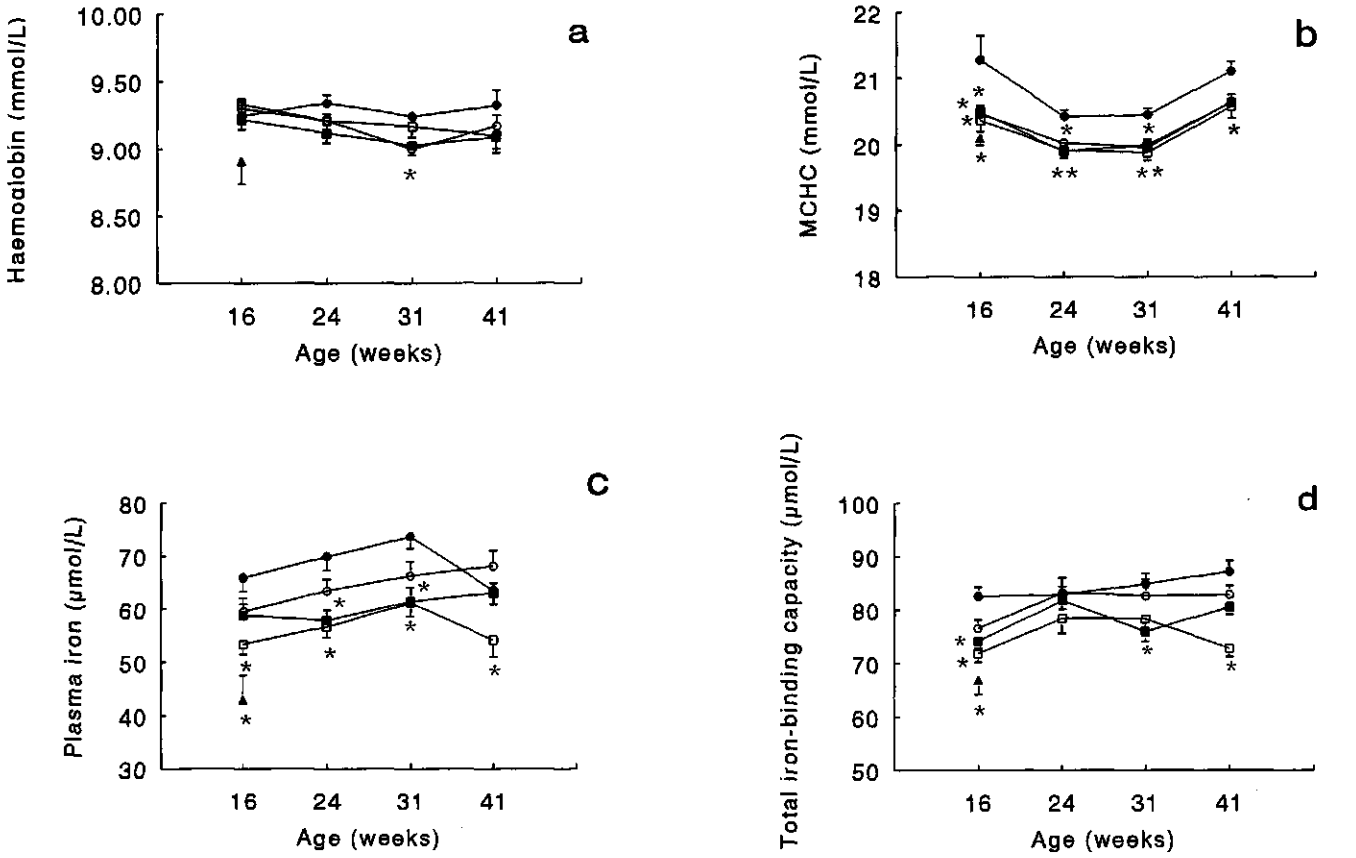

FIGURE 4 Blood haemoglobin concentration (a), mean corpuscular haemoglobin concentration (MCHC) (b), plasma iron concentration (c) and total iron-binding capacity (d) in female rats fed diets containing different amounts of vitamin A: ●, 1200 (control group); ○, 450; ■, 150; □, 75; and ▲, 0 RE/kg feed. Means of 12 animals per group and their standard errors are given. Differences versus the control group fed 1200 RE/kg feed: *, $p < 0.0125$ at the age of 16 weeks or $p < 0.017$ at the age of 24, 31 and 41 weeks.

4d). The groups given either 75 or 150 RE/kg also had a low total iron-binding capacity at the age of 41 weeks but not at the age of 24 weeks. Total iron-binding capacity and plasma iron were significantly associated with body weight at the age of 16 weeks, but not at older age (*Table 3*).

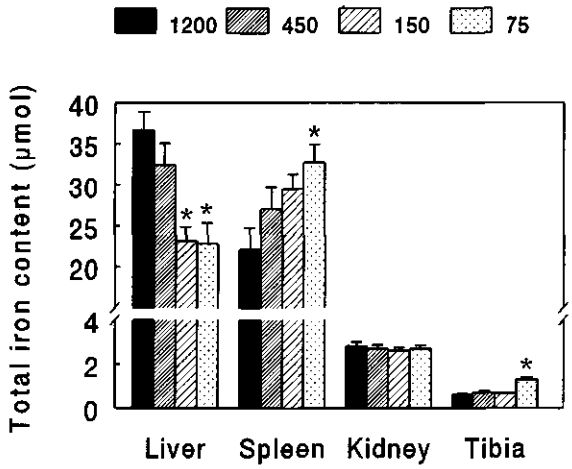

FIGURE 5 Total iron content in liver, spleen, kidney and tibia (mean of left and right tibia) in female rats fed diets containing different amounts of vitamin A: 1200 (control group), 450, 150 and 75 RE/kg feed. Means of 12 animals per group and their standard errors are given. Differences versus the control group fed 1200 RE/kg feed: *, $p < 0.017$. For tibia, data were log-transformed prior to analysis.

Total iron amount in organs at the age of 41 weeks is presented in *Figure 5*. Liver iron was reduced in the animals fed either 75 or 150 RE/kg when compared with the control group fed on the diet with 1200 RE/kg. Total iron in spleen and tibia was increased in the group given 75 RE/kg feed. There was no effect of dietary vitamin A on kidney iron (*Figure 5*). Differences in body weight affected the amount of iron in liver and tibia, but vitamin A intake remained a significant determinant after correction for body weight differences (*Table 4*).

DISCUSSION

The objective of this study was to produce rats with different, but more or less constant vitamin A status so as to simulate the situation in children with chronic vitamin A deficiency. Female rats were fed diets with different levels of vitamin A for a period of 41 weeks. With liver retinol concentration as indicator, the vitamin A status of the rats was shown to be affected by vitamin A intake in a dose dependent fashion. The low liver retinol concentration in the group fed the diet with 450 RE/kg, when compared with feeding the recommended level of dietary vitamin A (1200 RE/kg), was associated with an unchanged plasma

Table 3 P values of analysis of variance with body weight (BW) as covariate and vitamin A feeding (A) as independent variable for plasma retinol, haemoglobin, mean corpuscular haemoglobin concentration, plasma iron and total iron-binding capacity at the age of 16, 24, 31 and 41 weeks.

Age (w)	Plasma retinol		Haemoglobin		Mean corpuscular haemoglobin concentration		Plasma iron		Total iron-binding capacity	
	BW	A	BW	A	BW	A	BW	A	BW	A
16	<0.001	<0.001	0.888	0.006	0.001	0.018	0.001	0.001	<0.001	0.001
24	<0.001	<0.001	0.260	0.225	0.024	0.006	0.091	0.001	0.059	0.614
31	<0.001	<0.001	0.655	0.013	0.002	0.005	0.509	0.001	0.341	0.033
41	<0.001	<0.001	0.946	0.192	0.002	0.263	0.402	0.001	0.307	<0.001

Table 4 P values of analysis of variance with body weight (BW) as covariate and vitamin A feeding (A) as independent variable for liver retinol concentration, organ weights, and total organ iron contents at the age of 41 weeks.

Source of variation	Liver retinol concentration†	Organ weight			Total iron content			
		Liver	Kidney	Spleen	Liver	Kidney	Spleen†	Tibia
BW	<0.001	<0.001	<0.001	0.020	<0.001	0.023	0.571	0.001
A	<0.001	0.049	0.521	0.004	0.011	0.964	0.011	<0.001

† Data were log-transformed prior to analysis.

retinol concentration.

Liver retinol concentrations of the groups that received 150 and 75 RE/kg feed were very low and were associated with a different, low level of plasma retinol which was rather stable between 16 and 41 weeks of age. Thus, the chronic feeding of diets with 450, 150 or 75 RE/kg had produced rats with different, almost constant plasma retinol concentrations. It could be suggested that the rats fed the diets with different vitamin A concentrations had developed different steady states of vitamin A status.

Dietary vitamin A dosage affected body weight of the rats: the groups receiving either 150 or 75 RE/kg feed displayed a decrease in body weight. The variance of body weight explained part of the variation found in the vitamin A and iron status indicators as was anticipated on the basis of literature data [9-11]. However, analysis of variance with body weight as covariate showed that vitamin A intake was a significant determinant of vitamin A and iron status. It follows that vitamin A intake has a direct influence not only on vitamin A status, but also on iron status.

Blood haemoglobin concentrations were not systematically affected by vitamin A deficiency as induced after feeding either 150 or 75 RE/kg feed. In contrast, previous work has shown increased haemoglobin levels in vitamin A deficiency [8,12-14,**chapter 2**]. Possibly, haemoconcentration co-occurring with reduced growth and water imbalance due to severe vitamin A deficiency [9-11] was not so severe as in earlier experiments [8,15,**chapters 2,4**]. Haematocrit had not changed significantly, but mean corpuscular haemoglobin concentration was reduced in the vitamin A deficient groups, pointing to hypochromic red blood cells, as reported before [16]. In earlier studies we found no influence of vitamin A deficiency on mean corpuscular haemoglobin concentration [8,15,**chapters 2,4**], possibly because those studies had a relatively short duration. We have hypothesized [15,**chapter 4**] that impaired erythropoiesis might explain the changes in haematologic indices in vitamin A deficiency. In both humans [1-3,17] and rats with vitamin A deficiency [8,15,**chapters 2,4**] a decrease in total iron-binding capacity has been reported. It has been suggested that vitamin A deficiency interferes with the synthesis of the glycoprotein, transferrin, resulting in low total iron-binding capacity [18].

Vitamin A deficiency produced a reduction in the hepatic iron stores and an increase in the iron contents of tibia and spleen. These effects confirm earlier work [8,14,15,**chapters 2,4**]. The excess iron in spleen and tibia might be located in macrophages that are responsible for the breakdown of red blood cells and recycling of iron to the erythropoietic tissue. Perhaps, the metabolism

of macrophages is affected in vitamin A deficiency.

In this study we produced a rat model with different degrees of marginal vitamin A deficiency that are associated with almost constant plasma retinol concentrations. Vitamin A deficient rat models with almost constant plasma retinol concentrations [19] or unaltered growth rates [20] have been published before. Such a model may mimic better the situation in children with vitamin A deficiency than a rat model that is in a negative retinol balance and thus is gradually depleted of vitamin A. The effects of vitamin A deficiency on iron status found in the present model are similar to those seen before in rats with less chronic vitamin A deficiency [8,15,**chapters 2,4**]. The present model however, might not be affected by haemoconcentration. Analysis of variance with body weight as covariate indicated that the changes in iron metabolism due to vitamin A deficiency are not secondary to reduced growth. However, the underlying mechanisms are still unclear.

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CHAPTER 7

Indicators of erythrocyte formation and degradation in rats with either vitamin A or iron deficiency

ABSTRACT

Vitamin A deficiency produces anaemia and reduced iron status. In this study with rats the hypotheses were tested that vitamin A deficiency impairs erythropoiesis, leading to an increased red cell turnover, and that it inhibits the glycosylation of transferrin. Erythropoietic activity was assessed indirectly by determining the myeloid:erythroid ratio in bone marrow smears, the number of erythroid colonies in the red pulp of spleen, the blood reticulocyte index, zinc protoporphyrin and plasma transferrin receptor concentrations. Transferrin glycosylation was assessed by measuring the sialic acid content of transferrin. The effects of vitamin A deficiency were compared with those of iron deficiency. Iron deficiency produced anaemia and low iron levels in organs. Vitamin A deficiency was reflected by low levels of plasma and hepatic retinol and it induced decreased plasma total iron-binding capacity and raised iron levels in tibia and spleen. Early, but not longer term iron deficiency reduced the number of erythroid colonies in spleen: vitamin A deficiency had no influence. Neither iron nor vitamin A influenced the myeloid:erythroid ratio in bone marrow smears and the blood reticulocyte production. Plasma transferrin receptor and erythrocyte zinc protoporphyrin concentrations were not affected by vitamin A deficiency but increased with iron deficiency. Vitamin A deficiency did not stimulate erythrocyte breakdown as indicated by unaltered plasma lactate dehydrogenase activity and reduced plasma total bilirubin levels. Both vitamin A and iron deficiency raised the proportion of multiple sialylated transferrins in plasma. Thus we have not found evidence for vitamin A deficiency affecting erythropoiesis and erythrocyte turnover. Possibly the iron accumulation in spleen and bone marrow relates to reduced iron transport due to inhibition of transferrin synthesis rather than inhibition of transferrin sialylation.

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submitted for publication

INTRODUCTION

Vitamin A deficiency in man is associated with anaemia [1-5]. In rats, vitamin A deficiency reduces the incorporation of radioactive iron into erythrocytes [6,7], alters red blood cell morphology [8,9], produces mild anaemia [10-12,**chapter 2**] lowers plasma total iron-binding capacity [12-14,**chapters 2,4,5**], increases iron absorption (8,11,12) and causes iron accumulation in spleen [12-14,**chapters 2,4,5**,9] and bone [12-14,**chapters 2,4,5**]. Based on the rat studies, we have hypothesized [13,**chapter 4**] that vitamin A deficiency impairs erythropoiesis, so that mild anaemia with malformed red cells develops. The abnormal erythrocytes could be broken down at increased rates, causing accumulation of iron in the macrophages in spleen and bone marrow. The accumulation of iron could lead to low iron transport capacity as indicated by a decrease in plasma total iron-binding capacity. Or transferrin synthesis could primary be impaired in vitamin A deficiency.

We have tested this hypothesis in rats fed a diet deficient in vitamin A. The myeloid:erythroid ratio in bone marrow smears and the number of erythroid colonies in the red pulp of spleen were measured as indices of erythropoiesis. The ratio quantifies the rate of erythropoiesis relative to that of myelopoiesis [15]. Erythropoietic activity was also assessed by measuring plasma transferrin receptor concentration [16,17] and the reticulocyte index i.e. the number of circulating reticulocytes corrected for the degree of anaemia. Red cell degradation was estimated indirectly by measuring both plasma total bilirubin, which is derived from breakdown of haemoglobin, and plasma lactate dehydrogenase activity, which is in part derived from haemolysis [15]. Trapping of iron in the macrophages, so that it would be unavailable for erythropoiesis, was assessed by measuring zinc protoporphyrin, which is a pre-stage of haem in erythrocytes [18]. Transferrin synthesis in vitamin A deficiency might be impaired at the level of its glycosylation [19]; this possibility was checked by determination of the sialic acid content of transferrin.

In this study, the impact of vitamin A deficiency was compared and contrasted with that of iron deficiency so as to facilitate interpretation. Rats were sampled after 12 and 70 days of feeding the vitamin A or iron-deficient diet. It was anticipated that sampling at the two time intervals would allow the primary and secondary effects of vitamin A deficiency on the indicators of erythrocyte formation and degradation to be distinguished.

METHODS

This study was approved and supervised by the animal welfare officer of Wageningen Agricultural University.

Animals, housing and diets

Male Wistar rats (Cpb:WU), derived from a commercial breeder (Harlan CPB, Zeist, The Netherlands), were used. On arrival, the rats, aged 3 weeks, were housed in groups of six animals in wire-topped, polycarbonate cages (34.5 × 22.5 × 16 cm) with a layer of sawdust as bedding. During the pre-experimental period of 2 weeks, all rats were fed the diet (*Table 1*) without added vitamin A, but with adequate iron (35 mg added iron/kg feed). The vitamin A deficient diet was fed to lower vitamin A stores and thus facilitate the development of vitamin A deficiency during the experimental period.

After the pre-experimental period (day 0), the rats were divided into three groups of 24 rats each so that body weight distributions of the groups were similar. The rats were then housed in groups of four animals in stainless steel cages with wire mesh bases (30 × 42 × 19 cm). One group received the control diet. This diet (*Table 1*) was formulated according to the nutrient requirements of rats [20]. Another group continued to receive the vitamin A deficient diet. The third group was transferred to the iron-deficient diet containing 3.5 mg added iron/kg feed (*Table 1*). Analyzed iron concentrations of the diets were as follows (mg/kg feed): control diet, 45; vitamin A deficient diet, 45; iron deficient diet, 10. The purified diets were in powdered form and stored at 4°C until used for feeding. The animals had free access to feed and demineralised water. Feed intakes, corrected for feed spillage, were measured per cage twice weekly, and individual body weights once a week. A controlled light/dark cycle (light on: 0600-1800 h), temperature (20-22°C) and relative humidity (50-60%) were maintained in the animal room.

Collection of samples

On days 12 and 70, 12 animals taken from three cages of each dietary group were killed. For a period of seven days prior to days 12 and 70, the animals to be killed were housed individually in metabolism cages (314 cm^2 × 12 cm). During

the last four days while the rats were in the metabolism cages, feed and water intake were measured and faeces and urine collected. Faeces was stored at -20°C until analysis; urine was discarded. Between 09.00 and 11.00 h, heparinized blood was obtained from the non-fasting rats by orbital puncture while they were under diethyl ether anaesthesia. The blood was stored at 0°C for haematological examination, reticulocyte and differential white blood cell counting on the same day. The plasma collected was stored at -20°C until analysis, except for 250 μL which was stored at -80°C prior to analysis of retinol. Immediately after bleeding, the anaesthetized rats were decapitated. The kidneys, liver, spleen and the two hindlegs were removed. The organs were weighed and prepared for histology or stored at -20°C until analysis.

Table 1 Composition of the diets.

Ingredients	Control diet	Vitamin A deficient diet	Iron deficient diet
Casein (g)	151	151	151
Maize oil (g)	25	25	25
Coconut fat (g)	25	25	25
Glucose (g)	709.2	709.2	709.2
Cellulose (g)	30	30	30
$CaCO_3$ (g)	12.4	12.4	12.4
$NaH_2PO_4.2H_2O$ (g)	15.1	15.1	15.1
$MgCO_3$ (g)	1.4	1.4	1.4
KCl (g)	1.0	1.0	1.0
$KHCO_3$ (g)	7.7	7.7	7.7
$FeSO_4.7H_2O$ (mg)	174	174	17.4
Vitamin A preparation (mg)†	8	-	8
Mineral premix (g)‡	10	10	10
Vitamin premix (g)§	12	12	12

† Rovimix A 500®, 150 retinol equivalents/mg (F. Hoffmann-La Roche & Co. Ltd, Basle, Switzerland), consisting of retinyl acetate and retinyl palmitate.

‡ The mineral premix consisted of (mg): MnO_2, 79; $ZnSO_4.H_2O$, 33; $NiSO_4.6H_2O$, 13; NaF, 2; KI, 0.2; $CuSO_4.5H_2O$, 15.7; $Na_2SeO_3.5H_2O$, 0.3; $CrCl_3.6H_2O$, 1.5; $SnCl_2.6H_2O$, 1.9; NH_4VO_3, 0.2; maize meal, 9853.2.

§ The vitamin premix consisted of (mg): thiamin, 4; riboflavin, 3; niacin, 20; D,L-calcium pantothenate, 17.8; pyridoxine, 6; cyanocobalamin, 50; choline chloride, 2000; folic acid, 1; biotin, 2; menadione, 0.05; D,L-α tocopheryl acetate, 60; cholecalciferol, 2 (1000 IU); maize meal, 9834.15.

Histological examination

The right kidney, half of the spleen and a portion of the left lateral liver lobe were fixed in 10% neutralized formalin (Baker, Deventer, The Netherlands). Slides were prepared and coloured with a haematoxylin/eosine solution or by the Perls' Prussian Blue reaction (potassium ferrocyanide and neutral red) according to conventional techniques [21].

All slides were examined in a strictly standardized manner by one person who was blinded to treatment modality. Kidney slides were examined for the occurrence of calcification and basophilic cells, which may represent early stages in the morphogenesis of renal neoplasms [22]. In spleen slides the number of erythroid colonies were counted and liver slides were checked for portal or periportal roundcell infiltrations. The location of iron was described only for the histological slides corresponding to day 70 because iron was not detectable in the slides corresponding to day 12. The histological variables were quantified as scores (0,1,2,3) except for the number of erythroid colonies in the spleen and the percentage of hepatic iron that is located in macrophages.

Immediately after killing the rats, the right tibia was rid of adhering tissue and cut open with a small pair of scissors. The bone marrow was directly put on a glass slide with a scalpel and gently spread with another slide held at an angle of about 45°. After drying, the bone marrow smears were fixed and coloured according to either the May-Grünwald Giemsa (eosin-methylene blue/ azure eosin-methylene blue) colouring method or the Kaplan iron staining method (potassium ferrocyanide and safranine). A subsample of bone marrow smears was evaluated by differentially counting 300-400 cells. The location of iron was described.

Haematology

Haemoglobin concentration, haematocrit, red blood cell count, mean corpuscular haemoglobin concentration and mean cell volume were measured with a blood cell counter (Model K-1000, Sysmex, IJsselstein, The Netherlands). Zinc erythrocyte protoporphyrin was determined in whole blood with a haematofluorometer (AVIV Biomedical 206, Lakewood NJ, USA). Differential counting of white blood cells was done in blood smears. In a portion of the smears, the number of reticulocytes was counted per 1000 cells after staining with methylene blue. Reticulocyte production index was calculated as described by Hillman and Finch [23].

Chemical analyses

Plasma iron concentrations and total iron-binding capacity were determined spectrophotometrically using a commercial test kit (Roche Nederland, Mijdrecht, The Netherlands). Plasma total bilirubin, lactate dehydrogenase activity and aspartate aminotransferase activity were also determined with commercial test kits (Roche Nederland, Mijdrecht, The Netherlands). Plasma transferrin receptor concentrations were measured as described by Beguin et al [16]. Transferrin microheterogeneity was determined with an isoelectric focusing technique and densitometry [24]. Iron was measured by flame atomic absorption spectrometry following wet ashing with nitric acid for feed or dry ashing for liver, faeces, spleen, left kidney and tibia .

Plasma and liver retinol was measured by reversed phase high performance liquid chromatography (HPLC) as described by Roodenburg et al [12,**chapter 2**].

Statistical analysis

For each time interval, the groups fed either the vitamin A deficient or iron deficient diet were compared with the control group. For the continuous variables, contrasts with pooled variances based on Students' t-test were used for comparison of group means; the variables were found to be distributed normally as based on Kolmogorov-Smirnov goodness of fit tests. The effects of the experimental diets on the histological scores were evaluated with the non-parametric Mann-Whitney-Rank test. A pre-set p value of 5% was used throughout.

RESULTS

Vitamin A status

Plasma and hepatic retinol levels at day 12 were reduced in the vitamin A deficient group when compared to the control group *(Figure 1)*. After 70 days, liver retinol was undetectable and plasma retinol lowered by 85% in rats given the vitamin A deficient diet. In the rats fed the low iron diet, plasma, but not hepatic retinol levels were reduced *(Figure 1)*.

Table 2 Body and organ weights (g).

	Day 12			Day 70		
	Control diet	Vitamin A deficient diet	Iron deficient diet	Control diet	Vitamin A deficient diet	Iron deficient diet
Body weight	199.7 ± 4.9	203.5 ± 3.3	193.3 ± 5.2	370.3 ± 7.5	350.8 ± 7.8	371.7 ± 7.9
Liver	9.33 ± 0.31	9.23 ± 0.29	8.51 ± 0.26	12.28 ± 0.44	10.05 ± 0.20[c]	11.34 ± 0.22[a]
Kidney	0.75 ± 0.03	0.75 ± 0.01	0.70 ± 0.02	0.97 ± 0.08	1.05 ± 0.02	1.03 ± 0.02
Spleen	0.54 ± 0.04	0.48 ± 0.02	0.48 ± 0.03	0.64 ± 0.03	0.57 ± 0.03	0.66 ± 0.04
Tibia	0.275 ± 0.007	0.277 ± 0.004	0.268 ± 0.006	0.462 ± 0.012	0.482 ± 0.019	0.467 ± 0.018

Means are given with standard errors of the mean ($n = 12$). Organ and tibia weights refer to wet weight. When values differ significantly from the control group within time intervals, this is indicated as: [a] $p < 0.05$, [c] $p < 0.001$.

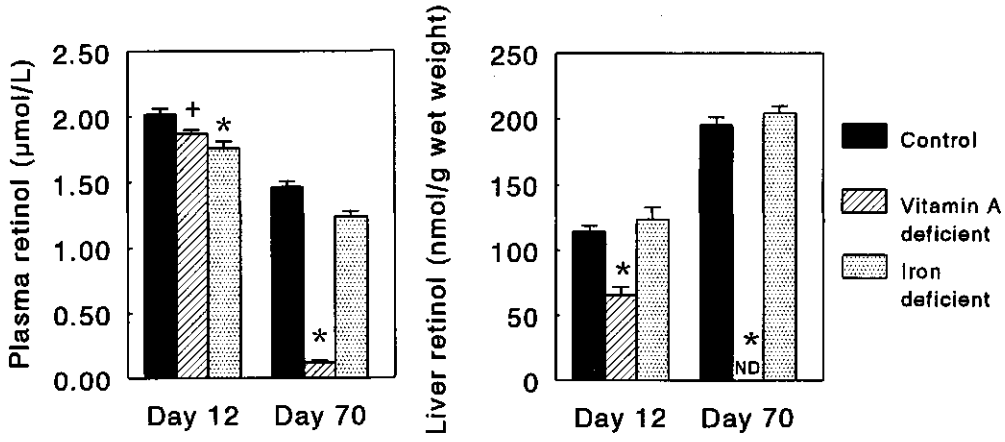

FIGURE 1 Plasma (a) and liver (b) retinol concentrations at days 12 and 70 of the experiment. Bars represent means of 12 animals per group and their standard error. Differences versus the group on the control diet: +, $p<0.05$; *, $p<0.001$, ND = not detectable.

Body weight, organ weights and feed intake

There were no diet effects on body weight and weights of kidney, spleen and tibia *(Table 2)*. Liver weight was reduced after either feeding the vitamin A or iron deficient diet for 70 days. Feed intake over the period of 67-70 days was reduced in both the vitamin A deficient (14.3 SE 0.4 g/d, $p<0.001$) and iron deficient groups (16.0 SE 0.3 g/d, $p=0.027$), when compared with the control group (17.3 SE 0.5 g/d).

Histology of organs

The spleen slides were examined to assess extra medullary erythropoiesis, and liver and kidney slides were studied to detect any pathological changes. Special attention was paid to possible calculi in kidney, because bladder and kidney stone formation may be stimulated by lack of vitamin A [25,26]. The location of iron in kidney, liver and spleen slides was described because it could provide clues as to mechanisms that underlie the impairment of iron metabolism in vitamin A

deficiency. No iron was detected in all slides from the iron deficient group.

Immunological activity as indicated by round cell infiltrations with or without cell necrosis was seen both at the portal site of the liver lobules and in the region of the terminal veins. There was no effect of diet on the incidence of round cell infiltrations. Iron was detectable only in the control and vitamin A deficient group and at day 70 of the experiment only, most of it being located in the Kupffer cells. Only a small percentage (2.5%) of the iron was in the hepatocytes, this percentage not differing between the vitamin A deficient and control group.

Calcification and basophilic cells were seen in the kidneys of all three groups at the two time intervals, the incidences increasing with age. There were no differences between the dietary groups. Iron was located mainly in the cortex in the tubuli contorti. At these sites iron was seen in the nucleus, as grains in the cytoplasm and in macrophages. Iron-positive stain was associated with the appearance of calculi, both in de medulla and the cortex. The presence of iron was not different between the vitamin A deficient group and the control group.

Extra medullary erythropoiesis was estimated by counting erythroid colonies in the red pulp of the spleen. Splenic erythropoiesis was more pronounced at day 12 than at day 70 of the experiment. The number of erythroid colonies, however, was relatively low in the iron deficient rats at day 12 ($p < 0.001$), this diet effect having disappeared at day 70. No effect of vitamin A deficiency on extra medullary erythropoiesis was detected. Splenic iron was located in the macrophages; no influence of vitamin A deficiency was observed.

Iron status

In the group fed on the low iron diet blood haemoglobin, haematocrit and mean cell volume were decreased, whereas zinc protoporphyrin was increased *(Table 3)*. Red blood cell count was increased and mean corpuscular haemoglobin concentration decreased after 70 days of feeding the low iron diet. Feeding the vitamin A deficient diet had no significant effect on haematological indices, except for the increase in reticulocyte count at day 12.

Plasma iron concentrations at day 12, but not at day 70, were reduced in rats fed the vitamin A deficient diet *(Table 4)*. Feeding the low iron diet produced low plasma iron concentrations at both day 12 and 70, the lowering being greater at day 12. Total iron-binding capacity at day 70 was significantly reduced in the vitamin A deficient rats. Total iron-binding capacity and plasma transferrin receptor levels were increased, whereas transferrin saturation was reduced after

Table 3 Haematological indices.

	Day 12			Day 70		
	Control diet	Vitamin A deficient diet	Iron deficient diet	Control diet	Vitamin A deficient diet	Iron deficient diet
Haemoglobin (mmol/L)	8.51 ± 0.07	8.38 ± 0.10	7.38 ± 0.11[c]	9.48 ± 0.07	9.61 ± 0.09	8.10 ± 0.16[c]
Haematocrit (%)	42.8 ± 0.6	42.6 ± 0.5	37.2 ± 0.7[c]	49.1 ± 0.4	49.4 ± 0.6	42.9 ± 0.7[c]
Red blood cell count ($*10^{12}$/L)	6.5 ± 0.1	6.2 ± 0.1	6.2 ± 0.1	7.8 ± 0.1	7.9 ± 0.1	8.2 ± 0.1[b]
Mean cell volume (fL)	66 ± 0.8	69 ± 0.9	60 ± 0.9[c]	63 ± 0.8	63 ± 0.8	53 ± 1.3[c]
Mean corpuscular haemoglobin concentration (mmol/L)	19.9 ± 0.1	19.7 ± 0.1	19.9 ± 0.1	19.3 ± 0.1	19.5 ± 0.1	18.9 ± 0.1[b]
Zinc protoporphyrin concentration (mmol/mol Hb)	0.32 ± 0.01	0.31 ± 0.01	0.37 ± 0.01[b]	0.30 ± 0.01	0.30 ± 0.01	0.42 ± 0.02[c]
Reticulocyte count (% of red cell count)	1.0 ± 0.6	3.0 ± 0.4[a]	2.2 ± 0.4	3.6 ± 0.7	3.3 ± 0.6	3.5 ± 0.4
production index†	1.0 ± 0.6	3.0 ± 0.5[a]	1.3 ± 0.2	3.7 ± 0.6	3.4 ± 0.6	2.1 ± 0.3

† Reticulocyte production index was calculated as {(reticulocyte count) * Ht / normal Ht} / maturation index, with normal haematocrit (Ht) taken to be 43% at day 12 and 49% at day 70. Maturation index, a measure for the prolongation of maturation time of circulating blood reticulocytes which occurs with anaemia, was 1 for control and vitamin A deficient groups and 1.5 in iron deficient groups [23].
Means are given with standard errors of the mean (n = 12) except for reticulocytes (day 12, n = 4 and day 70, n = 6). When values differ significantly from the control group within time intervals, this is indicated as: [a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$.

consumption of the low iron diet.

Iron concentrations of liver, kidney, spleen and tibia were reduced after 12 and 70 days feeding the low iron diet *(Table 5)*. The iron concentration of liver at day 12 and that of both spleen and tibia at day 70 was significantly increased in the vitamin A deficient group.

In the control and vitamin A deficient groups the efficiency of iron absorption was similar and decreased with time *(Figure 2)*. In the rats with iron deficiency the percentage of dietary iron absorbed was raised.

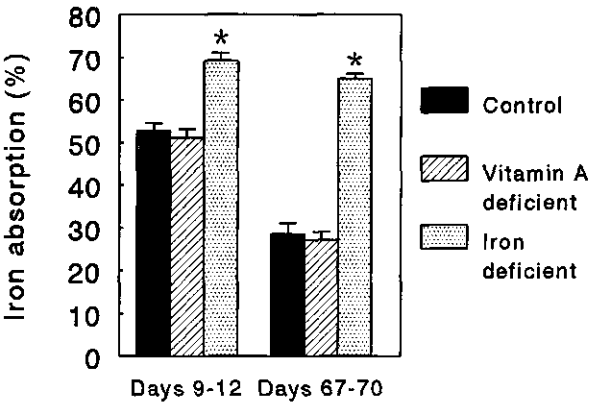

FIGURE 2 Apparent iron absorption for days 9-12 and days 67-70 of the experiment. Apparent absorption was calculated as iron ingested minus that excreted in faeces and expressed as percentage of intake. Bars represent means of 12 animals per group and their standard error. Differences versus the control group: *, $p<0.001$.

Blood smears

A differential counting of white blood cells was carried out as to indicate signs of infection. Vitamin A deficiency enhances the risk of infection [27]. The leucocyte composition in blood smears was as follows: basophils, 0%; eosinophils, 0.5% (range 0-3%); band cells, 0 %; poly-morphonuclear cells, 15.5% (range 2-36%); lymphocytes, 84% (range 64-98%) and monocytes, 0%. There were no systematic differences between dietary groups or between time intervals. The differential countings of each animal were within the range considered normal [28,29] so that the rats used appeared to be free of infection.

Bone marrow smears

The results of differential cell counting and localization of iron in bone marrow are given in *Table 6*. All values are within limits considered normal [30,31]. No differences between the dietary groups could be detected. Iron positive stain was not detectable in bone marrow slides from the iron deficient rats.

Table 6 Differential cell counts and location of iron-positive stain in bone marrow smears (% of total cells).

	Day 12	Day 70		
	Control diet	Control diet	Vitamin A deficient diet	Iron deficient diet
Total erythropoietic cells	29.0 ± 6.3	32.5 ± 3.5	29.3 ± 4.6	27.8 ± 4.3
Total myelopoietic cells	42.5 ± 7.0	43.0 ± 6.9	50.3 ± 6.4	47.3 ± 3.3
Myeloblasts	4.3 ± 0.9	3.8 ± 1.2	1.8 ± 0.3	4.5 ± 1.2
Promyelocytes	1.3 ± 0.3	1.3 ± 0.6	1.5 ± 0.8	1.3 ± 0.6
Myelocytes	2.0 ± 1.3	2.5 ± 0.3	4.3 ± 1.5	4.3 ± 1.3
Metamyelocytes	1.8 ± 1.0	0.0 ± 0.0	2.5 ± 1.4	1.3 ± 0.3
Bandcells	7.3 ± 1.1	7.0 ± 0.8	7.8 ± 1.7	8.5 ± 1.5
Segmented neutrophils	23.0 ± 6.3	25.3 ± 5.7	30.0 ± 8.2	25.5 ± 2.6
Eosinophils	3.0 ± 1.3	3.3 ± 0.9	2.5 ± 0.8	2.0 ± 0.8
Basophils	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Lymphocytes	27.0 ± 6.9	22.0 ± 7.6	18.3 ± 7.8	19.3 ± 7.5
Plasma cells	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.6
Monocytes	0.8 ± 0.6	1.3 ± 0.3	0.8 ± 0.6	1.8 ± 0.6
Myeloid/Erythroid-ratio	1.7 ± 0.6	1.4 ± 0.3	1.8 ± 0.3	1.8 ± 0.2
Iron located in				
Reticuloendothelium	trace	+ +/+	+ +	negative
Sideroblasts	+	trace	trace	negative
Siderocytes	+	trace	negative	negative

Means are given with standard errors of the mean (n=4 or 6).
Mast cells and megakaryocytes were not included in the differential counting of 300 to 400 cells per bone marrow smear.

Table 4 Plasma measures of iron status.

	Day 12			Day 70		
	Control diet	Vitamin A deficient diet	Iron deficient diet	Control diet	Vitamin A deficient diet	Iron deficient diet
Plasma iron (μmol/L)	45.9 ± 2.2	40.0 ± 1.8[a]	14.0 ± 1.0[c]	34.8 ± 1.0	35.5 ± 1.0	26.6 ± 2.5[b]
Total iron binding capacity (μmol/L)	100.9 ± 2.2	98.8 ± 2.3	125.5 ± 2.8[c]	97.7 ± 1.9	90.1 ± 1.7[b]	121.2 ± 2.1[c]
Transferrin saturation (%)	45.8 ± 2.7	40.7 ± 2.0	11.3 ± 1.0[c]	35.7 ± 0.9	39.5 ± 1.2	22.2 ± 2.2[c]
Plasma transferrin receptor concentration (ng/mL)	3333 ± 165	3233 ± 282	4242 ± 319[a]	2356 ± 193	2216 ± 139	3733 ± 261[c]

Table 5 Iron concentration in tissues (μmol/g dry weight)

	Day 12			Day 70		
	Control diet	Vitamin A deficient diet	Iron deficient diet	Control diet	Vitamin A deficient diet	Iron deficient diet
Liver	3.92 ± 0.26	5.62 ± 0.56[b]	2.00 ± 0.23[b]	8.01 ± 0.66	8.68 ± 0.32	2.35 ± 0.59[c]
Kidney	4.47 ± 0.18	4.36 ± 0.17	3.26 ± 0.10[c]	6.27 ± 0.45	5.39 ± 0.43	3.41 ± 0.20[c]
Spleen	10.74 ± 0.49	12.14 ± 0.69	8.63 ± 0.54[a]	29.75 ± 1.77	44.61 ± 6.35[b]	10.60 ± 0.34[b]
Tibia	1.12 ± 0.04	1.20 ± 0.04	0.78 ± 0.03[c]	1.28 ± 0.04	1.55 ± 0.08[b]	0.80 ± 0.05[c]

Tables 4 and 5: Means are given with standard errors of the mean (n = 12). When values differ significantly from the control group within time intervals, this is indicated as: [a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$.

Table 7 Plasma total bilirubin concentration, lactate dehydrogenase and aspartate aminotransferase activity.

	Day 12			Day 70		
	Control diet	Vitamin A deficient diet	Iron deficient diet	Control diet	Vitamin A deficient diet	Iron deficient diet
Total plasma bilirubin concentration (μmol/L)	15.4 ± 1.9	14.5 ± 1.4	16.4 ± 1.8	13.9 ± 2.2	8.1 ± 0.9[a]	11.4 ± 1.1
Plasma lactate dehydrogenase activity (U/L)	149.6 ± 10.5	157.7 ± 14.6	166.3 ± 31.8	245.9 ± 19.3	247.7 ± 26.8	208.5 ± 8.6
Aspartate aminotransferase activity (U/L)	62.7 ± 5.5	65.5 ± 5.0	78.0 ± 6.6	53.9 ± 1.5	50.5 ± 2.6	61.4 ± 1.8[a]

Table 8 Relative occurrence of sialo-transferrin (Tf) fractions (%), and the ratio 1+2 sialo-Tf:3+4 sialo-Tf.

	Day 12			Day 70		
	Control diet	Vitamin A deficient diet	Iron deficient diet	Control diet	Vitamin A deficient diet	Iron deficient diet
1-sialo Tf	2.9 ± 0.2	3.5 ± 0.2	3.8 ± 0.3	2.6 ± 0.3	2.4 ± 0.2	2.0 ± 0.1
2-sialo Tf	60.5 ± 0.8	59.8 ± 0.6	59.6 ± 1.0	63.7 ± 1.0	60.0 ± 1.0	59.6 ± 0.9
3-sialo Tf	31.3 ± 0.5	31.3 ± 0.6	31.6 ± 1.0	29.7 ± 0.7	32.7 ± 0.8	33.2 ± 0.7
4-sialo Tf	5.3 ± 0.3	5.5 ± 0.2	5.1 ± 0.4	4.0 ± 0.3	4.9 ± 0.2	5.2 ± 0.3
ratio 1+2 sialo:3+4 sialo-Tf	1.74 ± 0.05	1.73 ± 0.05	1.76 ± 0.10	1.99 ± 0.08	1.68 ± 0.07[b]	1.62 ± 0.06[b]

Tables 7 and 8: Means are given with standard errors of the mean (n = 10-12). When the values in table 7 or the ratio 1+2 sialo-Tf:3+4 sialo-Tf differs significantly from the control group within the time intervals, this is indicated as:[a] $p<0.05$, [b] $p<0.01$.

Indicators of erythrocyte breakdown and liver damage

Plasma lactate dehydrogenase activity, an indicator of erythrocyte degradation, was not affected by feeding the experimental diets, whereas the other indicator, plasma bilirubin was reduced in the vitamin A deficient group, but only at day 70 *(Table 7)*. Plasma aspartate aminotransferase activity, an index of tissue damage, especially liver damage [32], was not affected by diet type, except for the small increase at day 70 that was seen in the iron deficient group.

Transferrin microheterogeneity

The relative occurrence of the 1-, 2-, 3-, and 4-sialo transferrin fractions in plasma is given in *Table 8*. Feeding the experimental diets for 12 days did not affect the pattern of sialo transferrin fractions. However after 70 days of administrating either the vitamin A deficient or iron deficient diet there was a significant increase in (3 + 4) sialylated transferrins at the expense of (1 + 2) sialylated transferrins as illustrated by the decreased ratio (1 + 2)-sialo transferrin : (3 + 4)-sialo transferrin.

DISCUSSION

In essence, the observed effects of feeding the diets deficient in vitamin A or iron confirm in part our earlier findings [12,13, **chapters 2,4**]. The low iron diet produced anaemia within 12 days, reduced plasma and organ iron concentrations and increased apparent iron absorption and total iron-binding capacity. Vitamin A deficiency did not influence blood haemoglobin levels as shown previously [12,**chapter 2**], but the iron and vitamin A status of the rats in the present study, as indicated by the higher liver iron stores and slower progress of vitamin A deficiency was higher than in the previous study. Nevertheless, vitamin A deficiency did decrease total iron-binding capacity and increased the amount of iron in tibia and spleen, the iron being localized mainly in macrophages. The deficiencies of vitamin A and iron that were induced may be considered relatively mild. In the deficient rats, body weight gain was not affected and their kidneys and liver did not show pathological changes that differed from those in the control rats.

We have hypothesized that vitamin A deficiency impairs erythropoiesis resulting in the formation of abnormally shaped red cells. However, neither

haemoglobin concentration nor mean cell volume or mean corpuscular haemoglobin concentration were influenced by vitamin A deficiency. There was no evidence for changes in extra medullary erythropoiesis, as assessed by counting the number of erythroid colonies in spleen. In rats with mild iron deficiency anaemia, aged 18 weeks, there was an increase in early erythroblasts in spleen [33]. However, the number of erythroid colonies in the red pulp of spleen was decreased at day 12 in the iron deficient rats. Possibly, acute and long-term effects of iron deficiency anaemia on extra medullary erythropoiesis differ. The differential counting of bone marrow cells, with results within the normal range [29,30], revealed no effect of diet on the myeloid:erythroid ratio. The lack of effect of iron deficiency was unexpected because this condition is known to increase erythropoietic activity [34]. In any event, it can be concluded that contrary to our hypothesis, we were not able to demonstrate an effect of vitamin A deficiency on erythropoiesis.

Further measurements also indicated that vitamin A deficiency did not influence the activity of the erythropoietic system. Transferrin receptors in the body are mainly situated in bone marrow and circulating plasma transferrin receptor concentration is a measure for erythropoietic activity [16]. As would be expected plasma transferrin receptor concentration was increased with iron deficiency [16,17]. However, transferrin receptors in plasma were not affected by vitamin A status. In the vitamin A deficient rats, the blood reticulocyte index was raised at day 12, but was unaltered at day 70.

The rate of breakdown of red cells was assessed by measuring plasma lactate dehydrogenase activity and plasma total bilirubin concentration [15]. Lactate dehydrogenase was not affected by the diet and the bilirubin level was reduced rather than raised after feeding the vitamin A deficient diet. Thus, vitamin A deficiency cannot be shown to stimulate red cell degradation when measured either indirectly or with radioisotopes [7] disproving our hypothesis. This statement seems to be at variance with the observation that iron had accumulated in spleen and especially in bone marrow in the vitamin A deficient rats. Possibly iron was trapped in the macrophages and thus unavailable for haem production, but in that case erythrocyte protoporphyrin should be increased [18] as was seen in the iron deficient rats. With vitamin A deficiency, there was no increase in erythrocyte protoporphyrin concentration so that sufficient iron may have been available for erythropoiesis.

Vitamin A deficiency could reduce transferrin synthesis as indicated by the low plasma iron-binding capacity. To see whether transferrin glycosylation is affected by vitamin A, microheterogeneity of the transferrin molecules was determined. Vitamin A may assist in glycosylation of transferrin as found in *in vitro*

systems [19, 35-37], but the mechanism involved is still unclear. We found evidence for enhanced rather than reduced glycosylation in vitamin A deficiency, because the proportion of higher sialylated transferrin was increased. Iron deficiency had a similar influence. The physiological basis of transferrin microheterogeneity is obscure, but transferrin with low sialic acid content is associated with increased iron turnover and increased iron in hepatocytes [38]. Thus a high degree of sialylation could point to iron sparing which would be feasible for the condition of iron deficiency. However this is difficult to see for vitamin A deficiency with iron accumulation in macrophages of the reticuloendothelial system. Possibly, in vitamin A deficiency iron is less available, also triggering iron sparing. Glycosylation and synthesis of transferrin could be regulated independently [39] while there is evidence that *in vitro* transferrin gene transcription is stimulated by vitamin A [40,41]. Thus the low plasma iron-binding capacity in vitamin A deficiency could relate to inhibition of transferrin synthesis at the level of transcription.

In conclusion, we have found no evidence for an impaired erythropoiesis or an increased red blood cell turnover in rats with vitamin A deficiency. However the dynamic processes were measured only indirectly and qualitatively. Ferrokinetic studies might be more informative [34]. In this study there was no anaemia in vitamin A deficiency which could have prevented changes in erythropoiesis and erythrocyte turnover. However, the marginal vitamin A deficiency that was induced did lead to the accumulation of iron in spleen and bone marrow.

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CHAPTER 8

Effect of vitamin A supplementation for 3 days on iron metabolism, liver function indicator enzymes and differential cells counts in bone marrow of rats with severe vitamin A deficiency.

ABSTRACT

Rats with severe vitamin A deficiency were supplemented with vitamin A for 3 days; control rats were not supplemented. Vitamin A supplementation produced an increase in plasma retinol levels, but iron metabolism as assessed by haematological variables, plasma iron, total iron-binding capacity and organ iron contents was not affected. The high plasma concentrations of alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) in the control rats, which points to liver damage, were normalized by vitamin A supplementation. Differential counts of bone marrow cells revealed that vitamin A supplementation increased myelopoiesis relative to erythropoiesis.

We conclude that vitamin A supplementation for 3 days to rats with severe vitamin A deficiency corrects liver damage, influences blood cell synthesis, but does not affect iron status.

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INTRODUCTION

Vitamin A deficiency in man and animals produces a wide array of defects. In our studies with rats we have focused on iron metabolism and haematological variables. Vitamin A deficiency has been found to induce low plasma iron concentrations, low total iron-binding capacity, low liver iron levels, but high spleen and tibia iron levels [1-4,**chapters 2-4,7**]. Liver weight was reduced in rats with vitamin A deficiency, but the plasma activity of the liver function indicator enzyme ASAT, was unaltered [4,**chapter 7**]. In bone marrow smears from vitamin A deficient rats the ratio of myeloid:erythroid cells was unaltered [4,**chapter 7**]. The effects were studied in rats with a mild degree of vitamin A deficiency, i.e. without marked growth retardation and without clinical signs [4,**chapter 7**]. In essence, the changes mentioned were found to be reversible [2,3,**chapter 3,4**].

Current research by colleagues on the role of retinoids on spermatogenesis [5] offered the possibility to use a few rats with severe vitamin A deficiency, i.e. showing wasting and deformations of legs and tail. Some of the deficient rats were treated with vitamin A for 3 days and then sampled. The following questions were addressed. Does short-term vitamin A supplementation reverse the alterations of iron metabolism in rats with severe vitamin A deficiency? Does vitamin A deficiency affect liver function as assessed by measuring plasma activities of liver function enzymes? Does vitamin A supplementation for 3 days influence the differential cell counts in bone marrow smears?

METHODS

Animals and diets

Eleven, male vitamin A deficient Wistar rats (Cpb:U:WU) were obtained from a two-generation experimental design that has been described by Van Pelt and De Rooij [5]. When body weight began to decrease, at the age of 10-12 weeks, 7 rats were killed and each of 4 rats received a single intraperitoneal dose of 5 mg retinol acetate. The latter rats were also switched to a vitamin A-containing diet (RMH-B; Hope Farms, Woerden, The Netherlands, minimal vitamin A content, 5940 retinol equivalents/kg) and given it for another 3 days and were then killed [5]. Iron contents of the diets as reported by the manufacturers were for the vitamin A-free diet (Harlan Teklad, Madison WI, USA) 110 mg/kg and for the vitamin A-containing diet (Hope Farms, Woerden, The Netherlands) at least 165 mg/kg.

Collection of samples

The rats were killed by CO_2 inhalation and blood was collected immediately by aorta puncture. Blood was stored at 0°C for haematological examination on the same day. Plasma was collected and stored at -20°C until analysis. Immediately after bleeding, liver, spleen, one kidney and the two hindlegs were removed. The organs were weighed and stored at -20°C until analysis. One of the tibias was used to prepare bone marrow smears. The tibia was rid of adhering tissue and cut open with a small pair of scissors. The bone marrow was directly put on a glass slide using a scalpel and gently spread with another glass slide held at an angle of about 45°. After drying, the bone marrow smears were fixed and coloured according to a May-Grünwald Giemsa (eosin-methylene blue/ azure eosin-methylene blue) method. The bone marrow smears were evaluated by differentially counting 300-400 cells.

Chemical analysis

Haemoglobin concentration, haematocrit, red and white blood cell count, mean corpuscular haemoglobin concentration and mean cell volume were measured with a blood cell counter (Model K-1000, Sysmex, IJsselstein, The Netherlands). Plasma iron, total iron-binding capacity, alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT) and alkaline phosphatase were determined spectrophotometrically using commercial test kits (Roche Nederland, Mijdrecht, The Netherlands). Iron was measured by flame atomic absorption spectrometry following dry ashing for liver, spleen, kidney and tibia. Plasma retinol was measured by reversed phase high performance liquid chromatography (HPLC) as described by Roodenburg et al [1,**chapter 2**].

Statistical analysis

For all variables, contrasts with separate variances based on Students' t-test were used to compare group means. When the variances were not homogeneous (Levene statistic) or distributions not normal (Kolmogorov-Smirnov test), values were transformed logarithmically prior to statistical testing. A pre-set p value of 0.05 was used.

Table 1 Plasma iron, total iron-binding capacity and haematological indices.

Groups	Vitamin A deficient controls (n=7)	Vitamin A supplemented rats (n=4)	P value*
Plasma iron (μmol/L)	38.6 ± 4.0	28.6 ± 2.5	0.061
Total iron-binding capacity (μmol/L)	75.9 ± 3.1	70.7 ± 3.8	0.330
Haemoglobin (mmol/L)	9.4 ± 0.1	8.9 ± 0.2	0.114
Haematocrit (%)	48.5 ± 0.6	48.0 ± 1.2	0.671
Red blood cell count ($*10^{12}$/L)	8.5 ± 0.2	8.2 ± 0.3	0.437
Mean cell volume (fL)	57.5 ± 0.2	58.8 ± 1.6	0.533
Mean corpuscular haemoglobin concentration (mmol/L)	19.3 ± 0.1	18.6 ± 0.1	0.004
White blood cell count ($*10^{9}$/L)	10.8 ± 1.1	12.3 ± 0.8	0.292

Means are given with standard errors of the mean.

* Based on group comparison using Student's t-test.

Table 2 Plasma activities of liver-function indicator enzymes.

Groups	Vitamin A deficient controls (n=7)	Vitamin A supplemented rats (n=4)	P value*
Alkaline phosphatase (U/L)	89.8 ± 12.5	115.4 ± 7.9	0.116
Alanine aminotransferase (U/L)	132.0 ± 39.2	30.0 ± 0.4	0.003†
Aspartate aminotransferase (U/L)	304.4 ± 78.9	57.5 ± 4.2	0.001†

Means are given with standard errors of the mean.

* Based on group comparison using Student's t-test.

† Data had been log-transformed prior to statistical testing.

RESULTS

After three days of vitamin A supplementation, plasma retinol concentrations were 0.63 (SE 0.08) μmol/L and 0.01 (SE 0.001) μmol/L in the 7 non-treated, vitamin A deficient rats. Body weight did not differ between the control rats (144.6 SE 2.2 g) and the 4 supplemented rats (148.0 SE 10.1 g). Likewise there were no significant differences between organ weights of the two groups (*Figure 1*).

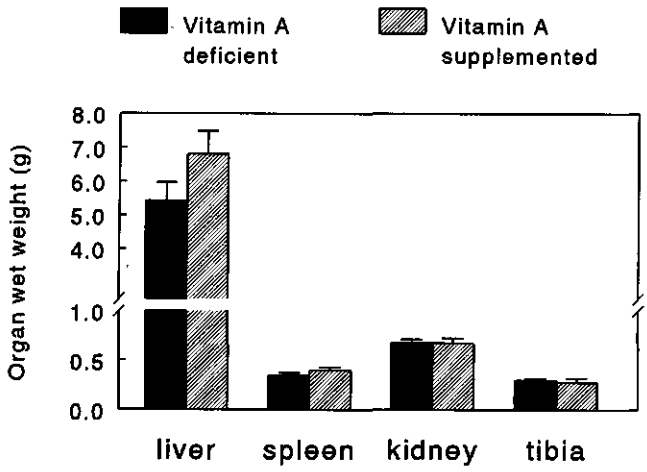

FIGURE 1 Organ weights of vitamin A deficient rats that were either not treated (controls, n = 7) or supplemented with vitamin A for 3 days (supplemented rats, n = 4). Results are expressed as means ± SE.

Iron status was not affected by vitamin A supplementation. There were neither group differences in iron content of organs (*Figure 2*) nor in total iron-binding capacity (*Table 1*). Vitamin A supplementation produced a reduction in plasma iron levels that almost reached statistical significance (*Table 1*). Of the haematological indices, including white blood cell count, only mean corpuscular haemoglobin concentration (MCHC = haemoglobin/haematocrit) was significantly reduced after vitamin A supplementation (*Table 1*). The reduction in the group mean values of haemoglobin and haematocrit seen after vitamin A supplementation was not significant (*Table 1*).

Vitamin A supplementation decreased plasma levels of alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT), but left alkaline

phosphatase activity unaffected (*Table 2*).

Differential counts of bone marrow smears revealed that vitamin A supplementation caused a shift towards more myeloid cells at the expense of erythroid cells. In particular, a higher percentage of the segmented neutrophils was present after vitamin A repletion (*Table 3*). The ratio myeloid/erythroid was not significantly different between the two groups. There was also a tendency towards more plasma cells after vitamin A supplementation (*Table 3*).

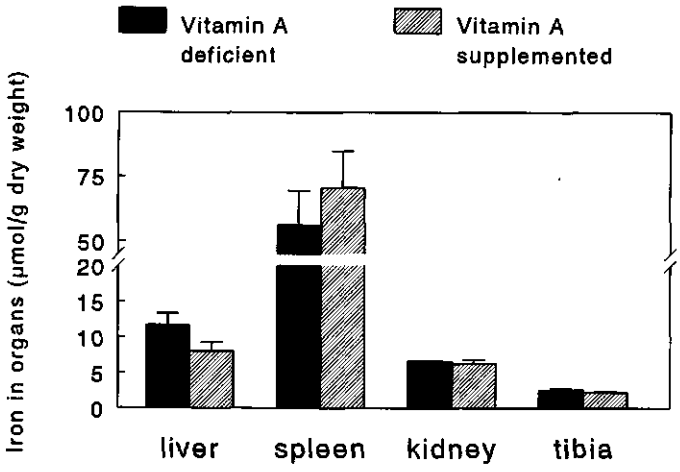

FIGURE 2 Iron concentrations in organs of vitamin A deficient rats that were either not treated (controls, n=7) or supplemented with vitamin A for 3 days (supplemented rats, n=4). Results are expressed as means ± SE.

DISCUSSION

In this experiment we have used vitamin A deficient rats that were losing weight and thus had a deficiency that was more severe [5] than that in our previous studies [1-4,**chapters 2-4,7**]. Vitamin A deficiency was at least partly reversed by supplementation with vitamin A, as shown by the increase in plasma retinol concentration. Liver retinol was not measured and thus it is not known to what extent retinol stores were repleted.

Table 3 Differential cell counts in bone marrow smears.

Groups	Vitamin A deficient controls (n=7)	Vitamin A repleted rats (n=4)	P value*
Total erythropoietic cells	20.9 ± 4.6	14.3 ± 1.9	0.219
Total myelopoietic cells	44.3 ± 3.7	57.5 ± 0.5	0.012
Myeloblasts	4.9 ± 0.6	5.0 ± 1.2	
Promyelocytes	1.8 ± 0.5	4.3 ± 1.7	
Myelocytes	5.7 ± 1.1	6.0 ± 1.6	
Metamyelocytes	1.0 ± 0.3	1.8 ± 0.9	
Bandcells	8.3 ± 0.7	6.8 ± 0.9	
Segmented neutrophils	20.1 ± 2.4	31.8 ± 3.6	0.040
Eosinophils	2.4 ± 0.3	2.0 ± 0.4	
Basophils	0.0 ± 0.0	0.0 ± 0.0	
Lymphocytes	25.6 ± 5.0	19.8 ± 1.3	0.295
Plasma cells	0.6 ± 0.3	2.3 ± 0.8	0.107
Monocytes	2.4 ± 0.4	2.3 ± 0.8	
Myeloid/Erythroid-ratio	2.9 ± 0.6	4.3 ± 0.6	0.152

Means are given with standard errors of the mean.
Mast cells and megakaryocytes were not included in the differential counting of 300 to 400 cells per bone marrow smear.
* Based on group comparison using Student's t-test.

Mean corpuscular haemoglobin concentration was reduced after vitamin A supplementation. However, iron status as assessed by other haematological indices, plasma iron, total iron-binding capacity and organ iron contents, was not significantly affected after 3 days of vitamin A supplementation. Dietary vitamin A and iron supplementation for a period of 10-12 days, after a period of both iron and vitamin A depletion, produced a reduction in haemoglobin, haematocrit and red blood cell count, increased plasma iron and total iron-binding capacity and reduced iron in spleen and bone [2,3, **chapters 3-4**]. Thus, 3 days of vitamin A supplementation to severely vitamin A deficient rats appeared not to be sufficient

to correct iron metabolism. The reduction in plasma iron, although not statistically significant, was probably caused by a lesser degree of haemoconcentration. In vitamin A deficient rats, haemoconcentration has frequently been observed [1-3,**chapters 2-4**,6-8]. The lack of effect of vitamin A supplementation on organ iron levels can be explained by the large iron stores in the present rats, the stores being 2-4 fold higher than those in our earlier studies [1-3,**chapters 2-4**]. In this study iron intake was about 3-10 times higher than in the studies with rats showing less severe vitamin A deficiency [1-3,**chapters 2-4**].

Vitamin A deficiency in rats or chickens may be associated with either unaltered [4,**chapter 7**,9] or raised [10,11] ASAT and ALAT activities in plasma and liver. In this study, the high plasma ALAT and ASAT activities in the vitamin A deficient controls had dropped markedly after 3 days of vitamin A supplementation. In rats, ALAT is considered a liver specific enzyme, whereas ASAT is distributed over various organs including the liver, and alkaline phosphatase occurs mainly in osteoblasts, kidney and intestine [12]. Thus, the levels of ALAT and ASAT could indicate that severe vitamin A deficiency had caused liver damage in the rats, this damage being reversed quickly by vitamin A supplementation. The values observed after vitamin A administration were in the normal range [12]. Group mean plasma activity of alkaline phosphatase was non-significantly raised by vitamin A supplementation. This observation corroborates the low activities of alkaline phoshpatase seen in livers, kidneys and spleens from vitamin A deficient rats [13].

Bone marrow smears of the severely vitamin A deficient control and supplemented rats contained more erythrocytes and less haematopoietic cells than in rats with milder vitamin A deficiency [4,**chapter 7**]. Thus, severe vitamin A deficiency possibly caused a reduction in bone marrow cells, leading to decreased haematopoiesis. The group mean myeloid:erythroid ratio was increased after vitamin A supplementation, suggesting that myelopoiesis was stimulated relative to erythropoiesis. The effect on myelopoiesis was caused mainly by an increase in the percentage of granulocytes, in particular the segmented neutrophils. In a recent experiment with vitamin A deficient Lewis rats, aged 9-11 weeks, vitamin A supplementation for 4.5 days selectively increased the levels of circulating lymphocytes in blood, but reduced those of granulocytes. As in this study, the total white blood cell count was not affected by vitamin A supplementation [14]. The vitamin A effects on white blood cell synthesis are supported by recent *in vitro* experiments, showing a retinoic-acid-induced granulocytic differentiation in human leukaemia cells [15] or marrow myeloid progenitors [16]. The effects of retinoic acid on erythroid differentiation were paradoxical [16,17]. Thus, vitamin A

supplementation to vitamin A deficient rats influences myelopoiesis and possibly also erythropoiesis.

In conclusion, after 3 days of vitamin A administration to rats with severe vitamin A deficiency, vitamin A status was increased and liver damage reduced. Vitamin A supplementation increased myelopoiesis relative to erythropoiesis. There were no clear effects on haematology and organ iron stores. The observed reduction in plasma iron levels after vitamin A supplementation might indicate that any raising effect as seen earlier [2,3,**chapters 3,4**], was overruled by a concurrent decrease in haemoconcentration.

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CHAPTER 9

A potential role for vitamin A in iron metabolism at the level of transferrin gene transcription

ABSTRACT

From studies with women and children in third world countries it is known that vitamin A deficiency is associated with impaired iron status. We have been studying mechanisms underlying the relationship between the metabolisms of vitamin A and iron using the rat as an animal model. One of the recurrent findings have been that vitamin A deficiency decreases transferrin concentration as measured by total iron-binding capacity. It is known that when retinoic acid regulates protein synthesis at the level of DNA transcription, a specific base sequence is located in the promoter of the gene, a so-called 'direct repeat'. Retinoic acid receptors can bind as dimers to this 'direct repeat', and thus regulate transcription. We hypothesize that vitamin A deficiency influences iron metabolism by reducing hepatic synthesis of the iron transport protein, transferrin. If transferrin synthesis is regulated by vitamin A, a 'direct repeat' in the upstream region of the transferrin gene is expected. We have analyzed the transferrin gene and other genes for proteins involved in iron metabolism, for direct repeats that could bind to retinoic acid receptors. Experimental data were obtained by incubating liver cells (HepG2) for 8, 24 or 48 hours with different concentrations of all-*trans* retinoic acid or 9-*cis* retinoic acid (10^{-7}-10^{-5}M). Transferrin expression was based on the mRNA content, quantified by PCR amplification. We have identified and located candidate retinoic acid response elements in potential regulatory regions in the genes for transferrin and erythropoietin. This suggests that vitamin A does in fact regulate transferrin and erythropoietin synthesis at the level of gene transcription. However there was no consistent effect of all-*trans* and 9-*cis* retinoic acid on transferrin gene transcription in cultured liver cells.

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INTRODUCTION

From studies with women and children in third world countries it is known that vitamin A deficiency is associated with impaired iron status [1-7]. Based on studies in rats on the mechanism underlying the relationship between vitamin A and iron metabolism, we have hypothesized that vitamin A deficiency impairs erythropoiesis [8-10,**chapters 2,4**]. In addition increased iron concentrations in liver [8,11] and spleen [8-10,**chapters 2,4**] and low plasma total iron-binding capacity or plasma iron concentrations in vitamin A deficient rats [9,10,12,**chapters 2,4**] or humans [2,4-6] may point to disturbed iron transport. The hepatic synthesis of transferrin, the iron transport protein or its subsequent glycosylation could be reduced with vitamin A deficiency [13-15]. Protein synthesis could be regulated at the level of gene transcription via nuclear receptors that bind retinoic acid, a product derived from vitamin A [16].

To date two receptors have been identified that bind retinoic acid. The retinoic acid receptor (RAR) binds all-*trans* retinoic acid and 9-*cis* retinoic acid, while the retinoid X receptor (RXR) only binds 9-*cis* retinoic acid [17-19]. These nuclear retinoic acid receptors affect transcription by binding specific sequences in the promoter region of target genes, known as hormone response elements. Retinoic acid response elements have been identified in the promoter of a variety of genes [20-25]. These response elements consist of a direct repeat (DR) of the half-site AGGTCA (or related sequence) separated by one to five nucleotides (designated DR1-5). Each half-site can occupy one molecule of RAR and RXR forming DNA-bound homodimers and heterodimers. The spacing between the direct repeat elements determines the identity of the bound complex. DR1 elements preferentially bind RXR homodimers. While DR2 and DR5 elements prefer RXR/RAR heterodimers [26].

Knowledge of the above mentioned specific sequences and spacings can be used to identify response elements, by using gene databases [16]. When genes and their promoters are characterized, an initial step in identifying a possible retinoic acid response element can be to search for the above mentioned direct repeats (DR1, DR2 or DR5). In the present study, the genes for transferrin and other proteins involved in iron metabolism were screened for potential retinoic acid response elements. These proteins were: erythropoietin (renal erythropoietic hormone), erythropoietin receptor, erythroid 5-aminolevulinate synthase (involved in haem-synthesis), ferritin and transferrin receptor. ß-actin was also included in the search, as it was used as the internal standard in the *in vitro* studies.

For transferrin, the presence of a retinoic acid response element is supported

by the increase of transcription *in vitro* after incubation with all-*trans* retinoic acid in cultured human liver cells [27,28]. We wanted to confirm whether 9-*cis* retinoic acid was as efficient as all-*trans* retinoic acid, using a human hepatoma cell line (HepG2). Liver cells were incubated with various concentrations of all-*trans* retinoic acid or 9-*cis* retinoic acid, for a period of 8, 24 or 48 hours. The expression of the transferrin gene was determined by reverse transcription of its messenger RNA (mRNA) to complementary DNA (cDNA) and subsequent amplification by the polymerase chain reaction (PCR) with specific primers. Transferrin mRNA levels were determined relative to the level of mRNA of β-actin, a housekeeping protein not regarded as being affected by retinoic acid [29].

METHODS

DNA screening

Information from the EMBL-Genbank (European Molecular Biology Laboratory, Heidelberg, Germany) has been used to identify retinoic acid response elements for ß-actin, erythropoietin, erythropoietin receptor, erythroid 5-aminolevulinate synthase, ferritin, transferrin receptor and transferrin. The search was directed at perfect direct repeats of any sequence of 6 bases with a spacing of 0 to 6 bases and repeat analogues to the sequence AGGTCA with two or less mismatches. Only direct repeats spaced by either 1,2 or 5 bases (DR1, DR2, DR5) located on a non-coding site of the gene (5' and 3' flanking regions, or introns) were selected as possible retinoic acid response elements.

Cell cultures and incubation

HepG2 liver cells were maintained in Minimum Essential Medium (MEM, Gibco, Paisley, UK) with L-glutamine, fetal calf serum (FCS, 3.5%, Gibco), sodium bicarbonate (2.2 g/L) and gentamycin (10 μg/mL, Gibco) in 25 cm^2 culture flasks (Costar, Cambridge, USA), placed in a humidified incubator with a constant flow of 5% CO_2 at 37°C. The medium was refreshed every 2-3 days. Incubation (8, 24 and 48 h) with all-*trans* and 9-*cis* retinoic acid, both kindly provided by Dr. Schüep and Dr Schierle (Hoffmann-La Roche, Basel, Switzerland) were performed on 6-day old cell cultures (75% confluent) in 6 wells clusters (35 mm diameter, Costar).

Solutions of each retinoid were added at a final concentration of 0, 10^{-7}, 10^{-6} and 10^{-5} M in dimethyl sulphoxide (DMSO, Merck, Darmstadt, Germany, final concentration < 1% v/v). The incubations were stopped by the addition of trypsin and the cells diluted to $4x10^5$ cells/mL. Possible toxic effects of the retinoids were examined by measuring cell growth after incubating cells with all-*trans* and 9-*cis* retinoic acid (10^{-5}M) for 48 h.

RNA isolation and reverse transcription of mRNA

Total RNA was isolated from the cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method [30].

The mRNA was reverse transcribed into first-strand cDNA. The primer-annealing mix containing 1 μL (0.3-0.5μg) RNA solution and poly(rA) tail primer Oligo(dT)$_{15}$ (1 μL, 50 μM, Pharmacia, Roosendaal, The Netherlands) and 10.5 μL double distilled water was incubated at 70°C for 10 min and chilled on ice. A mixture of RT-buffer (4 μL, 15 mM $MgCl_2$, 250 mM Tris-HCl and 375 mM KCl, Promega, Madison, USA), dNTPs (2 μL, 10 μM each, Pharmacia), rRNasin ribonuclease inhibitor (0.5 μL, 40 U/μL, Promega) and M-MLV Reverse Transcriptase (RT) RNase H Minus (1 μL, 200 U/L, Promega) were added and incubated in a Omnigene thermal cycler (Biozym, Landgraaf, The Netherlands) at 42°C for 50 min, soaked at 95°C for 5 min and chilled on ice. In control tubes Reverse Transcriptase (RT) RNase H Minus was replaced by double distilled water.

Polymerase chain reaction (PCR)

The RT reaction products were amplified by PCR using the following primers (Molecular Biology Department, Utrecht University Hospital, Netherlands): for

transferrin: upstream primer Tf3: 5'-TCTAAGCTTGCTGATGCCATGAGCTTGGATGGA-3'
downstream primer Tf4: 5'-CTATAATGCAGTTGTCGTCGT-GGA-3'

ß-actin: upstream primer β-act: 5'-GTGGGGCGCCCCGGCACCA-3'
downstream primer ß-act: 5'-CTCCTTAATGTCACGCACGATTTC-3'

Quality of PCR products was confirmed by electrophoresis of aliquots through an agarose gel (1.2%).

Quantization of PCR products by pyrophosphate (PPi) measurement

PPi, which is stoichiometrically released when nucleic acids are synthesized in PCR products was measured by the Homogenous Phase Pyrophosphate Measurement [29]. Per sample ATP luciferin-luciferase (100 μL, Lumac, Landgraaf, The Netherlands and Sigma, St. Louis, USA), ATP sulfurylase (12 μL, 1.9 U/mL in 0.025 M HEPES, pH 7.75 buffer, Sigma), 68 μL double distilled water and 10 μL of diluted PCR product were mixed. After determination of the blank value, adenosine 5'phosphosulfate (10 μL, APS, 5 μM, Sigma) was added. The maximum bioluminescence signal was registered, with a bioluminescence counter (Lumac). PCR products were diluted 25 or 3 times depending on whether luciferin-luciferase from Lumac or Sigma was used, respectively. PCR products from the same incubation period (8, 24 or 48 hours) were measured with the same luciferin-luciferase enzyme. When used for the same sub samples, both enzymes gave similar results. Possible differences between the enzymes of Lumac and Sigma were corrected for by the control incubation (0 M retinoic acid). A standard curve ($R > 0.99$) of different concentrations PPi in distilled water (100-1000 nM PPi, Lumac; 1.5-30 μM PPi, Sigma) was used.

Transferrin transcription was determined as the ratio of $[PPi]_{Tf}$: $[PPi]_{\beta\text{-act}}$ representing the ratio $mRNA_{Tf}$: $mRNA_{\beta\text{-act}}$ from which the log was calculated. One experimental unit comprised a concentration series of 0, 10^{-7}, 10^{-6} and 10^{-5} M all-*trans* and 9-*cis* retinoic acid measured in triplicate after an incubation time of 8, 24 or 48 h.

Statistical analysis

The mean of three control values per experimental unit expressed as log ratios, were used to correct individual values. Effect of retinoic acid concentration, incubation time and experiment as independent variables were evaluated using analysis of variance. After determining that interactions were not significant, interactions were removed from the model. From the analysis of variance, standard error of the mean (SE) was calculated as $\sqrt{}$RMSE/$\sqrt{}$n (RMSE, residual mean square error; n = number of samples) for the two retinoids. A pre-set p value of 5% was used.

RESULTS

Candidate retinoic acid response elements

Information about non-coding sequences was available for ß-actin and five proteins involved in iron metabolism, but information was not available for erythroid aminolevulinate synthase and transferrin receptor (*Table 1*).

On these non-coding DNA sequences, a staggered perfect direct repeat of any sequence of 6 bases spaced by 2 bases (DR2) was located in intron 1 (+503 bp) of the erythropoietin gene. A similar staggered perfect direct repeat, also in intron 1 (+606 bp), but spaced by 5 bases (DR5) was found in the transferrin gene. Perfect direct repeats were also located in the 5' flanking region of ß-actin: two DR1s (≈-100 and ≈-200 bp from the TATA-box) and as a staggered DR5 (≈-300 bp). The search for AGGTCA analogues in a direct repeat with two or less mismatches revealed an imperfect DR2 of the sequence '5-AGGTCAggAGTTCA-3' in the '5 flanking region of the erythropoietin gene (≈-200 bp). For transferrin, a perfect DR5: 5'-GGGTCAaatgaGGGTCA-3' was located at 611 bp in intron 1 and an imperfect DR4: 5'-GGATCAcctgAGGTCA-3' was located in intron 2 at +2302 bp. This DR4 could be a candidate thyroid hormone response element [16]. The location in intron 2 however, which is far from the transcriptional start, makes its importance for transcription regulation less likely. Also the direct repeats (DR1, DR5) found for ß-actin are probably less important for regulation of transcription by retinoic acid. They differ from the above mentioned repeates in that they are less diverse in base content and may represent GC islands in the sequence [43].

Legend to table 1 † The coding regions of the genes were not included. ‡ These include only perfect direct repeats spaced by either 1, 2 or 5 bases (DR1, DR2 and DR5), of any sequence or imperfect repeats of the sequence AGGTCA (DR1 to DR5) with 2 or less mismatches. * Sequence coordinates are relative to the transcriptional start. When this is unclear '≈' is used with the estimated distance from the transcriptional start (erythropoietin) of from the TATA-box (ß-actin).

Table 1 Candidate retinoic acid response elements in non-coding DNA sequences of several proteins involved in iron metabolism and ß-actin obtained from a gene database, the EMBL-Genbank.

Protein of interest	Information on non-coding sequences†	Possible retinoic acid response elements‡ (location)*				Reference
		DR1	DR2	DR5	AGGTCA analogue	
Erythroid-aminolevulinate synthase	not available	-	-	-		31
Erythropoietin	5'flank region >5000 bp intron 1-4 3'flank region 82 bp	-	AAGCTGatAAGCTG AGCTGAtaAGCTGA GCTGATaaGCTGAT CTGATAagCTGATA TGATAAgcTGATAA (intron 1, +503 bp)		AGGTCAggAGTTCA (5'flank, ≈-200 bp)	32-34
Erythropoietin-receptor	5'flank region -1050 bp	-	-	-	-	35
Ferritin: light chain	intron 1-4	-	-	-	-	36-39
heavy chain	intron 1-4	-	-	-	-	
Transferrin	5'flank region -963 bp intron 1-2	-	-	GAGGGTcaaatGAGGGT AGGGTCaaatgAGGGTC GGGTCAaatgaGGGTCA (intron 1, +606 bp)	GGGTCAaatgaGGGTCA (intron 1, +611 bp) GGATCAcctgAGGTCA (intron 2, +2302 bp)	40
Transferrin-receptor	not available	-	-	-	-	41
ß-actin	5'flank region >2000 bp	GCCCCCaGCCCCC (≈-200 bp) GAGGGGaGAGGGG (≈-100 bp)		GGGCAGgccggGGGCAG GGCAGGccgggGGCAGG GCAGGCcggggGCAGGC CAGGCCgggggCAGGCC (≈-300 bp)	-	42

Effect of all-trans- and 9-cis-retinoic acid on HepG2 cell growth

HepG2 cell growth was increased after 24 and 48 h in the presence of 10^{-5} M all-*trans* or 9-*cis* retinoic acid, when compared with cells to which no retinoic acid was added (*Figure 1*).

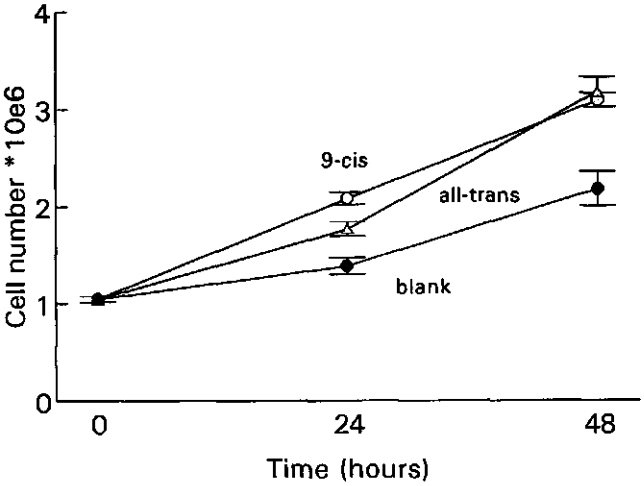
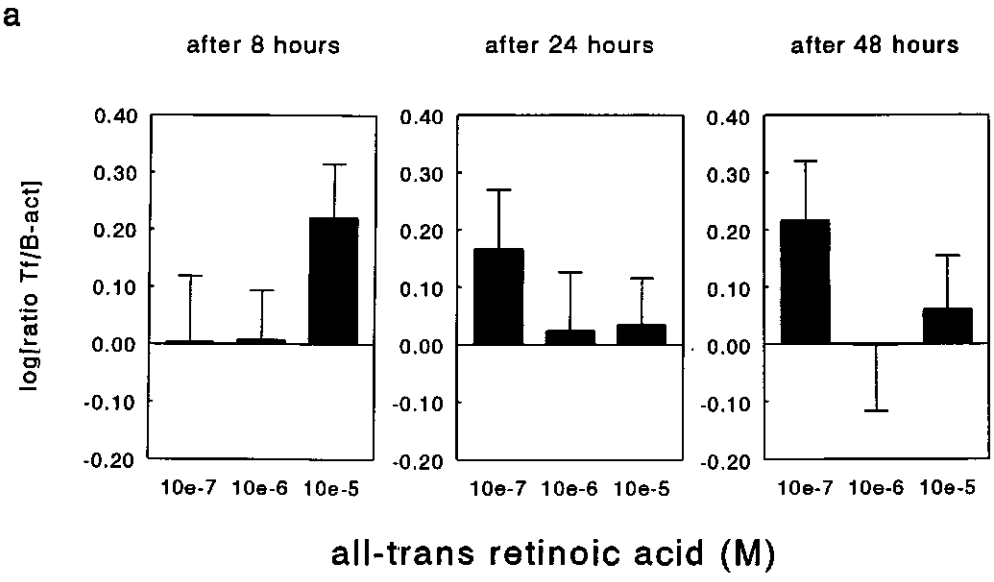


FIGURE 1 Effect of all-*trans*- and 9-*cis*-retinoic acid on HepG2 cell growth. HepG2 liver cells were incubated with (△) all-*trans*-retinoic acid and (○) 9-*cis*-retinoic acid (10^{-5} M in dimethyl sulphoxide, Merck for 48 hours). For the blank (●), cells were incubated with dimethyl sulphoxide. After 0, 24 and 48 h cells were counted. Values represent means and SE (n = 3).

Effect of all-*trans*- and 9-*cis*-retinoic acid on transferrin mRNA levels

Messenger RNA levels were measured after 8, 24 and 48 h incubation with 10^{-7}, 10^{-6} and 10^{-5} M of all-*trans* retinoic acid or 9-*cis* retinoic acid, in triplicate in two separate experiments. Means for the two experiments corrected for the control without added retinoid were averaged and are presented as bars in *Figure 2*. After 8 h, transferrin transcription increased with the highest concentration of all-*trans*-retinoic acid, but after 24 and 48 h the opposite effect was seen (*Figure 2A*). Thus analysis of variance with the log-transformed data revealed no effect of retinoid concentration, incubation time or experiment for all-*trans* retinoic acid. For 9-*cis* retinoic acid, there was a significant experiment effect ($p < 0.001$) and effect of retinoid concentration ($p = 0.05$): 10^{-6}M 9-*cis* retinoic acid giving a consistently lower transferrin transcription than did the two other concentrations at 8, 24 and 48 h (*Figure 2B*).



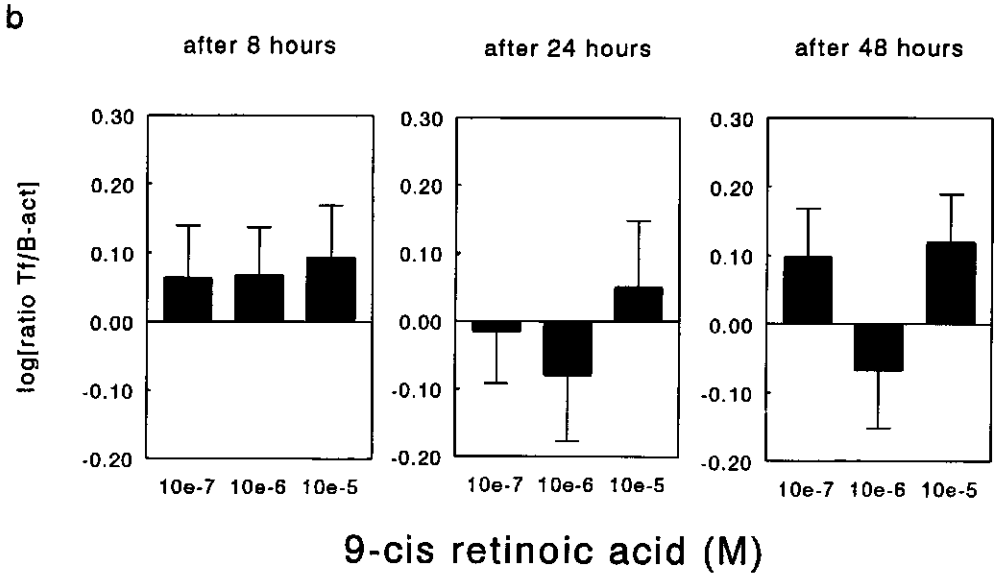


FIGURE 2 Effect of all-*trans* retinoic acid (A) and 9-*cis* retinoic acid (B) on transferrin synthesis determined as log [ratio $mRNA_{Tf}$: $mRNA_{\beta\text{-act}}$]. Messengers RNA levels were determined by pyrophosphate measurement of PCR products after respectively 8, 24 and 48 hours incubation with 10^{-7}, 10^{-6} and 10^{-5} M all-*trans* (A) or 9-*cis* (B) retinoic acid. Three blank values per incubation time were averaged and used for correction of the individual log ratios. Weighted means of the two experiments were averaged after correction for the blank condition. Bars represent means of two experiments with pooled SE.

DISCUSSION

Arising from our interest in the interrelationship between vitamin A and iron metabolism, we have screened non-coding sequences of genes of several proteins involved in iron metabolism for the existence of candidate retinoic acid response elements. Both the transferrin and the erythropoietin gene appear to have possible retinoic acid response elements. On the transferrin gene, a perfect direct repeat (DR5) of the AGGTCA-analogue is located in the first intron as part of the stagger. Location in the first intron and not in the promoter region suggests a less direct regulatory pathway than that for the retinoic acid response elements identified so far, which were all located in the promoter region upstream of the transcriptional start [20-25]. The first exon of the transferrin gene might encode a signalling peptide which could interact in transcription regulation of the transferrin gene. A likely candidate for a potential retinoic acid response element (DR2) is located in the promoter of the erythropoietin gene.

The occurrence of candidate retinoic acid response elements on the erythropoietin and transferrin genes supports the hypotheses that erythropoiesis might be impaired [8-10,**chapters 2,4**] and iron transport affected [2,4-6,9,10,12, **chapters 2,4**] in vitamin A deficiency. Although this provides a theoretical basis for the regulation of transferrin and erythropoietin synthesis by retinoic acid at the DNA transcription level, this need to be confirmed experimentally.

To confirm our theoretical finding of a retinoic acid response element (RARE, DR5) located 611 bases down-stream from the transcriptional start, we measured transferrin synthesis in liver cells (HepG2) after incubation with all-*trans* and 9-*cis* retinoic acid. This response element could be important in regulation of transferrin transcription. We hypothesized that both all-*trans* and 9-*cis* retinoic acid would stimulate transferrin transcription. Thus the level of transferrin transcription, after 8, 24 and 48 h incubation with 10^{-7}, 10^{-6} and 10^{-5} M of all-*trans* retinoic acid or 9-*cis* retinoic acid, was determined.

However, we did not find any consistent effect of all-*trans* retinoic acid on transferrin synthesis although transferrin synthesis was lower with 10^{-6} M 9-*cis* retinoic acid relative to the other two concentrations (10^{-7} and 10^{-5} M). This is in contrast to the findings of Hsu et al [27] who found that treatment of another hepatoma cell line (Hep3B) with all-*trans* retinoic acid (10^{-5} M for 48 h) resulted in an 8 fold increase in transferrin protein synthesis and a 10 fold increase in transferrin mRNA level. The induction of transferrin mRNA by all-*trans* retinoic acid was detected after 8 h of treatment [27]. Others have shown that incubation of 10^{-5} M all-*trans* retinoic acid in HepG2 hepatoma cells increased secretion of

transferrin 2.5 fold after 3 days [28]. Our method of quantifying PCR products by measuring PPi with ß-actin as internal standard, is more direct than Northern blotting of mRNA and audiography of radioactively labelled cDNA probes [27] or immunoelectrophoresis of the secreted proteins [28]. It is however possible that ß-actin is not a good internal standard, although there was no candidate retinoic acid response element on its 5' flanking region.

Incubation of HepG2 cells with all-*trans*-retinoic acid and 9-*cis* retinoic acid increased the growth rate of the liver cells. This is in contrast to the finding of Hsu and colleagues [27] that the growth rate of Hep3B cells was slightly inhibited by 10^{-5} M all-*trans* retinoic acid after 4-7 days. This discrepancy could possibly be explained by a difference in cell line or culture media which could have overruled the effect on transferrin gene transcription in the present study. Alternatively our culture medium with only 3.5% (v/v) FCS could have been iron deficient, resulting in an increased background transferrin synthesis which would mask a possible effect of retinoic acid.

The phenomenon of dedifferentiation as discussed by Kaptein et al [44] should also be considered. In our HepG2 cell line, this could have resulted in the loss of retinoic acid receptors or a mutation in the retinoic acid response element of the transferrin gene. The use of HepG2 cells in transfection assays studying the effect of both peroxisome proliferator-activated receptors (PPAR) and 9-*cis* retinoic acid receptors (RXR) suggest that this cell line should contain retinoic acid receptors [45].

In conclusion, with the use of a gene bank we were able to locate candidate retinoic acid response elements in potential regulatory regions in the genes for erythropoietin and transferrin. This supports the hypotheses that vitamin A affects erythropoiesis and iron transport. However, it was not possible using cultured liver cells incubated with either all-*trans*, or with 9-*cis* retinoic acid to confirm a role for vitamin A in transferrin gene transcription. This supports the implication that retinoic acid indirectly regulates transferrin transcription through a signalling protein or an intermediate transcription factor. The next step would be to optimalize the *in vitro* model of cultured liver cells for transferrin synthesis and to confirm the sensitivity to induction by retinoic acid of the located candidate retinoic acid response elements.

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CHAPTER 10

General discussion

The experiments presented in this thesis were carried out to simulate the interrelationship between vitamin A and iron metabolism found in children and women in third world countries [1-3] and elucidate the underlying mechanisms. In this chapter the main findings are summarized. The rat model is evaluated and possible mechanisms are discussed, leading to conclusions and directions for further research.

MAIN FINDINGS

Influence of vitamin A on iron metabolism

Effects of manipulation with dietary vitamin A and/or iron on vitamin A and iron status were studied in rats [**chapters 2-8**]. Consistent changes in iron metabolism with moderate vitamin A deficiency were iron accumulation in spleen and bone [**chapters 2-4,6-7**]; reduction in total iron-binding capacity [**chapters 2-7**], which is a measure of the transferrin level and reduced total liver iron, which is caused by a reduction in liver weight [**chapters 2-7**]. These effects were reversed by vitamin A supplementation for 10-12 days [**chapters 3-4**] but not for 3 days [**chapter 8**]. Mild vitamin A deficiency produced anaemia followed by increased iron absorption [**chapter 2**]. When vitamin A status further deteriorated, haemoglobin concentrations rose [**chapters 2-4**]. Simultaneously growth was reduced, implicating that the increased concentration of blood components could be ascribed to the well documented phenomenon of haemoconcentration in vitamin A deficient rats, which is a result of disrupted water balance and growth reduction [4-7]. Rats with chronic vitamin A deficiency may be less affected by haemoconcentration. But there was no anaemia in mature female rats with stable low plasma retinol levels (**chapter 6**).

Vitamin A deficiency versus iron deficiency

The effects of vitamin A deficiency on iron status differ from those of iron deficiency [**chapters 2,7**]. The slight lowering of haemoglobin concentration in early vitamin A deficiency has a metabolic different basis from the marked fall in haemoglobin observed in iron deficientcy [**chapters 2,7**]. The marked fall in haemoglobin was accompanied by increased erythrocyte protoporphyrin levels, indicating iron shortage in erythroid tissue, and increased plasma transferrin

receptor concentrations, indicating increased erythroid activity, which were both unaffected with vitamin A deficiency [**chapter 7**]. The rapid reduction in haemoglobin concentration and plasma and organ iron levels, and the increase in total iron-binding capacity with iron deficiency, was followed by an increased iron absorption efficiency [**chapters 2,7**]. With vitamin A deficiency, the increase in apparent iron absorption was associated with increased tissue iron concentrations and decreased total iron-binding capacity [**chapter 2**].

Possible mechanisms

As to the mechanism by which vitamin A might influence iron metabolism, it was speculated that with vitamin A deficiency, red blood cell synthesis is impaired, leading to increased destruction of malformed red blood cells and increased iron stores in the macrophages of tibia and spleen. The iron accumulation might be enhanced by reduced iron mobilization, arising from impaired synthesis of transferrin [**chapters 2-6**], possibly at the level of transferrin glycosylation [8-10]. The hypotheses could not be confirmed by measuring transferrin glycosylation, and indicators of red blood cell synthesis and breakdown in rats fed diets deficient in vitamin A [**chapters 7,8**].

Alternatively, transferrin synthesis might be regulated by vitamin A at the level of gene transcription [11,12]. With the use of a gene bank, a candidate retinoic acid response element for transferrin was located. However, it was not possible to confirm a role for vitamin A in transferrin gene transcription by using cultured liver cells incubated with all-*trans* and 9-*cis* retinoic acid [**chapter 9**].

THE ANIMAL MODEL

For ethical reasons it is not possible to carry out invasive studies in humans. Therefore the rat was used as model in our studies. The rat is among the best documented animal models for studying vitamin A deficiency. The effects of vitamin A status on vision [13] and growth [14] in the rat have been carefully described in the past. More recently, vitamin A needs of rats for reproduction [15] and lactation [16,17] and the kinetics of retinol metabolism influenced by vitamin A status [18,19] have been described. Also many rat studies have contributed to the present knowledge about vitamin A metabolism [20]. However, rats differ from humans in many aspects of which some concerning vitamin A and iron metabolism

will be discussed below.

Vitamin A metabolism

Vitamin A can be absorbed as preformed vitamin A (retinol or retinyl esters) or as pro-vitamin A (carotenoids) which can be converted into retinol in the intestine. After absorption, retinol is transported as retinyl esters in chylomicrons via the lymphatic system to the liver, where it is stored, or re-exported into plasma bound to retinol binding protein (RBP) from which it is delivered to target sites. Other tissues besides the liver play a role in retinol storage and mobilization [20]. In general, vitamin A is important in gene expression, cellular growth and differentiation.

Unlike humans, rats absorb intact pro-vitamin A only poorly, but convert it almost quantitatively to retinol [21]. This difference will be of little relevance to the studies described here. However another difference is the direct uptake by bone marrow of chylomicron remnants containing retinyl esters which does occur in primates and rabbits, but not in rats [22]. This species difference could be of importance for the regulation of red blood cell synthesis and thus affect iron metabolism. This possibly makes the rat a less suitable model for human bone marrow metabolism, in relation to vitamin A.

Iron metabolism

Iron is absorbed in the small intestine, the site where iron status is regulated. Absorption of iron increases profoundly as induced by iron deficiency [**chapters 2 and 7**]. The daily iron flux largely results from degradation of red blood cells in the reticuloendothelium (macrophages). This iron is taken up by the erythroid tissue for formation of new red blood cells. Iron is also necessary for oxygen storage in muscle (as myoglobin), in cytochromes and enzymes involved in oxidation/reduction reactions. The iron mentioned so far is all functional iron, the surplus is stored, mainly in liver but also in other organs [23].

Total body iron content in the rat is about 50 mg/kg body weight [24] which is similar to that in man [23]. This corresponds to about 17.5 mg for a 350 g weighing young growing male rat of 13 weeks, which was typical for these studies [**chapter 2**]. Red blood cell synthesis in adult humans mainly occurs in bone marrow of the flat bones [25] while in rats also the long bones and spleen are

involved [26, **chapters 8,7**]. The life span of red blood cells is about 50-60 days in rats [27] and 120 days in humans [25]. *Table 1* illustrates that young growing rats differ from humans in quantitative aspects of iron metabolism. Rats have a higher iron turnover, which is explained by the shorter lifetime of rat erythrocytes. Rats also absorb 5-30 times more iron, when expressed relative to their red cell iron content. The high iron absorption in young children, as compared to adults illustrates that growth is positively associated with iron absorption (*Table 1*).

The larger daily iron flux will make rats more sensitive to changes in iron metabolism than man. This will require careful standardization of rat experiments in order to detect changes in the dynamic process of iron metabolism.

Table 1 Quatitative differences in iron metabolism between rat and mans[¶].

	Rat	Human	
	350 g	Adult, 65 kg	Child, 1 y, 10 kg
Iron in red blood cells§, mg	12 (100)#	2200 (100)	270 (100)
Iron in plasma, mg	0.02 (0.2)*	3 (0.2)	
Iron flux, mg/day			
- from red blood cell breakdown	0.2 (1.6)‡	20 (0.9)	2.3 (0.9)
- absorbed	0.2 (1.6)†	1 (0.05)	0.7 (0.3)

¶ Data derived from chapter 2, for the rat and from references [23,28] for man. § Values in parentheses refer to proportion of iron in red blood cells, %. #Calculated as (blood volume, 25 ml)x(haemoglobin concentration, 8.5 mmol/L)x(M_{iron}, 56 g). * Calculated as (plasma volume, 14 ml)x(plasma iron, 30 μmol/L)x(M_{iron}, 56 g). ‡ Calculated as (total iron in red blood cells, 12 mg)/(lifetime red blood cells, 55 d). † Calculated as (apparent absorption, 25 %)x(feed intake, 18 g/d)x(iron in feed, 45 mg/kg).

Diets

The development of vitamin A deficiency progressed faster in second generation rats born from dams fed on purified diets [**chapter 4**] when compared with commercially purchased rats [**chapters 2,3 and 7**].

Composition of the purified diets was based on the Nutrient Requirements

for Laboratory Animals from the National Research Council. Recommended levels for dietary vitamin A and iron are 1200 retinol equivalents (RE)/kg and 35 mg/kg respectively [29]. Purified diets were fed to young commercially purchased rats from the age of 3 weeks onwards [**chapters 2,3,7**]. Alternatively, in a two-generation design these diets were fed to the dams, from the age of 10 weeks onwards, which was six weeks prior to the onset of pregnancy. And the diets were continued to be fed until the weaning of the pups [**chapters 4-6**].

Table 2 Body weight, liver retinol and liver iron of male Wistar rats (Cpb:WU) described in chapters 2,4 and 7 (mean ± SE).

	Chapter 2		Chapter 4	Chapter 7
Origin of the rats‡	Commercial		2nd generation	Commercial
Year	1990		1992	1993
	(n=12)	(n=12)	(n=8)	(n=12)
Dietary vitamin A, added (RE/kg)	1200		1200	0,1200†
Dietary iron, analyzed (mg/kg)	38		60	45
Age (w)	11	15	10	15
Body weight (g)	317 ± 9	371 ± 6	267 ± 7	370 ± 8
Liver retinol (nmol/g wet weight)	177 ± 7	181 ± 11	54 ± 4	195 ± 6
Liver iron (μmol/g dry weight)	3.5 ± 0.1	4.1 ± 0.2	3.3 ± 0.3	8.0 ± 0.7

‡ All rats entered the experiments when about 3 weeks old.

† Rats received during the 2w pre-experimental period feed without added vitamin A. SE = standard error of the mean, RE = retinol equivalents.

Dams of the Wistar rats (Cpb:WU) obtained commercially (Harlan, CPB, Zeist, The Netherlands) had been fed on commercially available diets (RMH-B, Hope Farms, Woerden, The Netherlands). According to the manufacturer, minimum levels of vitamin A and iron in the natural-ingredient diet were 5940 RE/kg and 165 mg/kg, respectively [**chapter 5**]. Both vitamin A and iron are stored essentially in the liver and accumulate there when dietary intake is higher than the amount required. Vitamin A and iron status of the dams influences the status in their pups [16,30-32, **chapter 5**]. However, nutritional status of 3 week old pups is not only influenced by the status of the dams, but also directly by consumption of the administered diets by the pups.

Liver stores of vitamin A and iron for control rats described in **chapters 2, 4 and 7** are listed in *Table 2*. The studies differed in both the origin of the rats as well as the experimental design, which makes comparison debatable. But after 7 to 12 weeks feeding of purified diets, commercially purchased rats [**chapters 2 and 7**] had greater vitamin A stores than did the second generation rats [**chapter 4**]. The analyzed iron content of the experimental diets do not explain the variable liver iron stores in commercially purchased rats [*Table 2*, **chapters 2,7**].

Thus, for optimal standardization of experiments with young growing rats involving nutrients such as vitamin A and iron, the diets fed to their dams should also be defined and controlled.

THE INFLUENCE OF VITAMIN A ON IRON METABOLISM

From the results presented in **chapters 2-6** we formulated the hypotheses that vitamin A deficiency:

- affects red blood cell metabolism, and
- reduces iron mobilization.

These are considered direct effects of vitamin A on iron metabolism. However vitamin A might also influence iron metabolism indirectly by increasing the incidence and severity of infection or by reducing growth and liver weight.

Red blood cell metabolism

Vitamin A deficiency in man has been associated with anaemia [1-3,7]. It was thus hypothesized [**chapters 3-4**] that the primary effect of vitamin A deficiency is to impair synthesis of red blood cells, leading to abnormally shaped cells [33,34] and mild anaemia [34-36, **chapter 2**]. A potential role for vitamin A in erythropoietin gene transcription corroborates this hypothesis [**chapter 9**]. Increased destruction of these abnormally shaped red blood cells can cause the accumulation of iron in the macrophages found in spleen and bone marrow [**chapters 2-7**, 34,37]. In turn this could lead to an increased stimulation of the erythropoietic tissue. However from **chapter 7** it follows that there was neither a change in red blood cell synthesis, increase in red blood cell degradation nor stimulation of erythropoietic activity with vitamin A deficiency. Ferrokinetic studies might provide more information enabling effects of vitamin A deficiency on the dynamic red blood cell turnover to be detected when compared to the indirect and

qualitative indicators of red blood cell synthesis or degradation that were used [**chapter 7**]. Such experiments will be methodological more complicated, but have been described [38,39].

Iron mobilization

The observed accumulation of iron in tissues [**chapters 2-6**] and reduced plasma iron and total iron-binding capacity [**chapters 2-6**] suggest that iron mobilization might be impaired in vitamin A deficiency. Synthesis of transferrin, which plays a central role in iron mobilization, might be reduced in vitamin A deficiency either at the level of glycosylation or gene transcription.

Vitamin A has been postulated to assist in glycosylation as retinyl-phosphate-mannose carrying saccharides to the transferrin polypeptide chain [8,9]. Chan and Wolf found no evidence for such a role of retinyl-phosphate-mannose, but did measure *in vitro* reduced glycosylation of microsomal proteins that were derived from vitamin A deficient rats [10]. This could have caused the accumulation of lipid-linked sugar intermediates as reported by others [8,9]. Alternatively, as reported by Guma and Bernard, vitamin A could activate the enzyme dolichyl-phosphomannose synthase and thus would stimulate glycosylation in this way [40].

Human transferrin has two binding sites for glycans on the polypeptide chain while rat transferrin has only one [41]. The glycans bound to transferrin can have several carbohydrate branches, terminating in sialic acid molecules (maximum for humans, 8 and for rats, 4). In **chapter 7**, plasma transferrin molecules were separated and quantified on the basis of the number of sialic acid residues [42]. There was evidence for enhanced rather than reduced glycosylation in vitamin A deficiency, because the proportion of higher sialylated transferrin was increased. Iron deficiency had a similar effect.

The physiological significance of transferrin microheterogeneity is obscure, but transferrin with a low sialic acid content is associated with increased iron turnover and increased hepatic iron stores [43]. Thus a high degree of sialylation could point to iron sparing which would be feasible for the condition of iron deficiency, but is difficult to understand for for vitamin A deficiency with iron accumulation in macrophages of the reticuloendothelial system. Possibly, in mild vitamin A deficiency the iron is less available, also triggering iron sparing [**chapter 7**]. However transferrin sialylation might be affected differently in severely vitamin A deficient animals with eye lesions or reduced growth, like those used in the

studies earlier mentioned [8-10].

Synthesis of transferrin and the subsequent glycosylation could be regulated independently [44] while there is evidence that transferrin gene transcription was stimulated *in vitro* by vitamin A [11,45]. Thus the reduced iron mobilization in vitamin A deficiency could be related to inhibition of transferrin synthesis at the level of gene transcription [12]. With the use of a gene bank, candidate retinoic acid response elements for transferrin and erythropoietin were located [**chapter 9**]. This supports the hypothesis that vitamin A affects not only iron transport but also red blood cell synthesis at the level of gene transcription. However, it was not possible to confirm a role for vitamin A in transferrin gene transcription by using cultured liver cells incubated with all-*trans*, and 9-*cis* retinoic acid. The next step would be to repeat the experiments described in **chapter 9** after optimalization of the *in vitro* model measuring transferrin synthesis. In addition the sensitivity for induction by retinoic acid of the located candidate retinoic acid response elements should be confirmed experimentally for example in *in vitro* reporter transfection assays.

Immune system and infection

Vitamin A deficiency is known to influence the immune system [46-49], to increase severity of infection [49-51] and to increase mortality and morbidity [51]. Infection affects iron homeostasis [52,53]. Especially in studies in women and children in third world countries, infectious diseases might have confounded the relationship observed between vitamin A and iron metabolism. In the experiments with rats [**chapters 2,7**] any infection was checked by differential counts of white blood cells. It was assumed that infection did not interfere in the studies.

Changes in iron metabolism under conditions of "anaemia of chronic disease and infection" are manifested by low haemoglobin levels, low plasma iron, low total iron-binding capacity and increased iron storage [52,53]. These are features similar to those of vitamin A deficiency. An exception is iron absorption, which is reduced in anaemia of chronic disease, but increased [33,36, **chapter 2**] or unaffected [54, **chapter 7**] in vitamin A deficiency. Thurnham [55,56] pointed to the acute phase response with infection, which also links vitamin A and iron metabolism. Both retinol binding protein and transferrin are negative acute phase proteins, and they are also reduced in vitamin A deficiency. This could possibly explain the interrelationship between vitamin A and iron metabolism. However, when vitamin A deficiency would cause an acute phase reaction, positive acute

phase proteins, such as α_2-macroglobulin would also be affected 10-100 fold [57]. In rat experiments, levels of α_2-macroglobulin remained unchanged with vitamin A deficiency [58]. This supports the concept that underlying mechanisms differ between the effects of chronic disease or infection on iron metabolism and those of vitamin A deficiency.

Vitamin A deficiency is characterized by various alterations in immunity [46]. Macrophage metabolism might also be altered [59] leading to impaired iron release and thus to the observed accumulation of iron in spleen and bone [**chapters 2-7**]. Thus another lead for further research would be to study the influence of vitamin A on iron release from macrophages [60].

Growth and liver weight

Vitamin A status affects growth both in humans [61,62] and rats [**chapters 3-6**]. Pair feeding was not applied and thus correction for the effects on body weight was done afterwards by covariate analysis, after which an effect of vitamin A status presisted. Liver weight appeared sensitive to vitamin A deficiency and fell by 20-50% [**chapters 2-7**]. Increased plasma levels of aspartate aminotransferase and alanine aminotransferase in vitamin A deficient rats were quickly reduced by vitamin A supplementation [**chapter 8**]. Thus vitamin A deficiency might lead to liver damage and impaired liver function, leading to a reduced synthesis of proteins including transferrin.

CONCLUSIONS AND DIRECTIONS FOR FUTURE RESEARCH

It is concluded that

- the effects of vitamin A deficiency on iron status differ from those of iron deficiency [**chapters 2,7**],
- young growing rats differ from humans in that rats have a higher iron turnover, and absorb 5-30 times more iron, relative to their red blood cell iron content (calculated from data in **chapter 2**),

- for optimal standardization of experiments with young growing rats involving nutrients such as vitamin A and iron, the diets fed to the dams should be defined and controlled [**chapters 2-7**],

- the inability to demonstrate changed red blood cell metabolism in vitamin A deficiency, by using indicators of red blood cell synthesis and breakdown does not exclude such changes, but should be followed up by ferrokinetic studies [**chapter 7**],

- macrophage metabolism might be affected, leading to impaired iron release and thus to the observed accumulation of iron in spleen and bone [**chapters 2-7**] so that future studies should focus on release of iron from macrophages derived from vitamin A deficient rats,

- both the location of a candidate retinoic acid response element in the first intron of the transferrin gene, and our inability to confirm a role for vitamin A in transferrin gene transcription by using cultured liver cells, might imply that vitamin A excerts its influence on transferrin synthesis through a signalling protein or an intermediate transcription factor [**chapter 9**],

- the experiments described in **chapter 9** should be repeated after optimalization of the *in vitro* model of cultured liver cells for measuring transferrin synthesis; and the sensitivity to induction by retinoic acid of the located candidate retinoic acid response elements [**chapter 9**] for transferrin and erythropoietin should be confirmed.

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Summary

The experiments presented in this thesis were carried out to simulate the interrelationship between vitamin A and iron metabolism, which has been illustrated in human studies in third world countries and to elucidate underlying mechanisms.

Effects of manipulation with dietary vitamin A on vitamin A and iron status were studied in rats. Recurrent effects on iron metabolism with moderate vitamin A deficiency were: iron accumulation in spleen and bone; reduction in total iron-binding capacity, a measure of transferrin and reduced total liver iron, caused by a reduction in liver weight. These effects were reversed by vitamin A supplementation for 10-12 days. Mild vitamin A deficiency produced anaemia followed by increased iron absorption. When vitamin A status further deteriorated, haemoglobin concentrations rose. Simultaneously growth was reduced, implicating that the increased concentration of blood components, could be ascribed to haemoconcentration in vitamin A deficient rats, which is a result of disrupted water balance and growth reduction. Rats with chronic vitamin A deficiency may be less affected by haemoconcentration. But there was no anaemia in mature female rats with stable low plasma retinol levels.

As to the mechanism by which vitamin A might influence iron metabolism, we have speculated that with vitamin A deficiency, blood cell synthesis is impaired, leading to increased destruction of malformed red blood cells and increased iron stores in the macrophages of bone marrow and spleen. The iron accumulation might be enhanced by reduced iron mobilization, arising from impaired synthesis of transferrin, possibly at the level of transferrin glycosylation. These hypotheses could not be confirmed by measuring transferrin glycosylation and indicators of red blood cell synthesis and breakdown in rats fed diets deficient in vitamin A.

Alternatively, transferrin synthesis might be regulated by vitamin A at the level of gene transcription. With the use of a gene bank, candidate retinoic acid response elements for transferrin and erythropoietin were located. However, it was not possible to confirm a role for vitamin A in transferrin gene transcription by using cultured liver cells incubated with all-*trans* and 9-*cis* retinoic acid.

Finally, evaluating the rat as animal model it can be deducted from these studies that for optimal standardization of experiments with young growing rats involving nutrients such as vitamin A and iron, diets fed to their dams should also be defined and controlled.

Samenvatting

De experimenten in dit proefschrift hadden als doel de relatie tussen het vitamine A- en ijzermetabolisme zoals die is aangetoond in studies met mensen in ontwikkelingslanden te simuleren en de mechanimsen op te helderen die hieraan ten grondslag liggen.

Het effect van het voeren van verschillende nivo's vitamine A op de vitamine A- en ijzerstatus van de rat werd bestudeerd. In ratten met een matige vitamine A-deficiëntie werden de volgende veranderingen in ijzerstatus gemeten: ijzerstapeling in milt en bot; verlaging van totale ijzerbindingscapaciteit, een maat voor de hoeveelheid circulerend transferrine en een verlaging van het leverijzer, veroorzaakt door een daling in levergewicht. Deze veranderingen in ijzermetabolisme werden genormaliseerd na vitamine A-suppletie voor een periode van 10-12 dagen. Milde vitamine A-deficiëntie veroorzaakte anemie en een verhoogde ijzerabsorptie. Wanneer vitamine A-status verder verslechterde, stegen de hemoglobine-concentraties. Gelijktijdig was de groei verminderd, hetgeen wees op hemoconcentratie, resultaat van een verstoorde waterbalans en verminderde groei in vitamine A-deficiënte ratten. Hemoconcentratie verhoogt de nivo's van de bloedcomponenten en bemoeilijkt het aantonen van eventuele anemie bij vitamine A-deficiënte ratten. Bij chronische vitamine A-deficiëntie zou hemoconcentratie van minder belang zijn, desondanks kon in volwassen vrouwelijke ratten met lage, stabiele plasma-vitamine A nivo's, geen anemie worden aangetoond.

Wat betreft de mechanismen via welke vitamine A het ijzermetabolisme beïnvloedt, werd gespeculeerd dat in vitamine A-deficiëntie, de rode-bloedcel-synthese is verslechterd. Dit zou leiden tot een verhoogde afbraak van misvormde rode bloedcellen en verhoogde ijzervoorraden in de macrofagen van beenmerg en milt. Daarnaast zou ijzerstapeling veroorzaakt kunnen worden door een verminderde ijzermobilisering, als gevolg van verlaagde transferrineproductie. Transferrine-synthese kan beïnvloed zijn op het nivo van glycosylering. Deze hypothesen konden echter niet worden bevestigd door het bepalen van transferrineglycosylering en indirecte parameters van rode-bloedcel-synthese en -afbraak in vitamine A-deficiënte ratten.

Een alternatieve verklaring is dat transferrinesynthese wordt gereguleerd op het nivo van DNA-transcriptie. Met behulp van een genenbank zijn kandidaat-"retinoic acid response elements" voor transferrine en erythropoietine gevonden. Het bleek echter niet mogelijk deze mogelijke rol voor vitamine A in transferrine-DNA-transcriptie experimenteel te bevestigen door levercellen te incuberen met all-*trans* en 9-*cis* retinolzuur.

Tenslotte, bij de evaluatie van de rat als diermodel kan worden afgeleid uit deze studies dat voor optimale standardisatie van experimenten met jonge groeiende ratten de voeders voor de moeders van deze ratten ook gestandardiseerd moeten worden wanneer het gaat om nutriënten zoals vitamine A en ijzer.

Nawoord

Eindelijk dan toch een hoofdstuk in de ik-vorm. Veel personen zijn betrokken geweest bij het tot stand komen van dit proefschrift. Logisch dat ik nog niet eerder "ik" geschreven heb. Ruim 5 jaar ben ik aangesteld geweest bij de vakgroep Proefdierkunde in Utrecht en gedetacheerd bij de vakgroep Humane Voeding in Wageningen. Een luxe! Twee werkplekken!! Het was iets langer dan dat de gemiddelde AIO erover doet, door twee zwangerschappen en een 80% aanstelling. Ik zal niet stoer beweren dat het niet zwaar was, die combinatie van moeder en AIO zijn. Maar het was goed te doen en dat heb ik te danken aan velen.

De eerste personen die ik wil bedanken in dit nawoord zijn mijn beide promotoren Anton Beynen en Clive West. Jullie hebben me van het begin af aan het vertrouwen gegeven dat dit alles moest kunnen. Ik kan me een van de eerste bijeenkomsten goed herinneren waarin jullie als ervaren vaders mij, als aankomende moeder, van de nodige adviezen voorzagen. Maar vooral op het vlak van de wetenschap heb ik veel van jullie geleerd. Anton, ik heb onze gesprekken in rustiger tijden zeer gewaardeerd. Hoewel je me altijd het idee hebt gegeven dat ik het schrijven nooit leer, ga ik er stilletjes toch vanuit dat ik er in deze jaren wat bijgeleerd heb. Ik heb met bewondering toegekeken hoe mijn schrijfsels zich met jouw en Clive's hulp vormden tot bondige, mooi samenhangende verhalen die ieder internationaal tijdschrift wel moet plaatsen. Clive, jij was een aanspreekpunt wanneer ik weer iets had. Jouw adviezen en stimulerende aanwezigheid waren erg belangrijk voor mij. Ik heb er bewondering voor hoe goed je stress en humor kunt combineren.

De beide vakgroepen hebben een voorzitter: Professor Josef Hautvast en Professor Bert van Zutphen. Professor Hautvast, bedankt voor de gastvrijheid op de vakgroep Humane Voeding. En Bert, bedankt voor de prettige gesprekken en natuurlijk voor jouw aandeel in dit onderzoeksproject.

Verschillende experimenten met ratten vormen de basis van dit proefschrift. De meeste dierproeven zijn uitgevoerd in het Centrum voor Kleine Proefdieren (CKP) in Wageningen: Gerrit van Tintelen, Frank van den Broek, Jo Haas en Maria Peters bedankt voor jullie hulp hierbij. Maar ook de medewerkers van het GDL in Utrecht bedankt voor de hulp. Shiguang Yu, you started at the same time as I did, but finished with great speed two years earlier. Despite your own work, you were always prepared to help with many things. Thank you! I wish you and your family all the best in the future. Ans van Pelt, het was een korte maar zeer prettige samenwerking!

Zonder vitamine A-deficiënt voer, geen vitamine A-deficiënte ratten: Piet Roelevelt en Karel Siebers van het ILOB/TNO in Wageningen, onmisbaar waren jullie nauwkeurig samengestelde rattevoeders.

De bijna zevenhonderd ratten die bijgedragen hebben aan dit proefschrift leverden een veelvoud van zevenhonderd te analyseren monsters. We analyseerden ijzer in Utrecht, en vitamine A in Wageningen. Veel ijzer-werk is uitgevoerd door Robert Hovenier. Robert, je bent een toffe collega, bedankt voor alle hulp. Inez Lemmens, je bent een drijvende kracht in het lab in Utrecht en zeker zo belangrijk, was je een vast aanspreekpunt voor mij. We hebben heel wat besproken en afgekletst. Wat betreft het analyseren van vitamine A wil ik Peter van de Bovenkamp en Jan Harryvan bedanken voor de vele hulp bij de vele (kreun) HPLC-perikelen en Jannie Bos voor het assisteren bij de vitamine A-analyses. Een deel van de analyses moest direct op de dag van de beëindiging van de proef in Utrecht bij de vakgroep Inwendige Ziekten en Voeding der Grote Huisdieren, (Faculteit Diergeneeskunde) gedaan worden. Dr. Theo Wensing, Marjory Polak en Ramon Cabrera, bedankt voor jullie hulp hierbij.

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Terugkijkend op de afgelopen 5 jaren, wil ik enkele gedenkwaardige gebeurtenissen in herinnering brengen, zoals het congres in Brighton waar we als vakgroep Proefdierkunde geïntimideerd werden door de plaatselijke actievoerders/dierenbeschermers. De ervaring was schokkend, maar een ervaring als deze schept wel een band. Door de sportieve teamgeest steeg het volleybalnivo van de vakgroep Proefdierkunde duidelijk in de loop der jaren. De epidemiologiecursus in Boston en de excursies met de Wageningse AIO's naar het buitenland waren van bijzonder aangename en nuttige aard. Het cabaret ter ere van het 25-jarige bestaan van de vakgroep in Wageningen heeft mij evenveel voldoening gegeven als het publiceren van 2 artikelen in een internationaal tijdschrift (impact 1.5).

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Annet

About the author

Annette Johanna Christina Roodenburg was born on the 13th December 1964, in Scharendijke, the Netherlands. In 1983 she finished secondary school at the 'Christelijk Lyceum' in Apeldoorn. In the same year she started the study 'Human Nutrition' at the Wageningen Agricultural University. During this study she worked for six months at the Laboratory of Human Nutrition of Dr. Vernon R. Young at the Massachusetts Institute of Technology in Cambridge, USA. She obtained her MSc degree in Human Nutrition in 1989 with main topics clinical nutrition, physiology and biochemistry. From January until August 1990 she worked on the development and performance of vitamin A determination in plasma with HPLC at the Department of Human Nutrition in Wageningen. In September 1990, she started as a PhD fellow (part time) at the Department of Laboratory Animal Science of the State University Utrecht, the Netherlands. This research which has been carried out in collaboration with the Department of Human Nutrition in Wageningen has been focused on the influence of vitamin A on iron metabolism in the rat, as an animal model. In February 1991 she obtained her official qualification as laboratory animal researcher. In July 1993 she attended the Annual New England Epidemiology Summer Program at Tufts University, Boston, USA. In January 1995 she attended the course in radiation hygiene at the Interfaculty Radiation Institute, Delft, the Netherlands and in March 1996 she will join the Third European Nutrition Leadership Programme in Luxembourg.

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