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

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To compare the changes in Fe metabolism during the development of vitamin A and Fe deficiencies, rats were given either a control diet with sufficient Fe (35 mg added Fe/kg feed) and retinol (1200 retinol equivalents/kg feed), a diet without added vitamin A or a diet with sufficient vitamin A but only 3·5 mg added Fe/kg feed. During a period of 10 weeks, indicators of vitamin A and Fe status were monitored. Neither vitamin A nor Fe deficiency produced clinical signs. Fe deficiency induced an immediate fall in blood haemoglobin concentration. Vitamin A deficiency produced a mild anaemia as the first change in Fe metabolism, pointing to impaired erythropoiesis. This effect was followed by a rise in Fe absorption and an increased amount of Fe in the spleen. By the end of the study, blood haemoglobin, packed cell volume, plasma Fe and Fe content in kidney and femur had increased above control levels, while total Fe-binding capacity had decreased. We speculate that the initial anaemia was masked later by haemoconcentration. The decrease in Fe mobilization, shown by lower total Fe-binding capacity, and the increase in Fe absorption may have caused the observed continuous rise in tissue Fe concentration in rats with vitamin A deficiency. In the rats with Fe deficiency, low tissue Fe levels coincided with high Fe absorption and high total Fe-binding capacity. Thus, changes in Fe metabolism with vitamin A deficiency differed from those with Fe deficiency.

Vitamin A: Iron: Anaemia: Rat

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. In later studies vitamin A deficiency led to moderate anaemia which was refractory to Fe but responsive to vitamin A (Sauberlich *et al.* 1974; Hodges *et al.* 1978). 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 (Hodges *et al.* 1978), pregnant women (Suharno *et al.* 1992) and children (Mejía & Arroyave, 1982; Bloem *et al.* 1989; Wolde-Gebriel *et al.* 1993 *a*, *b*). In addition, intervention studies in children (Mejía & Arroyave, 1982; Mejía & Chew, 1988; Muhilal *et al.* 1988; Bloem *et al.* 1990) and in adults (Panth *et al.* 1990) have shown that supplementation with vitamin A results in increased haemoglobin levels.

* For reprints.

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 (West & Roodenburg, 1992). 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 develops. This can be explained by water loss leading to haemoconcentration as the vitamin A deficiency proceeds (Koessler *et al.* 1926; McLaren *et al.* 1965; Mejía *et al.* 1979 *b*). At the present time little is known about the effects of vitamin A deficiency on Fe metabolism in rats, although it would appear that in vitamin A deficiency Fe absorption is increased (Amine *et al.* 1970; Beynen *et al.* 1991), liver Fe is raised (Mejía *et al.* 1979 *a, b*; Staab *et al.* 1984; Beynen *et al.* 1991), and the amount of Fe in bone is lowered (Beynen *et al.* 1991). It is not clear how these changes in Fe metabolism are interrelated.

A time-course study on the changes in various aspects of Fe metabolism which take place during the progression of vitamin A deficiency may allow us to distinguish between primary and secondary features of the altered Fe metabolism. Further clues to the mechanisms underlying the development of altered Fe metabolism as produced by vitamin A deficiency may be obtained by a comparison with the development of the anaemia as induced by Fe deficiency. Thus, we have compared the time-dependent changes in Fe metabolism of weanling rats as induced by deficiency of either vitamin A or Fe. We chose to produce marginal deficiencies of vitamin A and Fe because this would limit interference by non-specific influences on Fe metabolism such as those caused by reduced feed intake and retarded growth. In addition, marginal deficiencies of vitamin A and Fe 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 Fe metabolism (Scrimshaw *et al.* 1968), while animals deficient in vitamin A or Fe have increased sensitivity to infectious agents (Nauss *et al.* 1985; Keusch, 1990). In the above-mentioned rat studies on vitamin A deficiency and Fe metabolism, no information about the infectious status was provided. Thus, during the course of the present study we regularly checked the differential leucocyte 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 the 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 ($345 \times 225 \times 160$ mm) with a layer of sawdust as bedding. During the pre-experimental period of 2 weeks all rats were fed on the control diet containing adequate amounts of vitamin A (1200 retinol equivalents/kg feed) and Fe (35 mg added Fe/kg feed). The diet (Table 1) was formulated according to the nutrient requirements of rats (National Research Council, 1978). After the pre-experimental period (day 0) the rats were divided into three groups of ninety-six rats each. The rats were housed in groups of four animals in stainless steel cages with wire mesh bases ($300 \times 420 \times 190$ mm). 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 Fe-deficient diet containing 3·5 mg added Fe/kg feed (Table 1). Analysed Fe concentrations of the diets were as follows (mg/kg feed): control diet 38, vitamin A-deficient diet 38, Fe-deficient diet 10. The

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

Diet... Ingredients	Control	Vitamin A-deficient	Fe-deficient
Casein	151	151	151
Maize oil	25	25	25
Coconut fat	25	25	25
Glucose	709·2	709·2	709·2
Cellulose	30	30	30
$CaCO_3$	12·4	12·4	12·4
$NaH_2PO_4.2H_2O$	15·1	15·1	15·1
$MgCO_3$	1·4	1·4	1·4
KCl	1·0	1·0	1·0
$KHCO_3$	7·7	7·7	7·7
$FeSO_4.7H_2O$ (mg)	174	174	17·4
Vitamin A preparation* (mg)	8	—	8
Mineral premix†	10	10	10
Vitamin premix‡	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, pteroylmonoglutamic acid 1, biotin 2, menadione 0·05, D,L-α tocopheryl acetate 60, cholecalciferol 0·025, maize meal 9836·125.

purified diets were in powdered form and stored at 4° until fed. The animals had free access to feed and demineralized 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 06.00–18.00 hours), temperature (20–22°) and relative humidity (50–60%) was maintained in the animal room.

Collection of samples

On day 0, twelve animals were killed. After 1, 2, 4, 6, 8 and 10 weeks, twelve animals from three cages in each dietary group were killed. For a period of 7 d before being killed the animals were housed individually in metabolism cages (3140 mm² × 120 mm). Over the last 4 d of this period, feed and water intakes were measured. Faeces and urine of each rat were collected and stored at −20° until analysis. Between 09.00 and 11.00 hours, 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° for haematological examination and differential leucocyte counting on the same day. The plasma collected was stored at −20° until analysis, except for 250 μl which was stored at −80° before 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° until analysis. Organs were weighed before storage. After thawing and before ashing, spleen and liver were washed with saline (9 g NaCl/l) and the femur was cleaned of adhering tissue.

Blood volume determination

At 4 and 8 weeks of the experimental period, blood volume was determined in nine to twelve animals of each dietary group using a modified Evans Blue dye dilution method (Belcher & Harriss, 1957; van Waversveld & Van Bruchem, 1985). After the induction of

anaesthesia with an intramuscular injection of 0·3 ml Hypnorm (10 mg fluanison and 0·315 mg fentanylcitrate/ml; Janssen Pharmaceutics, Tilburg, The Netherlands), about 0·6 ml Evans Blue solution (3 mg/ml 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 (Belcher & Harriss, 1957), 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 packed cell volume. Animals were killed by asphyxiation with CO_2 after blood samples had been taken. After being allowed to stand for at least 10 min, the blood samples were centrifuged (3000 g, 10 min), the serum obtained was diluted 50-fold, and absorbance 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 absorbance of serum, a similar experiment was carried out with two 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 packed cell volume. The method was checked by measuring blood volume in seven animals after an average blood sample of 15·5 ml/kg body weight had been taken. In these animals blood volume was found to be 51·6 (SE 1·8) ml/kg body weight, whereas in eight non-bled animals blood volume was 63·7 (SE 1·5) ml/kg body weight. 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, packed cell volume, erythrocyte 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 leucocytes was done in blood smears.

Plasma Fe concentration and total Fe-binding capacity were determined spectrophotometrically using a test kit (Roche Nederland, Mijdrecht, The Netherlands). Fe was measured by flame atomic absorption spectrometry following wet ashing with HNO_3 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 Fe determination. All analyses for each rat were carried out singly, except for liver Fe which was done in duplicate. Fe in the femur was calculated as the mean of values for the left and right femurs. Absorption of Fe was calculated as Fe ingested minus that excreted in faeces and expressed as percentage of intake. Fe intake and excretion were measured over 4-d periods. Negligible amounts of Fe were found in urine, and thus the data are not given.

Plasma and liver retinol levels were measured by reversed-phase HPLC. Plasma (100 μl) was mixed vigorously with 400 μl ethanol (900 ml/l) and, after centrifuging (3000 g, 10 min), retinol was determined directly in the supernatant and calibrated against solutions of retinol in ethanol (720 ml/l). 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, 10 × 3 mm stainless steel packed with Chromguard reversed phase (Chrompack, Middelburg, The Netherlands); column, 100 × 3 mm stainless steel packed with Spherisorb-ODS (5 μm) cartridge (Chrompack);

isocratic pump (Spectra Physics, San Jose, CA, USA); u.v.–visible range 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. Liver homogenate (200 μl; liver:demineralized water, 1:5, w/v) was digested by heating at 100° for 15 min in 1500 μl 0·7 M-KOH in ethanol (500 ml/l, containing 6 g/l pyrogallol) and, after cooling, the mixture was extracted twice with 4 ml hexane. Standards with retinol acetate in absolute ethanol were processed identically and used for calibration. The upper layer of hexane extract was removed after centrifugation (3000 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 before 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 on the vitamin A-deficient or Fe-deficient diets 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 Fe 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 compared with control rats, feed intake (Fig. 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 kg 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 ($P < 0·001$; Fig. 1d). Body weight (Fig. 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, $P < 0·001$; weeks 8 and 10, $P < 0·001$; Fig. 1c). The Fe-deficient diet had no effect on body weight and feed intake (Fig. 1a, b), but reduced liver weight from week 2 ($P = 0·013$) until the end of the study ($P < 0·001$; Fig. 1c).

Blood volume

Blood volume, expressed as ml/kg body weight, measured at week 4 and 8 was affected neither by the vitamin A-deficient diet nor by the Fe-deficient diet (Table 2). There was a decrease in blood volume with age, which has been shown earlier (Belcher & Harriss, 1957).

Leucocyte counts

In the course of the experiment the relative numbers of leucocytes were as follows: granulocytes 1–48% (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 countings of all animals were within the normal range (Wirth, 1950). Thus, no signs of infection were detected.
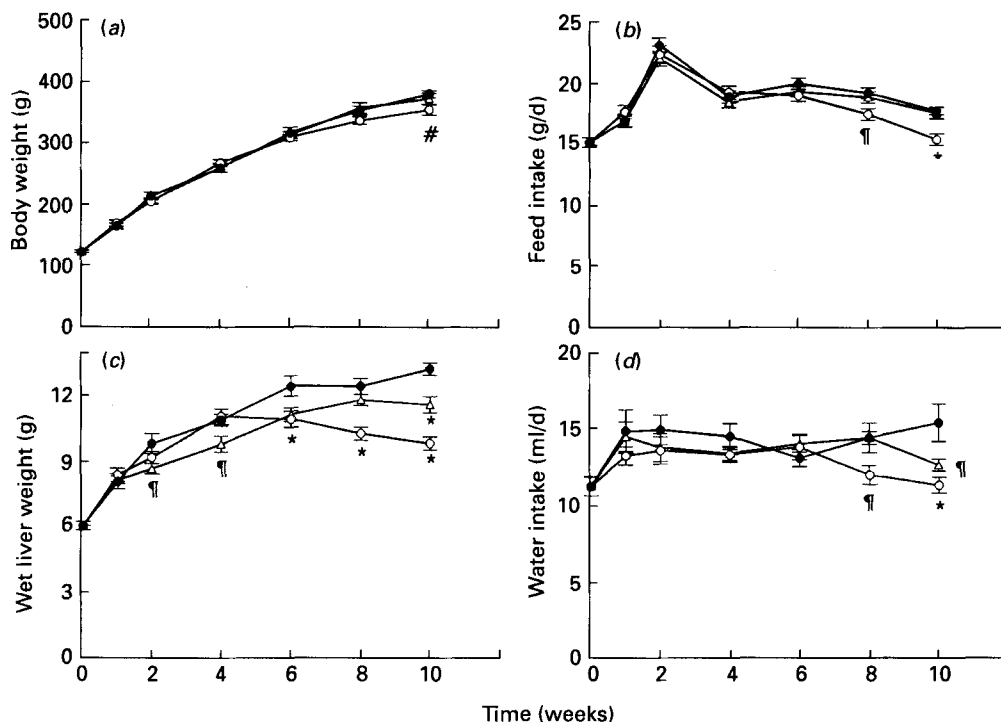
Fig. 1. Time course of body weight (a), feed intake (b), wet liver weight (c) and water intake (d) in rats given either the control diet (●), vitamin A-deficient diet (○) or Fe-deficient diet (△). Values represent means for twelve rats, with standard errors indicated as error bars. Differences between vitamin A-deficient or Fe-deficient and control groups: ¶ $P < 0.05$, # $P < 0.01$, * $P < 0.001$.

Table 2. *Blood volume (ml/kg body weight) in rats fed on the experimental diets**

(Mean values with their standard errors for nine to twelve rats per group)

Diet... Period of experiment	Control		Vitamin A-deficient		Fe-deficient	
	Mean	SE	Mean	SE	Mean	SE
Week 4	88·4	2·7	86·0	1·8	82·4	2·4
Week 8	70·4	2·1	70·6	1·6	65·5	1·5

* For details of diets and procedures, see Table 1 and pp. 688–690.

Vitamin A status

Total retinol in liver was decreased after 1 week of giving the vitamin A-deficient diet ($P < 0.001$; Fig. 2b). After 8 weeks, retinol in liver was not detectable. Plasma retinol concentration in vitamin A-deficient rats declined steadily from week 2 ($P < 0.001$; Fig. 2a). In the Fe-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 Fe deficiency affected vitamin A metabolism because it lowered plasma retinol concentrations, as was also found by Amine *et al.* (1970). 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).
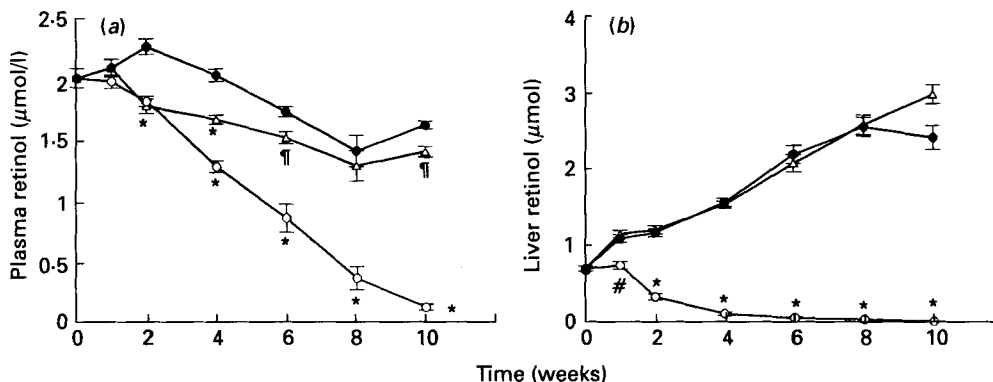
Fig. 2. Time course of plasma retinol concentrations (a) and liver retinol (b) in rats given either the control diet (●), vitamin A-deficient diet (○) or Fe-deficient diet (△). Values represent means for twelve rats, with standard errors indicated as error bars. Differences between vitamin A-deficient or Fe-deficient and control groups: ¶ $P < 0.05$, # $P < 0.01$, * $P < 0.001$.

Haematology

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

Anaemia was induced by Fe deficiency. After 1 week, haemoglobin and packed cell volume were significantly reduced (Fig. 3a, b). Haemoglobin concentrations and packed cell volume were lowest at week 2 and thereafter levels gradually increased, but remained lower than those of the control group ($P < 0.001$). Erythrocyte count in Fe-deficient rats was reduced during the first 2 weeks ($P = 0.032$ week 1 and $P = 0.003$ week 2), but was increased by the end of the study ($P < 0.001$; Fig. 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 Fe levels were not affected until week 10 when there was a rise ($P = 0.006$). Likewise, transferrin saturation suddenly rose at week 10 ($P < 0.001$; Fig. 3d, f). Total Fe-binding capacity remained unchanged in vitamin A-deficient rats until 10 weeks, when it dropped ($P < 0.001$; Fig. 3e).

The Fe-deficient diet produced an immediate decrease in plasma Fe and transferrin saturation and an increase of total Fe-binding capacity within 1 week. Both plasma Fe and transferrin saturation reached their lowest value after 2 weeks and then rose to control values at the end of the experimental period. Total Fe-binding capacity in Fe-deficient rats peaked after 4 weeks and then dropped, but remained higher than control values until the end of the experiment ($P = 0.008$).
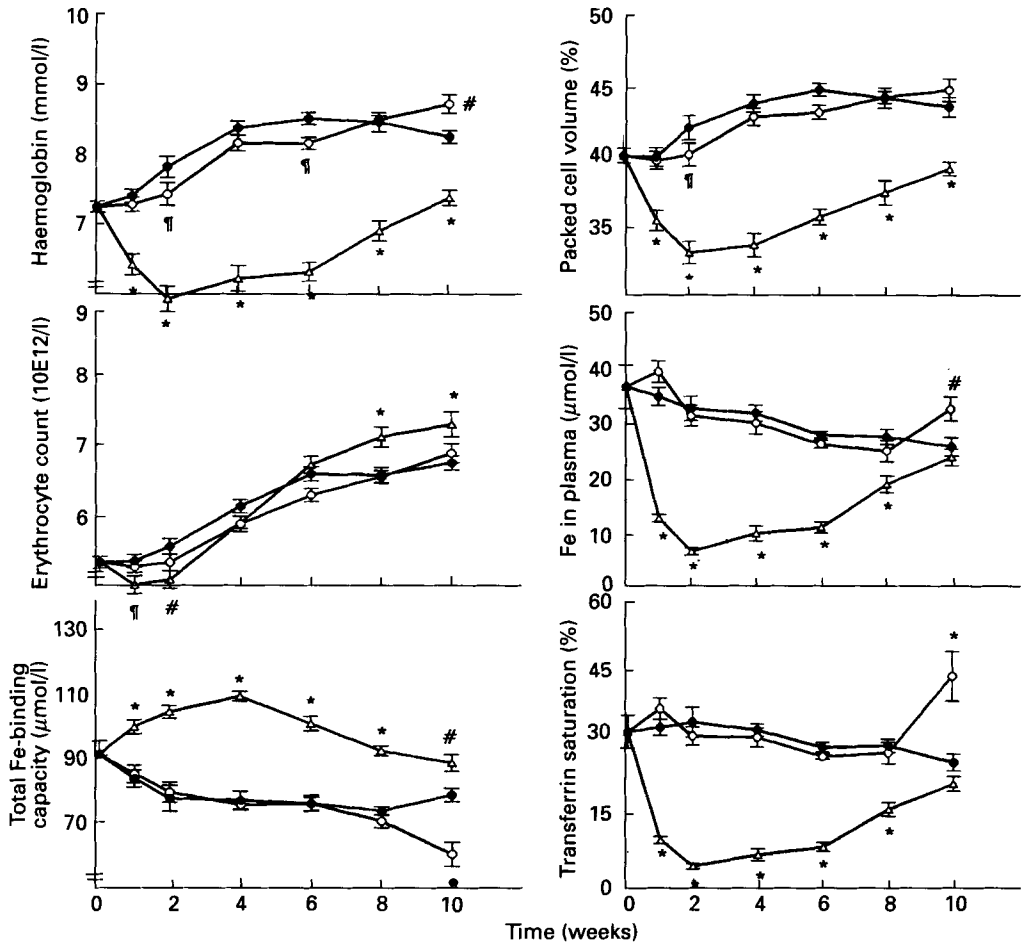
Fig. 3. Time course of haemoglobin concentrations (a), packed cell volume (b), erythrocyte count (c), Fe in plasma (d), total Fe-binding capacity (e) and percentage transferrin saturation (f) in rats given either the control diet (●), vitamin A-deficient diet (○) or Fe-deficient diet (△). Values represent means for twelve rats, with standard errors indicated as error bars. Differences between vitamin A-deficient or Fe-deficient and control groups: ¶ $P < 0.05$, # $P < 0.01$, * $P < 0.001$.

Iron in organs

After feeding the vitamin A-deficient diet, total Fe in liver decreased below control levels at week 8 ($P = 0.020$) and dropped further after week 10 ($P < 0.001$; Fig. 4a). This effect can be explained mainly by a reduction of liver weight (Fig. 1c) because hepatic Fe concentrations after 10 weeks were not significantly affected by vitamin A deficiency (4·36 (SE 0·15) μmol/g dry weight) compared with the 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 Fe 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 Fe content of spleen in vitamin A-deficient animals was already significantly raised after 8 weeks ($P = 0.011$; Fig. 4b–d). This can be explained by a rise in splenic Fe concentration in the vitamin A-deficient group (38·7 (SE 4·5) μmol/g dry weight) compared with the control group (27·4 (SE 2·3) μmol/g dry weight) at week 8 ($P = 0.001$). After 10 weeks, Fe concentration of both spleen and femur were higher in the vitamin A-deficient
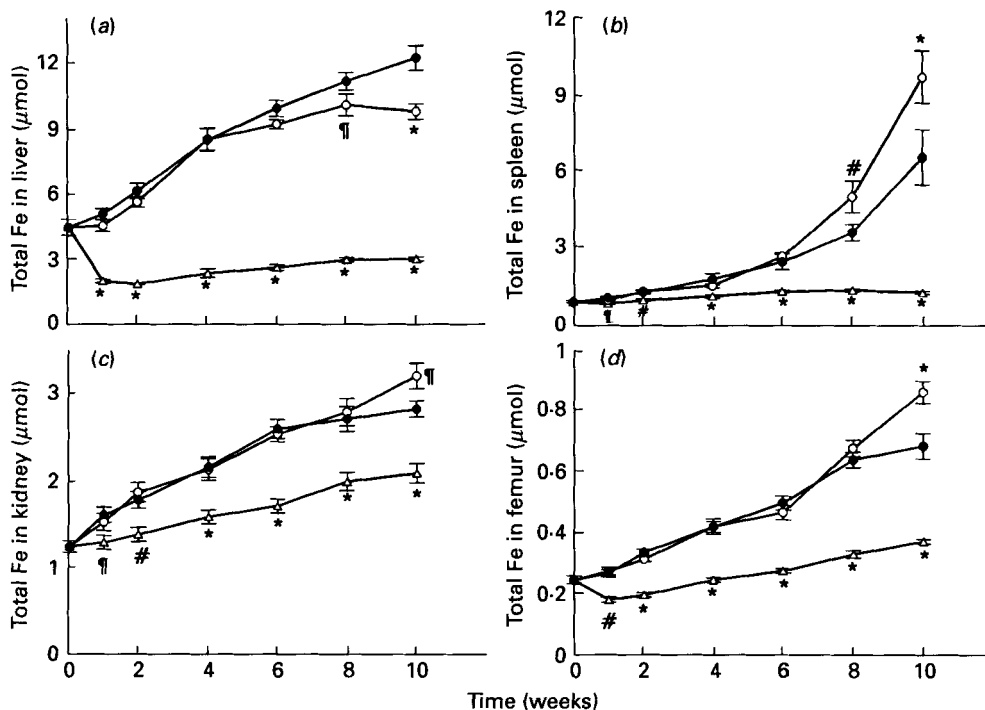
Fig. 4. Time course of total Fe in liver (*a*), spleen (*b*), kidney (*c*) and femur (*d*) in rats given either the control diet (●), vitamin A-deficient diet (○) or Fe-deficient diet (△). Values are expressed as μmol per organ and represent means for twelve rats, with standard errors indicated as error bars. Differences between vitamin A-deficient or Fe-deficient and control groups: ¶ $P < 0.05$, # $P < 0.01$, * $P < 0.001$.
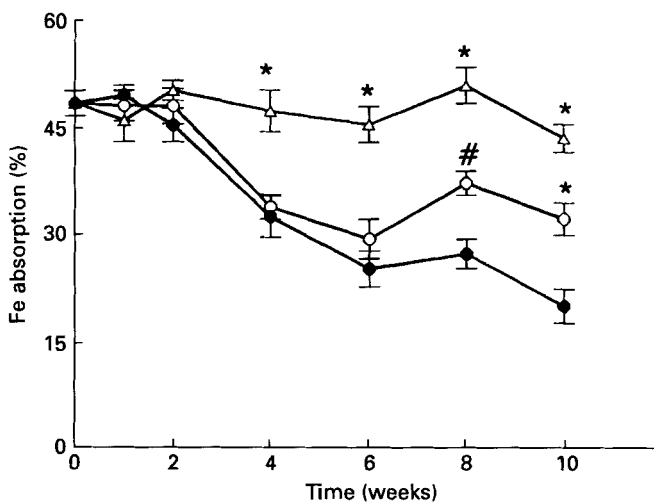

Fig. 5. Time course of Fe absorption, in rats given either the control diet (●), vitamin A-deficient diet (○) or Fe-deficient diet (△). Values represent means for twelve rats, with standard errors indicated as error bars. Differences between vitamin A-deficient or Fe-deficient and control groups: # $P < 0.01$, * $P < 0.001$.

group (72·8 (SE 8·41) and 1·78 (SE 0·09) μmol/g dry weight respectively) than in 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 Fe-deficient diet, total Fe content of liver, spleen, kidney and femur were significantly decreased below control levels after 1 week and remained low until the end of the experiment (Fig. 4 a–d). This is also demonstrated by the lower Fe concentrations in these organs after 10 weeks, when compared with 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 Fe 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 Fe 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 Fe absorption was raised in vitamin A-deficient animals (Fig. 5). In Fe-deficient rats, apparent Fe absorption was essentially constant during the entire experimental period and was significantly higher ($P < 0.001$) than in control rats from 4 weeks (Fig. 5).

DISCUSSION

The present study shows the time course of changes in Fe 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 and feed and water intakes declined. Blood volume, expressed as ml/kg 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 Fe 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 Fe metabolism. In the vitamin A-deficient rats, haemoglobin concentrations and packed cell volume 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 (Hodges et al. 1978; Mejía et al. 1979a; Sklan et al. 1986; Beynen et al. 1991). After 6 weeks, apparent Fe absorption was somewhat higher in the vitamin A-deficient than in the control rats; this effect became more pronounced later. The observed increase in Fe absorption as induced by vitamin A deficiency agrees with earlier work (Amine et al. 1970; Beynen et al. 1991). After 8 weeks, vitamin A deficiency was associated with raised Fe concentrations in liver and spleen. In other studies vitamin A deficiency has been shown to produce an increase in liver Fe concentrations (Mejía et al. 1979a; Staab et al. 1984; Sklan et al. 1986), but liver weights were not reported so that the effect on liver Fe mass cannot be ascertained. As found in the present study, Sijtsma et al. (1993) also observed that vitamin A deficiency reduced the total Fe content of liver. Unlike liver weight, vitamin A deficiency did not affect spleen weight. Thus, the increased splenic Fe concentration, which was also found by Mejía et al. (1979a), resulted in raised total Fe content of spleen.

When rats had been given the vitamin A-deficient diet for 10 weeks the amounts of Fe in kidney and femur were higher than in control animals, total Fe-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 Fe concentration were all increased above control levels. These effects, which have been

reported previously (Amine *et al.* 1970; Corey & Hayes, 1972; Mejía *et al.* 1979 *a, b*), may be explained by haemoconcentration (McLaren *et al.* 1965; Corey & Hayes, 1972; Mahant & Eaton, 1976; Mejía *et al.* 1979 *b*). 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 (Hodges *et al.* 1978).

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

The primary feature of altered Fe metabolism in vitamin A deficiency is probably impaired erythropoiesis. Indeed, Mejía *et al.* (1979 *b*) found in vitamin A-deficient rats that the rate of incorporation of intravenously administered ^{59}Fe into erythrocytes was depressed. The impaired erythropoiesis may stimulate, by some unknown mechanism, the absorption of Fe. The resulting enhanced influx of dietary Fe could have produced the higher Fe concentrations in liver and spleen followed by those in kidney and femur. Since the greater efficiency of Fe absorption may not overcome the depressed erythropoiesis as induced by vitamin A deficiency, Fe absorption remains enhanced and Fe amounts in spleen, and possibly also in kidney and femur, continue to rise. The excessive Fe status thus developed may inhibit transferrin synthesis as reflected by the decrease in total Fe-binding capacity. Fe loading, which also leads to excessive Fe status, has been shown to reduce total Fe-binding capacity (Worwood & Jacobs, 1972). The observed decrease in total Fe-binding capacity in vitamin A-deficient rats agrees with some studies with humans showing a positive relationship between plasma retinol concentration and either total Fe-binding capacity or transferrin in plasma (Mejía & Arroyave, 1982; Bloem *et al.* 1990), but other studies did not find such relationships (Mejía & Chew, 1988; Suharno *et al.* 1992). The reduced plasma concentration of transferrin in vitamin A-deficient rats might contribute to Fe accumulation in organs by reducing Fe 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 Fe-deficient rats. The Fe 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 Fe absorption. This partly corrected the haemoglobin and plasma Fe concentrations, transferrin saturation and packed cell volume, during the course of the experiment, but was not sufficient to raise tissue Fe concentrations to control levels. In vitamin A deficiency, in contrast to Fe deficiency, the increase in apparent Fe absorption was associated with increased tissue Fe concentrations.

In summary, we have provided evidence that the anaemia often seen in marginal vitamin A deficiency in both man (Hodges *et al.* 1978; Mejía & Arroyave, 1982; Bloem *et al.* 1989; Suharno *et al.* 1992; Wolde-Gebriel *et al.* 1993 *a, b*) and rats (Hodges *et al.* 1978; Mejía *et al.* 1979 *a*; Beynen *et al.* 1991) is not caused by impaired intestinal absorption of dietary Fe and thus has features different from those in Fe deficiency. In fact, vitamin A deficiency is associated with adequate Fe status as based on tissue Fe concentrations, which is caused by enhanced Fe absorption. This would suggest that the primary derailment of Fe metabolism in marginal vitamin A deficiency is probably impaired erythropoiesis.

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