Excessive vitamin D content of a standard iron-deficient diet for rats

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1. The observation that thyroid C cell hyperplasia occurred in rats given the iron-deficient diet described by McCall, Newman, O'Brien, Valberg & Witts (1962) prompted a closer study of the preparation and constituents of this diet.

2. It became apparent that there was a discrepancy between the amounts of fat-soluble vitamins in the dietary formulation reported and the supposed final content of the diet. A diet prepared as described by McCall *et al.* (1962) contains 1000 μg (40000 i.u.) ergocalciferol and 10 μg (14500 i.u.) retinyl palmitate/kg.

3. An experiment was designed to study the effect of Fe-deficient and Fe-supplemented, high-vitamin-D diets, and an Fe-supplemented, normal-vitamin-D diet, on thyroid C cell volume and serum calcium concentration.

4. Thyroid C cell volumes and serum Ca concentrations were significantly higher in both groups given excess vitamin D than in the group given the Fe-supplemented, normal-vitamin-D diet. It is evident therefore, that hypervitaminosis D was the cause of the morphological and biochemical changes found in rats given the McCall *et al.* (1962) diet.

The iron-deficient diet developed by McCall, Newman, O'Brien, Valberg & Witts (1962) (McCall diet) has found widespread application in studies of Fe deficiency and Fe metabolism (Bannerman, 1965; Sørensen, 1965; Burns & Spray, 1969; Dallman, 1969; Spry & Piper, 1969; Thomson, Shaver, Lee, Jones & Valdberg, 1971; Richmond, Worwood & Jacobs, 1972; Bedard, Pinkerton & Simon, 1973; Bailey-Wood, Blayney, Jacobs & Muir, 1975). The diet contains 650 g freeze-dried skim-milk powder and 205 g sucrose/kg, and the remaining essential dietary constituents (except Fe) are added in the pure forms. The Fe content of this diet is 1·2 mg/kg.

During experiments using this diet, both with and without added Fe, thyroid C cell hyperplasia was observed. These findings prompted a closer study of the constituents and preparation of the diet as originally described by McCall *et al.* (1962).

McCall *et al.* (1962) state, in the details of dietary supplements, that 1000 i.u. (25 μg) ergocalciferol and 8000 i.u. (4·403 mg) retinyl palmitate/kg diet are added. These vitamins are added (/kg diet) as a 1 ml mixture of fat-soluble vitamins taken from a stock solution of 50 ml prepared by mixing 0·4 g retinyl palmitate and 0·05 g ergocalciferol with 50 ml of a solution of α-tocopherol in refined soya-bean oil (350 g/l). Consequently 8000 μg vitamin A and 1000 μg vitamin D are added. It appears, therefore, that the values given for the dietary constitutents are μg amounts, which have been described mistakenly as i.u. Therefore, as 1 μg cholecalciferol is equivalent to 40 i.u., the McCall diet contains forty times as much vitamin D as intended. The excess of vitamin A that it contains is less than twofold (1 μg retinyl palmitate is

equivalent to 1·82 i.u. vitamin A). The calcium content of this diet (8·4 g/kg) is within normal limits for rat diets.

It was possible, therefore, that the initial observation of hyperplasia of the thyroid C cells resulted from an excess of vitamin D in the diet.

To study this, an experiment was designed to measure the effect of the McCall diet, with and without added Fe, on thyroid C cell volume and serum Ca concentration, using the Fe-supplemented diet with a normal concentration of vitamin D as control.

EXPERIMENTAL

Animals. Wistar rats (Turks Laboratories, Rayleigh, Essex) of both sexes were placed on their respective diets immediately on weaning at 21 d of age. They were kept in plastic cages (Associated Crates, Stockport, Cheshire) and provided with de-ionized drinking-water. Three groups of animals were used. One group of twelve rats was given the McCall (high-vitamin-D, Fe-deficient) diet; a second group of ten rats received the same diet supplemented with 180 mg Fe (added as $FeSO_4.7H_2O$)/kg (Fe-supplemented McCall diet), and a further group of twelve rats was given an Fe-supplemented McCall diet, containing 25 μg ergocalciferol/kg. The animals were given these diets *ad lib.* for 14 weeks.

Estimation of serum Ca. Serum Ca concentration was determined by an automated procedure using the method of Gindler & King (1972).

Microscopic examination of thyroid glands and estimation of cell volumes. Rats were anaesthetized with diethyl ether and exsanguinated by bleeding from the heart. The thyroid gland, together with the trachea, was then removed immediately and fixed for 24 h in 0·1 M-buffered phosphate–formol-saline (0·1 M-phosphate buffer–formaldehyde (400 ml/l) (9:1, v/v) containing 3·5 g magnesium chloride), pH 7·4. The lobes were then dissected from the trachea and processed in graded concentrations of ethanol and xylene using a Histokinette tissue processing machine (British American Optical Co. Ltd, Slough, Bucks.) before being embedded in Paramat wax (Gurr Products, High Wycombe, Bucks.). Sections were cut for examination 5–6 μm thick and stained in Mayer's haematoxylin and eosin.

The relative volume of C cells in the thyroid was estimated using a point-counting method which was essentially that of Haug (1955). C cells are not evenly distributed throughout the rat thyroid gland (Stux, Thomson, Isler & Leblond, 1961) and therefore a random counting method could not be used, and it was necessary to select a standard area for examination in each section. The proportion of the total thyroid volume occupied by C cells was quantified by systematic sampling of longitudinal sections taken through the vertical axis of each thyroid lobe. A series of fields were examined across the smallest axis of each section, midway between the superior and the inferior poles. A point-counting grid was attached to the screen of a Reichert Visopan microscope (C. Reichert Company, Slough, Bucks.) and a $\times 40$ objective was used. Volumes of follicular cells, stroma and colloid were measured, as well as that of C cells.

Statistical analysis. Results were expressed as mean values with their standard

errors. Differences between the results obtained for the three groups of animals were tested for statistical significance by the Student's unpaired t test (Croxton, 1959); values statistically different at $P > 0{\cdot}05$ were not considered significant.

RESULTS

Serum Ca levels. There was no significant difference between the values for the two groups of animals given the high-vitamin-D diets (Fe-deficient and Fe-supplemented). The mean values for the Fe-deficient and Fe-supplemented groups of animals were $2{\cdot}73 \pm 0{\cdot}046$ and $2{\cdot}70 \pm 0{\cdot}03$ mmol/l respectively. The combined mean value was significantly higher ($P < 0{\cdot}001$) than the mean for the animals given the control diet ($2{\cdot}56 \pm 0{\cdot}022$ mmol/l).

C cell volumes. Microscopic examination of thyroids from control animals (Plate 1 *a*) showed C cells, with their characteristic pale cytoplasm, lying singly or in small groups between the follicular cells and the basement membrane of the central thyroid follicles. In animals given the McCall diet (Plate 1 *b*; sections from rats given the Fe-supplemented McCall diet were similar in appearance to that shown in Plate 1 *b*) the cells more often formed small groups, and were more widely distributed through the thyroid lobe. Measurement of C cell volumes confirmed this pattern. The mean C cell volume (% total thyroid volume occupied by C cells) for the control group was $0{\cdot}98 \pm 0{\cdot}3$; values for the two high-vitamin-D-fed groups, $4{\cdot}7 \pm 0{\cdot}6$ and $5{\cdot}6 \pm 0{\cdot}8$ for the Fe-deficient and Fe-supplemented groups respectively, were not significantly different from each other. These combined values were significantly higher ($P < 0{\cdot}001$) than the mean value for the control animals.

There was no marked difference in volumes (% total cells) of the follicular cells, the stroma or the colloid in the three groups of animals.

DISCUSSION

It is difficult to determine what constitutes hypervitaminosis for a laboratory rat. Guldager (1936) states that the borderline for hypervitaminosis D toxicity in growing rats is between 300 and 700 i.u. (between 7·5 and 17·5 μg)/d. Each of the rats in the present experiment ate approximately 20 g food/d, and on this basis each animal given the McCall diet, made up according to McCall *et al.* (1962), received 800 i.u. (20 μg) ergocalciferol/d, an intake well above the borderline for hypervitaminosis D suggested by Guldager (1936). It is clear from the results of the present experiment that dietary Fe content makes no difference to either thyroid C cell volume or serum Ca levels with diets containing high concentrations of vitamin D and there is therefore every possibility that the changes found were due to the high intake of ergocalciferol itself. It is reasonable to suppose that the higher serum Ca concentration is the result of the known effect of ergocalciferol in increasing Ca absorption from the gut. The increase in C cell volume could also be a result of this, but the results of recent work have indicated that a high serum Ca concentration alone does not significantly increase the volume of these cells (Triggs, 1974). This raises the possibility that vitamin D, or one

of its metabolites, might act directly on C cells, and indeed it has recently been reported that ^{14}C-labelled cholecalciferol is concentrated by the mouse thyroid gland (Dencker & Tjälve, 1973).

The present results clearly have considerable significance to workers using the McCall diet. Although no adverse effects on the animals were observed by ourselves or presumably by previous workers, nevertheless it seems likely that results from previous work using this diet were obtained from hypercalcaemic rats. Clearly the composition of this must be altered, and we suggest a concentration of 25 μg ergocalciferol/kg diet.

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EXPLANATION OF PLATE

Longitudinal sections through the vertical axis of the thyroid gland from rats given (*a*) control diet, (*b*) high-vitamin-D, Fe-deficient diet. Hyperplastic areas are indicated by the arrows. For details of diets, see p. 278.

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