The effects of early zinc deficiency on DNA and protein synthesis in the rat

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1. The effects of early zinc deficiency on DNA and protein metabolism of the liver, kidneys, testes and spleen of the young rat were studied. The investigations were carried out in two phases: before food consumption and growth were affected, and afterwards.

2. The incorporation of [^{3}H]thymidine into DNA was markedly affected by differences of less than a week in the age of the rats.

3. Zn deficiency significantly reduced the incorporation of [^{3}H]thymidine into DNA of liver, kidneys and spleen before growth and food consumption were affected. The degree of inhibition was of the order of 50% in the first 5 d. A similar but non-significant trend was observed for the testes.

4. The incorporation of [^{14}C]lysine into protein was not significantly affected in liver and testes during the initial period of Zn deficiency; the incorporation into kidneys and spleen was significantly inhibited but the magnitude of the effect was only of the order of 20% in 5 d.

5. One week after the start of the second phase, the concentration of DNA in liver, testes, and spleen of Zn-deficient animals was not significantly different from that in pair-fed controls. The DNA content of the kidneys was significantly reduced by the deficiency but only to 97% of that in pair-fed animals given the Zn-supplemented diet. The incorporation of [^{3}H]thymidine into DNA was not significantly different between deficient and control groups in any of the four organs investigated.

Several groups of workers are at present investigating the lesions that develop in zinc-deficient rats, and the subject has been reviewed by Hurley (1969), Mills, Quarterman, Chesters, Williams & Dalgarno (1969), Oberleas & Prasad (1969) and Reinhold & Kfoury (1969).

The rapid and dramatic effect of Zn deficiency on growth has led several groups to investigate its effect on DNA synthesis (Fujioka & Lieberman, 1964; Williams, Mills, Quarterman & Dalgarno, 1965; Buchanan & Hsu, 1968; Macapinlac, Pearson, Barney & Darby, 1968; Becker, 1968 and Weser, Seeber & Warnecke, 1969). Fujioka & Lieberman (1964) induced Zn deficiency by EDTA infusion. Their results are of questionable relevance to deficiencies produced by diets low in Zn, since Becker & Hoekstra (1968) have shown that in vitro EDTA will remove 70% of the Zn from liver homogenates, whereas in vivo a low dietary Zn concentration seldom results in more than a 20–30% fall in liver Zn concentration. The other groups have all investigated deficiencies induced by diets low in Zn but their results are nevertheless conflicting, some groups finding an inhibition of DNA synthesis while others found no effect.

Work at this Institute with young rats has suggested that the course of Zn deficiency may be divided into three stages: an initial period of 4–5 d during which growth and food intake remain unaffected; an intermediate period of several weeks when reduced food intake and a failure to gain weight are the only obvious signs of the deficiency; a

terminal stage when further lesions develop before death occurs. During the intermediate period, food intake is erratic and is appreciably below the *ad lib.* intake of a normal animal of similar size.

In studies of the metabolic role of an essential nutrient, deficiency of which reduces food consumption, attempts are frequently made to eliminate the metabolic consequences of low food intake by paired feeding of control and experimental animals. However, Fábry & Braun (1967) and McCracken (1968*a*, *b*) have shown that pair-fed animals receiving restricted quantities of food consume it within a short period. They found that this 'meal-eating' effect caused modifications of the animals' metabolism which limited their value as controls. Experiments performed during the intermediate and terminal stages of Zn deficiency should therefore be so controlled that food restriction and meal-eating effects do not upset their evaluation. Furthermore, the terminal stages of the deficiency are characterized by changes, such as the appearance of skin lesions, which only appear after longer periods of Zn depletion than are required to affect growth and appetite. Considerable doubt must exist as to the primary role of Zn in producing some of these later lesions.

Examination of the procedures used by previous workers suggests, in many instances, inadequate control of the experiments in view of the effects of Zn deficiency on food intake. Furthermore, several groups have studied rats at the terminal stage of the deficiency, when the effects observed may be secondary consequences of the Zn-dependent lesions. To avoid such complications, the investigations now described were limited to the use of animals in the initial period or in the early stages of the intermediate period of the deficiency. In the former situation, the food consumption of the deficient animals was not affected and rats fed *ad lib.* on the Zn-supplemented diet were used as controls. To provide adequate controls after Zn deficiency reduced food intake, the pair-feeding regimen outlined below was adopted.

EXPERIMENTAL

Animals and their management

Male Hooded Lister rats of the Rowett Institute strain were used in all experiments. They were fed on either a semi-synthetic Zn-deficient diet (Williams & Mills, 1970), containing no more than 0·9 ppm Zn, or the diet supplemented with $ZnSO_4$ to give a dietary concentration of 40 ppm. The animals were individually housed in the Perspex-and-glass cages described by Quarterman, Williams & Humphries (1970). Pair-fed animals were given the Zn-supplemented diet from a rotating disc, which presented the dietary allowance uniformly over a 24 h period.

Experimental design and procedure

Expt 1. Thirty-six weanling rats, selected from litters all born within 2 d, were reared on the Zn-supplemented diet to a mean weight of 60 g and were then divided into six groups of six rats in such a way as to produce groups as uniform as possible with respect to weight. Each day for the next 5 d one group was transferred to the Zn-deficient diet. On the 6th day, all thirty-six rats were used for studies of

[^{3}H]thymidine incorporation into DNA of liver, kidneys, testes and spleen and also for [^{14}C]lysine incorporation into protein of the same organs.

Expt 2. Eighteen rats were reared on the Zn-supplemented diet to a mean weight of 70 g before being divided into two groups with similar ranges of body-weight. One group was given the Zn-deficient diet for 11 d and then used for studies of [^{3}H]thymidine incorporation into DNA. The second group was given the Zn-supplemented diet and pair-fed with the other group. This entailed a difference of 1 d between the equivalent intakes of the two groups, and the second group was therefore used for studies of thymidine incorporation after 12 d of pair-feeding.

Incorporation of [^{3}H]thymidine into DNA. The rats were injected intraperitoneally, 2 h before being killed by a blow on the head, with [^{3}H]thymidine (10 mCi/μmole) at 50 μCi/100 g live weight. Liver, kidneys, testes and spleen were removed and cooled to 0°. The organs were weighed and then processed as detailed below or were frozen in glass vials by immersion in acetone and solid CO_2 for storage before processing. Samples (1 g) of liver and the whole of the tissue of the other organs were homogenized in 0·2 N-$HClO_4$ at 0° in a Potter–Elvejhem all-glass homogenizer. After being left at 0° for 30 min, the homogenates were centrifuged and the pellets obtained were washed twice, by resuspension and centrifugation with ice-cold 0·2 N-$HClO_4$. The pellets were then extracted twice with 0·5 N-$HClO_4$ at 70° for 30 min and the extracts combined. Samples of these extracts were used for DNA determination by the method of Burton (1956) and the remainder was neutralized with KOH. Samples (1 ml) of the neutralized extract were added to 14 ml of a dioxane-based scintillator (Bray, 1960) and the radioactivity present was determined in a Packard liquid scintillation counter. An external standardization system was used to correct for quenching. In a preliminary experiment, the counting rate of the samples was found to fall slightly with time because of slow precipitation of radioactive material. In subsequent experiments, Cab-O-Sil (Packard Instrument Ltd, London) was added to Bray's scintillation fluid at a concentration of 5% (w/v) which resulted in a stable count rate being obtained. Results were expressed as disintegrations/min per mg of DNA phosphorus.

Incorporation of [^{14}C]lysine. In Expt 1, the incorporation of [^{14}C]lysine into protein was investigated at the same time as [^{3}H]thymidine incorporation into DNA. [^{14}C]lysine (8 μCi/μmole) was injected intraperitoneally at a dose rate of 5 μCi/100 g live weight 20 min before killing. The residues after hot $HClO_4$ extraction of the organs were used to prepare a protein fraction by washing them three times with acetone and once with ether. The pellets, after drying in air, were weighed and samples were taken for protein estimation by the method of Lowry, Rosebrough, Farr & Randall (1951), using bovine serum albumen as a standard.

Further 15 mg samples of the protein fraction were used for radioactivity determinations. The protein was dissolved in a mixture of 1 ml toluene plus 1 ml hyamine hydroxide by warming at 60° for 1·5 h. Toluene scintillator (10 ml) containing 50 mg 1,4-bis-2-(5-phenyloxazolyl)-benzene and 4 g 2,5-diphenyloxazole per l toluene was mixed with the hyamine solution and the resultant mixture was neutralized with conc. HCl. The method was subject to appreciable variation in counting efficiency owing to the colour produced by hyamine hydroxide solution, but this problem was

satisfactorily overcome by correcting for quenching as before. Differential counting procedures were used to correct for any contamination of the DNA fraction by ^{14}C and of the protein fraction by ^{3}H. The results were expressed as disintegrations/min per mg protein.

RESULTS

Incorporation of [^{3}H]thymidine into DNA and of [^{14}C]lysine into protein before failure of appetite caused by Zn deficiency

In a preliminary experiment, young rats (70 g), all of the same age, were offered the Zn-deficient diet *ad lib.* for up to 5 d before being killed. When compared with a

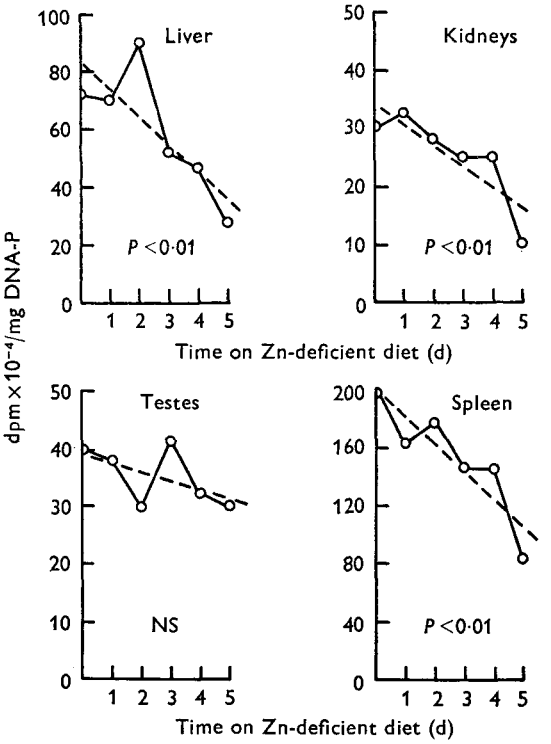

Fig. 1. Incorporation of [^{3}H]thymidine into DNA during the initial phase of zinc deficiency in rats. – – –, indicates the linear regression of incorporation on days of Zn deficiency. The *P* values refer to the statistical significance of the regression; dpm, disintegrations/min; NS, not significant.

control group killed at the start of the experiment, there was a linear decline in the incorporation of [^{3}H]thymidine into the DNA of the rats on the deficient diet. The reduction in incorporation amounted to approximately 46% after only 3 d on the Zn-deficient diet, but in a second control group killed at this time there was a 19% reduction of incorporation when compared with the original control group. It was found impossible to distinguish, with any statistical certainty, any effect of Zn deficiency over and above that which was associated with the fall in incorporation caused by the ageing of the animals.

Expt 1. As a consequence of the above findings, an experiment was designed so that all the rats were of the same age when killed. The final mean body-weights of the groups, which had been Zn-deficient for 0–5 d, were 83, 82, 94, 81, 83 and 77 g respectively. Only in the group deficient for 5 d was there any fall in food intake, a fact reflected by its slightly smaller mean weight.

The incorporations obtained in these groups are shown in Figs. 1 and 2. The results were analysed for any linear trend of incorporation with days on the deficient diet and for any effect of rat weight on the variability of incorporation. No significant differences were found in variability of incorporation between rats of different weights and an estimate of the general variance of the incorporation was used to test the significance of

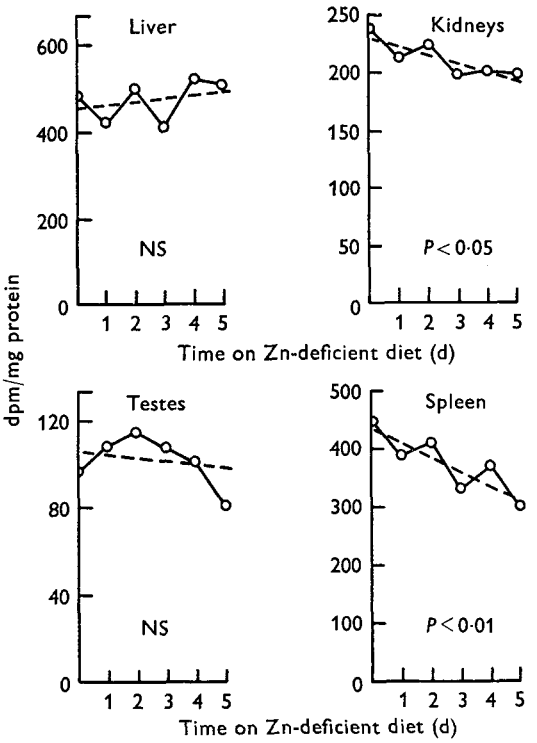

Fig. 2. Incorporation of [^{14}C]lysine into protein during the inital phase of zinc deficiency in rats. – – –, indicates the linear regression of incorporation on days of Zn deficiency. The *P* values refer to the statistical significance of the regression; dpm, disintegrations/min; NS, not significant.

the regression of incorporation on the duration of depletion. In each organ, incorporation of [^{3}H]thymidine into DNA tended to decrease with days on the deficient diet and these trends were statistically significant (at the 1% level) for all organs except testes. In most instances, the decrease in incorporation was of the order of 50% in 5 d.

No significant linear trend was found in the incorporation into protein in liver and testes. The corresponding trends for kidneys and spleen were significant (5 and 1% respectively) but the magnitude of the decrease in incorporation was only of the order of 20% in 5 d.

Tissue DNA concentrations and [^3H]thymidine incorporation into DNA after the fall in food intake caused by Zn deficiency

Expt 2. Table 1 shows the final DNA concentration in four organs of pair-fed rats receiving Zn-deficient or Zn-supplemented diets. In two tissues the DNA content was higher in the pair-fed animals; in the other two, the tissues of the Zn-deficient rats had the higher DNA concentration. Only the effect in the kidney was significant at the 5% level, and here the fall in DNA concentration with respect to the control was only 3%. The biological significance of such a difference remains doubtful.

Table 1. *DNA concentration in fresh tissues from zinc-deficient (−Zn) and pair-fed control (+Zn) rats*

(The Zn-deficient rats had been given the deficient diet for 11 d and weighed 85 g ± 9 g. The control rats weighed 89 g ± 10 g. There were nine male rats in each group)

	Mean DNA concentration				
Organ	−Zn (μg/g tissue) (A)	+Zn (μg/g tissue) (B)	Standard error of difference (μg/g)	A as % of B	Significance of difference
Liver	258	288	13	89	$P < 0.10$
Kidney	445	459	4	97	$P < 0.05$
Testes	303	289	8	105	NS
Spleen	794	776	36	102	NS

NS, not significant.

To separate any effects of food intake on the incorporation of [^{3}H]thymidine into DNA from the effects of Zn deficiency, the ratios of the specific activities of the Zn-deficient rats (disintegrations/min per mg DNA phosphorus) to those of their pair-fed controls were calculated. The means and standard errors for these ratios were: liver, 1·28 ± 0·20; kidneys, 0·91 ± 0·13; testes, 1·03 ± 0·15; spleen, 1·15 ± 0·17. None of these ratios was significantly different from 1·0 and thus there was no evidence of a difference of incorporation into the organs of the Zn-deficient rats compared with their pair-fed controls once Zn deficiency had induced a fall in food intake and, with it, a cessation of growth.

DISCUSSION

The results presented by Becker (1968) emphasized the difficulty of comparing Zn-deficient with control rats when the two types of animal had different food consumptions and growth rates. Becker attempted to solve this problem by using as controls deficient animals that had been injected with $ZnCl_2$. Weser *et al.* (1969) used a similar method but the results obtained by the two groups of workers were quantitatively different. Becker found a slight stimulation of incorporation in the Zn-injected controls but this was not statistically significant. Weser *et al.*, using liver from partly hepatectomized rats, found a qualitatively similar but much larger difference, which was significant at the 5% level. However, injection of Zn into Zn-deficient rats to provide control animals is of doubtful value since it might result in transient effects

of a different nature to those arising when the element is supplied continuously in the diet. Furthermore the rats used by both Becker (1968) and Weser *et al.* (1969) had been fed on the deficient diet for at least 4–5 weeks and the rats used by Weser *et al.* were showing signs of severe deficiency. With rats in this condition, some doubt must exist as to whether or not the observed effects were attributable to a primary Zn deficiency.

In our experiments, carried out during the initial phase of the deficiency, there was a definite trend of decreasing incorporation of thymidine into DNA as the deficiency progressed. Without further knowledge of the effect of Zn deficiency on nucleotide precursor synthesis and catabolism, it is not possible to claim that this effect necessarily indicates a partial failure in DNA synthesis, or that the defects noted are responsible for the cessation of growth. Further work on this point is necessary.

The experiments with strictly controlled feeding of the pair-fed group suggested no effect of Zn deficiency on incorporation of thymidine into DNA during the early part of the second phase of Zn deficiency, when voluntary food consumption had fallen. However, the decrease in *ad lib.* intake of the Zn-deficient rats was so severe that the pair-fed controls also ceased to grow. This cessation of growth of the control rats due to inanition may well have resulted in a fall in DNA synthesis which mimicked that of the deficient animal but had different origins.

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