The enhancement by dietary zinc deficiency of the susceptibility of the rat duodenum to colchicine

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1. The incidence of colchicine-induced lesions in the germinal epithelium of the rat duodenum was studied in young rats in an early stage of zinc deficiency and in their pair-fed controls. At both dose levels of colchicine used, a marked increase in the amount of cell damage was observed in the duodenum of Zn-deficient rats as compared with the pair-fed, control (Zn-supplemented) rats.

2. No statistical interaction between Zn and colchicine was demonstrable, and no lesions were found in the duodenum of animals that had not been treated with colchicine.

3. The results are discussed in relation to the effects of Zn deficiency in animals and the possible involvement of Zn in the maintenance of the integrity of microtubular structures.

In earlier work (Fell, Leigh & Williams, 1973) it was reported that the cytotoxic effects of colchicine on the rat pancreas were exacerbated under conditions of dietary zinc deficiency. In other tissues then examined (oesophagus, skin and liver) the effects of this drug appeared to be limited to metaphase arrest of dividing cells. That a tissue-specific response occurred is not surprising in view of the fact that in dietary Zn-deficiency the loss of Zn from different organs or tissues varies considerably (Prasad, Oberleas, Wolf & Horwitz, 1967; Reinhold, Kfoury & Thomas, 1967; Williams & Mills, 1970) and that the accumulation of colchicine also varies widely between different tissues (Back, Walaszek & Uyeki, 1951). The inference to be drawn from these earlier findings, i.e. that Zn may be able to exert some protective effect against colchicine toxicity, appeared to be related to the findings of Fujii (1954, 1955) that an important aspect of Zn metabolism is the association of this metal with the mitotic spindle and hence microtubules of the cell. Positive identification of Zn in the mitotic apparatus has been made (Morisawa & Mohri, 1972).

The cytotoxic effects of colchicine may be related to its ability to bind to the protein dimer subunit of the microtubular protein, tubulin (Weisenberg, Borisy & Taylor, 1968), and thus induce the disaggregation of the microtubular arrays of the cell (Taylor, 1965). The results of studies in vitro of Nickolson & Veldstra (1972) indicated that, of a number of heavy metals tested, Zn alone was able to inhibit the binding of colchicine to tubulin dimers and, at the same time, preserve the morphology of intact neurotubules.

In view of these findings it seemed desirable to determine whether, in the rat, dietary Zn status could be further related to the severity of the effects of colchicine toxicity. The choice of the duodenum as the tissue to be studied was influenced by the high mitotic rate of this tissue, the swift decrease in the activity of Zn-associated enzymes in the gut of Zn-depleted animals (Mills, Quarterman, Williams, Dalgarno & Panic,

1967; Williams, 1972) and the known accumulation of colchicine in the duodenum (Back *et al.* 1951).

EXPERIMENTAL

Animals and diets

Male weanling Hooded Lister rats of the Rowett Institute strain were housed individually in glass and Perspex cages and were given *ad lib.* an egg-albumen based diet containing 40 mg Zn/kg as previously described (Williams & Mills, 1970). When they had reached a weight of approximately 80 g, the rats were paired according to weight and one of each pair allocated at random to the Zn-deficient group and offered a similar diet with no Zn supplement (Zn content < 1 mg/kg). The other rat was pair-fed the Zn-supplemented diet. Growth of the low-Zn rats ceased on the 5th day and thereafter their food intake became erratic. From the 12th day after offering the low-Zn diet all animals were given a constant amount of food (6·5 g/d) for a further 4 d, which all could ingest, to obviate any effects of differing food intake on intestinal mitotic rhythms (Alov, 1963).

Treatments

Expt 1. Six pairs of animals, as described previously, were used. At the end of the 4 d constant food intake period, all the rats were injected, subcutaneously, with colchicine (BDH Ltd, Poole, Dorset), two pairs at each of the dose rates of 0·2, 1·0 or 2·0 mg/kg live weight. They were killed by a blow to the head 6 h later.

Expt 2. Nine pairs of animals were used. Preliminary studies of tissue from Expt 1 indicated that particular attention should be paid to the effects of the two higher dose rates of colchicine. In this experiment three Zn-deficient animals and their controls were injected with colchicine at a level of 1·0 mg/kg live weight, and three further animals on each diet received doses at 2·0 mg/kg live weight. The remaining six animals (three Zn-deficient and three controls) were not dosed with colchicine. Both colchicine-dosed and undosed animals were killed 6 h after administration of the drug.

Materials and methods

Immediately at death, the first 20 mm of the duodenum was excised and the end distal to the pyloric sphincter was diced to 1 mm^3 blocks under cold glutaraldehyde solution (50 g/l 0·06 M-sodium cacodylate buffer, pH 7·2) or in Palade's osmium tetroxide fixative, for electron microscopy. The remaining tissue was immersed in formaldehyde solution (40 g/l 0·15 M-sodium chloride) for subsequent paraffin-wax impregnation. Glutaraldehyde fixation was continued for 4 h at 4° before post-fixation in Palade's solution. Tissues so treated were subsequently stained en bloc with uranyl acetate before embedding in Araldite (BDH Ltd, Poole, Dorset). Survey sections 1 μm thick were cut vertically through the villi and stained with toluidine blue for light microscopy. Silver-gold sections from selected areas were stained with lead citrate before examination in an electron microscope AEI 801A (AEI Scientific Apparatus Ltd, Barton Dock Road, Urmston, Manchester). Three survey (1 μm) sections of two samples from each animal were assessed for incidence of necrotic bodies by using an

Table 1. *Expt* 1. *Incidence of lesions* in* 1 *μm sections from the duodenal crypts of groups of zinc-deficient and pair-fed control rats treated with different amounts of colchicine*†

	Colchicine (mg/kg live weight)	
	1	2
Zn-deficient	5·0	7·6
Controls	3·9	5·4

* Arithmetic mean of recognizable bodies per randomly selected square of side 50 μm.
† For details of experimental treatments, see p. 136.

eye-piece graticule to superimpose a net of 50 μm squares over the crypts of the mucosa. From each section, nine squares were selected at random and the number of enclosed bodies recognizable at a magnification of ×500 was recorded. The total count, for each animal, obtained by this method was analysed to determine the treatment effects and interaction. The effects were expected to be multiplicative rather than additive and so the logarithms of these totals were analysed statistically.

RESULTS

Colchicine administration resulted in the formation of rounded cytoplasmic inclusions within the rapidly proliferating crypt cells of the duodenal mucosa but none were evident in the mature cells of the villi. The bodies contained an eosinophilic background material in which Feulgen-positive basophilic inclusions were scattered.

Individual bodies ranged from 0·5 to 8·0 μm in diameter and were usually enclosed by a double membrane. Their contents showed a variable extent of morphological preservation but mainly consisted of mitochondrial figures (Plate (1)) together with traces of endoplasmic reticulum and, more rarely, nuclear material. Many of the inclusions were found in close proximity to a Golgi apparatus.

Lesions were found in the duodenal mucosa of all colchicine-treated animals but were absent from control Zn-deficient and Zn-supplemented animals. The incidence of lesions was calculated as the arithmetic mean of recognizable bodies per selected square of side 50 μm (Table 1). The increased incidence of necrosis, at 1 or 2 mg/kg live weight levels of colchicine administration, in Zn-deficient animals was strikingly evident in survey sections (Plate (2 and 3)) and confirmed by this quantitative assessment.

These experiments were designed so that the Zn effect and the Zn × colchicine interaction were based on within-pair comparisons and the colchicine effect was based on between-pair comparisons. It was noted that the rats on the lowest colchicine level (0·2 mg/kg live weight in Expt 1) had insufficient lesions for reliable assessment and so the statistical analysis was restricted to the results from the rats on 1 or 2 mg/kg live weight of colchicine (eight rats in Expt 1 and twelve in Expt 2). The variable examined in both experiments was the logarithm of the total count of lesions. One analysis was carried out on the sums for each pair, and one on the differences. For each analysis the residual mean square was combinable for the two experiments so the

Table 2. *Expts 1 and 2. Log no. of lesions in 1 μm sections from duodenal crypts of groups of zinc-deficient and pair-fed control rats treated with different amounts of colchicine**

(Mean value for each treatment averaged over both experiments; the derived number of lesions is given in parentheses)

	Colchicine (mg/kg live weight)		
	1	2	Mean for each group of rats
Zn-deficient	2·434 (272)	2·617 (414)	2·526 (336)
Controls	2·326 (212)	2·465 (292)	2·396 (249)
Mean for each colchicine treatment	2·380 (240)	2·541 (348)	—
		Individual treatment means	Overall colchicine or Zn means
SE of difference between mean values for colchicine treatments (6 df)		0·061	0·043
SE of difference between mean values for zinc treatments (6 df)		0·041	0·029

* For details of experimental treatments, see p. 136.

pooled value was used (with 6 df) to test whether there was any evidence that the treatment effects differed in the two experiments.

The colchicine effect was significant ($P < 0.01$), in that doubling the dose of colchicine increased the number of lesions by $46 \pm 10\%$ (SE). The Zn effect was also significant ($P < 0.01$). The presence of Zn led to a $26 \pm 6\%$ (SE) reduction in the number of lesions. There was no evidence that these effects differed between the two experiments or of any interaction between Zn and colchicine.

The means of the log values with the retransformed counts are given in Table 2.

DISCUSSION

The results of these experiments confirm the finding of Fell *et al.* (1973) that, in the rat, the Zn-deficiency state is associated with an increased sensitivity to colchicine toxicity, in certain tissues.

Colchicine is best known as an inhibitor of mitosis in dividing cells (Eigsti & Dustin, 1955), an effect which may now be attributed to the binding of colchicine with the dimer subunit of the microtubular protein, tubulin (Taylor, 1965; Borisy & Taylor, 1967*a*, *b*) thus disturbing the dynamic equilibrium between free and aggregated microtubular units. Differences in the sensitivity of various organs to colchicine poisoning may be related to the frequency of a polymerization–depolymerization cycle (Patzelt, Singh, Le Marchand, Orci & Jeanrenaud, 1975); an increased sensitivity to colchicine may, therefore, be associated with a reduced stability of intact microtubules and a concomitant increase in the amount of colchicine-binding material.

The high incidence of cytoplasmic inclusions observed in these experiments suggests that many more cells were affected than those in division. The influence of dosage on the incidence of these lesions has been studied by Dinsdale (1975).

The bodies observed are probably involved in the deletion of growing crypt cells and may result from the sequestration of damaged areas of the cytoplasm or from endocytosis of debris resulting from the death of a neighbouring cell (Dinsdale, 1975). Such a process was described in detail by Kerr, Wyllie & Currie (1972) and termed 'apoptosis'. Though occurring in normal tissues, the process is much more marked during atrophy or in response to noxious agents.

Whether a specific effect of colchicine on microtubular protein may be related to the effects we have observed cannot be stated with any certainty. Colchicine is reported to affect in vivo the activity of a wide variety of enzymes (Eigsti & Dustin, 1955; Myren, Luketic, Ceballos, Sachs & Hirschowitz, 1966). Significantly, however, Myren *et al.* (1966) were unable to demonstrate any inhibition in vitro of any of five dehydrogenases studied in mouse gut. It was suggested by these authors that a reduction in the in vivo activity of some of the enzymes, assessed by histological staining techniques, might be explained by a loss of enzyme into surrounding tissue or circulation. Increased xanthine oxidase activity (*EC* 1.2.3.2) in rat serum after colchicine administration appears to be related to the loss of this enzyme from liver tissue (Affonso, Mitidieri & Villela, 1961, 1962). Colchicine did not affect xanthine oxidase activity in a liver homogenate, but was a powerful inhibitor of the purified enzyme. An increase in rat liver alkaline phosphatase (*EC* 3.1.3.1) activity, after colchicine administration, was significantly higher 18 h after dosing (Ebner & Streckner, 1950). Substantial reductions in ATP levels were found in both normal and regenerating rat liver 8 h after colchicine administration (Wang, Greenbaum & Harkness, 1963) an effect which was still apparent 16 h later, by which time the anti-mitotic effects of the drug are essentially over (Clearkin, 1937). The lack of any time-dependent relationship between the effects of colchicine on enzyme function or substrate levels and mitotic arrest suggests that these properties of the drug are quite separable. It would, therefore, appear that colchicine-induced enzyme changes may be related to varying amounts of cell damage which may, initially, be attributable to the effects of the drug on the cell membrane; the distinction between cause and effect remains obscure.

The earliest directly observable clinical effect of Zn deficiency in the rat is a reduction in growth rate (Todd, Elvejhem & Hart, 1934). There is considerable evidence that Zn is required for DNA synthesis, or, more correctly, for processes involved in the incorporation of labelled precursors into the DNA of various tissues (Lieberman, Abrams, Hunt & Ove, 1963; Fujioka & Lieberman, 1964; Williams, Mills, Quarterman & Dalgarno, 1965; Weser, Seeber & Warnecke, 1968; Swenerton, Shrader & Hurley, 1969; Williams & Chesters, 1970). Paradoxically, however, an increase in the rate of cell division in the Zn-deficiency state was observed by Fell *et al.* (1973), notably in the oesophagus and pancreas, and a stimulation of [^{3}H]thymidine incorporation into the DNA of the thymus of Zn-deficient rats was noted by Stephan & Hsu (1973).

Mazia (1961) has pointed out that a high mitotic index may result from an increase

Table 3. *Duration of mitosis and intermitotic period (min) in selected tissues of the rat*

Tissue	Source	Intermitotic time (min)	Mitotic time (min)
Corneal epithelium	Mazia (1961)	14000	70
Jejunal epithelium	Mazia (1961)	2000	28
Oesphagus (control)	Present study	21000	108
Oesphagus (zinc-deficient)	Present study	1300	20

in the number of cells entering division, a protraction of the mitotic time or the existence of a wave of division.

An increase in the rate of cell division in certain tissues would result in a larger proportion of cells being susceptible to mitotic poisoning by colchicine, more cells would therefore be arrested at metaphase and subsequently destroyed. A close relationship between mitosis and apoptosis has been indicated in earlier studies (Kerr *et al.* 1972; Wyllie, Kerr & Currie, 1973) which suggests that an increased mitotic rate may be, in part, a response to increased cell deletion.

From the data of Fell *et al.* (1973) it can be calculated (Table 3) that the rate of cell division in the Zn-deficient rat oesophagus reverts to that characteristic of the lower gut, a finding consonant with the hypothesis of Chesters (1974) that de-differentiation of the genetic potential of the cell may occur in Zn deficiency, a process which may be more susceptible to Zn deficiency than is DNA synthesis. Searle, Lawson, Abbot, Harmon & Kerr (1975), suggest the possible involvement of 'apoptosis' in the deletion of abnormalities of DNA (gene?) expression.

If changes in mitotic times are considered a further paradox is demonstrable. Swenerton *et al.* (1969) reported that, while [^{3}H]thymidine incorporation in foetuses of Zn-deficient female rats was considerably reduced as compared with controls, a greater number of unlabelled mitotic figures were to be found in the neuroepithelium. This suggests that these figures resulted from a greater number of cells which had entered division before the administration of the isotope or in which mitotic cleavage had been delayed, in analogy to the effects of colchicine. In the severely Zn-deficient rat testis, Elmes (1974) observed abnormal mitotic figures, similar to those which may be induced by colchicine poisoning (Eigsti & Dustin, 1955), and in the Zn-deficient sheep colchicine administration resulted in gross disturbances to terminal nerve-endings in tongue epithelium, whereas a normal animal was not affected (Mann, Fell & Dalgarno, 1974).

Edstrom & Mattson (1975) have reported that Zn has a role in the microtubular-dependent axonal transport of protein in frog sciatic nerve.

It is suggested therefore in accord with the views of Nickolson & Veldstra (1972) that Zn has a role in the functioning of microtubular structures in the cell, and that, in the Zn-deficiency state certain manifestations may be a reflection of changes in the dynamic equilibrium of these important cell components, thus resulting in increased susceptibility to the effects of colchicine toxicity.

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EXPLANATION OF PLATE

(1) Electron micrograph of two intracellular necrotic bodies from the duodenum of a zinc-deficient rat 6 h after administration of 1 mg/kg colchicine. × 19000. (2) Survey section through the duodenum of a zinc-supplemented rat, 6 h after administration of 1 mg/kg colchicine, showing the presence of necrotic bodies. × 350. (3) Survey section through the duodenum of a zinc-deficient rat, 6 h after administration of 1 mg/kg colchicine, showing an increased incidence of necrotic bodies. × 350.

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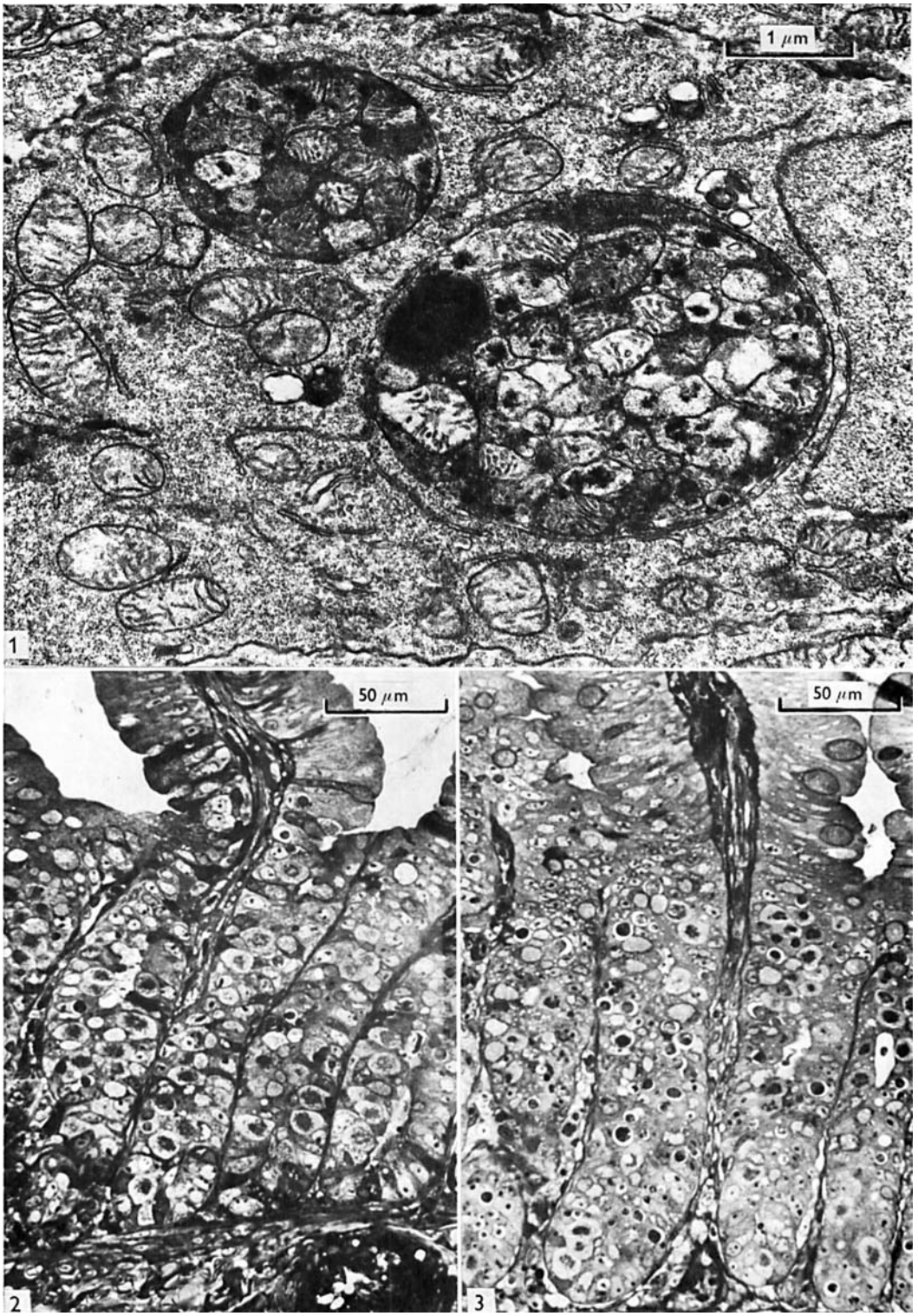

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