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A possible relation between dietary zinc and cAMP in the regulation of tumour cell proliferation in the rat

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1. The possibility of an effect of zinc on the rate of tumour cell division, mediated through a regulation of cellular cAMP concentration, was investigated in the present study in rats.

2. Dietary Zn deficiency (< 1·5 mg Zn/kg) but not Zn excess (500 mg Zn/kg) resulted in an increased cAMP concentration in transplanted hepatoma cells. Neither treatment had any effect on the cAMP concentration in regenerating liver or normal resting liver. Both the deficient and excess Zn diets resulted in a small reduction in tumour growth (not statistically significant).

3. The results seem to indicate that the relation investigated in the present study does not apply in the cell line used.

A dietary zinc deficiency results in, among other disorders, general growth retardation (Prasad, 1979; Huang *et al.* 1982) as well as reduced carcinogenesis and tumour growth rate in certain types of cancers (Duncan *et al.* 1974; Duncan & Dreosti, 1975; Minkel *et al.* 1979). In some cases these effects have also been shown to occur with a dietary Zn excess (Duncan & Dreosti, 1975; Minkel *et al.* 1979). Experiments to date have associated this regulatory role of Zn on tumour growth exclusively with DNA synthesis (Slater *et al.* 1971; Duncan & Dreosti, 1976; Minkel *et al.* 1979; Baker & Duncan, 1983). More recently however, studies indicating an importance of Zn for ω6-fatty acid metabolism (Cunnane, 1982; Huang *et al.* 1982) have led to proposals of an alternative (or additional) mechanism of regulation involving cellular cAMP (Horrobin, 1980). In this mechanism, as illustrated in Fig. 1, Zn is suggested to regulate the activity of Δ6-desaturase (a component of the

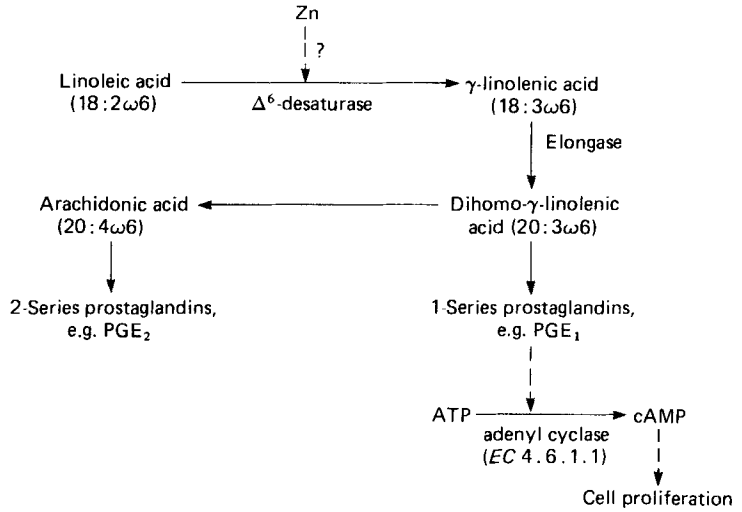

Fig. 1. An outline of the conversion of ω6-fatty acids to prostaglandins indicating the proposed site of action of zinc and the possible subsequent effect on the synthesis of cAMP and hence on cell proliferation (after Otten *et al.* 1971 and Huang *et al.* 1982). →, Metabolic pathways; ----→, suggested effects.

linoleoyl-CoA-desaturase complex), a key enzyme involved in the metabolism of linoleic acid to prostaglandins (Horrobin, 1980; Horrobin & Cunnane, 1980; Huang *et al.* 1982). Prostaglandin E_1 (PGE_1) in particular has a strong stimulatory effect on cAMP accumulation in cells (Zor *et al.* 1970; Oien *et al.* 1975; Hall & Behrman, 1982). cAMP in turn appears to have a regulatory role in cell proliferation and, in particular, in the transformation of certain cells (Otten *et al.* 1971; Sheppard, 1971, 1972). Studies concerning the previously mentioned relation are particularly significant considering that transformation of cells into cancer cells is often accompanied by a loss of Δ6-desaturase activity and a reduction in cAMP concentration (Otten *et al.* 1971; Sheppard, 1972; Dunbar & Bailey, 1973). Hence it would seem that attempts to normalize cancer cells should consider enhancement of this enzyme activity or increasing cellular cAMP concentration, or both, where these are affected.

The purpose of the present study was to investigate an aspect of this relation, namely the effect of dietary Zn on cAMP concentrations and the growth of hepatomas implanted as tumours subcutaneously in rats, and to compare this with the effect of dietary Zn on the cAMP concentrations of non-transformed tissue, namely resting and regenerating livers.

MATERIALS AND METHODS

Reagents

A radioactive dilution cAMP test kit was purchased from Boehringer Mannheim, Randburg. Water-saturated diethyl ether was prepared by vigorously mixing 1 vol. distilled and deionized water with 5 vol. diethyl ether for 2 min and then separating the water-saturated diethyl ether from the aqueous phase.

Animals and diets

Female Wistar rats weighing 140–150 g were obtained from the Natal Institute of Immunology, Pinetown. They were housed in stainless-steel and plastic cages and given distilled and deionized drinking water *ad lib.* to ensure that the diets were their only source of Zn. The essentially Zn-free (< 1·5 mg Zn/kg diet), biotin-enriched (4 mg/kg diet) basal diet consisted of (g/kg) 670 starch, 200 dried egg albumin, 80 maize oil, 40 salt mix, 10 vitamin mix (Mutch & Hurley, 1974) and represented the Zn-deficient diet. A control diet was prepared by adding $ZnSO_4.7H_2O$ to the basal diet to provide a level of Zn equivalent to 50 mg Zn/kg diet. The salt was added to a level of 500 mg Zn/kg diet in the Zn-excess diet.

In order to verify whether the results observed in the present study were a direct result of the Zn content of the diet or due to total food consumption, the effect of the diets on the growth of the rats was monitored in a separate study over 24 d.

Hepatoma cells

Type 350 hepatoma is a 3′-methyl-4-dimethylaminoazobenzene-induced transplantable hepatoma and was supplied by Nola Dippenaar of the Department of Physiology, Medunsa.

Surgical procedures

Hepatoma cells to be transplanted into rats were harvested from in vitro cultures and suspended in phosphate-buffered saline (5×10^6 cells/ml). Rats that had been feeding *ad lib.* on the experimental diet for 1 week were given subcutaneous injections of the cell suspension (0·1 ml in the abdominal area). After 4 weeks of continued feeding *ad lib.* on these diets, suitable-size tumours for the investigation under question had developed. These were dissected out, weighed and stored frozen (−20°).

In a separate experiment rats were fed *ad lib.* on the experimental diets for a period of 4 weeks after which they were anaesthetized with diethyl ether and partial hepatectomies (70%) performed (Higgins & Anderson, 1931). The fraction of liver removed served as the normal resting (non-regenerating) liver and the remaining fraction was allowed to regenerate over 24 h after which time the animals were killed and the remaining liver (regenerating) removed. Liver samples were stored at −20° for further analysis.

Sample preparation for cAMP determination

Approximately 0·5 g of each of the tissues (resting liver, regenerating liver and tumour) was accurately weighed and homogenized using a dounce homogenizer (ten passes with the loose and twenty passes with the tight plunger) in 5 ml cold 0·25 M-sucrose solution containing 3 mM-theophylline, an inhibitor of cAMP phosphodiesterase (*EC* 3.1.4.17) (Burk, 1968).

The procedure for the preparation of samples for cAMP determination was a modification of the methods of Gilman (1970), Walton & Garren (1970) and Wastila *et al.* (1971). The previously described tissue homogenate was transferred into a dounce homogenizer and 3 vol. cold trichloroacetic acid (TCA) solution (with 3 mM-theophylline), prepared so as to give a final concentration of 60 g TCA/l on addition, was added to precipitate proteins. This solution was further homogenized and allowed to stand at 0° for 10 min. It was then centrifuged at 46000 ***g*** at 0° for 10 min (J2-21 centrifuge and JA-21 rotor; Beckman). The supernatant fraction was removed and washed three times with 10 vol. water-saturated diethyl ether. Residual diethyl ether was removed in a stream of hot (70°) air, the disappearance of the diethyl ether odour marking the end-point.

The method used for the determination of cAMP concentration was a protein-binding assay based on the method of Gilman (1970) and described fully in the cAMP test kit.

Statistical analysis

The statistical significance of differences between means and their standard errors was tested by Student's *t* test.

RESULTS

Growth of animals

In a separate study it was found that the growth of rats fed on the Zn-deficient and Zn-excess diets was lower than that of the control group even when the controls were pair-fed with the Zn-deficient and Zn-excess groups (Fig. 2). In the main experiment, however, all dietary groups were fed *ad lib.* and growth of animals in this study was similar to that reported above.

Weight and cAMP concentration of tumours

The mean weights of tumours removed from rats fed on the Zn-deficient and those fed on the Zn-excess diets were both lower than of those from the control group, but this reduction was not statistically significant (Table 1). This was accompanied by an increase in the cAMP concentration of the tumours from both the Zn-deficient and Zn-excess animals of 40 and 30% respectively compared with the control group, that of the Zn-deficient group being statistically significant ($P < 0·05$).

cAMP concentration of normal resting and regenerating livers

There was essentially no change in the cAMP concentration of normal resting livers from both the Zn-deficient and Zn-excess animals when compared with the corresponding control (6 and 0·9% respectively; Table 2). The increase in cAMP concentration of

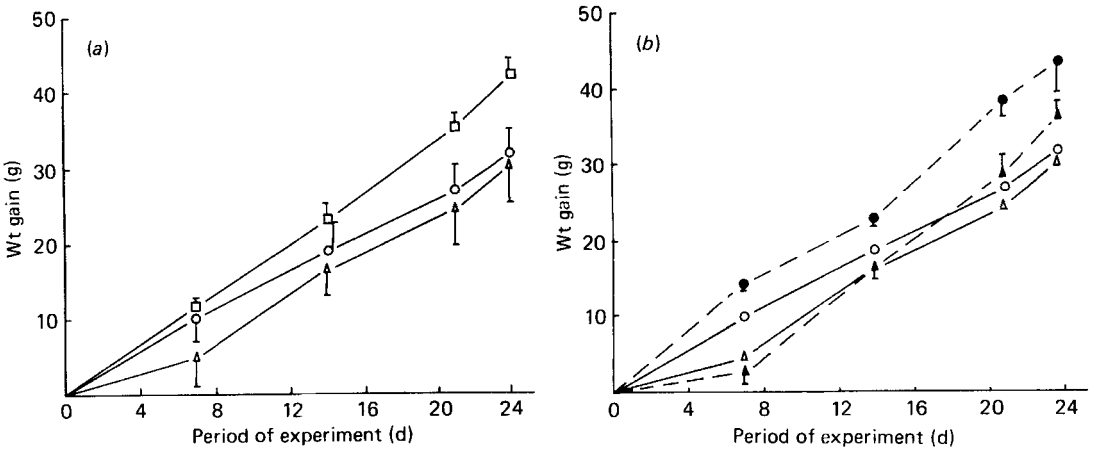

Fig. 2. Effect of dietary zinc on the weight gain of rats. (*a*) Groups 1 (○), 2 (△) and 3 (□) fed *ad lib.* on Zn-deficient, Zn-excess and control diets respectively. (*b*) Groups 4 (●) and 5 (▲) were given the control diet in an amount equivalent to that consumed by groups 1 (○) and 2 (△) respectively. Points represent means from four animals with their standard errors representated by vertical bars. For details of diets, see p. 438.

Table 1. *Effect of dietary zinc on the growth and cAMP concentration of type 350 hepatomas grown subcutaneously in rats for a period of 4 weeks*

(Mean values with their standard errors for eight samples)

	Weight of tumour (g)		cAMP concentration (pmol/mg tissue)	
Diet†	Mean	SE	Mean	SE
Zn-deficient	2·87	0·55	1·158*	0·131
Control (*ad lib.*)	4·97	0·96	0·825	0·074
Zn-excess	3·23	1·04	1·074	0·095

Significantly different from control value: * $P < 0{\cdot}05$. † For details, see p. 438.

Table 2. *Effect of dietary zinc on cAMP concentration in resting and regenerating rat livers from rats fed on a Zn-deficient, control or Zn-excess diet for 4 weeks*

(Mean values with their standard errors for eight samples)

	cAMP concentration (pmol/mg tissue)			
	Resting liver		Regenerating liver	
Diet*	Mean	SE	Mean	SE
Zn-deficient	0·564	0·052	0·760	0·061
Control (*ad lib.*)	0·532	0·037	0·641	0·025
Zn-excess	0·527	0·023	0·706	0·056

* For details, see p. 438.

regenerating livers from Zn-deficient and Zn-excess animals (19 and 10% respectively when compared with the corresponding control), though not significant, was greater than that of the normal resting livers but less than that of the tumours.

An interesting comparison was the relative concentrations of cAMP in the three tissue types examined. Both regenerating liver and tumour tissue had higher levels than normal resting liver, with that of the tumours being the highest (Tables 1 and 2).

DISCUSSION

The observed effect of the Zn-deficient diet in reducing the growth of rats is in agreement with results reported by other workers (Prasad, 1979; Huang *et al.* 1982). The similar effect of the Zn-excess diet is particularly interesting and supports earlier observations of a similar effect of these extreme dietary conditions (Duncan & Dreosti, 1975; Minkel *et al.* 1979). The fact that pair-fed controls had a moderately higher weight gain than the corresponding Zn-deficient and Zn-excess groups indirectly indicated that it was unlikely that the effects of the experimental Zn diets on the other variables examined were due to a difference in energy intake but were a direct result of the different Zn levels in the diets.

Increased cellular cAMP concentrations have generally been associated with reduced cell proliferation (Otten *et al.* 1971; Sheppard, 1971, 1972). Since both a dietary Zn-deficiency and Zn-excess have been found to result in reduced tumour growth (Duncan *et al.* 1974; Duncan & Dreosti, 1975) and, furthermore, since there have been indirect indications of a requirement for Zn by Δ6-desaturase (Cunnane, 1982; Huang *et al.* 1982), a relation between Zn, cAMP and cell proliferation, as indicated in Fig. 1, is implicated. In the present study although the reduction in hepatoma growth due to Zn deficiency was not significant, the latter was associated with a significant elevation in cAMP concentration. While Zn excess did reduce tumour growth and elevate cAMP levels to a certain extent this effect was not significant in either case. These findings suggest that it is only Zn deficiency and not Zn excess that has a stimulatory effect on cAMP synthesis, and that this effect is selective for the hepatoma cells. While the apparent inverse relation between cAMP and cell division would seem to be in support of the hypothesis being investigated, the fact that stimulation of cAMP accumulation resulted from a Zn deficiency rather than from a Zn excess would seem to contradict this hypothesis. It would therefore seem from these results that the regulation of cell division through the mechanism proposed earlier (p. 437) is not necessarily valid in the cells used in the present study. However, the possibility still exists that in other cell types Zn may regulate tumour cell growth through this mechanism. What the exact site of action of Zn may be remains to be identified by more detailed investigations of the pathways concerned.

Further studies should perhaps be done using in vitro cultured tumour cells to define more clearly the exact role of Zn in these cells and should attempt to investigate more directly the possible relation between Zn and Δ6-desaturase activity. Preliminary studies with this in mind have been carried out in this laboratory using another cancer-cell line and have indicated that Zn addition to the culture medium stimulates Δ6-desaturase activity (N. S. Skeef and J. R. Duncan, unpublished results).

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