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Effect of age, weight and adequacy of zinc intake on the balance between alkaline ribonuclease and ribonuclease inhibitor in various tissues of the rat

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1. Deficiency of zinc inhibits growth and also increases the activity of alkaline ribonuclease in certain tissues of the rat (Prasad & Oberleas, 1973). Zn could influence ribonuclease activity by direct effects on the enzyme or its natural inhibitor, or non-specifically as occurs when growth rate is affected by various other factors. These possibilities were studied.

2. Alkaline ribonuclease was shown to be inhibited by Zn in vitro, but the concentrations of Zn required were so high that the enzyme was probably not directly affected by changes in tissue Zn concentration caused by dietary deficiency.

3. At lower concentrations, Zn added in vitro increased the activity of alkaline ribonuclease in tissue homogenates probably by inactivating the inhibitor of the enzyme.

4. Age, weight and particularly food restriction caused tissue-specific alterations of ribonuclease and ribonuclease inhibitor concentrations in liver, kidney, oesophagus, testis and thymus.

5. The ribonuclease activities in liver, kidney and testis of Zn-deficient rats were unaltered in comparison with those of pair-fed rats. In thymus, which decreased in weight in the Zn-deficient animals, there was a concomitant increase in ribonuclease activity, but in oesophagus, the deficiency reduced the activity of ribonuclease.

6. The effects of Zn deficiency upon alkaline ribonuclease and its inhibitor are probably secondary consequences of reductions in food intake or growth.

Dietary zinc deficiency restricts the growth of animals (Mills, Quarterman, Chesters, Williams & Dalgarno, 1969) and has been found to cause a reduction in the RNA concentration of tissues (Macapinlac, Pearson, Barney & Darby, 1968; Somers & Underwood, 1969; Prasad & Oberleas, 1973). Macapinlac *et al.* (1968) did not, however, observe a corresponding reduction in the rate of RNA synthesis and suggested that the deficiency may result in an increased catabolism of RNA. Decreased availability of Zn also reduced the stability of 28S rRNA relative to that of 18S rRNA in phytohaemagglutinin-stimulated lymphocytes (Chesters, 1975). Prasad & Oberleas (1973) found increased activities of both acid and alkaline ribonucleases in kidney, thymus and testis of Zn-deficient rats and suggested that these may be partially responsible for the growth retardation found in Zn deficiency.

The activity of alkaline ribonuclease in tissues varies with age and growth rate (Kraft & Shortman, 1970*a*, *b*; Groves & Sells, 1971) and is determined by an interaction between the total concentration of alkaline ribonuclease and that of a natural inhibitor of the enzyme (Roth, 1956). In most tissues the balance is such that in addition to measurable alkaline ribonuclease activity there is also free ribonuclease inhibitor capable of inhibiting added pancreatic ribonuclease (*EC* 3.1.4.22) (Shortman, 1961). Both the enzyme and the inhibitor, which contains sulphydryl groups essential for its activity, can be inactivated in vitro by certain heavy metal ions (Roth, 1956; Eichhorn, Clarke & Tarien, 1969; Buri, Merlin & Vaillant, 1972). Dietary Zn deficiency might, therefore, influence ribonuclease activity by direct effects of metal ions on either the enzyme or its inhibitor. Alternatively, changes in enzyme activity might be consequences of the changes in growth rate induced by Zn deficiency. The significance of the enzyme in the reduction of growth caused by Zn deficiency depends on which of these possibilities is correct and an investigation of the alternatives is described below.

MATERIALS AND METHODS

Animals and their management

Groups of nine male Hooded Lister rats of the Rowett Institute strain were given the semi-synthetic, Zn-deficient diet described by Williams & Mills (1970) supplemented with 10 g inositol hexaphosphoric acid, sodium salt and 13·5 g calcium carbonate/kg diet. The basic diet contained less than 0·6 mg Zn/kg and for control groups it was supplemented with $ZnSO_4 . 7H_2O$ to give a dietary Zn concentration of 100 mg/kg. The animals were individually housed in Perspex-and-glass cages described by Quarterman, Williams & Humphries (1970). Two groups (*ad lib.* 1 and *ad lib.* 2) were offered the Zn-supplemented diet *ad lib.* for 6 and 36 d respectively and a third was offered the Zn-deficient diet *ad lib.* for 35 d. The rats of a fourth group (restricted controls) were given the Zn-supplemented diet for 36 d, but after the first day, were individually pair-fed with rats of the Zn-deficient group.

Supernatant fractions of tissue homogenates

At the end of the dietary treatments, the rats were killed by a blow on the head and the required tissues were removed, rapidly frozen in glass vials and stored at $-20°$ until required.

For assay for ribonuclease and its inhibitor, the whole organ, or approximately 1 g of liver, was weighed, suspended in 0·1 M-NaCl at 0° and twice homogenized at 0° for 30 s with a type X1020 homogenizer (International Laboratory Apparatus, Dottingen).

Centrifugation of the homogenate at 1500 *g* for 10 min at 4° yielded a supernatant fraction which contained all the alkaline ribonuclease and ribonuclease inhibitor activity of the homogenate. Before these activities were assayed the supernatant fractions were diluted appropriately with 0·1 M-NaCl.

Assay of alkaline ribonuclease and ribonuclease inhibitor

RNA and gelatin

Since heavy metals can interfere with the assay of ribonuclease and ribonuclease inhibitor activity (Shortman, 1961), it was necessary to purify the RNA and gelatin before use. Yeast RNA (Type VI, Sigma Chemical Co., London) was purified by the method of Shortman (1961) except that the initial procedure was replaced by that of Loening (1969).

Gelatin (4 g/l, British Drug Houses, Poole, Dorset) was dissolved in 100 mM-EDTA and then dialysed against three changes of distilled water to remove both EDTA and contaminating heavy metals.

Alkaline ribonuclease

The alkaline ribonuclease activity of a tissue preparation, the balance between total ribonuclease and ribonuclease inhibitor concentrations, was estimated by measuring the release of acid-soluble fragments from added high molecular weight RNA. Incubation mixtures were prepared in duplicate at 0° and contained: 0·25 ml 0·2 M-Tris-HCl pH 7·8, 0·1 ml of 50 mM-EDTA pH 7·8 (omitted in specified assays), 0·25 ml of diluted supernatant fraction and water to a final volume of 0·75 ml. For determination of the total ribonuclease concentration of the preparation, 0·1 ml 10 mM-*p*-hydroxymercuribenzoate was also included to inactivate the ribonuclease inhibitor. Assays were started by addition of 0·25 ml RNA (10 g/l), incubated at 37° for 30 min, then transferred to ice and 1 ml conc. HCl–ethanol–water (80:10:10, by vol.) was added. The tubes were centrifuged 15 min later and the extinctions of the supernatant fractions were measured at 260 nm. Extinctions were correc-

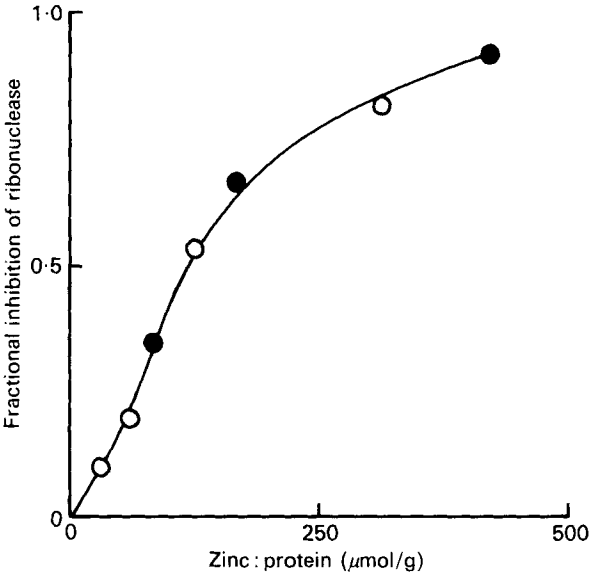

Fig. 1. Relationship between the inhibition of total ribonuclease activity from liver and testis and the ratio, zinc: total protein concentration in the incubation mixture. Activities were measured in the presence of *p*-hydroxymercuribenzoate but in the absence of EDTA and were expressed relative to the total ribonuclease activity measured in the absence of added Zn. (○—○), testis, total activity 1·4 U/assay, total protein 1·6 mg/assay; (●—●), liver, total activity 2·6 U/assay, total protein 1·2 mg/assay.

ted for values obtained when the mixtures were kept at 0° and the substrate was added at the end of the incubation period.

The unit of ribonuclease activity, as defined by Kraft & Shortman (1970*a*), was that quantity of enzyme which produced the same increase in extinction at 260 nm as did 1 ng pancreatic ribonuclease (*EC* 3.1.4.22; 4×crystallized, Koch-Light, Colnbrook, Bucks) assayed under the same conditions.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Free ribonuclease inhibitor

Concentrations of free ribonuclease inhibitor were estimated by the method of Kraft & Shortman (1970*a*) from the reduction in activity of pancreatic ribonuclease when assayed in the presence of the supernatant fraction. The incubation conditions were the same as for the assay of ribonuclease activity except for the inclusion of 50 μl pancreatic ribonuclease (40 μg/l) dissolved in gelatin (10 g/l). Under the conditions used there was a linear relationship between the quantity of supernatant fraction added and the inhibition of activity of the pancreatic enzyme, one unit of inhibitor being the amount required to halve the activity of 2 ng pancreatic ribonuclease (Kraft & Shortman, 1970*a*).

RESULTS

Effect of Zn on ribonuclease activity in vitro

With supernatant fractions from both liver and testis, addition of Zn reduced the total alkaline ribonuclease activity assayed in the presence of *p*-hydroxymercuribenzoate. Although both the initial enzyme concentration and the extent of inhibition by added Zn varied with the source of the enzyme preparation, the fractional inhibition of the enzyme by

Table 1. *Effects of adding Zn to the incubation mixture on the activity of ribonuclease from testis and liver of rats*

(Assays were performed in duplicate in the absence of EDTA and of *p*-hydroxy mercuribenzoate. The values for total ribonuclease were 1·43 and 2·58 U/assay for testis and liver respectively)

	Ribonuclease activity (U†/assay)				
Added metal ion concentration (μM) . . .	0	50	100	200	
Tissue					SE*
Testis	0·12	0·57	0·93	0·83	0·02
Liver	0·18	0·72	2·12	2·02	0·03

* Over-all estimate of SE of means obtained by analysis of variance.
† Defined by Kraft & Shortman (1970*a*).

Zn was the same for both tissues when the concentration of Zn added to the incubation mixture was expressed relative to that of the total protein from the supernatant fraction (Fig. 1).

In the absence of *p*-hydroxymercuribenzoate, addition of Zn increased ribonuclease activity (Table 1) probably as a result of inactivation of the ribonuclease inhibitor. At the highest concentration of added Zn, this stimulation of activity was partially counteracted by an inhibitory effect of Zn directly on the enzyme. The latter could be estimated, however, from the results described above and when allowance was made for it, there appeared to be a progressive inactivation of ribonuclease inhibitor by added Zn.

Effects of addition of EDTA to the assay mixtures

Since metal ions released during homogenization or present as contaminants can inactivate both ribonuclease and its inhibitor, Shortman (1961) suggested addition of EDTA to the incubation mixtures. However, when measuring in vitro the effects which heavy metals have produced in vivo, it would not normally be advisable to add a chelator to the assay mixture and therefore the effects of adding EDTA were investigated before it was used in later experiments.

These investigations showed that addition of EDTA to the incubation mixture did not reverse an inactivation of ribonuclease inhibitor if this had been caused by pre-incubation with Zn at 37°, but would substantially reduce that occurring during contact between inhibitor and Zn at 0°. Its addition to the incubation mixture also prevented direct inhibition of the enzyme by Zn regardless of the temperature of the pre-incubation.

Addition of EDTA seemed unlikely therefore to reverse an inactivation of the inhibitor by Zn which had occurred in vivo but it would reduce subsequent inactivation during homogenization and processing of the tissues at 0°. Since its addition was also beneficial to the estimation of total ribonuclease activity, EDTA was added to all subsequent incubation mixtures.

Effects of age, weight and food intake on the balance between ribonuclease and ribonuclease inhibitor

The effects of age and food restriction on the balance between ribonuclease and ribonuclease inhibitor were examined with three groups of rats, two of which had been fed *ad lib.* and the third restricted in food intake by being pair-fed with a group of Zn-deficient rats (Table 2). The restricted-fed rats were approximately the same weight as those of the younger group of animals fed *ad lib.* (*ad lib.* 1) but were the same age as those of the older and heavier group (*ad lib.* 2).

Table 2. *Effects of age, weight and food intake on ribonuclease activity, total ribonuclease concentration and free ribonuclease inhibitor in tissues of the rat*

(The rats of *ad lib.* 1 were 112 g mean body-weight, 35 d of age, *ad lib.* 2, 269 g mean body-weight, 65 d of age and the restricted group 133 g mean body-weight, 65 d of age. All assays were performed in the presence of 5 mM-EDTA. Results are expressed as means and standard errors for nine replicates from each group and the significances of the differences were determined by the Mann-Whitney *U* test)

		Liver		Kidney		Testis		Thymus		Oesophagus	
	Group	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Ribonuclease activity* (U/g tissue)	*Ad lib.* 1	10·1	$1·2^{a}$	865	46^{a}	2·0	$0·4^{a}$	3·3	$0·6^{a}$	3·4	$3·9^{a}$
	Ad lib. 2	8·3	$1·4^{a}$	486	21^{b}	1·7	$0·8^{a}$	2·7	$1·3^{a}$	12·9	$2·9^{ab}$
	Restricted	11·4	$0·7^{a}$	299	27^{c}	1·8	$1·0^{a}$	6·6	$2·0^{a}$	36·9	$12·4^{b}$
Total ribonuclease* (U/g tissue)	*Ad lib.* 1	304	8^{a}	1290	48^{a}	73·8	$3·4^{a}$	139	7^{a}	326	43^{ab}
	Ad lib. 2	280	9^{a}	929	19^{b}	65·8	$2·2^{a}$	144	4^{a}	402	35^{a}
	Restricted	349	9^{b}	728	37^{c}	44·7	$2·2^{b}$	165	4^{b}	223	34^{b}
Free ribonuclease* inhibitor (U/g tissue)	*Ad lib.* 1	3051	213^{a}	270	19^{a}	4843	157^{a}	3078	131^{a}	1066	131^{a}
	Ad lib. 2	3250	82^{a}	100	13^{b}	3206	87^{b}	2660	146^{a}	1056	91^{a}
	Restricted	2178	163^{b}	151	24^{b}	3833	174^{c}	2011	152^{b}	215	44^{b}

* Values without a common superscript letter were significantly different ($P < 0·05$).

Table 3. *Comparison of the ribonuclease activities, total ribonuclease concentrations and free ribonuclease inhibitor concentrations of various tissues of Zn-deficient and pair-fed control rats*

(The Zn-deficient rats (−Zn) were 109 g mean body-weight, 64 d of age and the pair-fed controls (+Zn) were 133 g mean body-weight, 65 d of age. All incubation mixtures contained 5 mM-EDTA. Results are expressed as means of nine values for each of the two groups together with the standard error of the differences between the individual values for paired rats. Significance was assessed by Student's paired *t* test)

		Liver	Kidney	Testis	Thymus	Oesophagus
Ribonuclease activity (U/g tissue)	+Zn	11·4	299	1·84	6·6	36·9
	−Zn	11·0	292	2·16	15·6	23·8
	SE	2·0	55	1·51	6·6	12·3
Total ribonuclease (U/g tissue)	+Zn	349	728	44·7	165	223
	−Zn	327	668	39·6	145	121
	SE	21	40	3·0	10	38*
Free ribonuclease inhibitor (U/g tissue)	+Zn	2178	151	3833	2011	215
	−Zn	2392	181	4365	1215	281
	SE	458	52	280	219**	61

Significance of differences: * $P < 0.05$, ** $P < 0.01$.

In agreement with the findings of Kraft & Shortman (1970*a*) the ratios of inhibitor to enzyme in four of the tissues, liver, oesophagus, testis and thymus, were such that only a small fraction of the total ribonuclease was active. In each of these tissues, restriction of food intake reduced the free ribonuclease inhibitor concentration compared with that of animals of the same weight fed *ad lib.* In testis alone, there was also a significant reduction in free inhibitor concentration in the heavier animals of *ad lib.* 2. Only in the oesophagus, however, did the reduced free inhibitor concentration associated with restriction of food intake cause a statistically significant increase in ribonuclease activity, the latter occurring despite a parallel decline in total ribonuclease concentration.

In contrast to the other tissues, there was little free inhibitor in kidney and a much higher proportion of the total ribonuclease was active. Increasing age and restricted food intake decreased both the ribonuclease activity and the total ribonuclease concentration of this tissue, the effect of restricted food intake being quantitatively more significant.

Effects of dietary Zn supply on the concentrations of ribonuclease and ribonuclease inhibitor

The ribonuclease activity, total ribonuclease concentration and free ribonuclease inhibitor concentration of liver, kidney and testis were unaltered in Zn-deficient rats when compared with pair-fed controls (Table 3). The thymus showed a marked decrease in weight with Zn deficiency, the mean weights being Zn-deficient, 0·16 g; Zn-adequate, 0·28 g with a SE of the difference 0·01 g. This was associated with a lower free inhibitor concentration and a markedly increased ribonuclease activity. In contrast, Zn deficiency resulted in reduced total ribonuclease concentration and lower ribonuclease activity in the oesophagus.

DISCUSSION

Inhibition of ribonuclease by Zn added in vitro has been confirmed in the present studies but its physiological significance is uncertain. It seems likely that the degree of inhibition was determined by the ratio, Zn:total protein in the incubation mixture (Fig. 1). In Zn-adequate rats, typical concentrations of Zn in vivo are 2·5 and 4 μmol/g protein for liver

and testis respectively (Williams & Mills, 1970) and, from the results presented in Fig. 1, it seems unlikely that these Zn concentrations would produce significant direct effects on ribonuclease activity. Furthermore, changes in Zn concentration caused by dietary deficiency of Zn are slight (Williams & Mills, 1970) and are even less likely to alter ribonuclease activity directly.

In biological systems, the effects of Zn and copper are often antagonistic (Davies, 1974) and Cu had been shown to inactivate ribonuclease inhibitor (Buri *et al.* 1972). If Zn had protected the inhibitor from inactivation by Cu, Zn deficiency could have resulted in lower free inhibitor concentrations and increased ribonuclease activity. In practice, however, Zn was found to inactivate the inhibitor. Low tissue concentrations of Zn would tend therefore to favour high values for free inhibitor and could not explain the high ribonuclease activities previously observed in Zn-deficient rats (Prasad & Oberleas, 1973).

The effects of age, weight and restricted food intake differed between tissues, those caused by food restriction generally being the most significant. The role of Zn in the balance between ribonuclease and ribonuclease inhibitor was, therefore, assessed by comparison of the activities of the Zn-deficient tissues with those from pair-fed animals. The effects of Zn deficiency on the activity of ribonuclease were less consistent in the present experiments than those found by Prasad & Oberleas (1973). The pair-fed rats in the latter investigations were, however, offered quantities of diet equal to the average daily intakes of the group of deficient rats and were not subject to the large fluctuations in daily intake which are characteristic of Zn-deficient rats (Williams & Mills, 1970) and which were experienced by the individually pair-fed animals of the present studies. Furthermore, although significant alterations to the balance between ribonuclease and its inhibitor were found in the present experiments in both thymus and oesophagus, the changes were in opposite directions in the two tissues. Regression of the weight of the thymus in the Zn-deficient rats was associated with increased ribonuclease activity but in oesophagus, there was a decrease in activity in the deficient group. In contrast to the over-all reduction in growth caused by Zn deficiency there is a large increase in the mitotic index of the oesophageal mucosa of deficient rats (Fell, Leigh & Williams, 1973). Zn deficiency also produces a rise in the mitotic index of buccal mucosa which is caused by an increase in the proportion of dividing cells in the basal layer rather than by an extension of the duration of mitosis (Alvares & Meyer, 1973). The decrease in oesophageal ribonuclease activity in Zn deficiency may therefore relate to an increase in the rate of cell division in the mucosa.

It seems likely that the effects of dietary Zn deficiency on tissue ribonuclease activities are manifestations of a generalized response to alterations in growth rate rather than direct effects of Zn on the enzyme system.

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