The effect of zinc deficiency on alkaline phosphatase (*EC* 3.1.3.1) and its isoenzymes

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1. Zinc deficiency in young rats reduced both the total alkaline phosphatase (*EC* 3.1.3.1) activity and Zn concentration in serum, kidney, small intestine and femur.

2. Addition of 0·01 mM-exogenous Zn had no greater activating effect with extracts of kidney, small intestine and femur from Zn-deficient than control rats, indicating that the main effect of the deficiency was on the amount of enzyme present rather than the efficiency of its operation. Exogenous Zn increased the activity of enzyme in serum of Zn-deficient rats, but it was still lower than in the serum of control animals.

3. Electrophoresis on polyacrylamide gel separated the alkaline phosphatase activity from all tissues into two bands. The bands had similar electrophoretic mobilities and appeared to be qualitatively identical in corresponding tissues from Zn-deficient and control rats.

4. Zn deficiency eliminated the first band found in serum from control rats and it had selective effects on the activity of individual bands in other tissues. The major inhibitory effect was on the first bands of enzyme activity in kidney and femur, but in small intestine only the second band was affected. In liver the activity of the first band was increased and that of the second band decreased by similar amounts.

Alkaline phosphatase (*EC* 3.1.3.1) is a zinc-metalloenzyme that also requires magnesium for activity and specific dietary deficiencies of either Zn (Prasad *et al.* 1967; Kfoury *et al.* 1968) or Mg (Loveless & Heaton, 1976) have been found to lower the alkaline phosphatase activity in serum and several cellular tissues from experimental animals. Controversy has, however, arisen as to whether the changes observed during Zn deficiency are due directly to the deficiency itself (Huber & Gershoff, 1973), or whether they are secondary to the associated inanition (Luecke *et al.* 1968).

It is generally accepted that isoenzymes of alkaline phosphatase occur in different mammalian tissues, but it now appears that different forms of the enzyme may also exist within the same tissue (Saini & Done, 1972; Ramadoss *et al.* 1977; Yokota, 1978). Recent work indicates that Mg deficiency has differential effects on the forms of alkaline phosphatase within the same rat tissue (Nehlawi & Heaton, 1979) and the present investigation was undertaken to determine whether Zn deficiency also has selective effects on individual components of the enzyme.

EXPERIMENTAL

Animal management

Twenty-four weanling male Wistar albino rats weighing approximately 70 g were randomly divided into Zn-deficient (2·8 mg/kg diet) and control (98 mg/kg diet) groups. They were housed, four to a cage, in stainless-steel cages within a room with 12 h light–12 h dark controlled illumination, and were fed on the appropriate diets for 29 d. All rats received an amount of food equal to that consumed by the Zn-deficient animals: this gave an initial intake of 10 g/rat per d reducing to 4 g/ rat per d. The food was supplied from automatic feeding apparatus (Loveless *et al.* 1972) to prevent any difference in feeding pattern and glass-distilled water was provided *ad lib.* Cages were cleaned with EDTA to remove traces of Zn and the synthetic diets were prepared by mixing (g/kg): casein 200, sucrose 660,

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arachis oil 80, cod-liver oil 20, salt mixture containing major minerals and trace elements 40, and purified vitamins, as described previously (Alfaro & Heaton, 1973). The diets contained 5 mg copper/kg and 120 mg iron/kg and were identical in composition except for the addition of zinc acetate to the control.

Rats were killed by exsanguination from the heart under light diethyl ether anaesthesia at the end of the experimental period and perfused through the aorta with 20 ml ice-cold 0·25 M-sucrose. Serum was separated and the liver, small intestine, spleen, both kidneys and both femora were removed as soon as possible. The lumen of the small intestine was flushed with distilled water to remove the contents and all tissues were stored individually at $-20°$ until taken for analysis.

The effect of storage on the electrophoretic mobility of alkaline phosphatase was investigated with tissues from four stock colony male rats weighing approximately 120 g that had received commercial animal cubes (41B; E. Dixon & Sons Ltd, Ware, Herts.) and been kept in glavanized cages.

Extraction of enzyme from tissues

The complete liver, small intestine, spleen and both kidneys were weighed and homogenized separately with 1 ml glass-distilled water/g at 0° for 5 min, using a top-drive homogenizer (MSE Scientific Instruments, Crawley, Sussex). A portion (2 ml) of each homogenate, corresponding to 1 g fresh tissue, was further homogenized with 5 ml 10 mM-Tris-hydrochloric acid buffer, pH 7·4, and 3 ml butan-1-ol for 10 min at 0° (Morton, 1953). After standing at 37° for 15 min, the homogenates were centrifuged and the lower aqueous layers separated and retained for enzyme assay. Other portions of the original distilled water homogenates were taken for Zn determination to avoid any contamination during the extraction of enzyme. Alkaline phosphatase was extracted from bone by grinding the complete left femur from each rat to a powder and allowing the powder to autolyse in 10 ml distilled water saturated with chloroform for 3 d at room temperature (Conyers *et al.* 1967); the clear extract obtained after centrifugation was used for assay.

Analytical methods

Serum was prepared for Zn analysis by deproteinization with HCl and trichloroacetic acid (Gubler *et al.* 1952). Portions of the soft-tissue homogenates in distilled water were dry ashed in silica crucibles by heating in a muffle furnace at 500° for 24 h. The right femur from each rat was dissolved in 1 ml concentrated nitric acid in a silica crucible, dried at 105° for 24 h and ashed at 500° for 24 h; the residue was redissolved in HNO_3 and the procedure repeated. Zn was determined in deproteinized serum or tissue ash dissolved in 2 M-HCl with an atomic absorption spectrophotometer (Unicam SP 90).

Alkaline phosphatase activity was measured at pH 10·0 in appropriately diluted serum and tissue extracts by a modification of the phenyldisodium phosphate hydrolysis method described by Wootton (1964). Assays were conducted in a basic reaction medium containing 20 mg Mg/l to provide uniform conditions for activation of the enzyme. One unit of enzyme activity is defined as the amount which liberated 1 μmol phenol/min at 37° and it is expressed per ml serum, per g liver, kidney, spleen or femur, and per mg intestinal protein. The effects of additional Zn were examined by adding Analar zinc acetate to the basic medium.

Protein was determined in homogenates of small intestine by the Lowry method (Lowry *et al.* 1951) using bovine serum albumin (Sigma (London) Chemical Co. Ltd, Poole, Dorset) as a standard.

The statistical significance of differences was assessed by Student's t test using group comparisons, except in the studies with exogenous Zn and the effect of storage on electrophoretic mobility where a paired analysis was employed.

Separation of alkaline phosphatase isoenzymes

Solutions of serum or tissue extracts containing approximately 0·24 enzyme units/ml were mixed with an equal volume of sucrose solution (0·3 g/ml) and 40 μl of each mixture was placed on top of a gel consisting of a 40 g acrylamide/kg spacer gel (20 × 5 mm) followed by an 80 g acrylamide/kg main gel (90 × 5 mm). The gels were prepared in 0·14 M-Tris-borate buffer, pH 9·5, containing 0·5 mM-magnesium chloride using acrylamide and N,N'-methylenebisacrylamide specially purified for electrophoresis (BDH Chemicals Ltd, Poole, Dorset). Electrophoresis was performed in a cold-room at 4° and the reservoir buffer was 0·05 M-Tris-borate, pH 9·5, containing 5 mM-sodium chloride and 2 mM-$MgCl_2$. Each run compared the same tissue from Zn-deficient and control rats. A current of 1 mA/tube was applied until the samples reached the end of the spacer gels, in approximately 10 min, and the current was then raised to 3 mA/tube and maintained constant for exactly 3 h.

Bands with alkaline phosphatase activity were visualized by incubating the gels with α-naphthyl phosphate (100 mg/l; Koch-Light Laboratories Ltd, Colnbrook, Bucks) and 10 mM-$MgCl_2$ in 0·05 M-borate buffer, pH 9·7, for 30 min at 37° and staining in the dark for 30 min at 4° with Fast Blue BB (300 mg/l; Sigma Ltd) and 10 mM-$MgCl_2$ in the same buffer. The gels were washed with distilled water and clarified with destaining solution containing methanol–acetic acid–glycerol–distilled water (100:20:6:100 by vol.). They were kept immersed in distilled water until measured.

The distances moved by the alkaline phosphatase bands were measured from the junction of the 40 and 80 g acrylamide/kg gels to the centre of each band. Intensity of staining was determined by scanning each gel with a Chromoscan recording densitometer (Joyce-Loebl, Gateshead) using light of wavelength 595 nm. The area under each peak on the resulting chart was measured with the Chromoscan integrator and related to the total for the whole gel; the enzyme activity associated with each band was obtained by applying the ratios for distribution in the gel to the known activity of enzyme that was placed on the gel.

RESULTS

Severe growth retardation was observed in the Zn-deficient rats whereas the control animals, which consumed the same amount of food, grew slowly but steadily throughout the experimental period. The mean ($\pm$ SEM) final body-weights (g) in the two groups were 67 ± 4 and 122 ± 2 respectively. Three deficient rats died shortly before the others were killed and visual signs of the Zn deficiency included rough, spiky hair, alopecia with dermal lesions and a kangaroo-like posture in some animals.

The Zn concentration decreased by approximately 30% in femur and 40–50% in serum, kidney and small intestine of the deficient rats (Table 1) and in all instances it was accompanied by an approximately corresponding reduction in alkaline phosphatase activity. No significant change was found in either Zn concentration or enzyme activity in the liver ($P > 0·3$ and $P > 0·1$ respectively) and the alkaline phosphatase in spleen was also unaffected by the deficiency ($P > 0·1$).

Isoenzymes of alkaline phosphatase

Electrophoresis on polyacrylamide gel separated the alkaline phosphatase from all tissues of control rats into two bands (Fig. 1). Only one band of enzyme activity was found in serum from Zn-deficient rats and this corresponded to the second, most mobile, band in control animals. All the other tissues from deficient rats gave two bands with alkaline phosphatase activity and detailed comparisons of the mobility of individual bands (Table 2) indicated that they were qualitatively identical in corresponding tissues from Zn-deficient and control rats.

Table 1. *Zinc concentrations and alkaline phosphatase (EC 3.1.3.1) activities in tissues from zinc-deficient and control rats*

(Mean values with their standard errors for nine rats in each group)

Tissue	Group	Zn (μg) Mean	Zn (μg) SE	Alkaline phosphatase (units†) Mean	Alkaline phosphatase (units†) SE
Serum (/ml)	Control	2·09	0·05	0·34	0·02
	Zn-deficient	1·27	0·06***	0·16	0·02***
Liver (/g)	Control	24·0	0·2	0·41	0·01
	Zn-deficient	23·4	1·3	0·39	0·02
Kidney (/g)	Control	19·0	0·4	20·5	0·5
	Zn-deficient	9·8	0·3***	10·6	0·4***
Small intestine (/mg protein)	Control	2·90	0·02	1·47	0·04
	Zn-deficient	1·70	0·04***	0·90	0·06***
Spleen (/g)	Control	—		1·68	0·01
	Zn-deficient	—		1·70	0·01
Femur (/g)	Control	153·8	2·3	62·0	0·9
	Zn-deficient	109·4	2·8***	34·7	1·3***

Value significantly different from control: ***$P < 0.001$.
† One unit of activity is defined as the amount which liberated 1 μmol phenol/min at 37 °.

Fig. 1. Distribution of alkaline phosphatase (*EC* 3.1.3.1) from serum and tissues of control rats after polyacrylamide gel electrophoresis. A, serum; B, liver; C, kidney; D, small intestine; E, femur.

Quantitative assessment of the staining intensity on the gels, however, indicated that Zn deficiency had selective effects on individual components of the enzyme. In addition to eliminating the first band found in the serum of control rats, it reduced the activity of the band corresponding to the second component (Table 3). The activities of both bands in kidney and femur were also significantly reduced, but in these tissues the major effect of Zn deficiency was again on the first band. Conversely in small intestine the total reduction in

Table 2. *Mobilities* (distance moved (*mm*) *from start of the main gel*) *of alkaline phosphatase* (*EC* 3.1.3.1) *isoenzymes* (*bands* 1 *and* 2) *from tissues of zinc-deficient and control rats on polyacrylamide gel electrophoresis*

(Mean values with their standard errors for nine rats in each group)

		Band 1		Band 2	
Tissue	Group	Mean	SE	Mean	SE
Serum	Control	26·6	0·4	31·6	0·4
	Zn-deficient	—		31·9	0·4
Liver	Control	16·9	0·3	27·1	0·3
	Zn-deficient	16·5	0·2	27·8	0·4
Kidney	Control	11·6	0·2	18·7	0·4
	Zn-deficient	12·1	0·2	18·4	0·2
Small intestine	Control	17·7	0·3	26·6	0·3
	Zn-deficient	17·5	0·4	26·8	0·4
Femur	Control	20·5	0·4	25·3	0·3
	Zn-deficient	21·1	0·4	24·9	0·3

Table 3. *Activity* (*units*†; *U*) *of individual alkaline phosphatase* (*EC* 3.1.3.1) *isoenzymes* (*bands* 1 *and* 2) *in tissues from zinc-deficient and control rats*

(Mean values with their standard errors for nine rats in each group)

		Band 1		Band 2	
Tissue	Group	Mean	SE	Mean	SE
Serum	Control	0·154	0·001	0·196	0·002
(U/ml)	Zn-deficient	—		0·164	0·001***
Liver	Control	0·306	0·001	0·128	0·004
(U/g)	Zn-deficient	0·331	0·002***	0·097	0·004***
Kidney	Control	12·25	0·06	9·27	0·07
(U/g)	Zn-deficient	2·52	0·04***	8·15	0·46*
Small intestine	Control	0·810	0·010	0·660	0·010
(U/mg protein)	Zn-deficient	0·780	0·010	0·120	0·010***
Femur	Control	46·2	0·2	22·6	0·2
(U/g)	Zn-deficient	16·3	0·2***	19·9	0·9*

Value significantly different from control: $*P < 0·05$, $***P < 0·001$.
† One unit of activity is defined as the amount which liberated 1 μmol phenol/min at 37°.

enzyme activity was due to the second band and no change was found in the least mobile component ($P > 0·1$). Liver was intriguing, because although there was no change in total alkaline phosphatase activity, Zn deficiency caused an increase in activity of the first band that was almost exactly balanced by a decrease in activity of the second band.

Addition of exogenous Zn

After completion of the electrophoretic studies the effect of additional Zn was examined with the remaining enzyme extracts. Addition of sufficient Zn to the sera from deficient rats (0·82 μg/ml) to raise the metal concentration to that found in control sera significantly increased the alkaline phosphatase activity from $0·14 \pm 0·01$ U/ml to $0·25 \pm 0·02$ U/ml, but it was still lower ($P < 0·01$) than the activity of $0·32 \pm 0·01$ U/ml found in sera from the control animals.

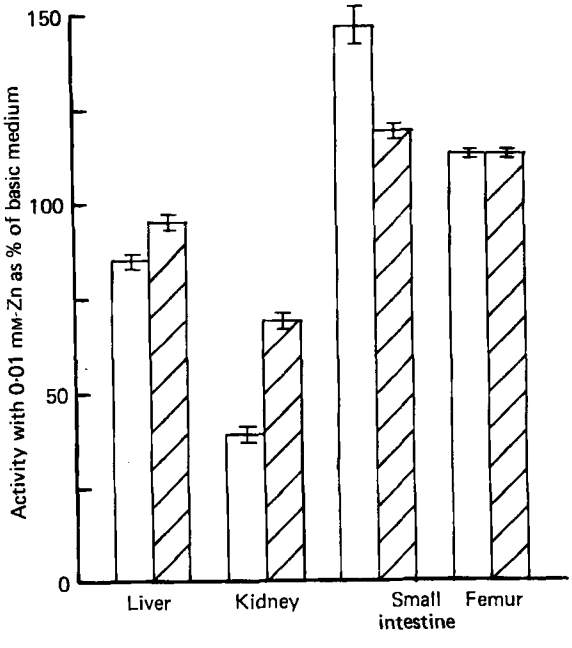
Alkaline phosphatase activity in the residual extracts of liver, kidney, small intestine and

Table 4. *Effect of exogenous zinc on alkaline phosphatase (EC 3.1.3.1) activities (units†; U) of tissues from Zn -deficient and control rats*

(Mean values with their standard errors; nine rats in each group for liver, five rats in each group for kidney, small intestine and femur)

Tissue	Group	Basic medium Mean	Basic medium SE	Basic medium +0·01 mM-Zn Mean	Basic medium +0·01 mM-Zn SE	Statistical significance of difference between activities with and without exogenous Zn
Liver (U/g)	Control	0·39	0·02	0·33	0·02	$P < 0·01$
	Zn-deficient	0·39	0·03	0·37	0·03	$P > 0·2$
Kidney (U/g)	Control	17·4	1·7	6·7	0·4	$P < 0·001$
	Zn-deficient	9·1	1·7***	6·3	0·9	$P < 0·01$
Small intestine (U/mg protein)	Control	1·15	0·12	1·69	0·28	$P < 0·01$
	Zn-deficient	0·67	0·10***	0·80	0·13***	$P < 0·01$
Femur (U/g)	Control	59·9	2·5	67·4	4·0	$P < 0·01$
	Zn-deficient	33·6	4·5***	37·9	5·2***	$P < 0·01$

Value significantly different from control: * $P < 0·05$; *** $P < 0·001$.
† One unit of activity is defined as the amount which liberated 1 μmol phenol/min at 37°.



Figs. 2. Effect of 0·01 mM-exogenous zinc on activity of alkaline phosphatase (*EC* 3.1.3.1) from tissues of control (□) and Zn-deficient (▨) rats. Mean values with their standard errors represented by vertical bars for nine rats for liver and five rats for kidney, small intestine and femur.

femur from control and Zn-deficient rats was measured both in the basic reaction medium and in medium containing 0·01 mM-Zn, the effect of exogenous Zn being assessed by paired *t* test. Additional Zn decreased the activity of enzyme from the liver of control rats and the

Table 5. *Mobilities (distance moved (mm) from start of the main gel) of alkaline phosphatase (EC 3.1.3.1) isoenzymes (bands 1 and 2) from tissues of stock rats on polyacrylamide gel electrophoresis after storage for various periods*

(Mean values with their standard errors for four rats)

		Period after killing (d)					
		0		7		33	
Tissue	Band no.	Mean	SE	Mean	SE	Mean	SE
Serum	1	26·6	0·1	24·7	0·1***	23·0	0·1***
	2	31·7	0·2	31·1	0·1*	27·5	0·1***
Liver	1	16·7	0·1	16·3	0·1**	16·2	0·1**
	2	21·9	0·1	23·2	0·4*	27·0	0·1***
Kidney	1	11·7	0·1	11·5	0·2*	11·1	0·1**
	2	19·0	0·3	18·0	0·1*	17·5	0·2**
Small intestine	1	26·5	0·1	21·9	0·3***	17·1	0·1***
	2	31·6	0·1	27·2	0·1***	26·5	0·2***
Femur	1	19·5	0·2	18·5	0·1*	18·4	0·1**
	2	25·8	0·1	24·2	0·1***	24·2	0·1***

Value significantly different from that on day of killing by paired t test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

kidney of both Zn-deficient and control rats (Table 4), but it increased the alkaline phosphatase activity from small intestine and femur of both groups of animals. The additional Zn had a proportionately greater effect in increasing the activity of enzyme from the small intestine and decreasing it with enzyme from the liver and kidney of control rats than it did with the corresponding tissues from Zn-deficient rats (Fig. 2), but it produced the same percentage change in activity with the enzyme from femur of both groups of rats.

Enzyme storage and electrophoretic mobility

Serum samples were subjected to electrophoresis on the day the experimental animals were killed, but because of the need to extract the enzyme from other tissues and the number of samples involved, an average delay of 7 d occurred between killing the rats and carrying out electrophoresis on extracts from cellular tissues. In order to determine whether storage of alkaline phosphatase affected the mobility of isoenzymes, tissues were obtained from four stock colony rats and portions of the extracts were electrophoresed on the day of killing and at various times thereafter.

The results, which are summarized in Table 5, showed that storage significantly altered the electrophoretic mobility of all enzyme bands and the effect usually appeared to be progressive with time. The mobility of liver band 2 increased with storage but that of all other bands decreased. The magnitude of the changes was only approximately 5% over the 33 d period of study with liver band 1 and both bands from kidney and femur, but with liver band 2 and both bands from serum and small intestine it was considerably greater and of practical significance in relation to the identification of particular bands.

DISCUSSION

The reductions in alkaline phosphatase activity observed in serum and several cellular tissues from Zn-deficient rats appear to be due directly to the Zn deficiency and not secondary to the associated general nutritional failure as suggested by Luecke *et al.* (1968). In the present investigation care was taken to standardize conditions and equate both the total food

intake of deficient and control animals and the circadian pattern of food consumption, because differences in feeding frequency have been found to affect alkaline phosphatase activity (Loveless & Heaton, 1976). However, the design of the experiment smoothed out any effect of the cyclical pattern of food intake observed in individual Zn-deficient rats (Chesters & Will, 1978), and it did not exclude the possibility that differences between the growth rates of the experimental and control rats might affect enzyme activity. Nevertheless the selective effects of Zn deficiency on particular isoenzymes of alkaline phosphatase, which will be considered in detail later, differed from those observed during Mg deficiency (Nehlawi & Heaton, 1979) in serum, kidney, small intestine and femur, indicating that the changes are likely to be caused by deficiencies of the specific metals rather than by more general secondary effects.

The activating effect of exogenous Zn added to the serum of deficient rats indicates that Zn deficiency reduces the efficiency of operation of the alkaline phosphatase in serum, but as the activity was still lower than that observed in serum from control animals it implies that the amount of enzyme present is also reduced and this is confirmed by the disappearance of the first electrophoretic band found in control sera. As additional Zn had no greater activating effect with extracts from the small intestine and femur of Zn-deficient rats than control animals (Fig. 2) it appears that the reduced alkaline phosphatase activity in these organs during Zn deficiency is due predominantly to a decrease in the amount of enzyme present. The inhibitory effect of additional Zn on kidney extracts implies that the reduced kidney alkaline phosphatase activity during Zn deficiency must also be due to a decrease in the amount of enzyme present.

Addition of sufficient Zn inhibits the alkaline phosphatase from all tissues, possibly by displacing Mg from its activating site (Cathala *et al.* 1975; Linden *et al.* 1977). The differences observed between tissues in response to exogenous Zn are difficult to interpret in detail, but they are consistent with the concept that intestinal alkaline phosphatase binds Zn less strongly than the enzyme from liver or kidney (Adeniyi & Heaton, 1979), with that from femur presumably occupying an intermediate position.

The well-defined electrophoretic mobilities of the various enzyme bands (Table 2) suggest the presence of at least seven different forms of alkaline phosphatase in the tissues studied. Whether the bands differ in sialic acid content or some other way is at present unknown. Identification of a particular isoenzyme from its electrophoretic mobility is complicated by the fact that the movement of all bands is affected by storage, even at low temperatures. For example, comparison of serum with tissue extracts when all were electrophoresed on the day the animals were killed (Table 5) indicates that the two serum bands both originate from small intestine, whereas in previous work, when comparisons were made after some unavoidable storage of cellular tissues, it appeared that serum band 1 corresponded to liver band 2 (Nehlawi & Heaton, 1979).

Unfortunately it is not possible to conclusively prove the identity of the alkaline phosphatase bands observed in the present work with those in the Mg-deficiency study (Nehlawi & Heaton, 1979). This is partly due to differences in ionic strength of the buffers but mainly to the fact that the distance travelled on electrophoresis is influenced by the purity of the reagents used to form the acrylamide gel and reagents of higher purity were used in this investigation than in the earlier work. Comparison of the distribution of enzyme activity between individual bands in the control rats of the two studies, however, indicates very close agreement between the components in most tissues and comparisons between the effects of Zn and Mg deficiency are therefore justified. The main exception is small intestine, where electrophoresis of the extract from the whole intestinal wall gave two bands in the present study, whereas the more concentrated extract from intestinal mucosa gave three bands in the previous work with one being a quantitatively minor component.

The decreased alkaline phosphatase activities observed during Zn deficiency in four of the tissues studied were in each instance due to a major effect of the deficiency on one component of the enzyme. In serum, kidney and femur the main effect was on the first band but in small intestine the second band was the only one affected (Table 3). If both alkaline phosphatase bands in serum do originate from small intestine, as discussed previously, the complete disappearance during Zn deficiency of the first band found in serum of control rats, while its parent band 1 in small intestine is unaffected, indicates that the deficiency preferentially inhibits the release of this form of enzyme into the blood. Mg deficiency also produced selective effects on individual components of the enzyme (Nehlawi & Heaton, 1979) but it differed from Zn deficiency in that its main effect on serum and kidney was to reduce the activity of the second alkaline phosphatase band, and it increased the activity of kidney band 1 and femur band 2 while Zn deficiency produced the opposite effect. Only in liver were the effects of the two deficiencies identical, increasing the activity of the first band and reducing the activity of the second band by a corresponding amount, which raises the possibility that the changes in liver may be secondary to a deficiency of the specific metals.

Zn deficiency therefore has selective effects on the individual components of alkaline phosphatase in many tissues. The differences between the individual enzyme bands are at present largely unknown and further work is necessary to determine whether they differ in affinity for Zn and to establish their characteristics.

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