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The Induction of Metallothionein in Rat Liver by Zinc Injection and Restriction of Food Intake

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The isolation of two forms of hepatic zinc-thioneins after either zinc injection into rats or partial restriction of their food intake is described. The proteins differed slightly in their amino acid composition and electrophoretic mobilities. Increases in liver zinc content after both treatments were synchronous with, and associated almost completely with, increased zinc-binding to these proteins. The time-course for the appearance and disappearance of the zinc proteins is shown. It is suggested that metallothionein is involved in the normal metabolism of zinc, perhaps in some temporary storage or detoxication capacity.

Although metallothionein was originally characterized as a cadmium- and zinc-binding protein from horse kidney (Kagi & Vallee, 1960, 1961), it is now evident that it occurs also in the livers of several species (Nordberg *et al.*, 1972; Winge & Rajagopalan, 1972; Weser *et al.*, 1973) and that its synthesis may be induced in kidney by administration not only of cadmium but also of mercury (Piotrowski *et al.*, 1974). The rapid incorporation and prolonged occurrence of these metals in this form stimulated the view that the role of the protein was the detoxication of heavy metals. However, metallothioneins which contain no cadmium or mercury, but only zinc and copper, have been isolated and characterized from the liver of the horse (Kagi *et al.*, 1974), sheep and calves (Bremner & Marshall, 1974*b*) and humans (Buhler & Kagi, 1974). Further, in sheep and calves the total amount of the hepatic copper and zinc occurring in this form is a linear function of the liver zinc content and therefore of the zinc status of the animals (Bremner & Marshall, 1974*b*). This suggests a possible involvement of zinc in the synthesis or stabilization of the protein and, moreover, a physiological role for the protein in the metabolism of these essential trace metals. The findings of Webb (1972) that injection of zinc salts into rats stimulated the rapid appearance of hepatic zinc in a similar low-molecular-weight form supports this view, although it was not unequivocally demonstrated that the protein produced was indeed metallothionein.

The present work, of which preliminary reports have been published (Bremner *et al.*, 1973; Davies *et al.*, 1973), describes the isolation and characterization of two forms of hepatic metallothionein in rats after either parenteral zinc administration or restriction of food intake. The time-course of appearance and disappearance of hepatic zinc from these forms is established and the possible role of the protein is discussed.

Experimental

Materials

Male Hooded Lister rats (Rowett Institute strain), usually weighing 150g, were used. The semi-synthetic diet given (Williams & Mills, 1970) contained less than 1mg of zinc/kg for zinc-deficient rats and 40mg of zinc/kg (added as $ZnSO_4$) for zinc-supplemented control rats. Sephadex G-75, DEAE-Sephadex A-25 and Blue Dextran were obtained from Pharmacia, Uppsala, Sweden, and Bio-Gel P-10 and Chelex-100 from Bio-Rad Laboratories, Richmond, Calif., U.S.A. All reagents were analytical-reagent grade and buffers were purified by passage through Chelex-100 columns. All glassware was acid-washed and rinsed with double-distilled water.

Copper and zinc determinations were made by atomic absorption by using a Techtron AA5 atomic absorption spectrometer (Varian Associates, Walton-on-Thames, Surrey, U.K.). All concentrations in liver refer to the fresh weight of tissue unless otherwise stated. Protein was generally measured by the method of Lowry *et al.* (1951), with bovine serum albumin [fraction V; Sigma (London) Chemical Co., London S.W.5, U.K.] as standard. The protein concentration of purified metallothionein solutions was calculated from their N content (×6.25). Superoxide dismutase activity was determined by the method of McCord & Fridovich (1969), and carbonic anhydrase activity by that of Roughton & Booth (1946). Thiol groups were measured by the method of Jocelyn (1962).

All preparative and column procedures were carried out at 0–4°C. Solutions were concentrated

by ultrafiltration under N_2 in a Diaflo cell, by using UM 2 filters (Amicon, High Wycombe, Bucks., U.K.). Polyacrylamide-gel electrophoresis was carried out by the method of Davis (1964), by using 105g of photopolymerized gel/litre which was run at 3mA/tube (80mm×5mm) until the Bromophenol Blue marker almost reached the end of the gel. The electrolyte contained 4.9mM-Tris and 38.5mM-glycine (pH8.3). Gels were stained with Amido Black. Amino acid analyses were performed by using a Locarte amino acid analyser (Locarte, London W12, U.K.) on samples hydrolysed at 100°C in 6M-HCl for 24h after performic acid oxidation (Moore, 1963).

Fractionation of metalloproteins: analytical studies

Pooled livers from three to five rats were homogenized in an Ultra-Turrax homogenizer (Janke and Kunkel KG, Staufen, Germany) at 1°C in 2.5vol. (v/w) of buffer, prepared by adjusting approx. 20mM-Tris to pH8.2 with acetic acid and diluting to 10mM. Homogenates were centrifuged immediately at 100000*g* ($r_{av.}$ 7.38cm) for 1h at 1°C in an MSE Superspeed 65 centrifuge. The supernatants were stored at −20°C where necessary before fractionation of 3.5ml samples on a column (900mm×26mm) of Sephadex G-75 equilibrated with 10mM-Tris–acetate (pH8.2) containing 100mg of NaN_3/litre. Metalloproteins were eluted with the same buffer at a flow rate of about 15ml/h, 5ml fractions being collected. The column was calibrated as described by Andrews (1965), by using the following proteins of known molecular weight: bovine serum albumin (68000), lactoglobulin (36800), chymotrypsin (25700), myoglobin (17200) and cytochrome *c* (12400).

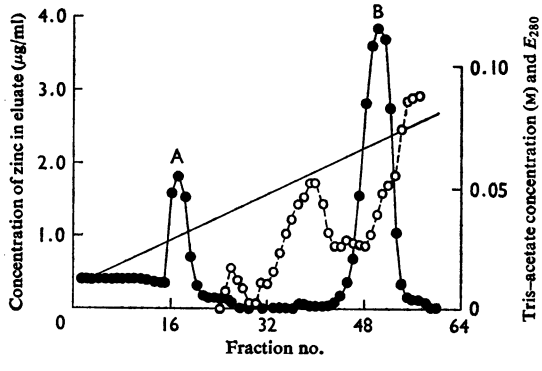

Fig. 1. *Separation on DEAE-Sephadex A-25 of two zinc-thioneins from rat liver*

The crude zinc-thionein fraction obtained by gel filtration on Sephadex G-75 was separated on DEAE-Sephadex A-25 into two components, A and B, as described in the Experimental section. Elution was with a gradient (——) of 10–80mM-Tris–acetate (pH7.4). The fractions collected were analysed for zinc (●) and E_{280} (○). The positions of fractions A and B are shown.

Isolation of metallothionein

There were slight variations in the procedures used for metallothionein isolation, mainly in the order of individual steps and in the concentrations of buffers, but, as the composition of the final products was invariably the same, only one procedure is described here.

Metallothionein production was induced in the livers of 25 rats either by intraperitoneal injection of 500μg of zinc (as $ZnSO_4$ in a solution containing 9g of NaCl/litre) 18h before death or by starvation of the rats for 36h. The livers were homogenized and centrifuged as described above, except that 1.5vol. (v/w) of 10mM-Tris–acetate (pH7.4) was used for homogenization. Initial fractionation of 50ml batches of supernatant on a column (600mm×50mm) of Sephadex G-75, equilibrated in the same buffer, gave three zinc-containing fractions. The third of these, eluted at between 720 and 860ml of eluent, contained metallothionein free from much of the other hepatic protein. This was concentrated by ultrafiltration to about 50ml and applied to a column (300mm×25mm) of DEAE-Sephadex A-25 pre-equilibrated in 10mM-Tris–acetate (pH7.4). The column was washed with the same buffer (500ml) and 11ml fractions were eluted with a linear gradient of 650ml of 10–80mM-Tris–acetate (pH7.4), at a flow rate of around 30ml/h. Two main zinc-containing fractions were eluted at Tris concentrations of about 30 and 70mM (Fig. 1). These were concentrated and desalted by repeated ultrafiltration and were further purified by chromatography on a column (900mm×26mm) of Bio-Gel P-10 equilibrated in 10mM-Tris–acetate (pH7.4). The zinc proteins were eluted with the same buffer at a flow rate of 10ml/h, 5ml fractions being collected (Fig. 2). The main zinc-containing fractions were combined, concentrated and desalted by repeated ultrafiltration. These solutions were used for characterization of the two zinc proteins.

Results

The object of the initial experiment was to determine the relationship between the zinc status of the rat, in particular its liver zinc content, and the occurrence of hepatic metallothionein. Unlike the situation in the ruminant animal (Bremner & Marshall, 1974*a*), it was found that very little zinc occurred in this form even in the normal rat. A typical separation of the supernatant from rat liver with mean zinc and copper contents of 29.6 and 8.5μg/g respectively is shown in Fig. 3. Three main

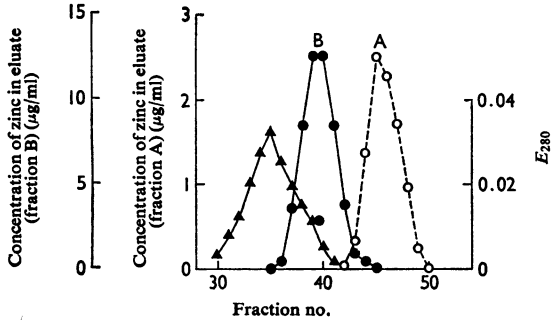

Fig. 2. *Separation on Bio-Gel P-10 of the two zinc-thioneins from rat liver*

The two zinc-thioneins from rat liver isolated after chromatography on DEAE-Sephadex A-25 were purified by gel filtration on Bio-Gel P-10 as described in the Experimental section. Elution was with 10mM-Tris–acetate, pH7.4. Concentrations of zinc for fractions A (○) and B (●) and E_{280} for fraction B (▲) are shown.

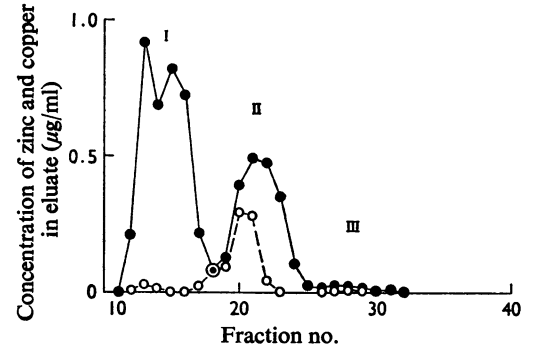

Fig. 3. *Fractionation on Sephadex G-75 of supernatant from livers of normal rat*

Livers were homogenized in 10mM-Tris–acetate buffer, pH8.2, then centrifuged at 100000*g* for 1h. The supernatant was then separated on Sephadex G-75 with the same buffer as eluent. Concentrations of zinc (●) and copper (○) are shown. The positions of fractions I–III are shown.

zinc- and copper-containing fractions (I–III) were identified, with approximate molecular weights as determined from their elution volumes of ≥65000, 35000 and 12000 respectively. Fraction I was not identified but was clearly heterogeneous. Fraction II was also heterogeneous and had both superoxide dismutase and carbonic anhydrase activities, the former enzyme associated mainly with the earlier copper-containing portion of the peak. Fraction III, a minor component accounting for less than 1.5% of the total hepatic zinc, showed little absorbance at 280nm and, as will be shown below, consisted mainly of metallothionein. The zinc contents in each of these fractions were 11.2, 5.5 and 0.4μg/g fresh wt. of liver respectively. The corresponding copper contents were 0.53, 2.47 and 0.12μg/g. These and subsequent values refer to the metal recovered in the 100000*g* supernatant. No correction was made for 'soluble' metal trapped in the pellet.

Effects of restriction of food intake

The rats used in the above study were given unrestricted access to the zinc-supplemented diet. However, in examining the effects of zinc deficiency on metalloprotein distribution, it was necessary to decrease the food intake of the control animals by about 50% to equal that of the zinc-deficient rats, which suffered a voluntary restriction of food intake 5 days after introduction to the low-zinc diet (Chesters & Quarterman, 1970). As a result of this the mean liver zinc content of the control rats increased within 1 day of food deprivation from 29.6 to 34.6μg/g and remained at about this value for the next 20 days. There was little change in the zinc content of fractions I and II, but a large proportion of the total increase could be accounted for by the increase of zinc associated with fraction III, which contained on average 2.1μg of liver, or 6% of the total hepatic zinc. In contrast, the voluntary restriction of food intake which occurred over the same period in the zinc-deficient rats, which had a mean liver zinc content of 28.1μg/g, did not increase zinc binding in fraction III, and the zinc distribution was identical at all times with that in control rats fed *ad libitum*. When rats, which had been given the zinc-supplemented diet in limited amounts for 20 days, were subsequently transferred to the zinc-deficient diet (7g/day), there was a gradual decline in zinc binding in fraction III over 6 days to the values found in rats fed *ad libitum*, suggesting this form of zinc was of limited biological stability.

The magnitude of the effect of restriction of food intake on hepatic zinc distribution depended on the degree of starvation and tended to be more evident in young rats. When the intake of a zinc-containing diet by rats weighing 70g was decreased from about 12g to either 7.8 or 2.1g in the 24h period before death, liver zinc contents increased and the proportion of the hepatic zinc present in fraction III rose to 12 and 19% respectively (Fig. 4). On total starvation of rats of weight 100–150g for 36–45h, this proportion increased to 19–26%.

As it had previously been observed that liver zinc contents were also increased during post-surgical trauma, an examination was made of rat livers taken 2 weeks after pancreatectomy or laparotomy. The proportion of hepatic zinc in fraction III had increased from less than 2% in the control rats to about 15%, whereas the zinc contents of fractions I and II were unchanged.

The contents of copper in the whole liver and in

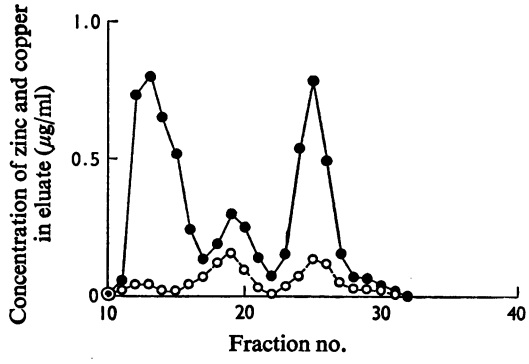

Fig. 4. *Fractionation on Sephadex G-75 of supernatant from livers of zinc-supplemented rats with restricted food intake*

Livers were obtained from rats whose food intake in the 24h before death was decreased by about 85%. The livers were homogenized in 10mM-Tris–acetate, pH8.2, and centrifuged at 100000*g* for 1h. The supernatant was then separated on Sephadex G-75 with the same buffer as eluent. Concentrations of zinc (●) and copper (○) are shown.

fraction III and of zinc in fractions I and II were only slightly affected by the above dietary treatments. There was, however, a tendency for the zinc content of fraction II to be greater in older rats.

Effects of zinc injection

As these results suggested a specific response in zinc-thionein production to these increases in liver zinc content above a basal value, the effects of injection of 300μg of zinc (as $ZnSO_4$ in a solution containing 9g of NaCl/litre) were investigated (see Bremner *et al.*, 1973; Davies *et al.*, 1973). In normal rats, fed *ad libitum*, there was a slight increase in the zinc content of both the whole liver and of fraction III within 2h of zinc injection. This continued rapidly to a maximum at 18h after injection, when 9.8μg of zinc/g of liver or 25% of the hepatic zinc was present in fraction III, but zinc concentrations started to fall thereafter. The zinc contents of fractions I and II were unchanged, and were identical with those found in control rats. Similar results were observed when zinc-deficient rats were studied. The distribution of zinc between the three fractions was the same as that in zinc-supplemented rats, and on zinc injection over 70% of the increase in liver zinc content was again associated with fraction III, fractions I and II being unaffected. In the zinc-deficient rats, however, maximum zinc contents in fraction III were attained earlier, at around 7h after injection, had decreased by about half by 24h and returned to control values by 3 days. Slight increases were also found in the copper contents of fraction III in livers with elevated zinc content.

The addition of zinc (10μg/ml as $ZnSO_4$) to either liver homogenates or supernatants several hours before fractionation did not increase the amount of zinc occurring in fraction III, suggesting that the apoprotein (thionein) was absent from the liver.

Isolation of zinc-thionein

Two forms of zinc-thionein were isolated from the livers of rats which had either been injected with zinc or had been starved for 36h. After preliminary separation on Sephadex G-75, fraction III was resolved by successive chromatography on DEAE-Sephadex A-25 and Bio-Gel P-10 into two zinc proteins which were homogeneous on polyacrylamide-gel electrophoresis. The distribution of zinc between these proteins appeared to be independent of the induction procedure. Zinc-thionein A, which accounted for 20–25% of the zinc in fraction III, was eluted first from the DEAE-Sephadex column, and had an electrophoretic mobility on polyacrylamide gels of 0.46 relative to the Bromophenol Blue marker. Zinc-thionein B had a mobility of 0.67 and, on the basis of its smaller elution volume from Bio-Gel P-10 columns (Fig. 2), had a slightly greater molecular weight than zinc-thionein A.

The amino acid composition of the two proteins and, for comparison, of sheep and human hepatic metallothioneins are shown in Table 1. The two rat proteins are basically similar insofar as they contain a very high proportion (30%) of cysteine residues. However, they differ in that isoleucine and leucine are present in only trace amounts in zinc-thionein A, which also contains less glutamic acid and alanine but more threonine, serine and glycine than protein B. In spite of these differences, both proteins are clearly of the metallothionein type. This is confirmed by the molar ratio of thiol group/Zn^{2+} in both proteins, about 3.2:1, and by the high zinc content of the protein, about 60mg/g.

Discussion

Zinc-thioneins have been isolated and characterized from the liver of ruminants (Bremner & Marshall, 1974*b*), humans (Buhler & Kagi, 1974) and the horse (Kagi *et al.*, 1974). Although it has been suggested that the zinc protein produced in rat liver in response to zinc administration is also of the metallothionein type (Webb, 1972; Bremner *et al.*, 1973; Chen *et al.*, 1974) this has never been proved. However, the isolation of two electrophoretically pure proteins with molecular weight, total metal content and amino acid composition similar to those of metallothionein, as originally isolated from horse kidney (Kagi & Vallee, 1960), provides unequivocal proof of the identity of those zinc-induced metal-binding species. The occurrence of more than one form is in accord with previous findings on hepatic

Table 1. *Amino acid composition of metallothionein from rat and human liver*

Rat zinc-thioneins A and B were isolated after injection of zinc or restriction of food intake. Amino acid analysis was carried out on samples hydrolysed in 6M-HCl for 24h after performic acid oxidation. The compositions of human zinc-thioneins 1 and 2 and of rat cadmium-induced metallothionein are taken from the papers of Buhler & Kagi (1974) and Winge & Rajagopalan (1972) respectively.

	Rat zinc-thionein		Human zinc-thionein		
	Form A (% of total residues)	Form B (% of total residues)	Form 1 (% of total residues)	Form 2 (% of total residues)	Rat metallothionein
Lysine	11.86	12.49	12.72	12.52	12.7
Arginine	0.15	—	—	—	0.8
Aspartic acid	7.11	6.74	4.71	6.54	7.6
Threonine	6.22	3.86	4.00	3.35	5.0
Serine	16.38	14.07	13.70	13.36	12.0
Glutamic acid	2.79	6.32	5.58	3.85	6.0
Proline	3.99	4.87	3.91	3.33	3.5
Glycine	9.71	7.34	7.94	8.27	8.5
Alanine	5.56	8.14	9.39	10.96	6.8
Cysteine*	29.3	27.2	30.2	33.1	28.0
Valine	3.17	2.37	3.49	1.80	3.4
Methionine†	1.99	1.75	1.35	1.40	1.7
Leucine	0.25	1.03	1.11	—	1.7
Isoleucine	0.34	1.74	1.92	1.53	1.7
Phenylalanine	0.15	0.54	—	—	0.8

* Measured as cysteic acid.
† Measured as methionine sulphone.

metallothioneins, whether induced by injection of cadmium in the rabbit (Nordberg *et al.*, 1972), or occurring naturally as the zinc protein in humans (Buhler & Kagi, 1974). In these cases, two forms of metallothionein were separated on the basis of different isoelectric points or by ion-exchange chromatography. However, no such heterogeneity has been demonstrated in horse, chick or rat hepatic metallothioneins (Kagi *et al.*, 1974; Weser *et al.*, 1973; Winge & Rajagopalan, 1972). All preparations have a typically high cysteine content of about 30% and usually no aromatic amino acid residues, but there are sometimes marked variations in the proportions of many other amino acids. For example, arginine, valine and leucine are present in only some preparations, and alanine and serine contents can range from about 6–16%. It is not known whether these variations result from species differences or whether the composition of the protein is dependent on the nature of the bound metals. As has been noted previously (Buhler & Kagi, 1974), microheterogeneity is indicated even in apparently pure preparations, since the numbers of mol of individual amino acids per mol of protein are frequently non-integers.

The molecular weights of both zinc proteins isolated from rat liver were calculated from gel-filtration behaviour in Sephadex G-75 to be about 12000, although their subsequent resolution on Bio-Gel P-10 may imply that their molecular weights differed slightly. These estimates agree well with similarly derived values for other metallothionein preparations (Kagi & Vallee, 1961; Weser *et al.*, 1973). More recently, however, it has been suggested that the molecular weight of the apoprotein as determined by sedimentation-equilibrium measurements and by gel filtration of oxidized apoprotein in the presence of guanidinium chloride is around 6000 (Buhler & Kagi, 1974; Kagi *et al.*, 1974). Such a discrepancy would be expected if metallothionein has a random-chain configuration, as has been suggested (Rupp *et al.*, 1974), since calibration of the Sephadex G-75 columns has always been by reference to globular proteins.

The ratio of thiol groups to zinc atoms was close to 3 in both rat liver proteins, in agreement with most previous findings on hepatic metallothioneins (Buhler & Kagi, 1974; Kagi *et al.*, 1974) and consistent with the view that each metal atom is bound to three cysteine residues in the protein (Kagi & Vallee, 1961). Other workers have suggested, however, that each metal atom is bound to two (Webb, 1972) or four thiol groups (Weser *et al.*, 1973). It is probable that the high metal-binding affinity of the proteins and their high metal content (6%) are a consequence of the high cysteine content.

These findings have therefore confirmed that the synthesis or stabilization of metallothionein can be influenced by zinc, an essential element (Webb, 1972; Bremner & Marshall, 1974*b*), despite the claim to the

contrary by Shaikh & Lucis (1970). Indeed, the production of the zinc protein after partial starvation of the rats suggests that it is involved in the normal metabolism of zinc. This may constitute one of the primary functions of metallothionein, and the binding of cadmium and mercury in this form may simply be a fortuitous consequence of the chemical similarities between all these elements.

Incorporation of cadmium and mercury into metallothionein (Nordberg *et al.*, 1971; Jakubowski *et al.*, 1970) and of copper into the analogous, but so far uncharacterized, protein in rat liver (Bremner & Davies, 1974) is preceded by binding of the metals to higher-molecular-weight proteins in the cytosol. When synthesis is induced by zinc, however, no such redistribution of zinc occurs and the increase in liver zinc content is synchronous with the appearance of most of that zinc as zinc-thionein. Metallothionein is therefore the major storage form of zinc in the liver regardless of whether it has been produced in acute or chronic experimental situations (Bremner & Marshall, 1974*b*). Unlike cadmium-thionein, which can persist in the liver for several months (Webb, 1972), zinc-thionein is readily mobilized and is probably metabolically available, as is shown by its disappearance a few days after production and by the apparent complete retention and utilization of an injected dose of zinc by zinc-deficient rats (N. T. Davies, unpublished work).

The rapid incorporation of zinc into metallothionein would be expected if zinc was merely binding to pre-existing thionein, but all attempts to demonstrate the presence of the apoprotein have failed. Further, studies on the inhibitory effect of cycloheximide on zinc-thionein production and on the incorporation of labelled lysine into the protein suggest that zinc induces the synthesis of metallothionein *de novo* (Davies *et al.*, 1973). Studies carried out *in vitro* with rat pancreas support these conclusions (Davies & Bremner, 1974), and show in addition that zinc-induced synthesis of the apparently similar protein is also inhibited by actinomycin D (N. T. Davies & I. Bremner, unpublished work). It cannot be excluded, however, that zinc serves to stabilize the apoprotein, which is being continually synthesized, but normally has a sufficiently short turnover time to prevent its accumulation in liver. Such a concept would be in accord with the known stabilizing effect of zinc on various macromolecules (Chvapil, 1973) and with its role in maintaining the conformational stability of enzymes such as alkaline phosphatase (Simpson & Vallee, 1969) and superoxide dismutase (Rotilio *et al.*, 1972).

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