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Zinc-Binding Protein in the Livers of Neonatal, Normal and Partially Hepatectomized Rats

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In the livers of rats after partial hepatectomy the zinc concentration began to increase soon after the operation, reached a maximum value at 14h, and decreased to the original value by 25h after the operation. In contrast, the plasma zinc concentration continued to decrease during the first 10h after the operation and remained depressed for at least 28h. The plasma and hepatic zinc concentrations were relatively unaffected by sham-operation. Synchronous with the increase in the hepatic zinc concentration after the partial hepatectomy, there was an appearance of zinc-binding protein (Zn-binding protein) in the liver cytosol. Studies with small doses of actinomycin D and cycloheximide suggest that both RNA and protein syntheses are necessary for the induction of Zn-binding protein after partial hepatectomy. A high content of the Zn-binding protein was found in neonatal rat liver. The Zn-binding protein, however, was undetectable 40 days after birth. The Zn-binding protein was also found in the adult rat liver when stimulated to proliferate after the administration of isoprenaline followed by glucagon. These findings indicate a close linkage between the appearance of Zn-binding protein in the liver cytosol and the regulation of DNA synthesis.

Zinc is one of the essential micronutrients for the growth of not only micro-organisms, but also animals including man (Underwood, 1971). An absolute requirement for zinc during DNA synthesis in animal cells has been indicated. The rate of DNA synthesis markedly decreased in the liver of zinc-deficient rats (Sandstead & Rinaldi, 1969). In partially hepatectomized rats, EDTA infusion resulted in the inhibition of the post-operative rise in the rate of DNA synthesis (Fujioka & Lieberman, 1964). This inhibition was specifically reversed by the addition of zinc in the infusate. Inhibition of increased DNA synthesis by EDTA and its reversal by zinc has also been found in cultured chick-embryo cells (Rubin, 1972) and primary cultures of rabbit kidney cells (Lieberman & Ove, 1962) as well as phytohaemagglutinin-stimulated lymphocytes (Chesters, 1972).

With regard to the total zinc concentration in the rat liver, Volm *et al.* (1974) reported an increase followed by decrease during the DNA-presynthetic period in partially hepatectomized rats.

In the present paper, we report that the changes in the hepatic zinc concentration after partial hepatectomy are synchronous with the appearance and disappearance of Zn-binding protein in the liver cytosol. The data reported herein also demonstrate that the Zn-binding protein appears in the liver of neonatal rats as well as in the liver of adult rats when DNA synthesis is induced by the administration of a combination of isoprenaline and glucagon. A preliminary account of this study has been published (Koga *et al.*, 1977).

Materials and Methods

Animals and reagents

Female rats of the Wistar strain (about 150g body wt.) were used for most of the experimental work. The animals were kept in separate cages with free access to food and water and with illumination between 08:00 and 20:00h each day.

[*methyl*-^{3}H]Thymidine (50Ci/mmol) was purchased from New England Nuclear Corp., Boston, MA, U.S.A. Actinomycin D (Makor Chemical, Jerusalem, Israel) was dissolved in a small volume of ethanol and diluted with physiological saline (0.9% NaCl) to the desired concentrations. Cycloheximide was from Boehringer Mannheim G.m.b.H., Mannheim, Germany. DL-Isoprenaline hydrochloride was from Sigma Chemical Co., St. Louis, MO, U.S.A. Glucagon was from Calbiochem, La Jolla, CA, U.S.A. Pronase E was from Kaken Chemical Co., Tokyo, Japan. All chemicals used were of reagent grade. Buffers and reagents were prepared in resin-deionized water.

Treatment of animals

For studies on the regenerating livers, rats were partially hepatectomized under light diethyl ether anaesthesia by removing about 70% of the liver

(Higgins & Anderson, 1931). The remaining lobes were examined at different time periods after the operation. Sham-operated rats were used as controls.

For studies on the livers of neonatal rats, the litters were allowed to stay in the same cage with the mother for 19 days after birth; then they were placed in individual cages.

For studies on the induction of hepatic DNA synthesis in the intact animal, each rat was injected subcutaneously with a saline solution of isoprenaline hydrochloride (40μg/ml; 1ml/100g body wt.) at zero time and at 1h intervals for the next 4h; glucagon (100μg/ml; 1ml/100g body wt.) was administered subcutaneously as a single injection at 6h, as described by the previous report of Hasegawa & Koga (1977), as scheduled in Table 3. The final injections were performed between 16:00 and 17:00h. DNA synthesis reached a maximum value at 20h after the first injection of isoprenaline.

Treatment of liver

Determination of total zinc concentration. Rats were killed by cervical dislocation at various time periods after partial hepatectomy or sham operation. The livers were immediately removed and sliced into about 1-mm thickness with a razor blade. The liver slices were blotted on a filter paper to remove blood. The zinc in the liver slices was extracted with 0.1M-HNO_3 overnight at room temperature. The concentration of zinc was determined by an atomic-absorption spectrophotometer (Shimadzu model MAF). Plasma was diluted with deionized water and the zinc concentration was measured directly by atomic-absorption spectrophotometry.

Subcellular fractionation. For studies on the intracellular distribution of zinc in liver fractionation was performed by the differential centrifugation method described by Appelmans *et al.* (1955). The zinc in each fraction was extracted overnight with 0.1M-HNO_3 and measured as described above.

Isolation of Zn-binding protein

Zn-binding protein was isolated from the liver cytosol fraction by the isolation method for hepatic zinc-thionein as reported by Feldman & Cousins (1976), with slight modifications as described below. Liver samples were immediately rinsed in ice-cold 0.25M-sucrose. All subsequent steps were carried out at 4°C. The tissue was weighed, minced and homogenized in 4vol. of ice-cold 0.25M-sucrose/0.01M-Tris/HCl buffer, pH8.2, in a Potter–Elvehjem-type homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 140000$g_{av.}$ for 60min to obtain the soluble (cytosol) fraction. A portion (usually 1.5–2.0ml) of the soluble fraction obtained from each liver sample was applied to a column (1.5cm×36cm) of Sephadex G-75 which had been equilibrated with 0.05M-Tris/HCl buffer, pH8.2. Elution was with the same buffer. The eluate was collected in fractions of 1.5ml. The concentration of zinc in each fraction was determined by atomic-absorption spectrophotometry. A_{280} of each fraction was also measured.

Pronase digestion

The peak-III fraction (see Fig. 2) was pooled and digested with Pronase. To the mixture containing 0.6ml of the peak-III fraction (42μg of Zn equivalent/ml of 0.17M-Tris/HCl buffer, pH8.2) and 0.2ml of 0.001% (w/v) of $CaCl_2,2H_2O$, 0.1ml of Pronase solution (0.5mg of Pronase/ml of 0.01M-Tris/HCl buffer, pH8.2) was added twice, at 0 and 10h. Digestion was carried out at 37°C for 30h. After the digestion, the whole reaction mixture was applied directly on a Sephadex G-50 column (1.2cm×38cm) which had been equilibrated with 0.1M-Tris/HCl buffer, pH8.2. The same buffer was used as eluent. The eluate was collected in fractions of 1.5ml. The concentration of zinc in each fraction was determined as described above.

Estimation of DNA synthesis and content

For studies on DNA synthesis in the rat liver, [*methyl*-^{3}H]thymidine (5μCi per rat) was given via the tail vein at 20h after the partial hepatectomy or after the first injection of isoprenaline. Then 1h later, the rats were killed and each liver was immediately homogenized in 10vol. of ice-cold 0.1M-citric acid. The radioactivity incorporated into nuclear DNA was measured by the method of Short *et al.* (1972).

DNA, extracted as described by Schmidt & Thannhauser (1945), was determined by the diphenylamine reaction (Burton, 1955).

Results

Zn-binding protein in regenerating livers

The changes in the plasma and hepatic zinc concentrations were followed in partially hepatectomized rats during the first 28h after the operation (Fig. 1*a*). The concentration of plasma zinc decreased gradually during the first 10h after partial hepatectomy, and remained depressed for at least 28h. A maximum decrease of a 40% was observed by 14h. Sham-operated rats showed a decrease of 15%. Fig. 1(*b*) shows the changes in the concentration of hepatic zinc in rats after partial hepatectomy. The concentration of hepatic zinc began to increase immediately

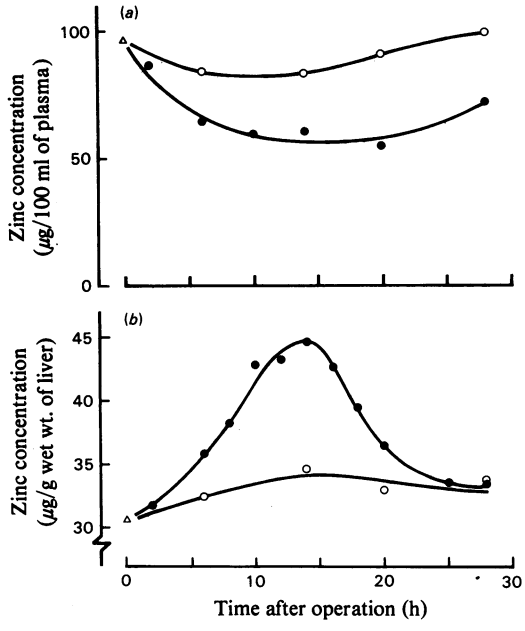

Fig. 1. *Changes in the plasma and hepatic zinc concentrations after partial hepatectomy*

The operation was carried out at zero time and the plasma and hepatic zinc concentrations were determined at intervals after operation as described in the Materials and Methods section. (*a*) Plasma zinc concentration after partial hepatectomy (●) or sham-operation (○). (*b*) Hepatic zinc concentration after partial hepatectomy (●) or sham-operation (○). △, Zero time value. Each point represents the average of results from three to five rats.

after the operation and reached a maximum of 50% increase at 14h. After this it returned to the original value by 25h. In contrast, only a slight increase was observed in the sham-operated rats.

The distribution of zinc among the subcellular fractions of rat liver was investigated in the next series of experiments (Table 1). Most of the increase in the total zinc concentration observed after the operation was accounted for by the increase in the concentration of zinc in the cytosol fraction. No significant change was observed in the particulate fractions.

In order to obtain further information about the increase in the hepatic zinc concentration after partial hepatectomy, the liver cytosol was analysed by the gel-filtration procedure on a Sephadex G-75 column. The elution profile of liver cytosol fraction obtained 14h after the operation showed that zinc was associated with three fractions (Fig. 2). Although the amounts of zinc in peaks I and II were similar in livers of normal, partially hepatectomized and sham-operated rats, there was a marked increase in the

Table 1. *Zinc distribution among rat subcellular fractions*

Zinc in each fraction was extracted with 0.1 M-HNO_3 and measured by atomic-absorption spectrophotometry. These values are typical of results obtained in three independent experiments.

Subcellular fraction	Zinc (μg/g wet wt. of liver) Normal liver	Regenerating liver	Change (%)
Original homogenate	30.4	50.4	+66
Nuclei (crude)	6.9	7.2	+ 4
Mitochondria	2.8	2.5	−11
Microsomes	5.2	5.5	+ 6
Final supernatant	14.8	31.4	+112
Recovery	29.7	46.6	

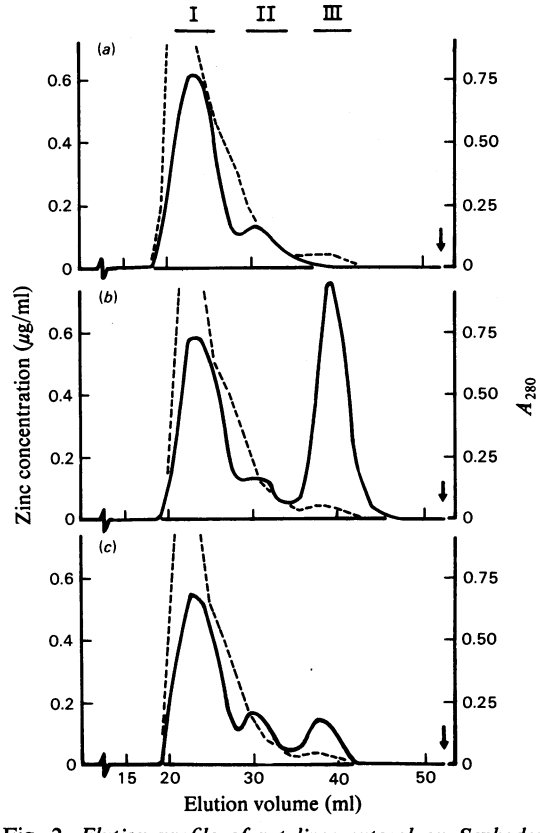

Fig. 2. *Elution profile of rat liver cytosol on Sephadex G-75 column*

Concentration of zinc (——), A_{280} (----) and the position of sucrose eluted (↓) are shown. (*a*) Normal rat liver; (*b*) partially hepatectomized rat liver; (*c*) sham-operated rat liver.

amount of zinc in peak III of partially hepatectomized rat liver. These three peaks represent 95% or more of the total zinc concentration placed on the

column, suggesting that there was little or no 'free zinc' in the liver cytosol of partially hepatectomized rats.

To examine whether a protein(s) is involved in the binding of zinc in the peak-III fraction, the fraction was treated with Pronase and subsequently analysed by the Sephadex G-50 fractionation (Fig. 3). After the treatment, the amount of zinc in peak III was markedly decreased and the distribution of zinc was shifted to more slowly eluting fractions. The accumulation of free zinc was also observed by the prolonged digestion extending to 30h. These results indicate that the zinc occurring in peak III is bound, in some way, to a protein(s) of low molecular weight.

The time course of appearance and disappearance of this Zn-binding protein in the liver cytosol fraction was followed in partially hepatectomized rats during the first 28h after the operation (Fig. 4). The Zn-binding protein content (expressed as μg of zinc equivalent/g wet wt. of liver) began to increase immediately after the operation, reaching a maximum value (8.5 times that found in sham-operated rats) at 14h and then declined gradually. The time course of the increase in the Zn-binding protein content was exactly the same as that of total zinc (see Fig. 1*b*).

In the next series of experiments, the effect of small doses of actinomycin D on the appearance of Zn-binding protein was examined in the liver of rats after partial hepatectomy. Rats were injected with actinomycin D or saline at the time of operation, and again 2h later, and killed 14h after the operation. A small dose of actinomycin D (5μg/100g body wt.) that is sufficient to delay the initiation of the post-operative rise in DNA synthesis (Fujioka *et al.*, 1963) had no inhibitory effect on the appearance of Zn-binding protein after the operation. In contrast, 20μg of actinomycin D/100g body wt. decreased the Zn-binding protein content to 64% of the control value (8.50μg of zinc equivalent/g wet wt. of liver in rats given actinomycin D as compared with 13.25μg of zinc equivalent/g wet wt. of liver in saline-treated rats). At 40μg of actinomycin D/100g body wt., the content decreased to about 35% of the control value.

The effects of cycloheximide on the appearance of Zn-binding protein and on the rate of DNA synthesis after partial hepatectomy are shown in Table 2. Injections of a small dose of cycloheximide (5μg/

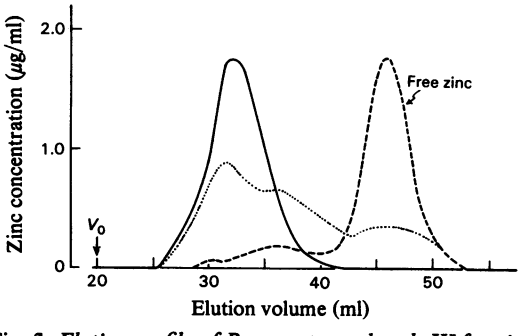

Fig. 3. *Elution profile of Pronase-treated peak-III fraction on a column of Sephadex G-50*
The conditions of the Pronase treatment and the fractionation of treated samples are described in the Materials and Methods section. The control was the zero-time value (——). Pronase treatment for 5h (····) and 30h (----) are shown.

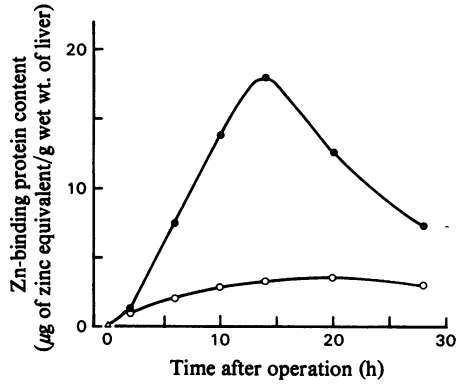

Fig. 4. *Changes in the Zn-binding protein content in the liver after partial hepatectomy*
The Zn-binding protein content in the liver cytosol was estimated from each elution profile on the Sephadex G-75 column as shown in Fig. 3. Zn-binding protein content in the liver after partial hepatectomy (●) and that in the liver after sham-operation (○) are shown. △, Zero time value.

Table 2. *Effects of cycloheximide on the Zn-binding protein content and on the rate of DNA synthesis in livers after partial hepatectomy*
Rats were injected intraperitoneally with cycloheximide (5 or 20μg/ml of saline per 100g body wt.) or saline at the time of operation and again 2h later. The Zn-binding protein content was determined at 14h after the operation as described in the Materials and Methods section and the legend to Fig. 3. The incorporation of [*methyl*-^{3}H]thymidine into nuclei was determined at 20h after the operation. Each value represents the mean±S.E.M. and the numbers of rats used are given in parentheses.

Injection	Total dosage (μg/100g body wt.)	Thymidine incorporation (d.p.m./mg of DNA)	Zn-binding protein content (μg of zinc equivalent/g wet wt. of liver)
Saline	—	47460±5030 (4)	12.21±1.37 (4)
Cyclo-heximide	10	17990±3610 (3)	7.96±0.35 (3)
	40	1170± 470 (3)	3.30±0.75 (3)

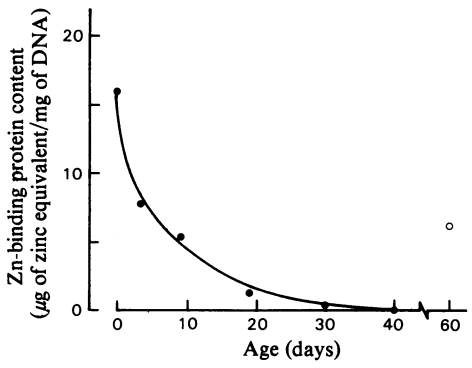

Fig. 5. *Changes in the hepatic Zn-binding protein content during development*

These are typical results of four independent experiments. The Zn-binding protein content in the developing liver (●) and that in the liver of 60-day-old adult rats determined at 14h after partial hepatectomy (○) are shown. Each point represents the average of results from two neonatal rats.

100g body wt.) at the time of operation and again 2h later reduced the Zn-binding protein content as well as the rate of DNA synthesis to 65 and 38% of the control values respectively. At 20μg of cycloheximide/100g body wt., the Zn-binding protein content decreased to 27% of the control values. This dose of the inhibitor entirely blocked the post-operative rise in the rate of DNA synthesis.

Zn-binding protein in developing livers

Zn-binding protein was also found in the liver cytosol of neonatal rats. To examine a linkage between the Zn-binding protein content and the liver development, the change in the Zn-binding protein content was studied in liver of rats of different age groups from early postnatal life to adulthood. Fig. 5 shows that the Zn-binding protein content is high in the liver of newborn rats. During the subsequent development, the content decreased rapidly and it became undetectable by 40 days after birth. The Zn-binding protein content in the intact newborn rat liver was 2.5 times that in the liver of partially hepatectomized adult rats at 14h after the operation.

Zn-binding protein in the liver of intact rats after the induction of DNA synthesis by isoprenaline and glucagon

In order to examine a possible significance of Zn-binding protein in the regulation of hepatic DNA synthesis, the appearance of Zn-binding protein was

Table 3. *Induction of DNA synthesis and Zn-binding protein content in liver of rats after administration of isoprenaline followed by glucagon, or partial hepatectomy*

Administrations of isoprenaline at 1h intervals for 4h followed by glucagon at 6h were performed as described in the Materials and Methods section. The dose of each compound was: DL-isoprenaline hydrochloride, 40μg/100g body wt.; glucagon, 100μg/100g body wt. At 14h after the first injections or after partial hepatectomy, the liver samples were removed and the Zn-binding protein content was determined as described in the Materials and Methods section and in the legend to Fig. 3. The incorporation of [*methyl*-^{3}H]thymidine into nuclei was determined at 20h after the first injections or after partial hepatectomy. Each value represents the mean± S.E.M. and the numbers of rats used are given in parentheses.

Treatment of animals	Thymidine incorporation (d.p.m./mg of DNA)	Zn-binding protein content (μg of zinc equivalent/g wet wt. of liver)
None	1720± 320 (4)	<0.05
Isoprenaline, glucagon	15040±1300 (3)	8.35±0.38 (4)
Partial hepatectomy	43830±4350 (4)	11.07±0.79 (4)

further investigated in the liver of intact rats when DNA synthesis was induced by injections of isoprenaline followed by glucagon (Hasegawa & Koga, 1977). Table 3 shows that isoprenaline followed by glucagon caused large increases in the Zn-binding protein content and in the rate of DNA synthesis (75 and 34% respectively of the values of partially hepatectomized rats).

Discussion

In the present study it is shown that the increase in the hepatic zinc concentration in the DNA-presynthetic period after partial hepatectomy is synchronous with the appearance of Zn-binding protein in the liver cytosol (see Figs. 1*b* and 4). Zn-binding protein is therefore the major storage form of zinc in the liver after the operation. In contrast with the hepatic zinc, a significant decrease in the plasma zinc concentration occurred simultaneously in rats after partial hepatectomy. These findings seem to indicate that a primary source of the zinc, which accumulates in the liver after the operation, is the plasma. Richards & Cousins (1975) reported that administration of zinc salts produced a transient increase in plasma zinc concentration and this increase caused the synthesis of hepatic zinc-storage protein, metallothionein. The discrepancy in the plasma concentration of zinc between our results and those reported by Richards & Cousins (1975) may be attributed to the initial stimuli used in each study,

although the mechanism(s) by which zinc is transferred from blood carrier(s) to intracellular ligand is unknown at present.

The appearance of Zn-binding protein in the liver of rats after partial hepatectomy is sensitive to small doses of actinomycin D and of cycloheximide. Moreover, in unoperated rats the addition of zinc (as $ZnSO_4$) to either liver homogenates or supernatants $\frac{1}{2}$h before the Sephadex fractionation did not increase the amount of zinc in peak III (data not shown). These findings suggest that the binding protein (apoprotein) is absent from the liver and a synthesis *de novo* of this protein occurs after partial hepatectomy.

In the liver of newborn rats, a high content of Zn-binding protein is observed in the present experiments and the Zn-binding protein decreased rapidly to undetectable contents by 40 days after birth. Ferdinandus *et al.* (1971) reported that in the liver of newborn rats the rate of DNA synthesis was very high and decreased to very low rates in the adult. The time course of the decrease in the rate of hepatic DNA synthesis reported by them has a strong resemblance to the time course of the reduction in the Zn-binding protein content as shown in the present experiments. These findings suggest that the Zn-binding protein content in the liver correlates positively with the activity of the hepatic DNA synthesis.

In the liver of intact rats, the DNA synthesis could be induced efficiently by subcutaneous injections of isoprenaline followed by glucagon. These findings confirm the previous report by Hasegawa & Koga (1977), in which it was shown that DNA synthesis reached a maximum value at 20h and mitoses were observed at 28–30h after the first injection of isoprenaline. In the present experiments, a considerable amount of Zn-binding protein was found at 14h after the start of administration of isoprenaline followed by glucagon. These findings indicate that Zn-binding protein appears in the neonatal rat liver as well as in the DNA-presynthetic period of normal adult rat liver stimulated to proliferate.

Kägi & Vallee (1960, 1961) isolated metallothionein, soluble cadmium- and zinc-containing proteins, from equine renal cortex. The protein had a strong metal-binding activity, especially to zinc and cadmium, and contained a high amount of cysteine. It is now evident that the metallothioneins are also present in the liver and kidney of various animals including man (Nordberg *et al.*, 1972; Winge & Rajagopalan, 1972; Weser *et al.*, 1973; Bühler & Kägi, 1974).

It has been reported that zinc-thioneins occur also in the liver of rats after administration of zinc, and zinc bound to thionein (apoprotein) is indeed mobilized during a metabolic need for zinc (Bremner & Davies, 1975; Richards & Cousins, 1975; Chen *et al.*, 1977). From these findings, zinc-thioneins have been supposed to be involved in the normal metabolism of zinc, perhaps in some temporary storage, transport or detoxication capacity. The elution profile of liver cytosol from a partially hepatectomized rat on the Sephadex G-75 column as shown in the present study is similar to that of liver cytosol from a rat given zinc as reported by Richards & Cousins (1975). Although purification and full characterization of the Zn-binding protein remains to be established, it is possible that the Zn-binding protein in the present study is of a metallothionein type.

Although the function of Zn-binding protein in the DNA-presynthetic period is not known at present, the removal of zinc from the culture medium with EDTA impaired the synthesis of both thymidine kinase and DNA polymerase, and inhibited the DNA synthesis in primary cultures of rabbit kidney cells (Lieberman *et al.*, 1963). Speculation on the possible molecular basis for the zinc requirement ought to take into account the occurrence of zinc in several enzymes associated with nucleic acid synthesis, including DNA polymerase I (Slater *et al.*, 1971), RNA polymerase (Scrutton *et al.*, 1971) in *Escherichia coli*, DNA polymerase in sea urchin (Slater *et al.*, 1971), and the reverse transcriptase in several tumour viruses (Auld *et al.*, 1975). From these findings, it is suggested that Zn-binding protein is important in the synthesis or activity of enzymes required for nucleic acid metabolism as well as in the regulation of DNA synthesis.

Vallee (1977) reported important roles of zinc at critical points of metabolism, especially in nucleic acid metabolism, in normal and neoplastic growth processes. In contrast with the cultures of rabbit kidney cells, the depletion of zinc with EDTA had no inhibitory effect on the DNA synthesis in L cells and HeLa cells (Lieberman & Ove, 1962). Chick-embryo cells infected with Rous sarcoma virus were also reported to be less sensitive to inhibition by EDTA than normal cells (Rubin, 1972). Normal leucocytes contained substantial quantities of zinc, whereas leukaemic cells contained less than 10% of the zinc found in normal leucocytes (Vallee, 1959). We also found that a small amount of Zn-binding protein is present in actively proliferating ascites hepatoma cells (results not shown).

On the basis of these findings, it is hypothesized that zinc and Zn-binding protein may be involved in the regulation of normal growth rather than of neoplastic growth processes. The implications of these findings to normal and abnormal growth are currently being examined.

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