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BINDING OF BERYLLIUM AND OTHER DIVALENT METAL IONS TO FERRITIN IN RELATION TO METAL REQUIRING ENZYMES

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Daniel Jonathan Price

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ABSTRACT

The ability of ferritin to bind various divalent metal ions (Be^{2+}, Cd^{2+}, Zn^{2+} and Cu^{2+}) was documented. Information obtained concerning amounts of metal bound and affinity with which they were bound was quantitated for each metal binding to ferritin or apoferritin. Enzyme activity studies were conducted to test ferritin's ability to serve as a metal donor for enzymes activated by the metal or as a metal acceptor for enzymes inactivitated by the metal.

For studies of Zn^{2+} exchange, ferritin was isolated from Cd^{2+} injected, Zn^{2+} injected and control rats. Such ferritins contained between 1-9 gram atoms Zn^{2+} (ferritin-control < ferritin-Zn < ferritin-Cd). This ferritin bound Zn^{2+} was donated to Zn^{2+} requiring apo-enzyme forms of alkaline phosphatase, yeast phosphoglucomutase and yeast aldolase, resulting in the reactivation of these enzymes. Accumulation of Zn^{2+} in ferritin also suggested its role as a Zn^{2+} detoxicant for enzymes sensitive to Zn^{2+} excess.

The ability of ferritin and apoferritin to bind Be^{2+} was quantitated and compared to the binding of other divalent metal ions (Zn^{2+}, Cd^{2+} and Cu^{2+}). For each metal, binding affinities (K_D) and binding capacities (n) were determined. Because of the exceptional chemical properties of Be^{2+} in solution, it was necessary to use different procedures for such determinations than were used for other metals. Although the affinity of Be^{2+} for ferritin was similar to other metals ($K_D \approx 10^{-6}M$), significantly more Be^{2+} was bound (>800 gram atoms Be vs. < 400 gram atoms of

other metals). Based on comparison of the Be^{2+} binding of apoferritin and holoferritin it was concluded that Be^{2+} was bound mainly to the iron-oxyhydroxide phosphate core. This was in contrast to the other metals (Zn^{2+}, Cd^{2+} and Cu^{2+}) which bound the core to a lesser extent.

It was shown that ferritin protected three Be^{2+} sensitive enzymes in vitro. These included alkaline phosphatase, Na^{+}-K^{+} ATPase and rabbit muscle phosphoglucomutase. Ferritin was shown to reactivate phosphoglucomutase inactivated with Zn^{2+}, Cd^{2+}, Cu^{2+} or Be^{2+}. Calculations were made concerning the theoretical reactivation of Zn-PGM by ferritin. A model was presented to explain the observed binding of Be^{2+} to ferritin.

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LIST OF ABBREVIATIONS

SDS sodium dodecyl sulfate

SSA sulfosalicylic acid

PGM phosphoglucomutase

Fer ferritin

CHAPTER I

LITERATURE REVIEW

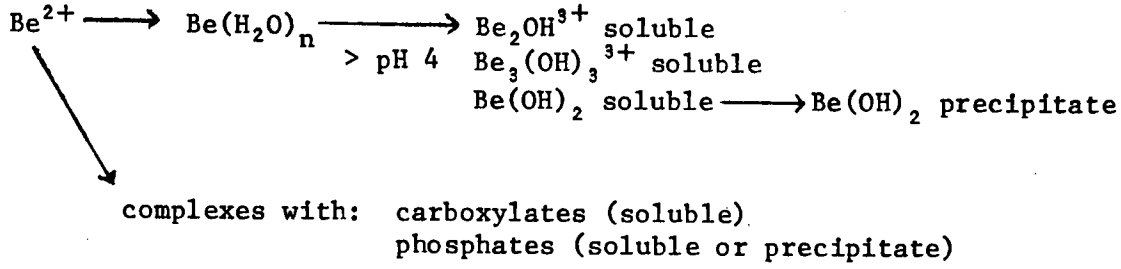
A. PHYSICAL AND CHEMICAL PROPERTIES OF BERYLLIUM

Beryllium is the lightest of the divalent metal ions and also the smallest in terms of ionic radius. The most comprehensive review of beryllium chemistry is that of Everest (19), and much of the information which follows is from that volume. The Be atom has an outer electronic valency of $1S^1 2S^2$. Thus, like other group IIA metals, it can exist as a stable metal with two complete S shells. It can give up its two outer electrons to form halogen salts or oxides and hydroxides. The forming of chemical bonds promotes 2S electrons to the 2P level and results in two linear hybrid sp bonds. Unlike other group IIA metals such as Mg^{2+}, beryllium cannot form hexameric complexes because of its small size and lack of d electrons. The high ratio of nuclear charge to ionic radius (z/r) causes beryllium to form bonds of significant covalent character compared to other group IIA elements. For this reason beryllium (z/r = 6.45) forms chemical bonds very similar to aluminum (z/r = 6.00). The ionization potential of beryllium is unlike other group IIA elements but is similar to that of Zn and Cd. Thus, similar complexes are formed such as Zn_2SiO_4 and Be_2SiO_4. In light of these unique atomic properties, it is important to consider the behavior of Be^{2+} ions in solution. Another consequence of the high nuclear charge to ionic radius is that Be^{2+} is a strong order producing ion (25) which brings water molecules

in much closer proximity. Thus, Be^{2+} has a lower partial molar entropy ($\bar{S}°$) compared to other group IIA ions. The tendency of $Be^{2+}(H_2O)_n$ to lose hydrogen ions is very strong above pH 4-5. This results in a complex equilibrium involving Be^{2+}, Be_2OH^{3+}, $Be_3(OH)_3^{3+}$ and $Be(OH)_2$ (35). The free Be^{2+} predominates below pH 4. Between pH 4 and 6, the major species is $Be_3(OH)_3^{3+}$ with Be_2OH^{3+} as a minor component. It is only above pH 6 that $Be(OH)_2$ is a major component. $Be(OH)_2$ has a very limited solubility in water (38). In order to better define the types of complexes that Be^{2+} can form, Kosel and Neuman (38) did pH titrations of $BeSO_4$ in the presence of various potential Be chelators. When the pH of a solution of Be^{2+} plus chelator was raised with NaOH, eventually Be^{2+} dissociated and formed insoluble $Be(OH)_2$. However, the precipitation occurred at a higher pH than it would have if Be^{2+} were free in solution. Thus, it was possible to screen a variety of molecules for chelating ability. A number of mono- and di-carboxylic acids were found to have affinity for Be^{2+}. Among these were lactate, oxalate and malonate. Hydroxy-dicarboxylic and tricarboxylic acids such as tartarate and citrate also had a high affinity for Be^{2+}. Be-citrate is thought to be one of the complexes formed when Be^{2+} is injected intravenously (62). Sulfhydryl or amino containing compounds showed no affinity for Be^{2+} as tested above.

A number of phosphate compounds formed complexes with Be^{2+}. Among these were orthophosphoric and α-glycerophosphoric acids. By other methods it has been shown that aromatic hydroxy acids have a high affinity for Be^{2+}. Among these are salicylic acid, sulfosalicylic acid (17), aurintricarboxylic acid (40) and tyrosine (58).

The chemical and solution properties outlined here for beryllium point to a much greater complexity of interaction than would be expected for other metal ions. The possible fate of Be^{2+} ions in solution is summarized below:



In light of this we can expect that Be^{2+} would be distributed in many forms in living systems. The measurement of Be^{2+} complexes <u>in vitro</u> is also greatly complicated by the formation of hydroxides at neutral pH. This problem is dealt with in greater detail in Chapter III.

B. PHYSIOLOGICAL ASPECTS OF BE TOXICITY

Systemically, Be is toxic to a number of tissues (68). Among these are lung, liver, spleen, and kidney. Clinical symptoms from beryllium disease (berylliosis) vary in type and severity depending on the degree of Be exposure. Tepper et al. (68) have documented clinical courses of acute, subacute, chronic, and later chronic beryllium disease. Frieman and Hardy (21) found that high Be doses delivered over a short period resulted in immediate symptoms and rapid fatality, while low doses produced milder symptoms after a latency period and greater probability of recovery.

Almost all cases of human Be disease involved industrial exposure, predominantly to pulmonary tissue. As recorded in U. S. Beryllium Case

Registry through 1977 (65), most beryllium disease resulted from exposure during Be smelting and metal production (57.2%). Another 31% of cases resulted from breathing of Be containing phosphors used in the fluorescent light bulb industry from the late 1930's up to 1950. Initially, Be in the lungs produced pneumonitis and dypsnea, coughing but with little or no sputum (26). Postmortem lung histopathology showed noncaseated granulomata containing abnormally high levels of Be (>0.02μg/g tissue) (65). Systemic involvement from Be redistribution brought about severe weight loss related to difficulty in digestion and a negative nitrogen balance (26). Hypercalciuria resulting from higher gut absorption of Ca^{++} was also observed in these patients. This resulted in stone formation (kidneys, salivary glands and lungs) as well as increased calcification of bones. In a number of the fatal cases of Be toxicity, enlarged livers and spleens were observed. Autopsy confirmed that abnormal liver function was a contributing cause of death (65). The pathogenesis of Be exposure has been shown to have an immunological component leading to a Be antigen-antibody reaction and subsequent inflammation (67). Possibly related to this was the fact that in a number of Be-toxicity cases, conditions of rheumatoid arthritis were exacerbated (26). The triggering of Be disease by stress (such as surgery, childbirth, or combat fatigue) has been related to an adrenal imbalance. The lack of steroid production was shown to produce lysosomal instability and a release of Be previously stored there (14). Cortisol and cortisone were found to be antiinflammatory and increased stability of the lysosomes. Mineralocorticoids such as aldosterone decreased lysosomal stability and resulted in translocation of Be to the liver.

Beryllium compounds are also potent carcinogens. Injection of Be phosphors into rabbit bone marrow produced osteosarcoma in 100% of the animals whereas injection of all other minerals failed to produce the tumors (22). Exposure of rats to aerosol $BeSO_4$ for 6 months produced lung cancer in 39% of the animals (57) within 18 months of initial exposure. Although the level of exposure in these experiments (33 $\mu gBe/m^3$) was probably about 10 times the level encountered in cases of human Be disease, statistical studies have shown a higher incidence of lung cancer among beryllium industry workers. For white males entered in the Beryllium Case Registry, Infante et al. (31) found a doubling of deaths due to lung cancer over the expected number for that group. Lung cancer risk seemed higher for short term Be exposure as these patients did not die from the toxic effects of the metal, but retained the metal in their systems for up to 20 years following the exposure (64). Thus, even though most cases of acute Be toxicity have been eliminated by the limiting of Be production and use, it is still feared that low levels of Be would be carcinogenic over a long period of time.

C. CELLULAR AND BIOCHEMICAL ASPECTS OF BE TOXICITY

Although Be^{2+} was toxic to a number of tissues (68) as mentioned in the previous section, after injection of $BeSO_4$ it was primarily the liver which accumulated Be^{2+} and suffered necrosis (2). Much of the injected $BeSO_4$ was converted to Be-phosphate in the blood and this particulate Be-phosphate was accumulated by the liver reticuloendothelial system (RES). Witschi and Aldridge (83) determined the subcellular

location of Be^{2+} which accumulated in the liver after intravenous injection of $BeSO_4$. Sixty three percent of the total Be^{2+} was accumulated by the liver cell nuclei, and about 5% was trapped in the lysosomes.

The latter was confirmed by the copurification of Be and acid phosphatase known to be present only in the lysosomes. It was initially thought that the hepatotoxicity was due to accumulation of Be-phosphate by liver Kupffer cells (RES) and then redistributed to the parenchymal cells where necrosis occurred. However, the data of Skilleter and Price (60) showed that uptake of Be-phosphate by Kupffer cells and uptake of soluble Be^{2+} (as $BeOH_2$ or Be-citrate) by parenchymal cells were independent of each other. In another study, Skilleter and Price (61) showed that following accumulation of Be-phosphate in lysosomes, 60-70% of the accumulated Be is released. The Be^{2+} released was shown to be mainly transported to other organs such as bone and spleen (60). Although Be was shown to induce lysosomal instability in cultured Kupffer cells (62), the release of hydrolytic enzymes by the lysosomes was shown to be a small component of the liver toxicity. Thus, the major hepatic necrosis was due to Be accumulation by parenchymal cells.

The cell death seemed to result from Be^{2+} binding to nuclear components and to a lesser extent binding to cytosolic proteins. Thomas and Aldridge (69) first showed the selective inhibition of four cytophasmic enzymes at micromolar concentrations of Be^{2+} (alkaline phosphatase, Na^+-K^+ ATPase, phosphoglucomutase and hexokinase). Other reports in greater detail have been published

for individual enzymes: alkaline phosphatase (40), Na^+-K^+ ATPase (70) and phosphoglucomutase (29). Of these enzymes, phosphoglucomutase was most susceptible to inactivation and appeared to be irreversibly inhibited. As shown by Witschi and Aldridge (83) injection of Be did not depress several biochemical functions, but did prevent rats from adjusting to a 24 hour starvation period. Thus, reduced phosphoglucomutase activity may prevent normal carbohydrate metabolism in cases of Be toxicity. This abnormal metabolism may also be due to absence of enzymes induced for gluconeogenesis.

The effect of Be on nuclear metabolism was of great importance because most of the injected Be was shown to accumulate in nuclei (83). Previous to this, it had been shown by Chevremont and Firket (12) that Be^{2+} interfered with normal cell division. Bassleer (7) confirmed this and showed that while DNA synthesis was inhibited in these cells, RNA synthesis was not affected. Witschi (82) has observed the effect of Be^{2+} on a number of DNA synthesizing enzymes in regenerating rat liver. He found that after partial hepatectomy, Be^{2+} injection blocked the induction of particular DNA synthesizing enzymes (thymidine kinase, thymidylate kinase, thymidylate synthetase, deoxycytidylate deaminase and DNA polymerase). Although the lack of induction of the enzymes listed seemed not to be due to a cessation of RNA synthesis, as is the effect of actinomycin D, it was not clear at what stage the induction was prevented. Recently, Parker and Stevens (46) have shown that the accumulation of Be^{2+} by nuclei was due to a specific phosphorylated non-histone fraction. It was further shown that Be^{2+} did not bind to

DNA, to histones, or to nonhistones which eluted at low salt concentration. The work of Allfrey (3) showed that phosphorylated nonhistone proteins are involved in transcription and DNA replication. Further work by Stein et al. (66) has shown that tissue specific patterns of transcription are dependent on the population of phosphorylated nonhistone proteins particular to that tissue.

As a natural resistance to Be, it was likely that protective factors might exist in the liver to lessen the inhibitory effect of Be^{2+} on enzymes. It had been shown by Fleming (20) that phosphoglucomutase in crude homogenates was far less susceptible to inhibition by Be^{2+} than the pure enzyme. Thus it seemed possible that a Be^{2+} chelator in crude homogenates could be responsible for this protection. The experiments presented in chapter III identify ferritin as a major Be^{2+} binding protein and characterize the nature of this binding. It is further shown in chapter IV that ferritin can protect enzymes from Be^{2+} inhibition and in chapter II that it can serve as a chelator or donor for Zn^{2+} and perpahs a number of other divalent metal ions.

D. FERRITIN AS A GENERAL DIVALENT METAL CHELATOR

Ferritin is a protein of 480,000 m.w. (iron not included) consisting of 24 subunits arranged as a hollow sphere (1). Subunits have molecular weights of between 18,500 and 21,000 depending on the source and method of determination. Up to 4000 iron atoms can be accumulated by ferritin within this shell as a ferric-oxyhydroxide phosphate complex. The ability of ferritin to sequester and release

iron is well known and has been reviewed most recently by Harrison et al. (27). It has also been shown by Macara et al. (43) that apoferritin binds a number of divalent or trivalent metal ions (Zn^{2+}, Cd^{2+}, Cu^{2+}, Mn^{2+} and Tb^{3+}). As shown by pH titrations, of the total of 5 metal ions bound per subunit, there were $\sim$2 metal ions per subunit strongly bound and $\sim$3 metal ions per subunit weakly bound to apoferritin. The exception to this was Mn^{2+} which bound 0.5 metal ions per subunit strongly and 2 metal ions per subunit weakly. It was later reported by Treffry and Harrison (73) that iron containing ferritin also bound 2 Zn^{2+} per subunit, but Zn^{2+} at 0.5 sites seems to be more tightly bound than at the remaining sites. It was found by Wetz and Crichton (80) that modification of 11 carboxyl groups on apoferritin abolished iron incorporation. Such modification also eliminated the ability of Zn^{2+} or Tb^{3+} to bind to apoferritin (71). Thus, carboxyl residues were implicated with both Zn^{2+} binding and iron incorporation. X-ray diffraction studies have located the positions of divalent binding to be at the diad axis of subunit-subunit interaction (15). It is still not clear whether these sites are for nucleation of the iron micellar core or for iron incorporation into the growing iron core.

Very little is known about the <u>in vivo</u> significance of Zn^{2+} binding to ferritin. In cultured hepatocytes, Zaman and Verwilghen (84) have shown that from 1.45 to 1.89 Zn^{2+} ions per subunit are bound to ferritin as determined by antibody precipitation. This was a very small fraction ($1/400^{th}$) of the total cellular Zn^{2+}. However, given that most Zn^{2+} is tightly bound to proteins within the cell, this ferritin bound

Zn^{2+} may represent a readily available source of Zn^{2+} for newly synthesized Zn^{2+} requiring enzymes. Experimental evidence for this is described in the following chapter.

CHAPTER II

FERRITIN: A ZINC DETOXICANT AND A ZINC ION DONOR

A. INTRODUCTION

The observation that ferritin is a good chelator for Zn^{2+} (43, 55, 84) *in vivo* and *in vitro* led us to speculate that such binding would be of physiological significance. Along this line, we investigated the possible dual role for ferritin as a Zn^{2+} detoxicant as well as a Zn^{2+} donor to enzymes which require it for activity.

In this chapter we show that Zn^{2+} when injected into rats is, at least in part, sequestered by ferritin in the liver. The bound zinc is readily available to some zinc requiring apoenzymes. These include alkaline phosphatase from calf intestine, yeast phosphoglucomutase and yeast aldolase. As zinc sources we utilized ferritins from Zn injected (ferritin-Zn), Cd injected (ferritin-Cd) and noninjected rats (ferritin-control). These ferritins contained various amounts of Zn (ferritin-Cd > ferritin-Zn > ferritin-control). The reactivation of zinc requiring enzymes was proportional to the amount of zinc in each case.

B. EXPERIMENTAL PROCEDURE

Male Sprague-Dawley rats weighing between 250-275 g were injected subcutaneously with 1 mg Zn^{2+} or 2 mg Cd^{2+} (as $ZnSO_4$ or $CdSO_4$) per Kg body weight/day. Each group contained six rats. After 7 injections the animals were sacrificed, the livers were homogenized and

ferritin was isolated from liver as described by Linder and Munro (41). It was homogenous as judged by sucrose density gradient centrifugation, polyacrylamide electrophoresis and reaction with antibodies. The latter were a generous gift of Dr. I. Listowsky of Albert Einstein School of Medicine, N.Y. Yeast phosphoglucomutase was isolated from Fleischmann's yeast as described previously (18) and aldolase as described by Rutter and Hunsley (56). Calf intestinal alkaline phosphatase was purchased from Sigma Chemical Company. Electrophoretically pure metallothionein I and II were isolated from Cd^{2+}-injected rats as described by Vander Mallie et al. (78). Protein concentrations were determined by the method of Lowry et al. (42) using bovine serum albumin as the standard with a 29% upward correction for ferritin as shown by Linder and Munro (41).

Apoenzymes were prepared and used on the same day. A sephacryl-200 column (1 × 18 cm) was washed with 50 ml of 0.1 M EDTA, pH 7.6 (by addition of Tris base) and EDTA was removed by washing the column with 0.01 M Tris, pH7.6 which was made metal-free by treatment with chelex-100 (5g chelex was suspended in 1000 ml of buffer and stirred overnight at 4°). Aliquots of alkaline phosphatase and aldolase were incubated for 24 hr and 4 hr respectively with 0.015 M EDTA pH 7.6 in a final volume of 0.3 ml, passed through the sephacryl S200 column and eluted with metal-free Tris buffer as above. After such a treatment the enzymes had lost 98% of their activity.

Yeast phosphoglucomutase was incubated with 0.5 mM EDTA and 0.04 M Tris pH 7.6 for 30 min prior to the passage through the column. The eluted enzyme was less than 5% active without any added metal, 100%

active with 5 mM Mg^{2+} and 85% active with 0.4 mM Zn^{2+}. Activity of phosphoglucomutase was measured by coupled assay as described by Joshi et al. (33).

Yeast aldolase was assayed according to the method of Rutter and Hunsley (56). Alkaline phosphatase was assayed according to the method of Garen and Levinthal (23). Metal analyses of different samples were performed by Stewart Laboratories, Knoxville, Tennessee. All reagents were of analytical grade and were purchased from Sigma Chemical Company or Baker Chemical Company. Chelex-100 was purchased from Bio-Rad, Incorporated. Only glass distilled water passed through a deionizer was used.

C. RESULTS

The three enzymes chosen for the study of zinc transfer from ferritin were yeast phosphoglucomutase, yeast aldolase, and alkaline phosphatase from calf intestine. Of these alkaline phosphatase and aldolase contain tightly bound Zn^{2+} and the holoenzymes do not require added metal, however their apoenzymes are inactive. In contrast pure yeast phosphoglucomutase does not contain any bound metal ion but requires the presence of added divalent metal ion for activity. It is most active with 5.0mM Mg^{2+}. The relative maximal activities with other metal ions were Zn^{2+} (at 0.5mM) 84%, Co^{2+} (at 2.0mM) 95%, and Mn^{2+} (at 0.5mM) 62% (18).

Reactivation of Yeast Phosphoglucomutase

Results in Figure 2.1 confirm and extend our earlier observations. As seen, the demetallo-enzyme was less than 1% active without any added metal ion. Although 2 μM Cd^{2+} activated the metal-free enzyme the activity elicited was only 10% of that obtained with 0.4 mM Zn^{2+} and 8% of that obtained with 5 mM Mg^{2+}. Simultaneous presence of 0.4 mM Zn^{2+} and 2 μM Cd^{2+} was as effective as 0.4 mM Zn^{2+} alone. Furthermore, activity elicited by Mg^{2+} or Zn^{2+} was unaffected by 0.156 μM concentration of ferritin-control, ferritin-Zn or ferritin-Cd. Thus under these conditions the metal ions were available to the enzyme. However, as seen in Figure 2.1, the presence of ferritin from metal injected rats during assay activated the apoenzyme in a concentration dependent manner. The metal transfer was complete within one minute because preincubation of metal free yeast phosphoglucomutase with ferritin-Zn or ferritin-Cd for longer periods did not yield any additional phosphoglucomutase activity. As expected, ferritin-control was a poor activator and ferritin-Cd was a better activator than was ferritin-Zn.

Activation of Apo-alkaline Phosphatase from Calf Intestine

This enzyme was also activated by ferritin (Figure 2.2). Similar to yeast PGM, ferritin-Cd and ferritin-Zn were better sources of Zn than was ferritin-control. However, unlike yeast phosphoglucomutase, the rate of activation was slower and only a maximum of 25% of the expected activity was recoverable. Measurement of the amount of metals bound to various ferritins (Table 2.1) showed that reactivation of

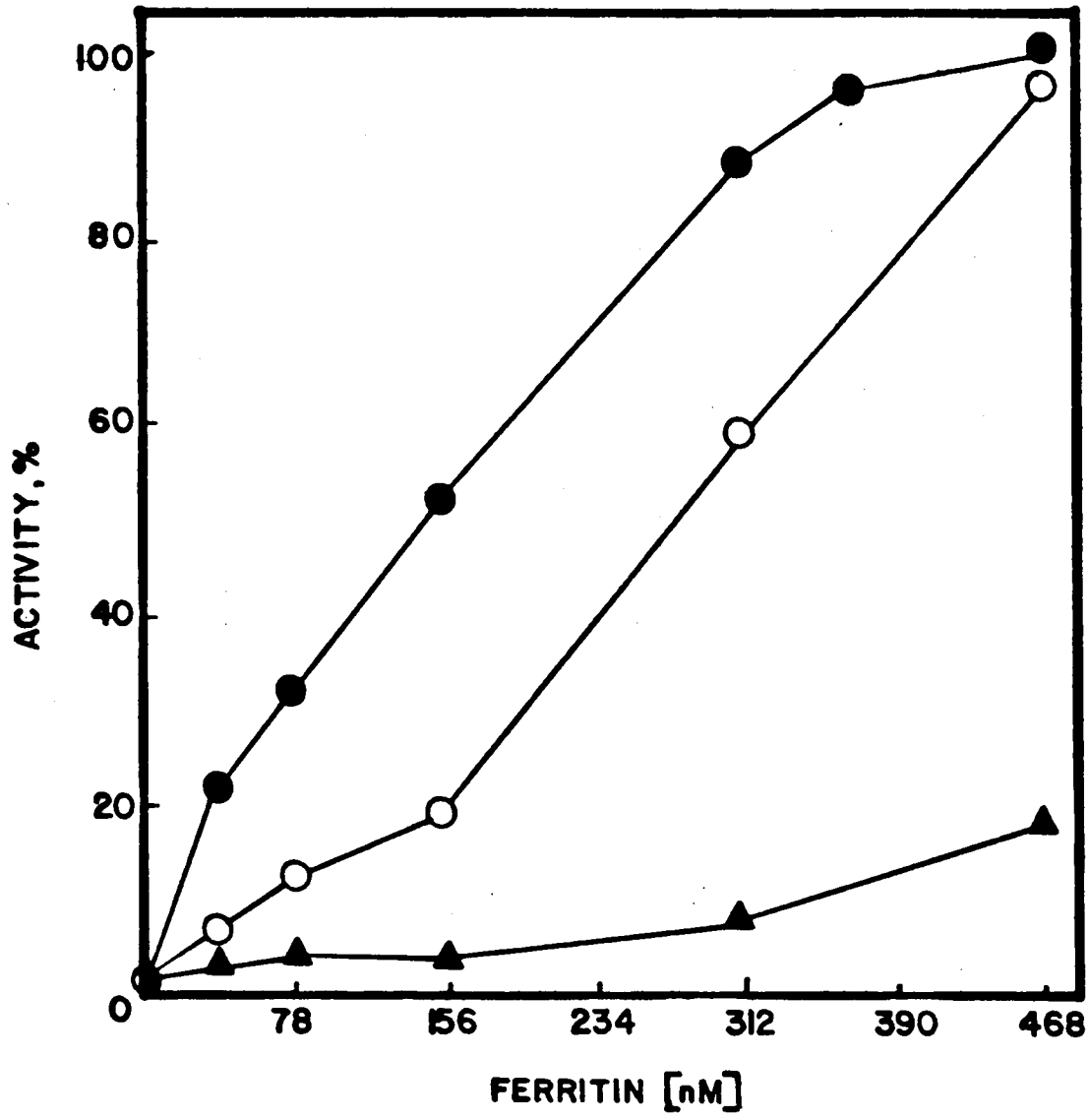


Figure 2.1. Effect of various preparations of ferritin on phosphoglucomutase from yeast. The activity was assay by coupled assay (8) in the presence of varying concentrations of ferritin-control (△), ferritin-Zn (○) or ferritin-Cd (●). The concentration of enzyme was 18.7nM. Activity observed in the presence of 0.5 mM Zn is equal to 100%.

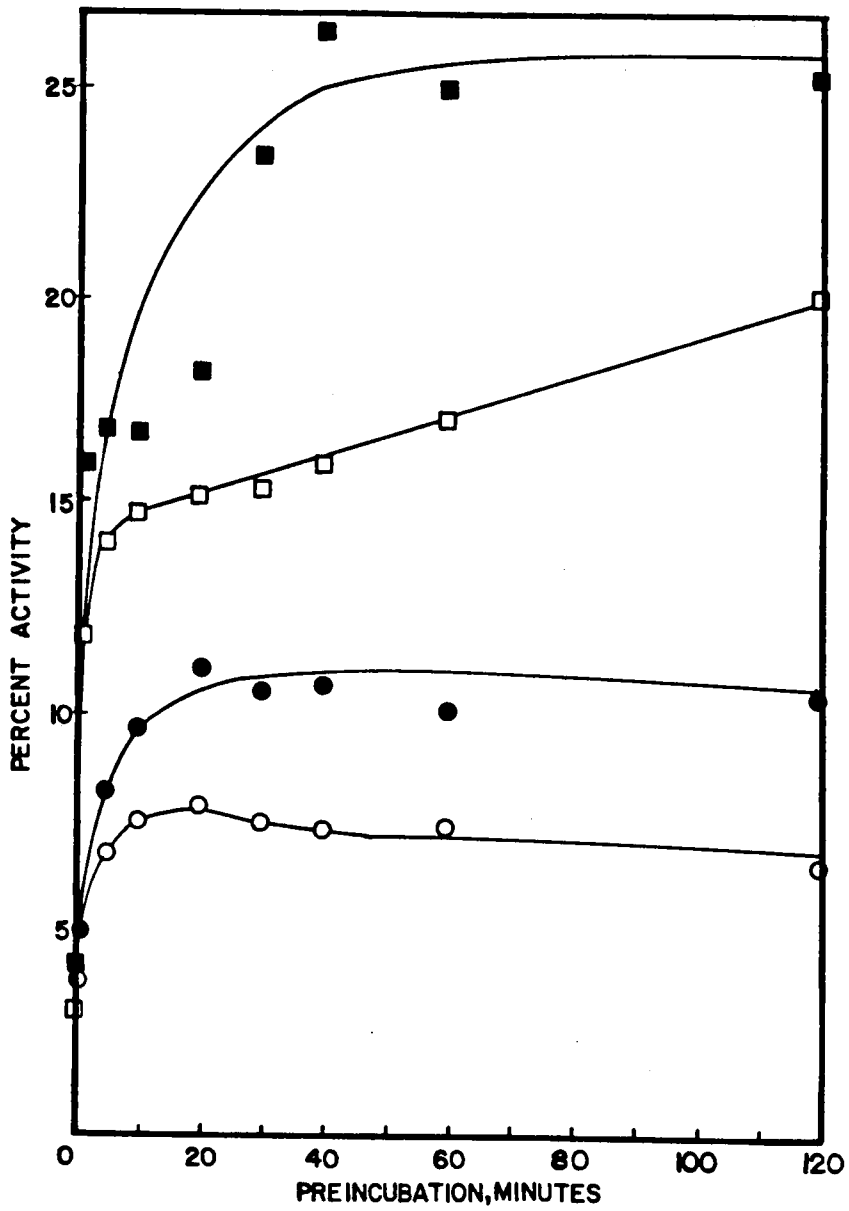


Figure 2.2. Reactivation of Alkaline Phosphatase. Apo-alkaline phosphatase (calf intestine) was incubated at 0.97 μM in a final volume of .5 ml of 40 mM Tris buffer, pH 7.4 with various activators:
(○) ferritin-control at final .78 μM protein, 1.05 μM bound zinc.
(●) ferritin-Zn at final .78 μM protein, 1.05 μM bound zinc.
(□) ferritin-Cd at final .78 μM protein, 7.31 μM bound zinc.
(■) $ZnSO_4$ at final concentration of 7.3 μM.
Aliquots of 5 1 were assayed at various time intervals in 1 mM p-nitrophenyl phosphate, 1.0M Tris pH 8.0. Under such conditions 100% alkaline phosphatase activity represented as A^{414}nm/minute of 1.34.

TABLE 2.1

Metal Analysis of Different Preparations of Ferritin.
g atoms/mole of ferritin

	Zn	Cd	Fe	Protein Recovered mg ferritin/g liver
Zn injected rat ferritin	3.88	0	1045.7	.255
Cd injected rat ferritin	9.32	0.68	851.7	.203
Noninjected rat ferritin	1.33	0	840.9	.258

alkaline phosphatase was proportional to the amount of zinc in ferritin. As seen, ferritin-control contained the least amount of zinc. Significantly, ferritin-Cd had eight times more zinc and ferritin-Zn had three times more zinc than ferritin-control. This excess of bound zinc was thus available for enzyme reactivation. When $ZnSO_4$ was used as an activator at metal concentration equivalent to that bound to ferritin-Cd, the course of reactivation was similar although occurring at a faster rate and to a slightly higher final level of reactivation. Similar results were obtained with $ZnSO_4$ levels equivalent to other ferritins.

Activation of Apo-aldolase from Yeast

The results of these experiments are seen in Table 2.2. This enzyme, like yeast phosphoglucomutase was also completely reactivated by ferritin-Zn, or ferritin-Cd. The activation was instantaneous and dependent upon the concentration of ferritin. In response to ferritin-control 23% of aldolase activity returned while only 14% of yeast phosphoglucomutase activity returned under similar conditions. Rate of reactivation by ferritin-Cd was only slightly greater than with ferritin-Zn.

D. DISCUSSION

Zinc, a biologically essential trace element, serves as a structural stabilizer for certain proteins such as bovine superoxide dismutase, and as an essential cofactor for several enzymes (13). In some proteins such as alkaline phosphatase it serves a dual role of a cofactor and a

TABLE 2.2

Effect of Various Preparations of Ferritin on the Activity of Aldolase and Apo-aldolase from Yeast. The Activity was Assayed by Coupled Assay (9). Concentration of Ferritin was 1.47 10 M. This Reprepresented Final Zn^{2+} Concentrations of 0.15 µM (Ferritin-control), 0.35 µM (Ferritin-Zn), and 1.05 µM (Ferritin-Cd)

Conditions	% Activity
Holoenzyme	100.0
Holoenzyme + Ferritin-control	98.6
Holoenzyme + Ferritin-Zn	100.0
Holoenzyme + Ferritin-Cd	97.2
Apoenzyme	4.2
Apoenzyme + Ferritin-control	22.9
Apoenzyme + Ferritin-Zn	91.9
Apoenzyme + Ferritin-Cd	

stabilizer. However, high doses of zinc are toxic (76). As a defense against these toxic levels, animals synthesize metallothionein which in turn sequesters Zn^{2+}(34). Indeed, the same protein is synthesized in response to other metal ions such as Cd^{2+} or Ag^{2+}; the amount of protein synthesized varies with the metal ion. Thus under comparable conditions Cd^{2+} is a far better inducer than Ag^{2+} or Zn^{2+}. Until recently, the physiological role of metallothionein was considered only to be that of a metal detoxicant. However, Udom and Brady (74) have recently observed that metallothionein can serve as a zinc donor in vitro to several zinc requiring apoenzymes. The amount of activity restored due to metal transfer varied with the apoenzyme. Thus yeast apo-aldolase was fully and instantaneously reactivated, yeast apo-alcohol dehydrogenase was not activable at all, whereas apo-thermolysin, and apo-alkaline phosphatase were partially reactivated to submaximal levels (74). Thus it appeared essential to examine whether yeast phosphoglucomutase is activated by metallothionein and whether metallothionein I or II were equally effective in this respect. Accordingly metallothionein I and II were isolated from the Cd-injected rats and 1.56×10^{-8} M apo-yeast phosphoglucomutase was assayed in the presence of a four-fold molar excess of either of the metallothioneins. In each instance full and instantaneous reactivation was observed.

The data presented here shows that in addition to metallothionein, ferritin can also function as a metal chelator and donor for other proteins. Although the synthesis of metallothionein is induced to varying degrees by several metal ions, that of ferritin is known to be specifically induced by iron salt (24). The degree of induction is

dependent upon the age of the animal, amount of injected metal ion and duration of the dosage (28). The fact that ferritin-Cd was a better Zn donor than was ferritin-Zn led to measurement of Zn and Cd in these preparations. As seen in Table 2.1, page 17, the amount of ferritin isolated from normal or Cd- or Zn-injected rats was very similar, yet they varied in the metal content. This suggests that the existing ferritin absorbed, at least in part, the injected Zn and spared the animal the biologically expensive procedure of making metallothionein. As stated earlier, zinc though toxic at high concentrations is indispensible for several biological processes. In contrast, Cd^{2+} is nonessential and toxic (75). Upon its entry into the animal not only did it induce the synthesis of metallothionein as observed by Winge et al. (81), but it was also deposited into ferritin (Table 2.1). Of particular interest is the fact that Cd injections mobilized zinc from other locations and caused its deposition into ferritin (Table 2.1). Although the source of this zinc remains to be determined, it is clear that once bound to ferritin it is available to the apoenzymes as evidenced by increased reactivation of apoenzymes by ferritin-Cd than by ferritin-Zn. Ferritin-control, which contained the least amount of zinc, was a poor zinc donor.

The interrelationships between different divalent metal ions and metallothionein is well-recognized (37) and continues to be a subject of active interest. For example, Brown et al. (10) recently studied the metabolism of Zn^{2+}, Cu^{2+} and Cd^{2+} in pretumorous livers from diethyl-nitrosamine (DEN) exposed mice. They observed that DEN exposed mice had

lower concentrations of Cu^{2+} and Zn^{2+} in cytosol of tumorous as well as pretumorous tissue and that this reduction was from a "heat stable, high molecular weight" protein pool. Exposure of Cd^{2+} alone resulted in its accumulation in metallothionein whereas simultaneous exposure of Cd^{2+} and DEN resulted in the accumulation of the metal ion in a "heat stable, high molecular weight protein pool." We have found that high molecular weight protein pools of comparable heat stabilities contain predominantly ferritin (50, 51). Thus, carcinogens such as DEN seem to cause redistribution of metal ions amongst metallothionein and high molecular weight protein(s), such as ferritin. Both ferritin and metallothionein represent potential metal chelator-donors. Ferritin is known to be present at concentrations of at least .25 mg/g liver (Table 2.1, page 17) and it can bind at least 50 g atoms of Zn^{2+} (84). In contrast, methallothionein binds a maximum of only 8 gm atoms of zinc and is present only in nanogram quantities in either control or zinc treated rats at the levels used here. Stimulation of the synthesis of Zn-thionein by zinc injections required at least 5 mg zinc/kg body wt., and even at this level, the amount of methallothionein stimulated was only a fraction of that stimulated in response to cadmium (data not shown). Thus, ferritin, because it is present at high constitutive levels is probably more suited to handle transient fluctuations in levels of divalent ions. In turn, binding of divalent ions to ferritin may also interfere with normal iron storage and mobilization. Indeed, deposition of iron into cellular ferritin was inhibited by Zn^{2+} in a dose dependent manner (84) and Pb^{2+} bound to ferritin or ferritin-like substances in

liver, spleen, and kidney of Pb-poisoned cattle suggesting an alteration in the storage of iron (55). In the case of such extreme toxicity, synthesis of metallothionein might serve to handle excess divalent metals and allow ferritin to continue to function as an iron storage protein.

The *in vitro* data presented here suggests an analogous role for ferritin as a zinc donor *in vivo*. Since ferritin is known to bind Cu^{2+} (43), it is tempting to speculate that it may function as a donor of Cu^{2+} to the corresponding apo-enzymes. Plants contain phytoferritin and yeast also contain a ferritin-like protein (16). These are large molecular weight proteins. In addition to these, rat liver and plants are known to contain smaller molecular weight proteins or even peptides capable of binding metal ions (36, 52). Perhaps such proteins may also function as divalent metal donors in a manner similar to that described for ferritin in this chapter.

CHAPTER III

BINDING OF BERYLLIUM AND OTHER DIVALENT METAL IONS TO FERRITIN

A. INTRODUCTION

As noted in Chapter I, beryllium is the most toxic of the divalent metal ions. The toxicity was found to be due to binding of Be^{2+} to both nuclear and cytoplasmic proteins. In the cytoplasm, liver enzymes which are inhibited at micromolar concentrations of Be include alkaline phosphatase (40), Na^{+}-K^{+} ATPase (70), and phosphoglucomutase (29). Of these, the inhibition of phosphoglucomutase has been shown to be complete and only partially reversible (29). The inactive enzyme contained one gm atom of Be^{2+} per mole of enzyme. Further experiments in vitro showed that under comparable concentrations of protein and beryllium, the inhibition of phosphoglucomutase was the highest with pure preparations followed by the dialyzed homogenates and the least with crude liver homogenates (20). This suggested that in crude extracts the enzyme is protected by components of different molecular weights, the dialyzable with low molecular weights and the nondialyzable with high molecular weights.

Our search for the nondialyzable component led us to the isolation of a high molecular weight beryllium binding protein. In this chapter we show that it is the iron storage protein, ferritin. Further, binding of beryllium to ferritin is compared to that of Zn^{2+}, Cd^{2+}, and Cu^{2+}.

Ferritin binds more than 800 gm atoms of Be^{2+}, a significantly greater amount than is the case for other divalent metal ions. The majority of these Be^{2+} ions appear to bind to the iron core of ferritin. However, binding of beryllium to the protein shell is likely because apoferritin bound up to 160 gram atoms of Be^{2+}. Speculation is made as to how the physical and chemical characteristics of beryllium would give rise to the observed binding. It is shown in chapter IV that in vitro ferritin protects the three enzymes phosphoglucomutase, alkaline phosphatase and Na^{+}-K^{+}ATPase against inhibition by beryllium. A number of the above findings were first presented in abstract form (50, 51).

B. EXPERIMENTAL PROCEDURE

$^{7}BeCl_2$ (carrier free, in 0.1 M HCl) and $^{109}CdCl_2$ (carrier free, in 0.1 M HCl) were purchased from Amersham. ^{64}Cu (10 mCi/mg copper, in 1M HNO_3) and $^{65}ZnCl_2$ (carrier free, in 0.5 M HCl) were purchased from New England Nuclear. Sephacryl S200, Sephacryl S300, Sephadex G100 (medium), Sephadex G-75 (medium), Sepharose CL-6B, and the molecular weight protein standards were purchased from Pharmacia. Horse spleen ferritin was purchased from Sigma and further purified as described below. Agar diffusion plates were purchased from Travenol Laboratories. PM30 filters, UM2 filters, and Centrifree micropartitions were purchased from Amicon. All other reagents were purchased from Sigma or Baker. Rabbit antise4um against horse spleen ferritin and sheep antiserum against human ferritin were kindly provided by Dr. I. Listowsky of Albert Einstein School of Medicine, New York, NY.

Eluates from columns were monitored at 280 nm. Whenever necessary protein concentration was measured by either the method of Lowry et al. (42) or Bradford (9) using bovine serum albumin as a standard and multiplying by appropriate correction factors. For the Lowry assay, protein values for ferritin and apoferritin were multiplied by 0.7 correction factor to get the best approximation. For the method of Bradford, ferritin protein values were multiplied by 0.73 for ferritin and 0.82 for apoferritin to get the best approximation. The correction factors were determined by comparing the values obtained colorimetrically and by Kjeldahl nitrogen analysis (4).

Iron in ferritin or apoferritin was determined by atomic absorption spectroscopy on an IL157 Instrumentation Laboratories spectrophotometer. Radioactivity was measured with a Beckman gamma counter. Protein samples for phosphate analysis were first digested in H_2SO_4 and were then analyzed for inorganic phosphate by the method of Bartlett (6).

Purification of Metallothionein

^{109}Cd-thionein was purified from rat liver according to the procedure of Vander Mallie and Garvey (78) with some modifications. Six adult male albino rats (Charles River CD) were given daily injections of $CdSO_4$ in 0.85% NaCl (1.0 mg Cd/Kg body wt/per rat on the first day and 2.0 mg Cd/Kg body wt. per rat daily for the following 6 days). Another group of six rats was injected with $BeSO_4$ in 0.85% NaCl (1.0 mg Be/Kg body wt. per rat on the first day and 2.0 mg Be/Kg body wt. per rat daily for the following 6 days). Rats were sacrificed and their livers were homogenized

in 5 volumes of 0.1 M Tris-HCl buffer, pH 7.4 containing 0.1 mg/ml phenylmethyl sulfonyfluoride. The homogenate was spun at 7,000xg for 30 minutes and the pellet was discarded. The supernatant was heated to 80° in a boiling water bath, cooled immediately and centrifuged at 27,000xg for 10 minutes. To the heat stable supernatant was added either $^{109}CdCl_2$ tracer (for Cd injected animals) or $^{7}BeCl_2$ tracer (for Be injected animals). To the radioactively labeled heat stable supernatants was added solid ammonium sulfate (24.3 g/100 ml) and after 30 minutes the precipitate was centrifuged at 27,000xg for 10 minutes. The precipitate was saved for radioactivity counting, and to the supernatant was added solid ammonium sulfate (37.5 g/100 ml). After 30 minutes, the precipitate was centrifuged at 27,000xg for 10 minutes. The resulting pellet was dissolved in minimal amount of 5 mM Tris-HCl buffer, pH 7.4. To this solution was added cold acetone (-15°) to final concentration of 50%. After centrifugation at 27,000xg for 10 minutes more cold acetone was added to the supernatant to give a final concentration of 80% acetone. The solution was then centrifuged at 27,000xg for 10 minutes, and the pellet was dissolved in 0.5 mM Tris-HCl, pH 7.4. Further purification was accomplished by sephadex G-75 chromatography and DEAE cellulose chromatography as described by Vander Mallie and Garvey (78). Whenever required, apo-thionein was also prepared according to Vander Mallie and Garvey (79).

Purification of "Beryllium Binding Protein"

Four adult albino rats (Charles River CD) were sacrificed, their livers removed, rinsed free of blood and homogenized in 5 volumes of

0.1 M Tris pH 7.4 containing 0.1 mg/ml of phenylmethylsulfonyl fluoride. The homogenate was centrifuged for 30 minutes at 70,000xg at 4° and pellet discarded. The supernatant was heated at 80°C for 1 minute in a boiling water bath, cooled on ice, and then centrifuged at 27,000xg for 10 minutes. $^{7}BeCl^{2}$ tracer was added to the heat stable supernatant and the solution was incubated overnight at 4°. To this solution was added solid ammonium sulfate (24.3g/100 ml) and after 30 minutes the precipitated protein was removed by centrifugation (27,000xg, 10 min.). The precipitate was dissolved in 0.5 mM Tris pH 8.5 and applied to a sephacryl S200 column (3 × 70 cm). The sample was then eluted with the same buffer. Column fractions of 5 ml. each were collected and assayed for protein and radioactivity. Fractions eluting with the void volume which were of constant specific radioactivity (CPM/A^{280}) were pooled and concentrated using a UM2 filter and an Amicon concentrator.

Purification of Rat Liver Ferritin

Ferritin was purified by the procedure of Linder and Munro (41) with slight modifications. Purification was the same as that for the beryllium binding protein up to and including the steps leading to the heat stable supernatant. The heat stable supernatant was brought to pH 4.8 with dropwise addition of 0.15 M acetic acid. After 1 hour at 5° the denatured precipitate was centrifuged. Solid ammonium sulfate was added to the supernatant (31.3 g/100 ml). After 1 hour the precipitate formed was centrifuged at 2400xg for 20 minutes. The pellet was dissolved in 0.05M phosphate pH 7.0 and applied to a Sephadex G100

(medium) column (3 × 70 cm) equilibrated and eluted with the same buffer. Ferritin, which eluted with the void volume, was concentrated using a UM2 filter and Amicon concentrator.

Purification of Horse Spleen Ferritin

Ferritin purchased from Sigma was first dialyzed against 1000 volumes 0.01 M Tris pH 8.5 containing 0.5 mM EDTA overnight at 5° and applied to a Sepharose CL-6B column (3 × 7 cm) equilibrated and eluted with 0.01 M Tris pH 8.5 containing 0.5 mM EDTA. Five milliliter fractions were collected and absorbance at 280 nm was monitored. Fractions in the major peak were pooled and dialyzed twice at 5° against 1000 volumes of 0.05 M Tris-HCl pH 8.5. Final preparations of electrophoretically pure horse spleen ferritin were stored in plastic vials at 5°.

Preparation of Horse Spleen Apoferritin

Approximately 6 mg of horse spleen ferritin was dialyzed for 3 hours at 5°C against 2 liters of 1% thioglycolate in 0.1 M acetate buffer, pH 5.6. The thioglycolate was removed by further dialysis against 4 liters of 0.1 M acetate buffer, pH 5.6 for 3-4 hours at 5°, followed by dialysis against 2 liters of 0.05 M Tris-HCl, pH 8.5 with Chelex 100 to remove residual iron. In spite of this treatment, the final preparation contained betwee 24 and 30 gram atoms of iron per native apoferritin or 1-1.3 iron atoms per subunit.

Analytical Techniques

Visible and ultraviolet absorption spectra were recorded on a Cary 15 double beam spectrophotometer or a Beckman DU7 spectrophotometer.

Ouchterlony immunodiffusion precipitation was done by applying 10 µl of protein samples (1 mg/ml) to the surrounding wells and the antibody in the center well of the agarose gel plate. After precipitin bands became visible, the gel was washed extensively in 0.05M phosphate pH 7.4 in 0.85% NaCl, stained with 0.5% coomassie blue in methanol: acetic acid: water (5:1:5) followed by destaining with the same solvent. Isoelectric focusing was conducted as described by Jeppsson (32). A 30 µg aliquot of protein was applied to precast LKB gels, (5% acrylamide pH 3.5 - 9.5) electrofocused at 1500 V (20mA) for 1.5 hours and stained with coomassie brilliant blue R-250. Total amino acid composition of acid hydrolyzed proteins was determined using a Beckman amino acid analyzer with standard techniques (63). Sedimentation velocities were determined by sucrose density gradient centrifugation as described by Martin and Ames (44). A 5.0 mg protein sample was layered on a 20-50% sucrose gradient in 0.01 M phosphate, pH 7.0 and centrifuged at 70,000xg for 9 hours in an SW50.1 rotor. The gradient fractions were assayed for protein by absorbance at 280 nm.

Electrophoresis of native protein containing $^{7}Be^{+2}$ was done in duplicates on 5% polyacrylamide tube gels with bromphenol blue as the dye marker. One tube gel was stained with coomassie brilliant blue R-250 and the other cut into 4 mm sections. Each section was digested for 12 hours at 60° in capped vials containing 200 µl of 30% H_2O_2. To each of these vials were added 4 ml of scintillation fluid [5.5 gm Permablend (Packard) per liter of toluene and one-third volume of Triton-X100] and counted for radioactivity.

Subunit molecular weight was determined by electrophoresis on SDS-polyacrylamide gels using the method of Laemmli (39). Proteins were dissolved in 0.05 M Tris-HCl pH 6.8 containing 2% SDS and 2% β-mercaptoethanol. Samples were heated for 5 minutes in a boiling water bath and electrophoresed with appropriate standards on 12.5% acrylamide slab gels and stained with coomassie brilliant blue R250.

Equilibrium gel filtration of horse spleen ferritin and apoferritin was carried out using the method of Pieterson et al. (47). For each column run approximately 0.6 mg of protein was lyophilized and redissolved in 0.3 ml of metal containing buffer. For Zn^{2+}, Cd^{2+}, and Cu^{2+} the metal buffer consisted of 0.2 mM of the respective metal salt and 0.05 M Tris-HCl pH 7.4 buffer. For Zn^{2+} and Cd^{2+} radioactive tracer metal was used to monitor metal concentration. Concentrations of Cu^{2+} were determined by atomic absorption spectroscopy. In each case, protein was monitored by absorbance at 280 nm. Protein eluting with the void volume was pooled and assayed for protein by Lowry assay and for radioactivity by gamma counter. An aliquot of the pooled samples was dialyzed twice against 1000 volumes of 0.01 M Tris-HCl pH 7.4 to remove loosely bound metals. A second aliquot was dialyzed against 0.01 M Tris-HCl, pH 7.4 and 0.2 mM EDTA to quantitate tightly bound metal ions.

In order to quantitate both the affinity and the maximal amount of metals that could be bound to ferritin, we used the Centrifree micropartition technique (Amicon) for separation of bound and free ligands. Ferritin or apoferritin from horse spleen were incubated for 15 minutes with various amounts of individual metal ions (Zn^{2+}, Cd^{2+} or Cu^{2+}).

Free metal was separated from the bound metal by centrifugation in the Centrifree filter for 15 minutes at 3100 rpm in a fixed angle clinical centrifree separation centrifuge. In all cases, metal concentration was quantitated by radioactive tracer.

Solubility of $BeSO_4$ decreases with increase in pH (19). Thus the following procedure was employed to prepare Be buffers. To 0.5 mM unlabeled $BeSO_4$ (unbuffered) was added radioactive $^7BeCl_2$ (carrier free). This solution was then adjusted to pH 6.3-6.5 with a dropwise addition of 0.1 M Tris base. The solution was then filtered through a microporous filter (0.45 μm, Amicon). The concentration of Be was quantitated and the solution was used immediately for binding studies.

Horse spleen ferritins containing various amounts of bound Be^{2+} were prepared by using the microcentrifuge desalting technique of Helmerhorst and Stokes (30). A small amount of glass wool was placed in a 10 ml plastic syringe body and then packed with G-75 sephadex (medium) in 0.05 M Tris-HCl pH 6.5 and 0.005% azide to produce a bed volume of 5 ml. Columns were precentrifuged for 3 minutes at 1960 rpm in swinging bucket type clinical centrifuge. Samples containing horse spleen ferritin were incubated with 0.05 M Tris-HCl pH 6.5 and various amounts of $^7BeSO_4$ (as prepared above). Incubation mixtures were concentrated approximately 10 fold by ultrafiltration (PM 30 filter) and the concentrated protein was applied to the precentrifuged column. The column was then centrifuged as before, but for 2 minutes. The eluted Be-ferritin was then quantitated for amount of Be^{2+} bound. Again Be^{2+} concentration was measured by gamma counting. Protein was measured by absorbance at

280 nm, utilizing extinction coefficients of 16.90 A^{280}/mg ferritin and 1.235 A^{280}/mg apoferritin. These extinction coefficients were determined by Lowry (42) and A^{280} measurement.

The beryllium chelator, sulfosalicylic acid (SSA), was used to remove Be^{2+} from Be-ferritins. ^{7}Be-ferritin, prepared as described above, was incubated with varying amounts of SSA and then the ^{7}Be-SSA was separated from $^{7}Be^{2+}$ which remained bound to ferritin by centrifugation in Centrifree filters for 15 minutes at 3100 rpm. Measurements of the radioactivity in the filtrate quantitated the amount of Be^{2+} removed from ferritin by SSA.

The affinity of Be^{2+} for SSA was measured by spectrophotometric titrations. Varying amounts of $BeSO_4$ were added to SSA in 0.05 M Tris pH 6.5 buffer in a final volume of 3.0 ml and the increase in absorbance at 310 nm was measured with a Beckman DU-7 spectrophotometer.

C. RESULTS

Isolation of a High Molecular Weight "Be^{2+} Binding Protein"

As stated earlier (20), in crude liver extracts phosphoglucomutase is partially protected by dialyzable small molecular weight factors and nondialyzable large molecular weight factors. We first tested to see whether metallothionein could be one of these factors, because it binds divalent metal ions such as Zn^{2+}, Cd^{2+} and Cu^{2+} and its synthesis is induced by these metal ions (37). Accordingly, groups of rats were injected with Cd^{2+} or Be^{2+} and the isolation of liver metallothionein

was attempted. Although our procedure for induction and purification of Cd-thionein yielded the expected protein, injection of beryllium failed to induce metallothionein synthesis. However, 25% of the soluble ^{7}Be appeared in the 0.4 ammonium sulfate precipitable fraction, a fraction in which metallothionein does not precipitate (79). The remainder of the Be^{2+} was free or bound elsewhere. A procedure developed for the purification of this "Be^{2+} binding protein" is described in the methods. The purified protein was brick red in color. It eluted in the void volume of a sephacryl S200 column, suggesting a molecular weight of at least 150,000. When subjected to native gel electrophoresis (Figure 3.1), the protein migrated as a single band containing the bound beryllium.

Identification of the "Be^{2+} Binding Protein" as Ferritin

In 20-50% sucrose gradients our "Be^{2+} binding protein" sedimented very closely to the horse spleen ferritin which was used as a standard (Figure 3.2). To test the identity of ferritin with the "Be^{2+} binding protein," rat liver ferritin was purified by the established procedure of Linder and Munro (41). Sucrose density sedimentation showed that the two proteins migrated as broad peaks within 3 svedberg units of each other. Ferritin sedimented as 64.8 S and "Be^{2+} binding protein" sedimented as 61.4 S (Figure 3.2). U.V. and visible absorption spectroscopy indicated that both proteins had a broad absorbance below 400 nm, although the rate liver ferritin had a greater absorbance per milligram protein (Figure 3.3, top panel). However, these differences disappeared when the proteins were treated with thioglycolate and

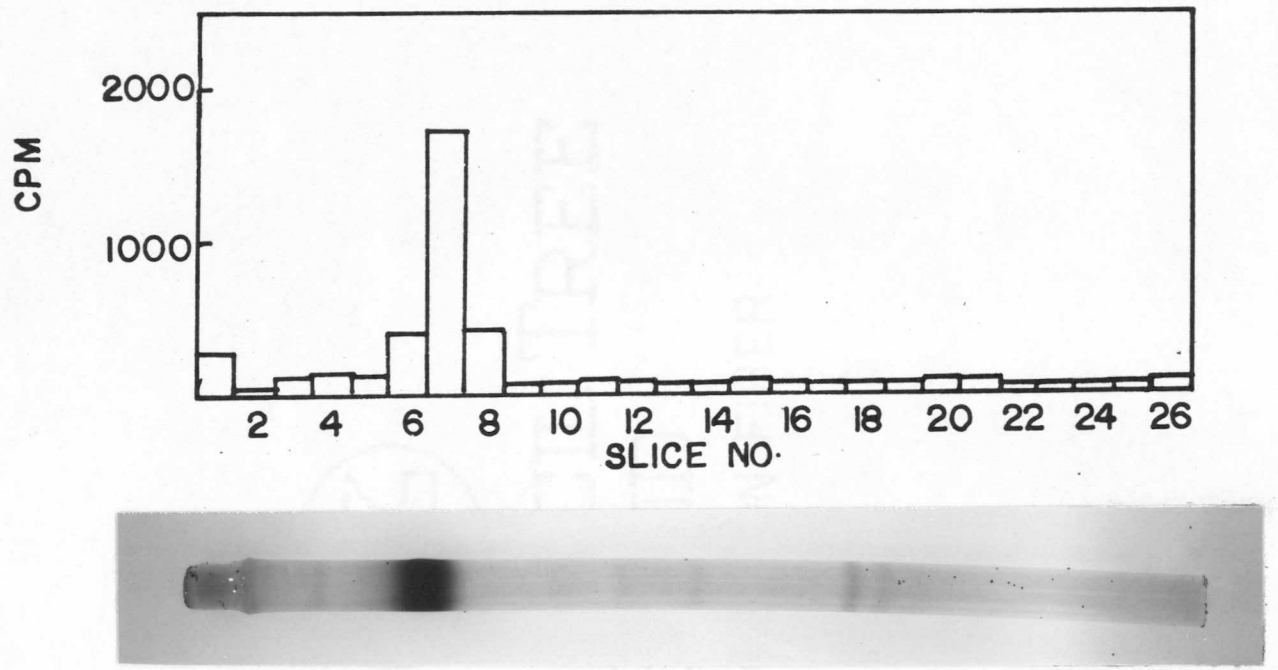


Figure 3.1. Native polyacrylamide gel electrophoresis of Be^{2+} binding protein. 80 µg Be^{2+} binding protein was electrophesed in duplicate on 5% polyacrylamide tube gels. One gel was stained for protein and the other cut into four mm thick slices and the radioactivity counted.

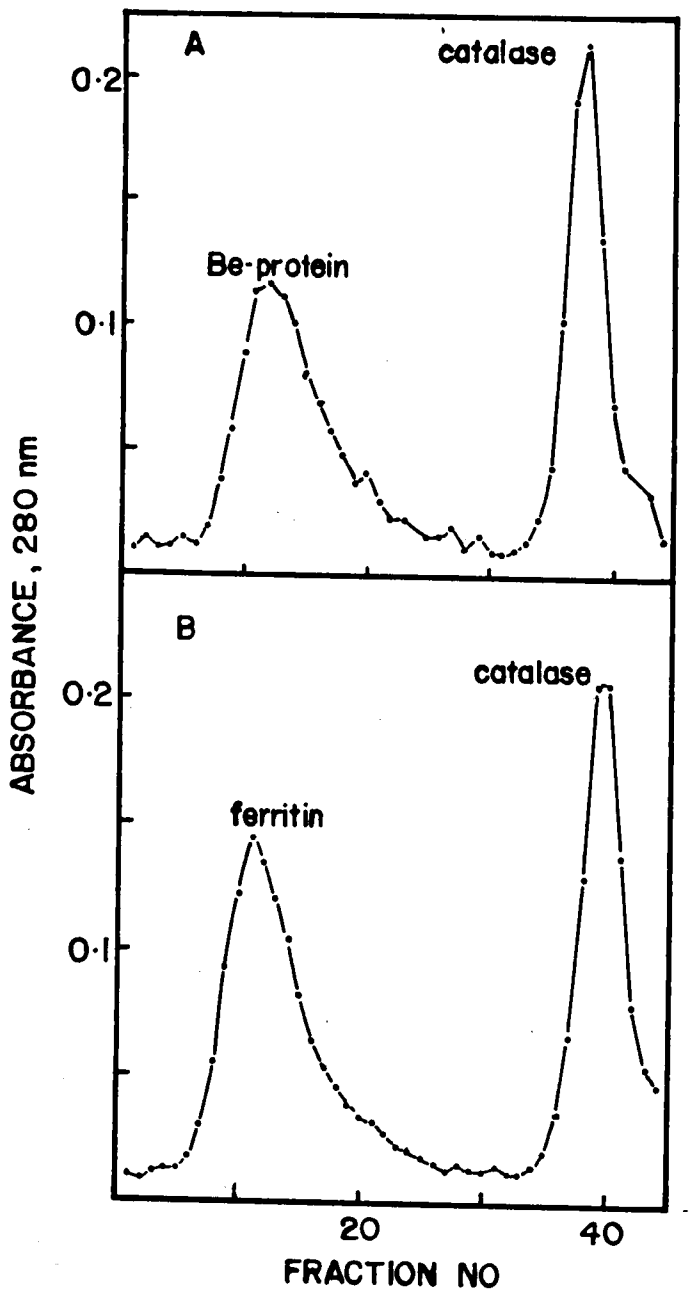


Figure 3.2. Sucrose density sedimentation of Be^{2+} binding protein and ferritin. (A) Be^{2+} binding protein plus bovine catalase, (b) Rat liver ferritin plus bovine catalase. Samples we centrifuged as described in "Experimental Procedure." Assuming an $S_{20,w}$0.725 for catalase of 11.3S, $S_{20,w}$0.725 values of 61.4S and 64.8S were calculated for Be^{2+} binding protein and rat liver ferritin respectively.

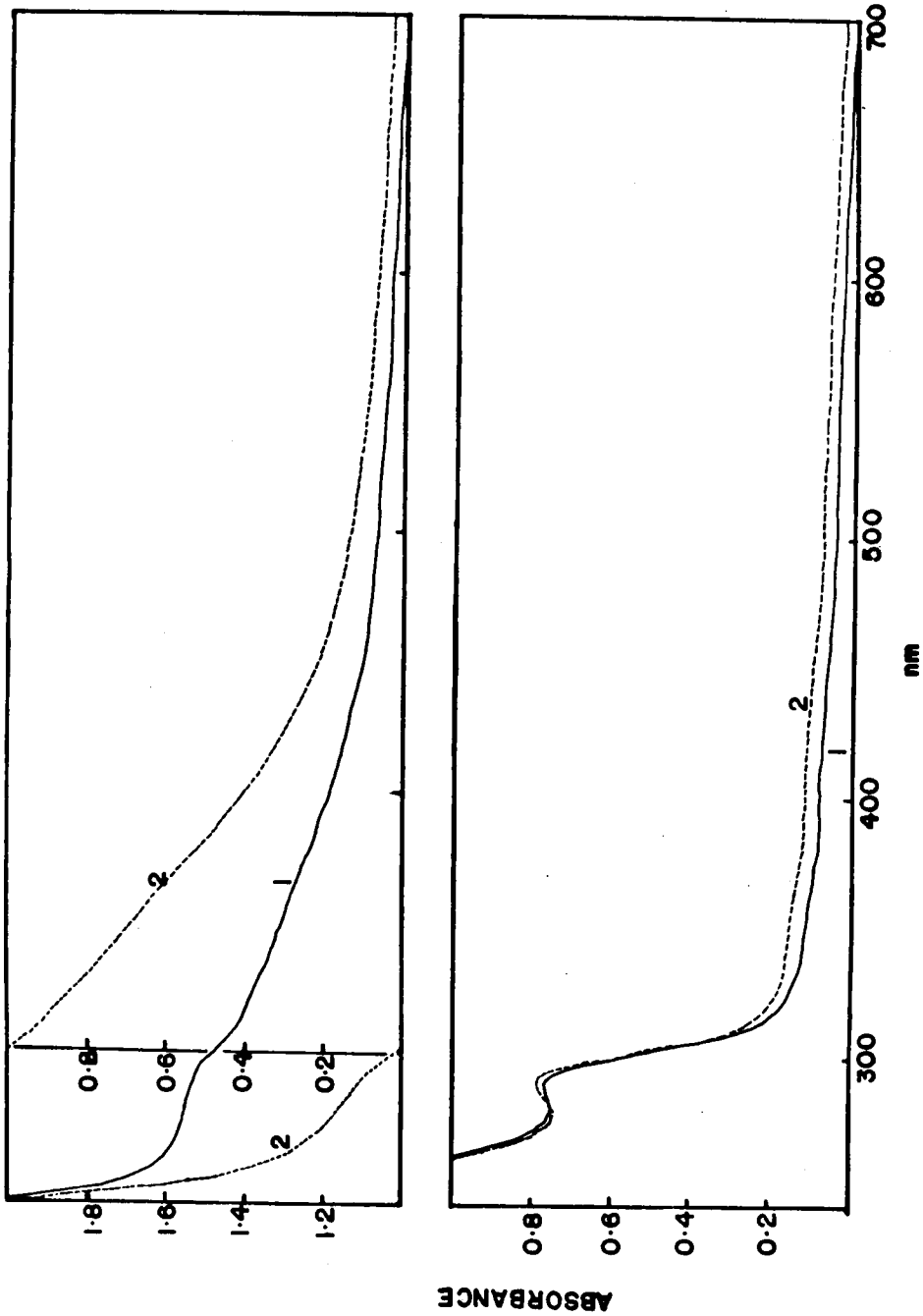


Figure 3.3. UV-visible absorption spectroscopy of (1) Be^{2+} binding protein and (2) ferritin from rat liver. Top panel shows spectra of native iron containing samples at 0.1 mg/ml protein in deionized water. Expanded scale in left rectangle applies to ferritin (2) only. Bottom panel shows spectra of iron free Be^{2+} binding protein (1) at 0.94 mg/ml protein and rat liver apoferritin (2) at 1.00 mg/ml protein in 0.005 M Tris-HCl, pH 8.5.

dialyzed to remove iron (Figure 3.3, bottom panel). Both proteins had a subunit molecular weight of about 20,000 (Figure 3.4) which is expected for ferritin (8). The two proteins had similar amino acid composition as well as electrophoretic patterns on isoelectric focusing gels (data not shown).

The identification of "Be^{2+} binding protein" as ferritin was established by their identical antigenic properties. The procedure involved Ouchterlony immunodiffusion using either rabbit anti-horse spleen ferritin or sheep anti-human ferritin (Figure 3.5). In all cases, the rat liver ferritin and "Be^{2+} binding protein" formed continuous precipitin lines and formed a single spur when adjacent to horse spleen ferritin.

Thus, the two proteins were indistinguishable as judged by sucrose density sedimentation, SDS-polyacrylamide gel electrophoresis, total amino acid composition, isoelectric focusing, and the reaction with antibodies.

Quantitation of the Binding of Zn^{2+}, Cd^{2+}, Cu^{2+} and Be^{2+} to Ferritin or Apoferritin

Once the Be^{2+} binding protein was identified as ferritin, horse spleen ferritin was used for further studies. First, the equilibrium gel filtration method of Pieterson (47) was employed to determine equilibrium binding of metals to ferritin or apoferritin at 0.2 mM of Zn^{2+}, Cd^{2+} or Cu^{2+}. Figure 3.6 shows a typical elution profile for ferritin run on the Zn^{2+} equilibrated column. The Zn^{2+} bound to ferritin observed in the protein peak is absorbed from the metal containing elution

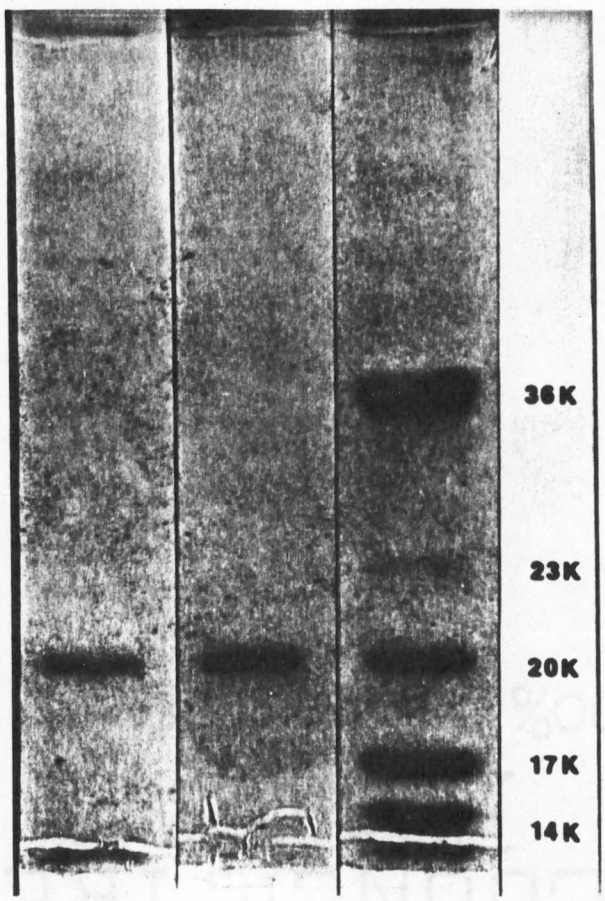


Figure 3.4. SDS-polyacrylamide gel electrophoresis of Be^{2+} binding protein and ferritin from rat liver. Subunit molecular weights of Be^{2+} binding protein (left) and rat liver ferritin (center) were determined to be approximately 20,000 each. Right lane indicates Be^{2+} binding protein plus standards, glyceraldehyde 3-phosphate dehydrogenase (36K), myoglobin (17K), and lysozyme (14 K).

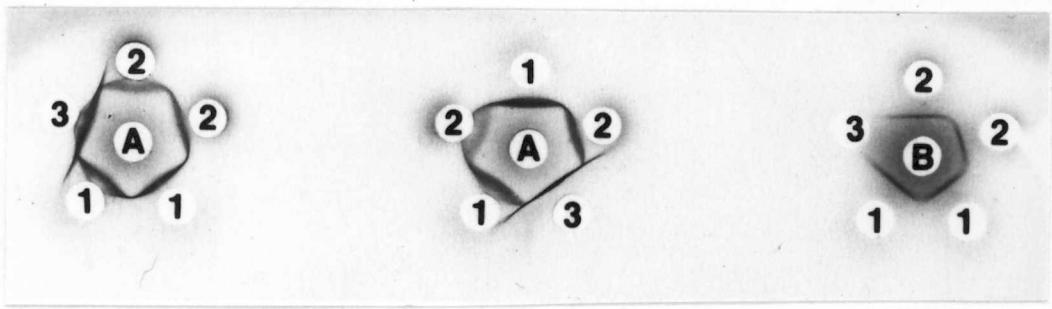


Figure 3.5. Immunological precipitation of ferritin and Be^{2+} binding protein with anti-ferritin antibodies. Ouchterlony gel diffusion plates reacting (A) rabbit anti-horse spleen or (B) sheep anti-human ferritin with (1) rat liver ferritin, (2) Be^{2+} binding protein, (3) horse spleen ferritin.

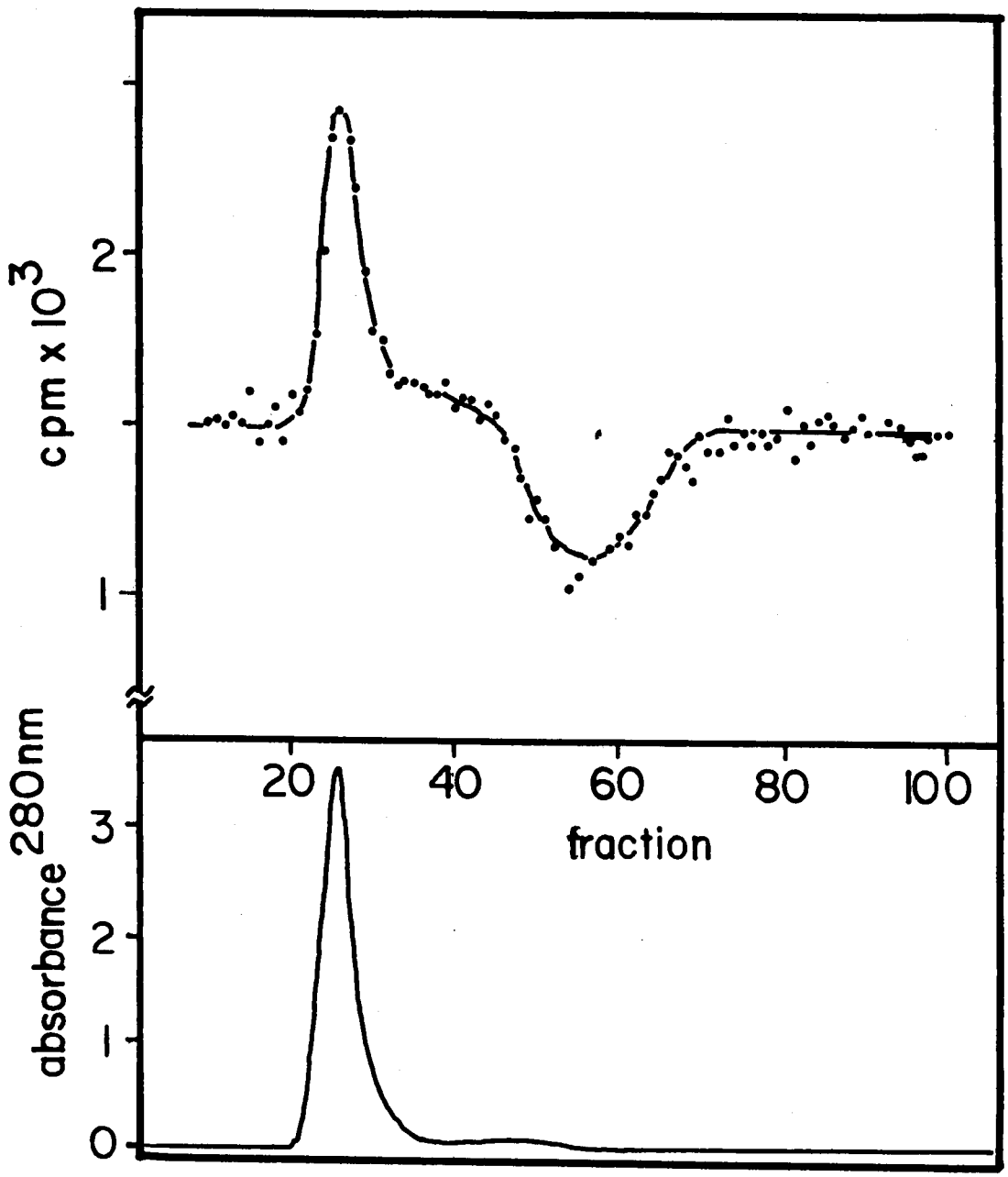


Figure 3.6. Zn-equilibrium gel chromatography of horse spleen ferritin. The protein sample was dissolved in Zn containing buffer, applied to a G-75 sephadex (coarse) column, and eluted as described in "Experimental Procedure." Protein was monitored by absorbance at 280 nm and Zn concentration was measured by a 65Zn tracer in a gamma counter.

buffer and this results in a valley in the metal profile at the position of the included volume of elution. Similar elution profiles were obtained for Cd^{2+} and Cu^{2+}. Fractions containing protein bound metal ions were pooled and dialyzed against Tris-HCl buffer or Tris-HCl, EDTA buffer to distinguish the loosely and tightly bound metal ions (Table 3.1).

As seen, under equilibrium conditions ferritin bound 175 gram atoms of Zn^{2+} and 224 gram atoms of Cd^{2+} but only 58 gram atoms of Cu^{2+}. After dialysis against Tris-HCl, 64 gram atoms of Zn^{2+}, 83 gram atoms of Cd^{2+} and 34 gram atoms of Cu^{2+} remained bound to ferritin. Dialysis against Tris-HCl containing EDTA removed all but a small percentage of the bound metal and different ferritin metal complexes were not distinguished on this basis.

In contrast to ferritin, apoferritin bound much less of Zn^{2+} and Cd^{2+}. The amount of Cu^{2+} bound to apoferritin (50 gram atoms) was similar to that bound to ferritin (58 gram atoms). Dialysis of metal-apoferritin complexes against Tris-HCl reduced all metals bound by about 50%. Dialysis against EDTA + Tris-HCl reduced Cd^{2+} and Cu^{2+} to 3 gram atoms and 11 gram atoms respectively, similar to the levels remaining in ferritin. Zn^{2+} however was not significantly removed by EDTA + Tris-HCl dialysis.

In another series of experiments the equilibrium binding of metals to ferritin or apoferritin was studied by Centrifree separation. This method is less time consuming and allowed us to vary the concentration of metal incubated with the protein. The Scatchard plots of the data obtained are shown in Figure 3.7. From this figure, the dissociation

TABLE 3.1

Gram Atoms of Divalent Metals Bound to Ferritin and Apoferritin in Equilibrium Gel Filtration

	Ferritin			Apoferritin		
	Undialyzed	Buffer Dialyzed	Buffer + EDTA Dialyzed	Undialyzed	Buffer Dialyzed	Buffer + EDTA Dialyzed
Zn	175 ± 11	64 ± 9	11 ± 6	36 ± 6	----	34 ± 8
Cd	224 ± 20	83 ± 4	3 ± 0.2	71 ± 31	38 ± 5	3 ± 0.8
Cu	58 ± 3	34 ± 7	9 ± 3	50 ± 4	20 ± 2	11 ± 1

The experiment was done at the saturating concentration of metal ions, which for Zn, Cd and Cu involved 0.2 mM Divalent metal and 0.5 M Tris pH 7.4.

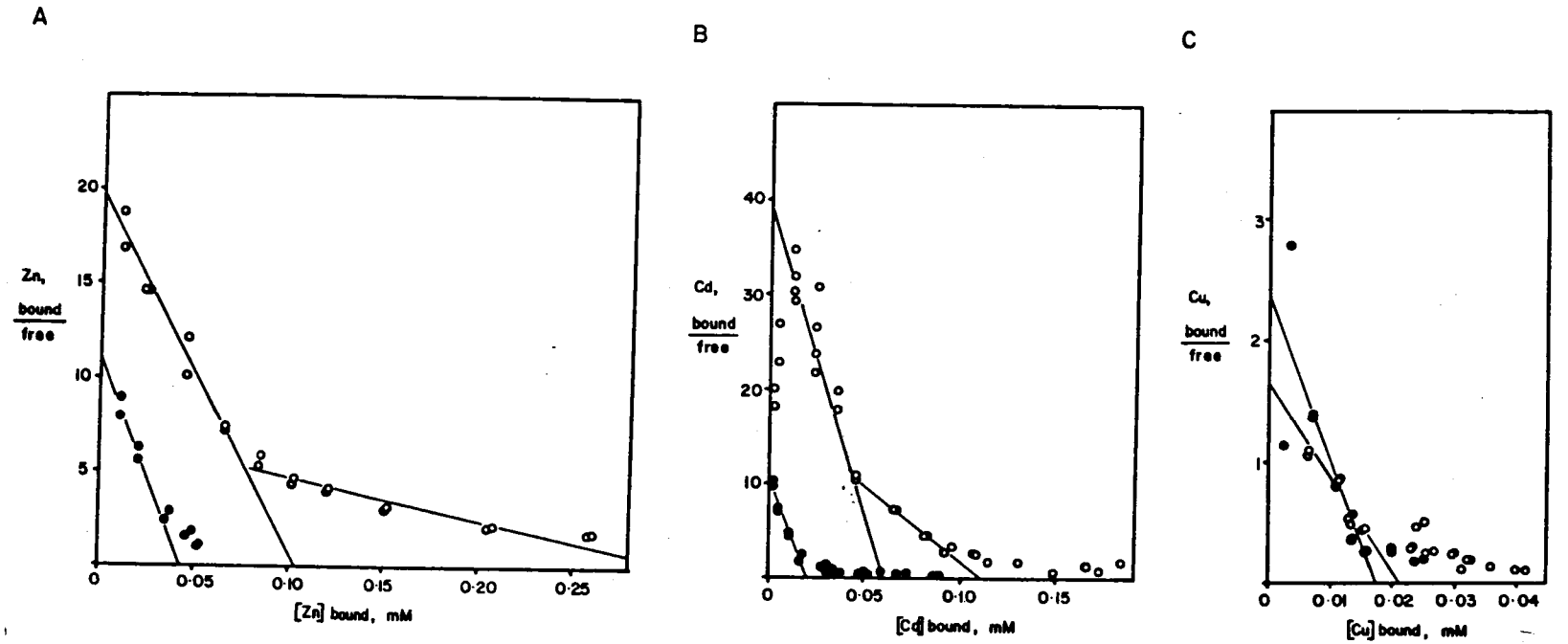


Figure 3.7. Scatchard analysis of divalent metal binding to ferritin (O) or apoferritin (●). Metal ions tested for binding by the Centrifree method were Zn^{2+} (panel A), Cd^{2+} (panel B) and Cu^{2+} (panel C). These plots were then analyzed for gram atoms of metal bound (n) and dissociated constant (K_D) as shown in Table 3.2.

constants, K_D (-1/slope) and the binding capacities, n (x-intercept/ protein concentration) for Cd^{2+}, Cu^{2+} and Zn^{2+} were calculated (Table 3.2). As seen, ferritin has two classes of binding affinities for Zn^{2+} and Cd^{2+}, but only one for Cu^{2+}. For apoferritin, Zn^{2+}, Cd^{2+} and Cu^{2+} each showed single classes of binding sites. The affinities of all the metals were of the magnitude of 10^{-6}M (Table 3.2). Compared to apoferritin, ferritin bound more of each metal except Cu^{2+}. As was seen in equilibrium gel filtration, ferritin and apoferritin bound similar amounts of Cu^{2+}. Some discrepancy between the results obtained by the Centrifree method (Table 3.2) and by equilbrium gel filtration is apparent. In these cases, we would judge that the Centrifree method is probably more reliable because the values represent extrapolation to the maximum number of binding sites.

Equilibrium gel filtration technique was unsuitable for Be^{2+} binding studies because as discovered later ferritin bound at least 800 gm atoms of Be^{2+} (see below) and at the protein concentration required for such studies, the amount of soluble Be^{2+} was insufficient to saturate the system. Therefore, binding of Be^{2+} to ferritin or apoferritin was studied by the Centrifree method. Analysis of data by the Scatchard method did not give a value for binding affinity. Figure 3.8 shows the beryllium binding data plotted with gram atoms on the y axis and total Be^{2+} concentration on the x axis. As seen, apoferritin bound a maximum of 160 gram atoms of Be^{2+} but ferritin was not saturated even after it bound 800 gram atoms of Be^{2+}. When binding of Be^{2+} was monitored by the microcentrifuge desalting method we expected to

TABLE 3.2

Binding Parameters of Divalent Metals for Ferritin and Apoferritin as Determined by Micropartition followed by Scatchard Plot

Metal	Ferritin		Apoferritin	
	n	$K_D \times {}^{-6}M$	n	$K_D \times 10^{-6}M$
Zn	135.2 251.0	5.25 44.1	65.3	3.93
Cd	76.7 61.6	1.56 6.11	29.0	1.92
Cu	26.4	13.0	26.0	7.65

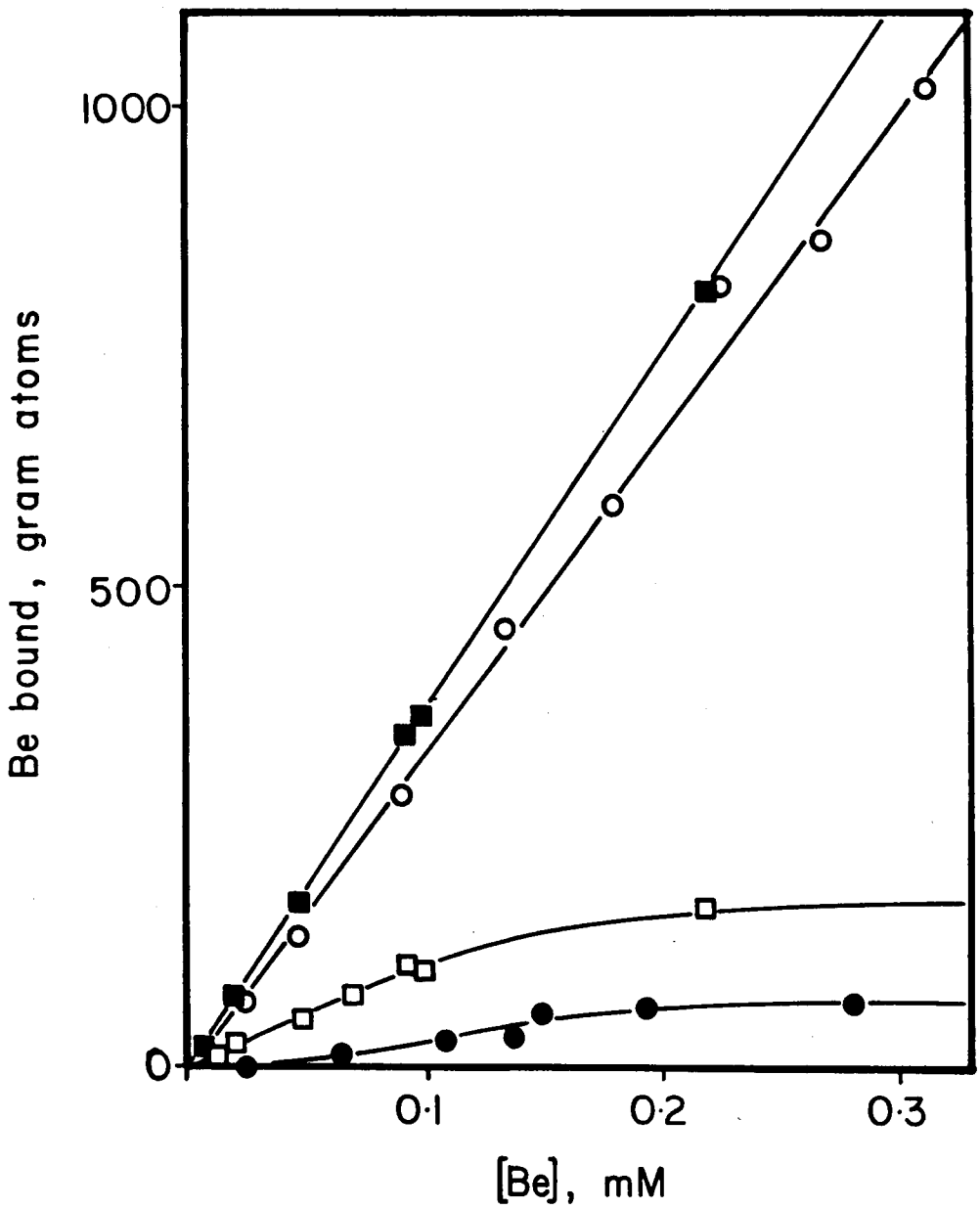


Figure 3.8. Binding of Be^{2+} to ferritin and apoferritin. Amounts of Be bound as a function of Be concentration are shown by Centrifree technique (ferritin, ■; apoferritin, □) and by microcentrifuge desalting technique (ferritin, ○; apoferritin, ●).

measure only those metal ions which were tightly bound (Figure 3.8). Amounts bound to ferritin by this method were only slightly less than those observed by the Centrifree method. At 0.22 mM total Be^{2+}, ferritin bound 827 gram atoms by the Centrifree method and 750 gram atoms by the desalting method. In contrast apoferritin under similar conditions bound 160 gm atoms by Centrifree method and 60 gm atoms by the desalting method. Measurement of iron showed no change in the amount of iron bound after Be was bound to ferritin or apoferritin. This ruled out a replacement of iron by Be^{2+} in these proteins.

To determine whether Be^{2+} either precipitated or dissociated after it was bound to ferritin, aliquots of this proteins containing different amounts of $^{7}Be^{2+}$ were centrifuged in a sucrose gradients (Figure 3.9). For Be-ferritin containing 128 gram atoms, all the ^{7}Be migrated with ferritin during the centrifugation. For higher amounts of Be in ferritin (377 or 599 gram atoms) about 20% of the ^{7}Be remained on top of the gradient and the remainder migrated with the ferritin. This suggests that some bound Be^{2+} dissociated due to dilution, and that the amount of dissociable Be^{2+} is dependent on the amount bound. Quantitation of the dissociation of Be^{2+} from ferritin was accomplished by using sulfosalicylic acid (SSA), a known reversible chelator for Be^{2+}.

Affinity of Be^{2+} for Sulfosalicylic Acid

To carry out the above experiment, it was first necessary to determine the dissociation constant of Be^{2+} for sulfosalicylic acid at pH 6.5. The results of the spectrophotometric titration of sulfosalicylic acid with Be^{2+} are shown in Figure 3.10. Binding of

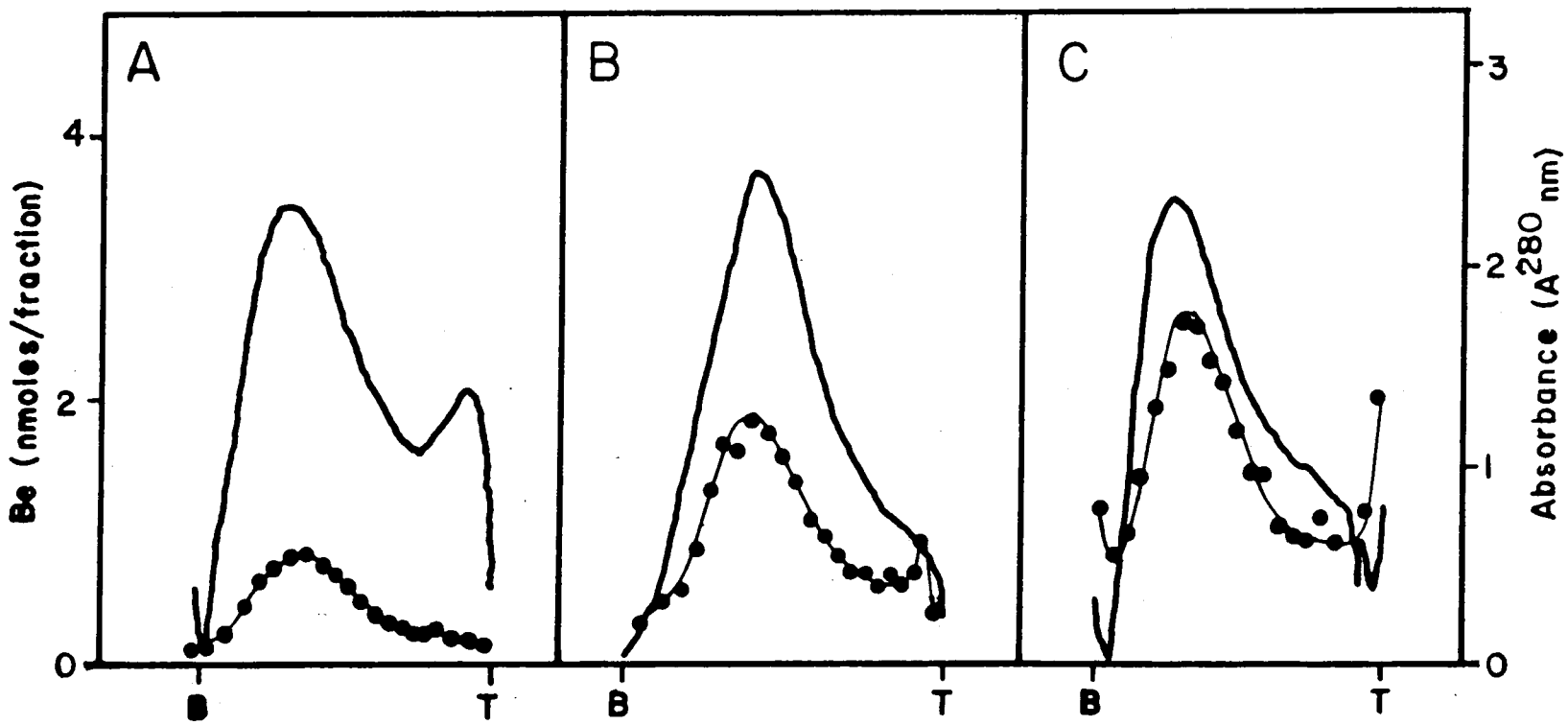


Figure 3.9. Sucrose density sedimentation of Be-ferritins with increasing Be^{2+} content. Be-ferritins of different Be^{2+} content were prepared using ^{7}Be as tracer. The protein bound Be^{2+} was separated from unbound Be^{2+} by the microcentrifuge desalting method. Be-ferritin samples containing 128 gram atoms Be^{2+} (A), 377 gram atoms Be^{2+} (B) and 599 gram atoms Be^{2+} (C) were centrifuged in 20-50% sucrose gradients as described in "Experimental Procedure" except that 0.05 M Tris-HCL pH 6.5, not 0.01 M phosphate pH 7.0 was used as buffer. Fractions (0.2 ml each) were monitored for protein (A^{280nm}) and Be^{2+} content as measured by specific radioactivity (T = top, B = bottom).

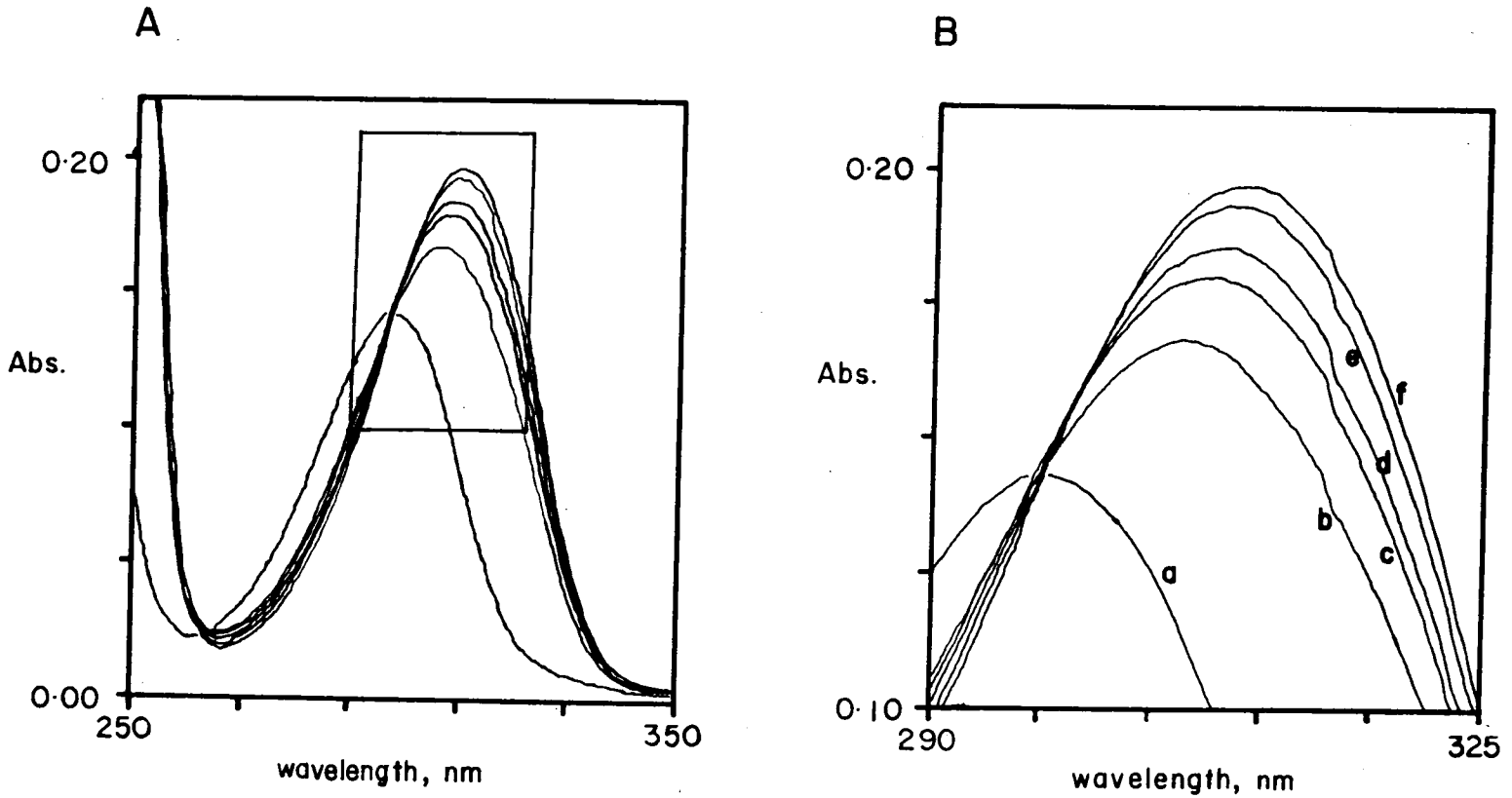


Figure 3.10. U.V. absorbance spectrum of SSA with increasing levels of Be^{2+} added. Panel B is an enlargement of the rectangle shown in panel A. The concentration of SSA was 0.05 mM in each sample. The final concentrations of Be^{2+} were 0 mM (a), 0.04 mM (b), 0.05 mM (c), 0.06 mM (d), 0.073 mM (3) and 0.10 mM (d). Absorbances at 310 nm were measured for quantitation of bound and free Be^{2+} (Figure 3.11).

Be^{2+} causes a decrease in absorbance at 300 nm and an increase in absorbance at 310 nm. The concentration of Be-SSA complex was determined by the equation:

$$[\text{Be-SSA}] = \frac{(\text{Abs}^{310} - \text{Abs}_{\text{SSA}}{}^{310})}{E^{310\text{nm}}} \qquad (1)$$

$E^{310\text{nm}}$ = molar extinction coefficient for Be-SSA = 4.088×10^3

The term $\text{Abs}_{\text{SSA}}{}^{310}$ is equal to the absorbance of free SSA at 310 nm expressed as a fraction of a total of 0.05 mM total SSA. This is calculated as follows:

$$\text{Abs}_{\text{SSA}}{}^{310} = \text{Abs}_{\text{o}}{}^{310} \left(\frac{5 \times 10^{-5} - [\text{Be-SSA}]}{5 \times 10^{-5}} \right) \qquad (2)$$

where $\text{Abs}_{\text{o}}{}^{310}$ = absorbance of 0.05 mM SSA alone at 310 nm = 0.842.

Combining equations 1 and 2 and solving for [Be-SSA] we write:

$$[\text{Be-SSA}] = \frac{\text{Abs}^{310} - \text{Abs}_{0}{}^{310}}{\left(E^{310\text{nm}} - \frac{\text{Abs}_{0}{}^{310}}{5 \times 10^{-5}} \right)} \qquad (3)$$

Using equation 3 and the absorbance values at 310 nm, we have calculated the amount of Be-SSA complex and amount of free SSA.

From the Scatchard plot of the data (Figure 3.11) the $^{\text{SSA}}K_{\text{D}}{}^{\text{Be}}$ at pH 6.5 was calculated to be -1/slope = 5.04×10^{-6}M.

Affinity of Be^{2+} for Ferritin

The competitive removal of ^{7}Be by SSA was determined by adding increasing amounts of SSA to ^{7}Be-ferritin followed by the separation

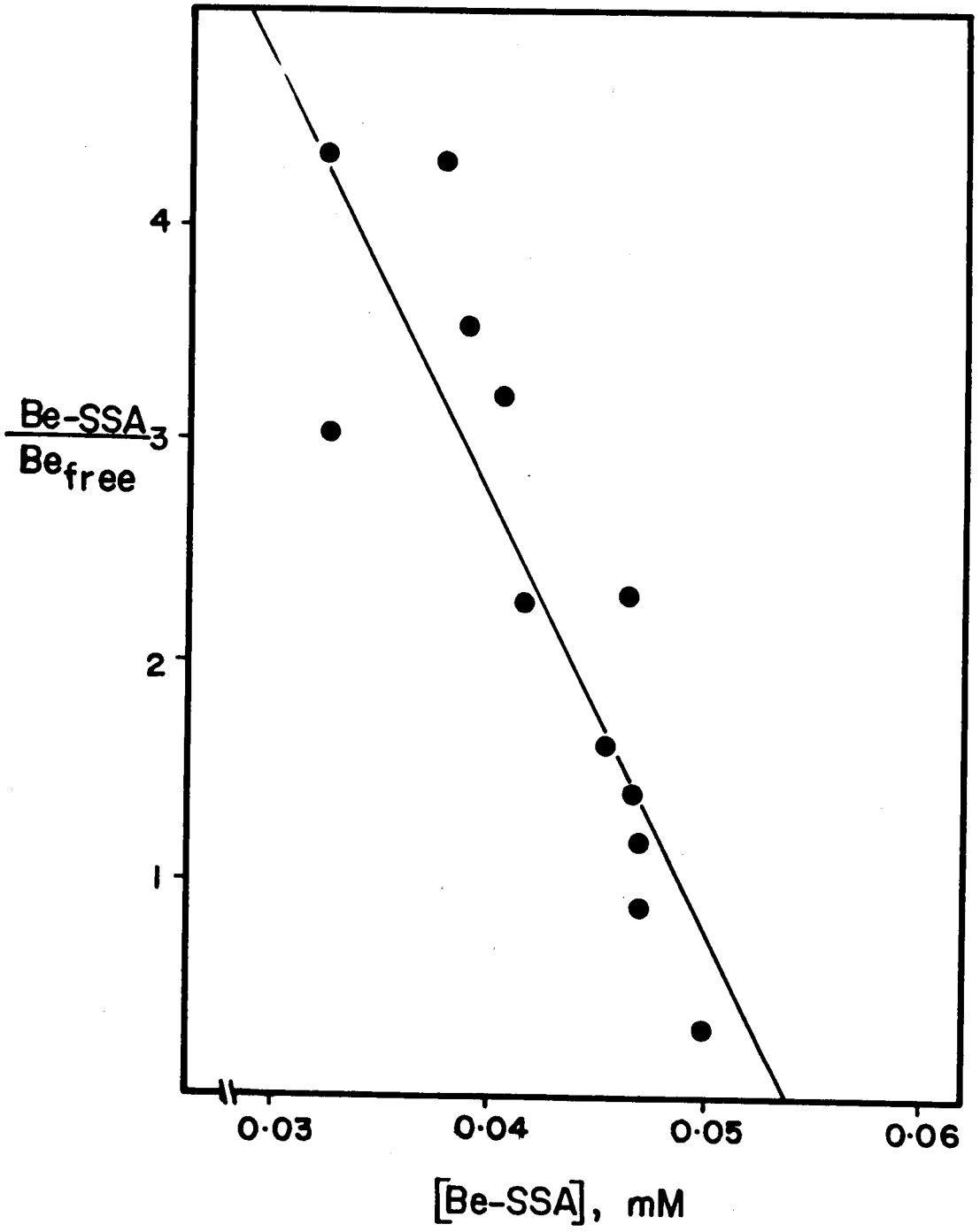


Figure 3.11. Scatchard plot of Be^{2+} binding to SSA. Amounts of bound and free Be^{2+} were determined from spectrophotometric data (Figure 3.10) as described in the "Results" section. From the least squares fit line we determined the dissociation constant ($^{SSA}K_D^{Be}$) to be -1/slope = 5.04 $\times 10^{-6}$M.

of the ^{7}Be-SSA from ferritin by Centrifree method. Figure 3.12 shows the increase in ^{7}Be removal from ferritin as a function of increasing SSA concentration. Three ^{7}Be-ferritins were utilized. They contained 83, 335, and 698 gram atoms of Be^{2+} respectively. Horizontal lines represent the maximum amount of $^{7}Be^{2+}$ that could be removed from each Be-ferritin. As can be seen in the three curves, SSA removed all but 20-40 gram atoms of Be^{2+}. The affinity of Be^{2+} for ferritin was calculated with the use of the following relationships:

$$\text{Be-ferritin} \rightleftharpoons \text{Be} + \text{ferritin} \qquad {}_{Be}K_{D}Fer = \frac{[Be]\ [ferritin]}{[Be\text{-}ferritin]} \qquad (5)$$

$$\text{SSA} + \text{Be} \rightleftharpoons \text{Be-SSA} \qquad {}_{Be}K_{A}SSA = \frac{[Be\text{-}SSA]}{[Be]\ [SSA]} \qquad (6)$$

Sum

$$\text{Be-ferritin} + \text{SSA} \rightleftharpoons \text{ferritin} + \text{Be-SSA}$$

$$Keq = \frac{[ferritin]\ [Be\text{-}SSA]}{[SSA]\ [Be\text{-}ferritin]} \qquad (7)$$

Thus, the equilibrium expression (equation 7) of the reaction carried out is the sum of two individual components (equations 5 and 6).

From these three reactions,

$$\frac{[ferritin]\ [Be\text{-}SSA]}{[SSA]\ [Be\text{-}ferritin]} = {}_{Be}K_{D}Fer \cdot {}_{Be}K_{A}SSA \qquad (8)$$

When half of the total Be^{2+} is removed from ferritin, the concentrations of ferritin and Be-ferritin are equal. Therefore, equation 8 simplifies to:

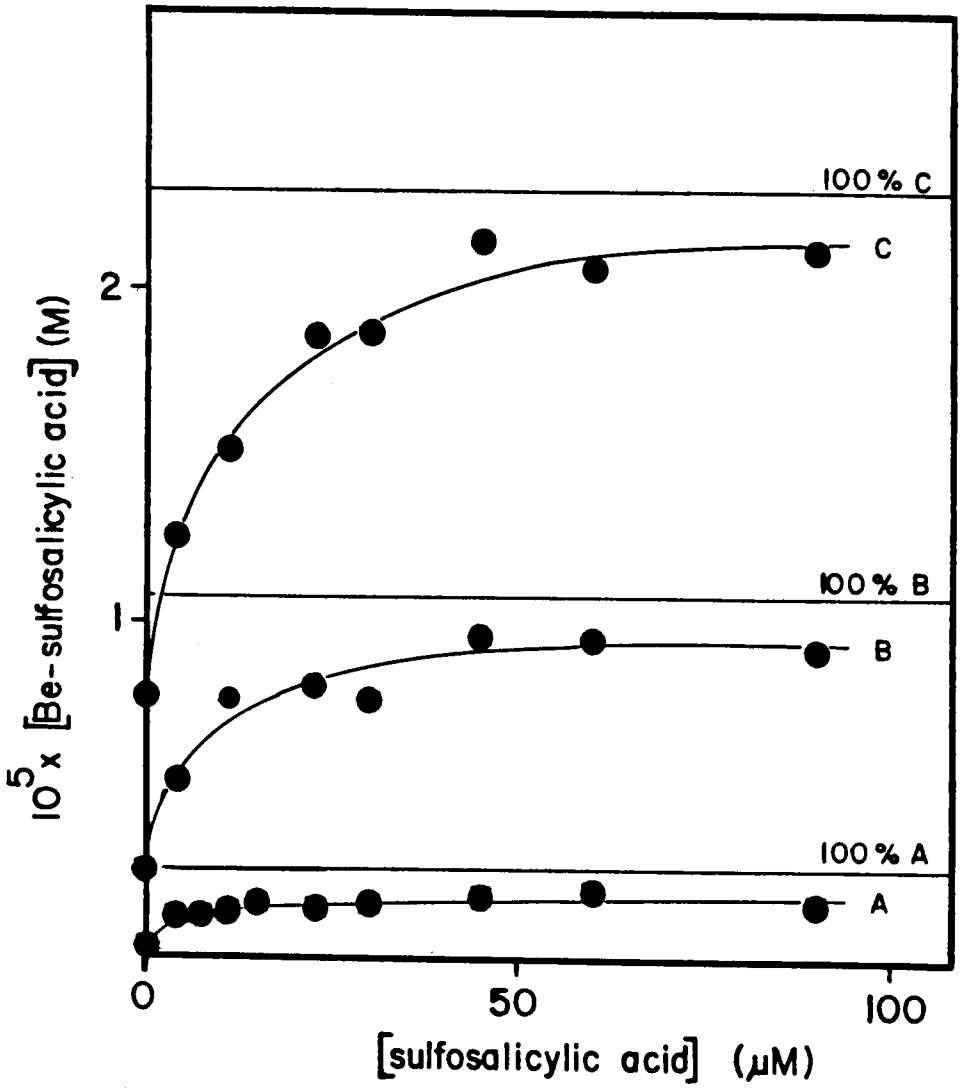


Figure 3.12. Removal of Be^{2+} from ferritin by SSA. ^{7}Be-ferritins of three different Be contents were made containing 83 gram atom Be^{2+} (A), 335 gram atoms Be^{2+} (B) and 698 gram atoms Be^{2+} (C) according to the method described in Figure 3.9. ^{7}Be-ferritin (final 20 nM protein) and increasing levels of SSA (final 0-.1 mM) were incubated in 0.5 ml final volume with .05 M Tris-HCl pH 6.5 buffer for 2 hours at 20°. Ferritin bound Be^{2+} was then separated from SSA bound Be^{2+} by the centrifree method. Horizontal lines represent the expected Be-SSA levels if all Be^{2+} were removed from ferritin.

$$\frac{[\text{Be-SSA}]}{[\text{SSA}]} = {}^{\text{Be}}K_D\text{Fer} \cdot {}^{\text{Be}}K_A\text{SSA} \qquad (9)$$

From the data for Be-ferritin containing 698 gram atoms of Be^{2+} (Figure 3.12) half maximal removal of Be^{2+} was achieved at 3.7×10^{-5}M SSA. We now simplify the left half of equation 9 and substitute the known values:

$$\frac{[\text{Be-SSA}]}{([\text{SSA}]\ \text{total} - [\text{Be-SSA}])} = \frac{(1.55 \times 10^{-5}\text{M})}{(2.7 \times 10^{-5}\text{M} - 1.55 \times 10^{-5}\text{M})} = 1.35$$

The value of ${}^{\text{Be}}K_A\text{SSA}$ in equation 9 is calculated as the reciprocal of the dissociation constant of Be for SSA as determined in the previous section:

$${}^{\text{Be}}K_A\text{SSA} = \frac{1}{{}^{\text{Be}}K_D\text{SSA}} = 1.98 \times 10^{-5}\ \text{M}^{-1}$$

We then substitute the values of ([Be-SSA]/[SSA]) and ${}^{\text{Be}}K_A\text{SSA}$ into equation 9 and solve for the dissociation constant of Be^{2+} for ferritin (${}^{\text{Be}}K_D\text{Fer}$):

$${}^{\text{Be}}K_D\text{Fer} = \frac{1.35}{1.98 \times 10^{5}} = 6.80 \times 10^{-6}\text{M}$$

Thus, by an indirect method the affinity of Be^{2+} for ferritin is calculated.

D. DISCUSSION

The data presented here clearly establish ferritin as one of the major beryllium binding proteins in liver cytoplasm. As shown by Piotrowski and Szymanska (48), we found that metallothionein was not induced by Be^{2+}. We also found that metallothionein induced by Cd^{2+} and then made devoid of Cd^{2+} was unable to bind Be^{2+} (unpublished data).

This is not surprising in that other divalent metals form mercaptides with metallothionein (37) and Be^{2+} is not able to form such bonds (38).

Ferritin contains carboxylate and phosphate functional groups (28) which are known to bind Be^{2+} in certain cases. It has been shown that beryllium forms complexes with carboxylic acids such as aurintricarboxylic acid (59), citrate, and malonate (38). Ferritin contains carboxyl residues which are able to bind Zn^{2+} and Tb^{2+} (71). Chasteen and Theil have shown that vanadyl ions, VO^{2+}, compete with Zn^{2+}, Fe^{3+} Fe^{2+}, and Tb^{2+} for the binding to apoferritin (11). The fact that EPR signals of VO^{2+} complexed with malonate or apoferritin are similar underscores the importance of carboxylate residues for metal binding. Beryllium also seems to form complexes with various nuclear nonhistone phosphoproteins (46) and other phosphate compounds (38). The well documented presence of phosphate in the iron core of ferritin (72) makes this protein a good candidate as a beryllium chelator. We have observed in one of our preparations that horse spleen ferritin containing 1500 gm atoms of iron had 322 gm atoms of phosphate whereas its apoprotein containing 29 gm atoms of iron had no detectable phosphate. Thus, most if not all of the phosphate in ferritin resides in the iron core.

As mentioned in chapter I, at neutral pH beryllium forms insoluble hydroxides. This presents a problem when measuring the binding of Be to biological substrates. Therefore, Parker and Stevens used the Be-SSA complex as a Be^{2+} donor to determine the affinity of Be^{2+} for nuclear acidic nonhistone proteins (46). In calculating these affinities,

however, the affinity of SSA for beryllium appeared to have been ignored. It was therefore not surprising that SSA-Be and citrate-Be complexes gave very different results for the affinity of Be^{2+} to nonhistone proteins. We have determined the affinity of Be^{2+} for SSA spectrophotometrically ($^{SSA}K_D^{Be} = 5.04 \times 10^{-6}M$). In our calculations, we have assumed a 1:1 binding of SSA to Be^{2+}. Das and Aditya have shown 1:1 binding of SSA to Be^{2+} at pH 4.5 and 4.0 (17). They have reported that at higher pH (9-11), SSA binds to Be^{2+} in a 2:1 ratio. Since our pH (6.5) was much closer to 4.5, we concluded that the binding in our experiments was primarily in a 1:1 ratio. The fact that our calculated $^{SSA}K_D^{Be}$ ($5.04 \times 10^{-6}M$) was much smaller than that determined by Das and Aditya ($5.4 \times 10^{-5}M$ at 0.05 ionic strength and 29.5°) may be due to increased ionization of SSA at the higher pH. Using our value for $^{SSA}K_D^{Be}$, we were then able to determine the affinity of Be^{2+} for ferritin.

In our initial experiments we used 0.5 mM Be pH 6.5 where Be^{2+} is in solution. We found that solutions of 0.5 mM $BeSO_4$ would remain soluble at pH 6.3-6.5 when used immediately after pH adjustment. Even under these conditions, we suspect that some of the beryllium exists as soluble $Be(OH)_2$. For this reason binding to the chelator may be less than expected for completely ionized solution. Initially, we attempted to do binding studies of Be^{2+} to ferritin by equilibrium gel filtration. This was successfully employed to determine binding capacities for Cu^{2+}, Zn^{2+} and Cd^{2+} to ferritin or apoferritin (Table 3.1, page 43). However, the results for Be^{2+} were inconsistent. Therefore, we used two techniques, the Centrifree micropartition and the microcentrifuge

desalting methods. The Centrifree method yielded the maximal amount of Be^{2+} bound because it was carried out under equilibrium conditions. The desalting method showed only tightly bound Be^{2+} since it was equivalent to dialysis against Be^{2+} free buffer. The large amount of Be^{2+} bound to ferritin is especially noteworthy. Ferritin bound greater than 800 gm atoms of Be^{2+} under both equilibrium and dialyzed conditions. We hypothesize that this large amount of tightly bound Be^{2+} bound is due to association with phosphate (322 gram atoms) that is present in the iron core. We were also able to get as high as 1200 gram atoms of Be^{2+} bound to ferritin at higher total Be^{2+} concentration. The high levels of Be^{2+} bound to ferritin under these conditions may be an artifact if a loss of subunits from the ferritin shell has occurred. If the calculation of molar quantity of ferritin molecules is erroneously low due to this damage, then it would appear that the gram atoms of Be^{2+} were greater than were actually bound to each ferritin molecule. It may be that Be^{2+} tightly associated with the core disrupts the iron oxyhydroxide-phosphate lattice and results in the partial breakdown of the ferritin molecule.

Apoferritin binds substantially less Be^{2+} than did ferritin and of the 160 gram atoms bound only 50-60 are tightly bound. Since the percentage of tight binding Be^{2+} is so much lower for apoferritin than for ferritin, one would suspect a different mode of binding. Thus, rather than binding to the residual iron ($\sim$29 gram atoms) or phosphate (none detected) of apoferritin, we suspect that Be^{2+} is bound to amino acid residues such as carboxyl residues or the hydroxyls of tyrosine on the protein shell.

We have analyzed the binding of other divalent metals (Zn^{2+}, Cd^{2+}, and Cu^{2+}) to ferritin or apoferritin in order to draw some general conclusions about divalent metal binding to these proteins. It was then possible to compare and contrast the Be^{2+} binding with the characteristics found for these other divalent metal ions.

The binding of various metal ions such as Zn^{2+}, Cd^{2+}, Cu^{2+}, Mn^{2+}, and Tb^{3+} to apoferritin has been analyzed by Macara et al. (43). It was found for all these metals except Mn^{2+}, that $\sim$2 metals per subunit were tightly bound and $\sim$3 metals per subunit were loosely bound. For Mn^{2+} there is only 0.5 metal ion tightly bound and two metal ions loosely bound per subunit. Our results for apoferritin binding of Zn^{2+}, Cd^{2+}, and Cu^{2+} were very similar to the results of Macara (43). Although we obtained a hyperbolic Scatchard plot for these metals as did Macara, we have only determined values for what are the most tightly bound metals. Our results for the binding of Zn^{2+}, Cd^{2+}, and Cu^{2+} to ferritin show some different trends. Zn^{2+} and Cd^{2+} binding to ferritin showed an increased number of low affinity sites, but there was also about a 50% increase in the high affinity sites compared to apoferritin. Cu^{2+} binding is not increased for ferritin as was the case for the other metals. Harrison has reported that ferritin and apoferritin have the same number of high affinity sites for Zn^{2+} (73). Our results suggest that during the formation of ferritin from apoferritin some new high affinity Zn^{2+} sites are created.

The binding of Be^{2+} to apoferritin was of particular interest when compared to the binding of other metals. We show that apoferritin binds

a total of 6.7 gram atoms per subunit and of these only 2.1-2.5 are tightly bound. Thus, our findings of loosely and tightly bound Be^{2+} to apoferritin are in reasonable agreement with those observed for other metals by Macara (43). It is therefore likely that in ferritin molecules the carboxylate residues are required for Be^{2+} binding just as they have been shown for the chelation of Zn^{2+} (71).

The binding of Be^{2+} to ferritin appears to be different from that of other metals. Although all the metal ions except Cu^{2+} showed binding which could be attributed to the iron core, beryllium binding was by far the highest in amount and affinity. This binding could be to either phosphate or ferric oxyhydroxide components of the core. However, since phosphate is known to bind Be^{2+}, this is the most likely possibility. Experiments are now in progress to make this distinction. To quantify the tightness of the binding of Be^{2+} to ferritin, we have measured the removal of the metal ion from ferritin by a known chelator, SSA. This technique was analogus to that of Vallee and Coombs (77) who estimated the binding of Zn^{2+} to alcohol dehydrogenase. Accordingly the affinity of Zn^{2+} for 1,10-phenanthroline was first measured spectrophotometrically, and then the ability of 1,10-phenanthroline to remove Zn^{2+} from alcohol dehydrogenase was observed. Based on our findings, the affinity of Be^{2+} for sulfosalicylic acid ($^{SSA}K_D^{Be} = 5.04 \times 10^{-6}M$) and for ferritin ($^{Fer}K_D^{Be} = 6.80 \times 10^{-6}M$) are very similar if not identical.

The sequestering of Be^{2+} by ferritin is significant not only because of the large amount bound and the tightness of binding, but also because the bound metal would have only limited accessibility to other proteins.

X-ray data of Banyard et al. (5) has shown that the only access to the ferritin core is through pores of 10 Å diameter on the fourfold axis of the protein shell. Thus, formation of beryllium complexes in the iron core of ferritin would make Be nonaccessable to other proteins which might have a higher affinity for beryllium. In particular, enzymes which are susceptible to inhibition by beryllium could be protected by this sequestration. This property is the subject of chapter IV.

The tissues shown to accumulate highest amounts of injected beryllium, liver and spleen (83), are also rich sources of ferritin (28). We have reported previously that Be^{2+} injected into rats is sequestered at least in part by ferritin (36). It is not claimed that ferritin is the only Be^{2+} binding protein capable of a protective effect. Since we initially searched for a Be^{2+} chelator in the heat stable fraction, we may have overlooked Be^{2+} chelators which were heat labile. Nevertheless, based on the properties of beryllium binding shown here, ferritin may represent a natural detoxicant for beryllium.

CHAPTER IV

DIVALENT METAL BINDING TO FERRITIN AND THE PROTECTION OF ENZYMATIC ACTIVITY IN VITRO

A. INTRODUCTION

As shown in chapter III, ferritin binds Be^{2+} as well as other metal ions. This observation led to the possibility that ferritin might be a natural detoxicant for Be^{2+} and other metal ions. The concept of using Be^{2+} chelator to alleviate Be^{2+} toxicity was first investigated by Lindenbaum et al. (40). These workers injected the beryllium chelator, aurintricarboxylic acid, into Be^{2+} intoxicated rats and noted a reversal of the Be^{2+} inhibition of alkaline phosphatase. At micromolar concentrations Be^{2+} also inhibits the membrane bound Na^{+}-K^{+} ATPase (70), and the cytoplasmic enzyme phosphoglucomutase (29). All three enzymes require a divalent metal ion for activity and catalyze a phosphate transfer. Inhibition of Na^{+}-K^{+} ATPase is reversible by Na^{+} (69) and that of alkaline phosphatase is reversible by aurintricarboxylic acid (40) but inhibition of PGM is reported to be irreversible (29). This enzyme binds a maximum of one gm atom of a divalent metal ion. PGM-Mg complex is most active; other metal ions form less active complexes (54) but PGM-Be complex is inactive. Furthermore, several metal chelators such as cysteine; EDTA or imidazole facilitate Be-PGM binding because they chelate other metal ions which compete for the metal binding site (29). Because ferritin

has a very high affinity for Be^{2+}, the ability of ferritin to reactivate the Be-PGM complex was investigated.

Ray (54) has studied the effect of various divalent metal ions on PGM reaction. In chapter III it is shown that Be^{2+} binding to ferritin is different from the binding of other divalent metals. The data presented here compares the reactivation by ferritin of PGM complexed with Be^{2+}, Zn^{2+}, Cd^{2+} or Cu^{2+} and suggests that the reactivation of PGM complexed with Be^{2+} is different from reactivation of Zn^{2+}, Cd^{2+} or Cu^{2+}-PGM complexes.

B. EXPERIMENTAL PROCEDURE

Glucose 1-phosphate, glucose 1,6-diphosphate, glucose 6-phosphate dehydrogenase, para-nitrophenyl phosphate, adenosine 5^1-triphosphate, bovine serum albumin and *E. coli* alkaline phosphatase were purchased from Sigma. Carrier free $^7BeCl_2$ was purchased from Amersham. Na^+-K^+ ATPase from pig kidney was a gift from Dr. Robert Post of Vanderbilt University. Chelex-100 and Bradford assay reagent were purchased from Bio-Rad. Rabbit antihorse spleen ferritin was purchased from U.S. Biochemicals. All other chemicals were of reagent quality and were purchased from Sigma. All buffers were made in double deionized water. Whenever necessary, metal free buffers were made by adding Chelex-100 with stirring for a number of hours and then filtering the solution through Whatman 2 filter paper. Whenever possible, plastic-ware was used for storage of solutions to avoid leaching of metals from glassware.

Metal Assays

Concentrations of metals were determined on an Instrumentations Laboratories IL157 atomic absorption spectrophotometer.

Protein Assays

Protein concentrations of ferritin and apoferritin were measured by the method of Lowry et al. (42) or Bradford (9) using bovine serum albumin as a standard. Appropriate correction factors were applied as described in chapter III. Protein concentrations of PGM and alkaline phosphatase were determined by absorbance at 278nm using extinction coefficients $\epsilon_{PGM} = 0.77\ A^{278}/mg$ (45) and $\epsilon_{Alkaline\ Phosphatase} = 0.72\ A^{278}/mg$ (49).

Enzyme Assays

PGM activity was measured by the coupled assay as described by Joshi et al. (33). For PGM assays involving Zn^{2+}, Cd^{2+} and Cu^{2+}, the assay medium contained 0.04 M Tris-HCl, pH 7.4 as buffer. In PGM assays involving Be^{2+}, 0.04 M Imidazole pH 7.4 was utilized as buffer. Alkaline phosphatase activity was measured spectrophotometrically by the method of Garen and Levinthal (23) and Na^{+}-K^{+} ATPase, activity was measured as described by Toda et al. (70).

Enzyme and Protein Preparations

Rabbit muscle PGM was purified according to the procedure of Joshi et al. (33). Metal-free PGM was prepared by incubating the enzyme for 30 minutes with 0.1 mM EDTA in 0.04 M Tris-HCl, pH 7.4 followed by dialysis against 1000 volumes of metal free buffer (0.01 M Tris-HCl pH 7.4 containing 10 gms Chelex-100) for 18 hours at 5° with one change after 12 hours. When this preparation was compared with the equivalent enzyme in the presence of chelating agent (0.1 mM

EDTA), activity assay indicated that the preparation was 83% metal free. Ferritin from horse spleen (Sigma) was further purified as described in chapter III. Be^{2+} containing ferritin or apoferritin was prepared by first dissolving 1 mg of the protein in 0.3 ml of 0.8 mM $BeSO_4$ in 0.2 M Tris-HCl pH 6.5 buffer (Be^{2+} buffer). The sample was then applied to a column (Sephadex G25 coarse, 0.6 × 15 cm) equilibrated with the same Be^{2+}-buffer and then eluted with Be^{2+}-buffer. The fractions containing protein were pooled and the unbound Be^{2+} removed by dialyzing twice against 1000 volumes of metal free buffer (0.01 M Tris-HCl, pH 6.5) for 12 hours each time at 5°. ^{7}Be labeled ferritin containing 360 gram atoms of Be^{2+} and labeled with $^{7}BeCl_2$ was prepared by a procedure utilizing microcentrifuge desalting (30) as outlined in chapter III.

Immunoprecipitation of Be^{2+} containing ferritin involved addition of 30 µg antihorse spleen ferritin to 8.4 µg of ^{7}Be-ferritin in a total volume of 1 ml and buffered with 0.04 M Tris-HCl pH 6.5. After incubation for 2.5 hours at 37°, samples were incubated 12 hours at 5°. To isolate precipitin, samples were centrifuged for 2 minutes at 3000 rpm. The supernatants were pipeted off, and the pellet resuspended in 1.0 ml of 0.2M Tris-HCl pH 6.5. Samples were then centrifuged as before, and the supernatant was pipeted off. The precipitins were then counted in an Intertechnique gamma counter.

C. RESULTS

Protective Effect of Ferritin Against Inhibition of Enzymes by Be^{2+}

These studies were conducted with the three enzymes: alkaline phosphatase from *E. coli*, Na^+-K^+ ATPase form pig kidney and PGM from rabbit muscle. Figure 4.1 shows that ferritin prevented the inhibition of alkaline phosphatase and that the reactivation of the inhibited enzyme was dependent upon the concentration of ferritin. Similarly, ferritin preserved 97.8% of Na^+-K^+ ATPase activity when exposed to Be^{2+} levels sufficient to inhibit all but 1.2% of the activity (Table 4.1). It is noteworthy that at equivalent concentrations apoferritin did not protect either enzyme against inhibition by Be^{2+} or reactivate the enzymes which were already inhibited by Be^{2+}. In contrast to alkaline phosphatase and Na^+-K^+ ATPase, binding of Be^{2+} to PGM is reported to be irreversible (29). Thus, the role of ferritin in reactivating such an enzyme was studied in greater detail. Accordingly, mixtures of PGM and varying amounts of ferritin were exposed to 1×10^{-5}M $BeCl_2$ and the enzyme activity measured at various times. The results showed (Figure 4.2A) that ferritin afforded partial protection against inhibition and that this protection was dependent on its concentration. The protective effect was specific because bovine serum albumin at the same concentration was without effect (Figure 4.2B). In a separate series of experiments PGM was preincubated with Be^{2+} and EDTA. To this fully inactivated complex was added varying amounts of ferritin and aliquots were assayed for activity. Parallel experiments were conducted

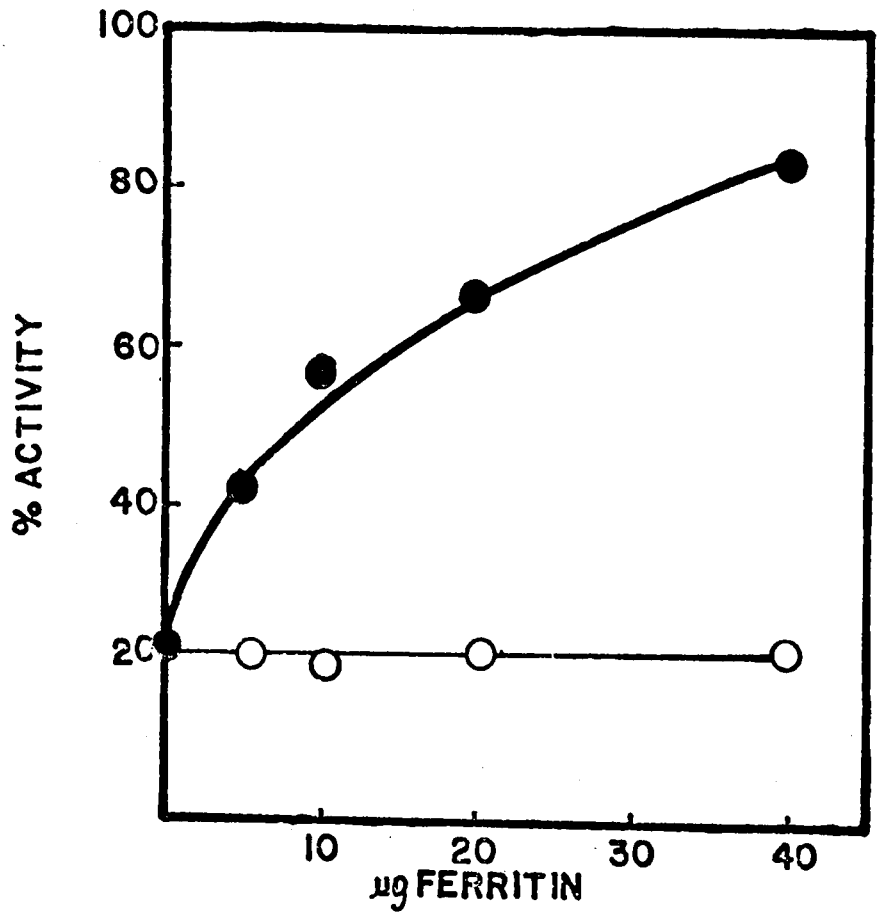


Figure 4.1. Effect of Ferritin on Be-inactivated alkaline phosphatase. 10 µg of alkaline phosphatase form E. coli was combined with 0.1 M Tris-HCl pH 8.0 and various amounts of ferritin (●) or apoferritin (○) followed by incubation with 1.0 mM $BeSO_4$ in a total volume of 1 ml. After 60 minutes at 4°C, enzyme activity was assayed in 5 µl aliquots.

TABLE 4.1

Effect of Beryllium and Ferritin on Na^{+}-K^{+} ATPase. Enzyme Was Incubated with 40mM Triethanolamine-HCL, pH 7.4, 12mM Mg^{++}, 16mM KCl and with 27.5µ of Ferritin or Apoferritin and 10µM Be^{++} As Desired in a Final Volume of 1.0ml for 45 Minutes at Room Temperature and ATPase Assayed

Preincubation Condition	% Activity Remaining
Control	100
Control + Be	1.2
Control + Ferritin + Be^{++}	97.8
Control + Apoferritin + Be^{++}	1.5

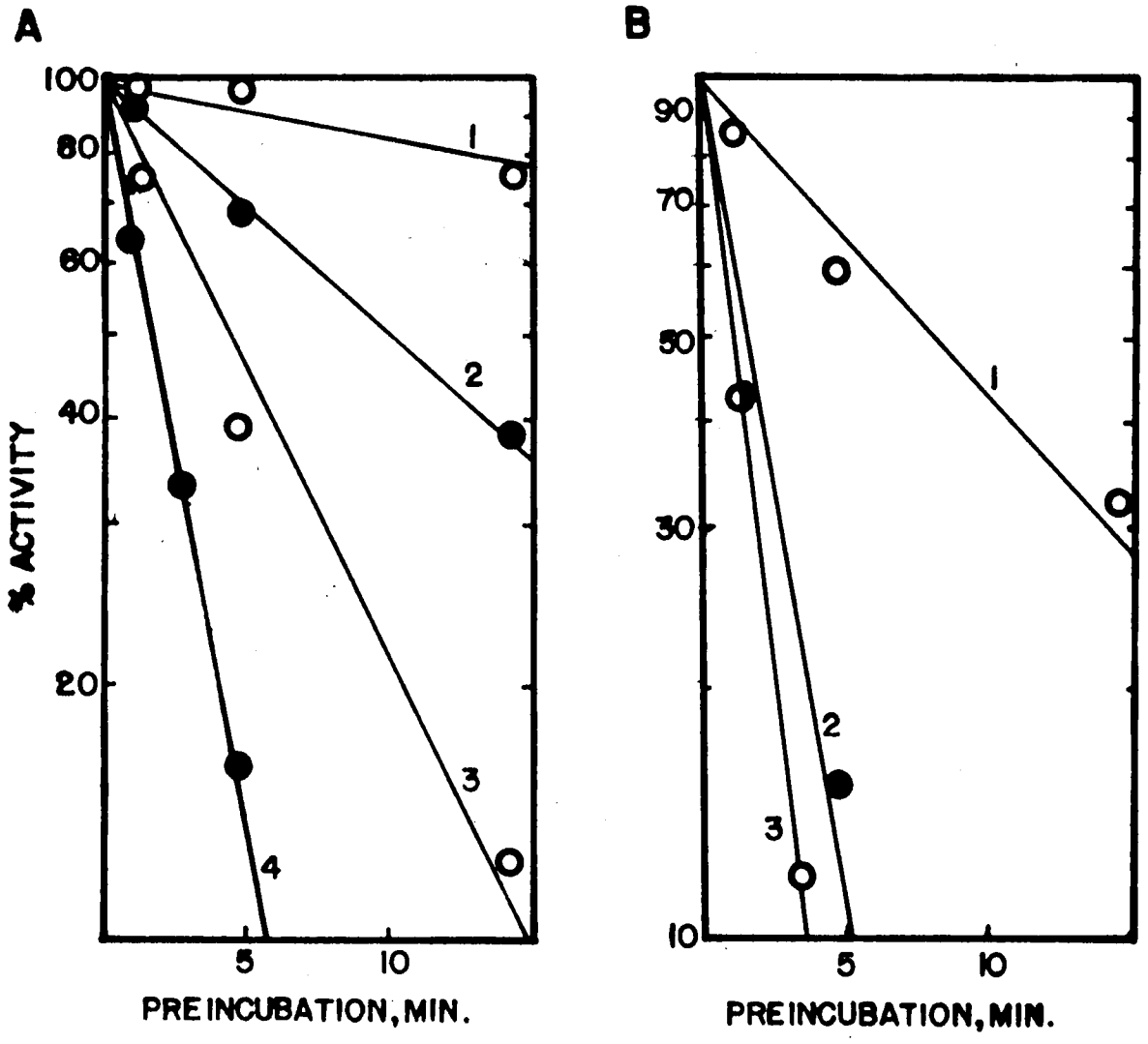


Figure 4.2. Effect of ferritin on the inhibition of phosphoglucomutase by beryllium. 0.5 m µmole of phosphoglucomutase was combined with 0.04 M Tris-HCl, 0.1 mM EDTA and varying amounts of ferritin. $BeCl_2$ was added to each to a final concentration of 0.01 mM in a volume of 1 ml. After incubation at 20°C for various times, 10 µl aliquots were taken for assay. Panel A (1) 1.04 m µmole ferritin; (2) 0.416 m µmole ferritin; (3) 0.204 m µmole ferritin; (4) without ferritin. Panel B (1) 0.416 m µmole ferritin; (2) 4.7 m µmole bovine serum albumin; (3) without ferritin or BSA.

with Cd^{2+}, Cu^{2+} and Zn^{2+}. Hyperbolic curves obtained for all the metal ions are shown in Figure 4.3A, B, C, and D. The reactivation of the Be-PGM complex was significant because it showed that the Be-PGM complex can be dissociated to generate an active enzyme and that a natural chelator, ferritin, can be used to accomplish this function. For all metal ions the reactivation of the inhibited enzyme by ferritin was time dependent, reaching a maximum within 3 hours and about 50% of the activity was recovered by 4 μM ferritin. As seen in Table 4.2, second column, similar amounts of ferritin were required for 50% reactivation of PGM-metal complexes. However, far less ferritin sufficed to regain 25% activity with the Be-PGM complex than with Cd^{2+}, Zn^{2+} or Cu^{2+} (Table 4.2, first column). The first-order rate constants for reactivation of PGM-metal complexes by ferritin (Table 4.2, third column) show that the Be-PGM complex is reactivated 2.5-4.4 times slower than that of other metals.

Inactivation of Metal Free PGM by Be-ferritin or Be-apoferritin

We have shown that Zn^{2+} injected into rats is sequestered at least in part, by ferritin in liver and that *in vitro* the bound Zn^{2+} is transferred to Zn^{2+} requiring apoenzymes. Thus, the binding of Zn^{2+} to ferritin is reversible (53). In view of this observation, the *in vitro* transfer of Be^{2+} from ferritin to PGM was investigated. Accordingly, samples of ferritin and apoferritin containing 346 gm atoms and 16 gm atoms of Be^{2+} respectively were prepared. Demetallo-PGM was incubated with either of the two proteins or $BeSO_4$. The concentration of Be^{2+} in each instance was maintained at 1×10^{-6}M. Thus,

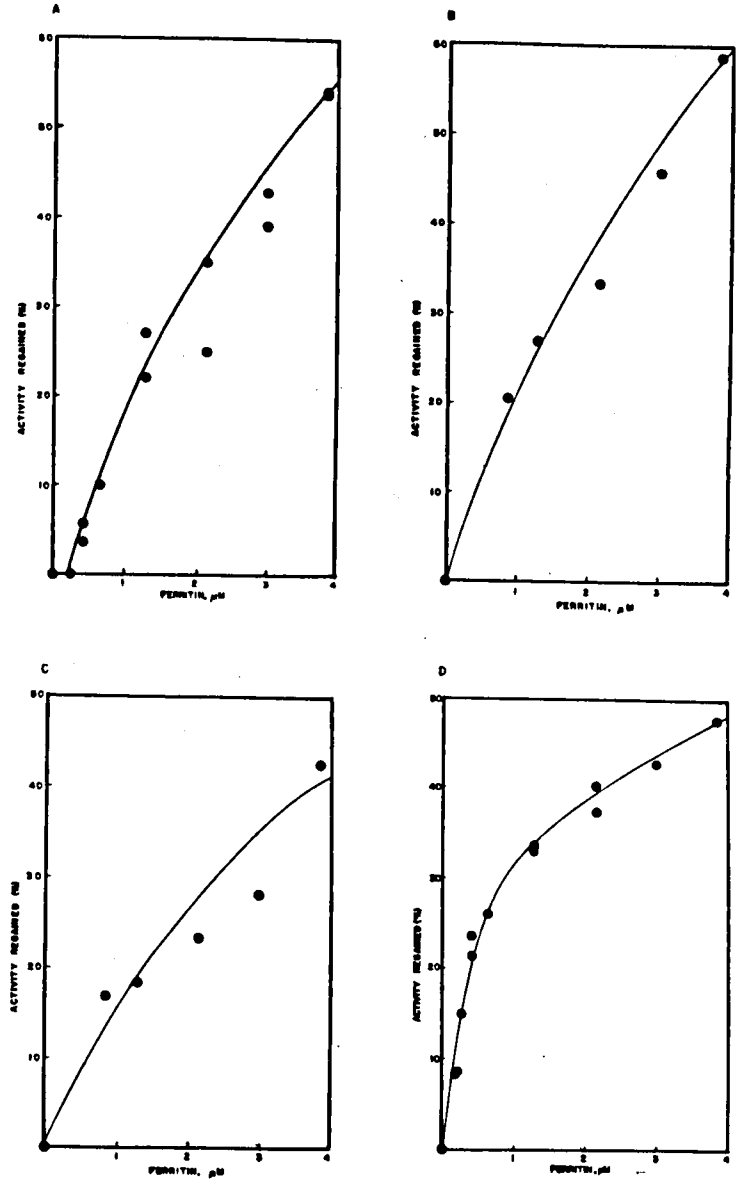


Figure 4.3. Reactivation of metal-phosphoglucomutase complexes by addition of ferritin. Metal free phosphoglucomutase in .024 M Tris-HCl, pH 7.4 was inhibited by 0.19 mM metal in a final volume of 262 µl. For reactivation 10 µl of inactive phosphoglucomutase was incubated with 0.04 M Tris-HCl, lysozyme to total protein concentration of 1.0 mg/ml., and various amounts of ferritin in a final volume of 100 µl. After 180 minute incubation at 20°C, 5 µl aliquots were taken for assay. (A) Zn-phosphoglucomutase; (B) Cd-phosphoglucomutase; (C) Cu-phosphoglucomutase; (D) Be-phosphoglucomutase.

TABLE 4.2

Reactivation of PGM-metal Complexes by Ferritin.

Metal	Concentration Ferritin Required for 25% Reactivation (μM)	Concentration Ferritin Required[1] for 50% Reactivation (μM)	Rate Constant[2] SEC^{-1} M^{-1}
Zn	1.4	3.4	3810
Cd	1.2	3.0	4580
Cu	1.9	4.5	6800
Be	0.5	4.4	1550

[1]For double reciprocal plots determining ferritin concentration for 50% reactivation, correlation coefficients ranged from 0.922 to 0.985.

[2]For log plots determining rate constant, correlation coefficients ranged from 0.953 to 0.975.

proportionately more of apoferritin than holoferritin was required. Aliquots of each incubation were assayed for activity at timed intervals. As expected (Figure 4.4) $BeSO_4$ inactivated PGM faster than similar amounts bound to either apoferritin or holoferritin. Significantly, the rate of inactivation of PGM by Be^{2+} transfer from the two proteins was indistinguishable.

Removal of Be^{2+} from Ferritin by Excess PGM

In the above experiments the concentration of Be^{2+} was far in excess of that of PGM. Thus, it was possible that the easily available Be^{2+} complexed with either ferritin was too large and therefore quantitatively indistinguishable by the procedure employed. Therefore, a separate series of experiments were conducted. Accordingly, 8.4 µg of ferritin containing 360 gm atoms of $^{7}Be^{2+}$ was incubated with varying amounts of PGM for 4 hours at 5°. Because PGM binds a maximum of one gm atom of Be^{2+}, the concentrations of PGM used in these experiments represented up to 11 fold molar excess over Be^{2+} bound to ferritin. After the incubation, ferritin was precipitated with antiferritin antibody and the radioactivity in the precipitate was determined (see methods). As seen in Figure 4.5, when in molar excess, PGM readily removed Be^{2+} from ferritin such that less than 10% of the Be^{2+} remained bound to ferritin. Indeed, at molar excess beyond 3 fold of PGM over Be^{2+} the amount of Be^{2+} in ferritin remained constant and could not be reduced below 10% of the total Be^{2+}.

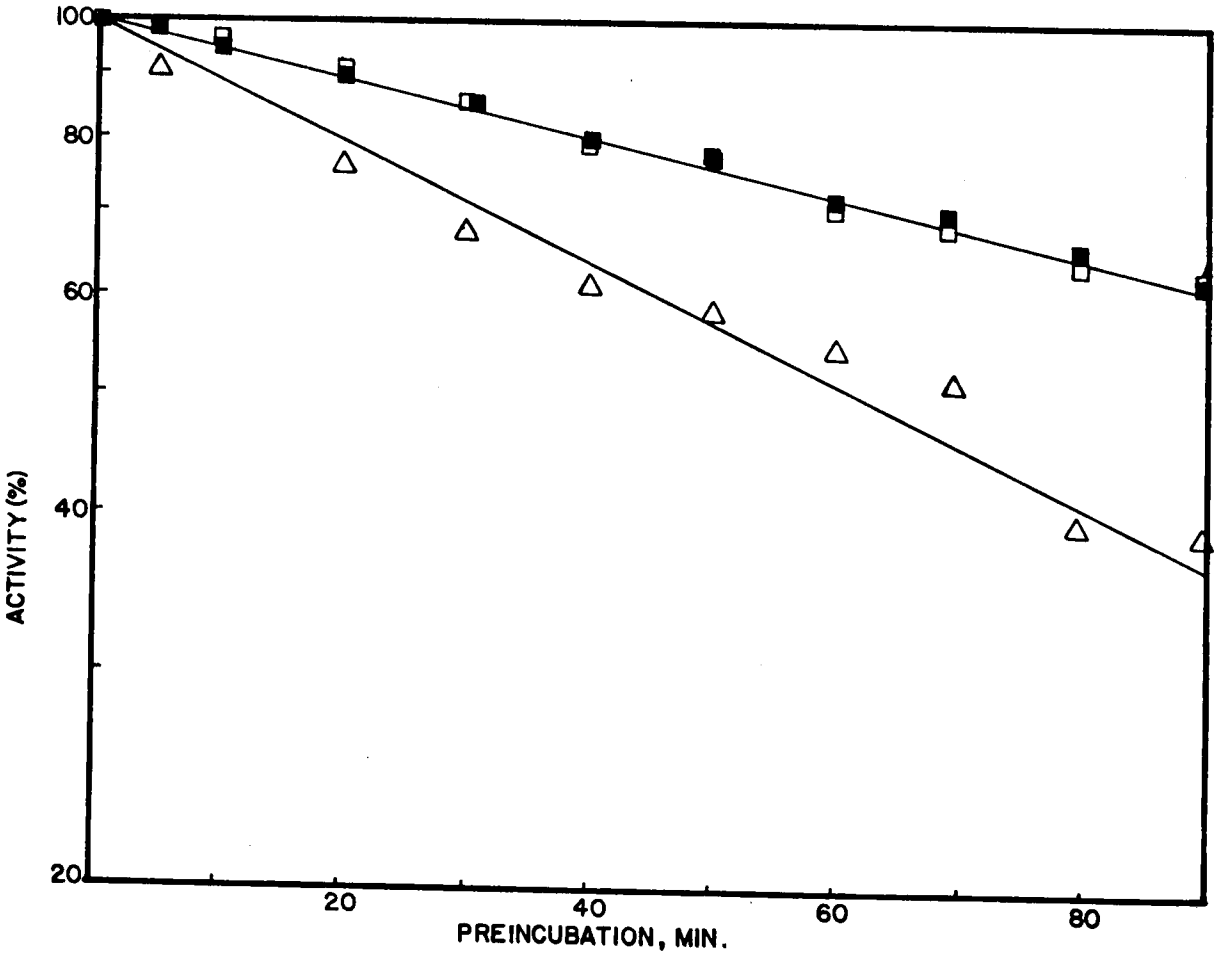


Figure 4.4. Inactivation of metal-free PGM by Be-ferritin or Be-apoferritin. Phosphoglucomutase (21 µg) was preincubated with final concentrations of 0.1 mM EDTA, 1 mg/ml BSA and 0.04 M Tris-HCl, pH 7.4 for 15 minutes in order to fully activate the enzyme. To preincubation samples as prepared above were added Be-ferritin (■), Be-apoferritin (□), or $BeSO_4$ (△) to a final Be concentration of 1×10^{-6}M and a final volume of 0.5 ml in each case. Be-ferritin and be-apoferritin samples used contained 346 and 16 gram atoms of Be^{2+} respectively so that proportionately more apoferritin protein was added to achieve the same concentration Be^{2+}. 5 µl of each sample was assayed for PGM activity at time intervals up to 90 minutes after addition of Be^{2+} sources.

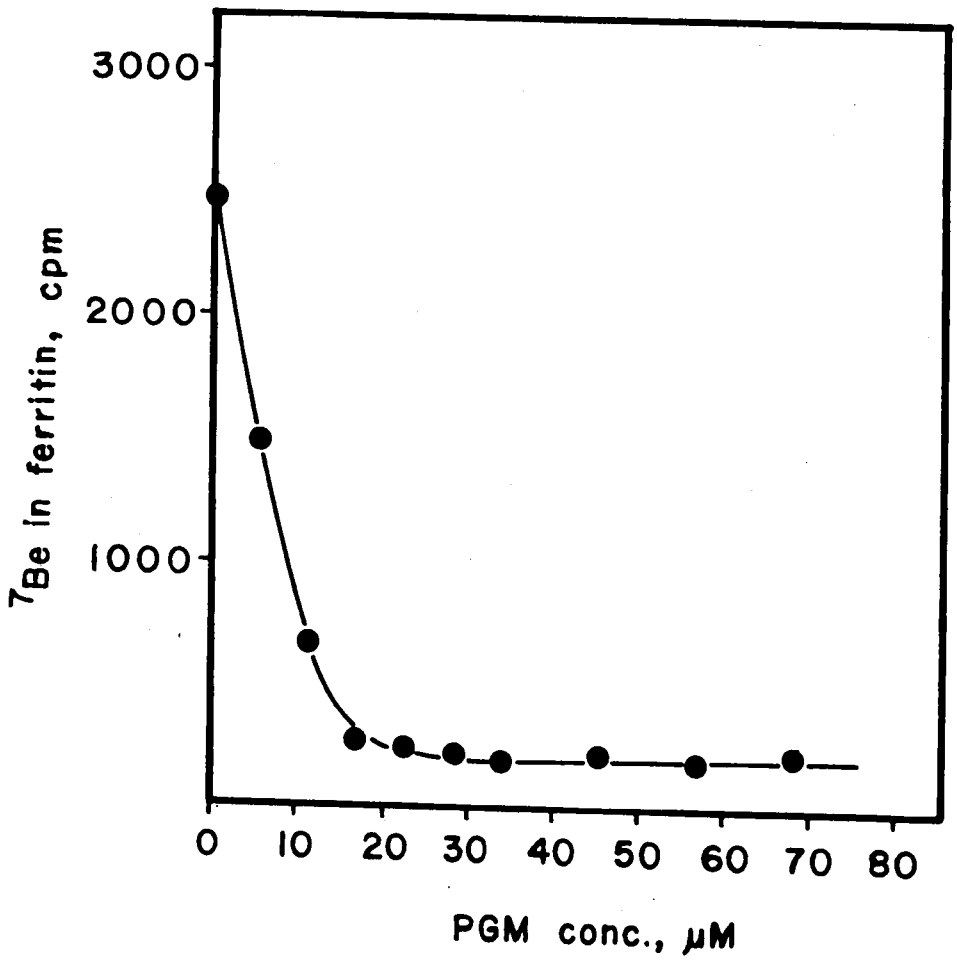


Figure 4.5. Removal of Be^{2+} from Be-ferritin by PGM. ^{7}Be-ferritin (8.4 μg) containing 360 gram atoms Be^{2+} was incubated with up to 4.4 mg of PGM in a final volume of 1.0 ml. Incubations contained final concentrations of 1.0 mg/ml BSA, 0.1 mM EDTA, and 0.04 M Tris-HCl, pH 6.5. Samples were incubated at 4° for four hours and then ^{7}Be which remained bound to ferritin was precipitated with antiferritin antibody as described in "Experimental Procedure." Amounts of Be^{2+} remaining in ferritin were quantitated by the radioactivity.

Calculation of the Theoretical Reactivation of Zn-PGM by Ferritin

The dissociation constants (K_D's) and numbers of available sites for two classes of Zn^{2+} binding sites on horse spleen ferritin are determined in chapter III. Ray (54) has determined the dissociation constant (K_D) for Zn^{2+} binding to PGM, and has also developed an equation to relate the activity of the PGM to the concentration of Zn^{2+} and Mg^{2+} present. Thus, one may calculate the expected reactivation by ferritin of PGM inhibited by Zn^{2+}. For ferritin we first formulate equilibrium expressions for the two types of binding sites I and II:

$$Zn\text{-}Fer_I \rightleftharpoons Fer_I + Zn^{2+}, \qquad {}^{Zn}K_{D_I}^{Fer} = \frac{[Fer_I]^{free}\,[Zn^{2+}]^{free}}{[Zn\text{-}Fer_I]} \tag{1}$$

$$Zn\text{-}Fer_{II} \rightleftharpoons Fer_{II} + Zn^{2+}, \qquad {}^{Zn}K_{D_{II}}^{Fer} = \frac{[Fer_{II}]^{free}\,[Zn^{2+}]^{free}}{[Zn\text{-}Fer_I]} \tag{2}$$

In these equations, the concentrations of protein represent the total concentrations of binding sites either occupied by Zn^{2+} or free of Zn^{2+}. Thus, we assume that in the multivalent binding of Zn^{2+} to ferritin, there is no interaction of the various sites. By adding equations 1 and 2 and solving for $[Zn^{2+}]^{free}$ we write:

$$[Zn^{2+}]^{free} = \frac{{}^{Zn}K_{DI}^{fer} + {}^{Zn}K_{DII}^{fer}}{\left(\dfrac{[Fer_I]^{free} + [Fer_{II}]^{free}}{[Zn\text{-}Fer_I] \qquad [Zn\text{-}Fer_{II}]}\right)} \tag{3}$$

In order to solve for the concentrations of Zn^{2+}-free sites we use the following general equation:

$$[Fer_i]^{free} = \left([Fer]_{total} \cdot \frac{\#\ of\ sites_i}{molecule}\right) - \left([Zn^{2+}]_{total} \cdot P_i\right) + \left([Zn\text{-}PGM] \cdot P_i\right) \quad (4)$$

The term $[Zn^{2+}]_{total} \cdot P_i$ represents the amount of Zn^{2+} bound to the i^{th} class of sites. The term $[Zn\text{-}PGM] \cdot P_i$ is a correction for the amount of Zn^{2+} bound to PGM. Thus, the actual concentration of Zn^{2+} bound to the i^{th} class of sites is represented by:

$$[Zn\text{-}Fer_i] = \left([Zn^{2+}]_{total} \cdot P_i\right) - \left([Zn\text{-}PGM] \cdot P_i\right) \quad (5)$$

P_i is the fraction of Zn^{2+} binding to the i^{th} class of sites with respect to the total number of binding sites. It is directly related to the number of sites in the i^{th} class and is inversely related to the dissociation constant for that class.

Thus, of the total ferritin-Zn binding, we can calculate the fraction of binding to sites I and II by the equations:

$$P_I = \frac{\left(\frac{\#\ sites_I}{K_{DI}}\right)}{\left(\frac{\#\ sites_I}{K_{DI}} + \frac{\#\ sites_{II}}{K_{DII}}\right)} \quad (6A)$$

$$P_{II} = \frac{\left(\frac{\#\ sites_{II}}{K_{DII}}\right)}{\left(\frac{\#\ sites_I}{K_{DI}} + \frac{\#\ sites_{II}}{K_{DII}}\right)} \quad (6B)$$

For the binding of Zn^{2+} to ferritin, the numbers of sites and dissociation constants determined in chapter III are given as follows: $^{Zn}K_{DI}^{Fer}$ = 5.25×10^{-6}M; $^{Zn}K_{DII}^{Fer}$ = 44.1×10^{-6}M; # sites I = 135.2 gm atoms Zn^{2+}; # sites II = 251.0 gm atoms Zn^{2+}. We have thus calculated the proportions of Zn^{2+} bound to sites I and II as $P_I = 0.82$ and $P_{II} = 0.18$. To obtain the 50% reactivation of Zn-PGM by ferritin (Figure 4.3A, page 71), the following concentrations were used:

$[Zn^{2+}]^{total} = 1.89 \times 10^{-5}M$

$[Ferritin]^{total} = 2.86 \times 10^{-6}M$

$[PGM]^{total} = 4.68 \times 10^{-7}M$

$[Zn\text{-}PGM] = 2.34 \times 10^{-7}M$

Substituting these values and the proportions P_I, P_{II} into equations 4 and 5 we then calculated the concentrations of Zn^{2+} occupied and unoccupied sites for classes I and II of ferritin:

$[Fer_I]^{free} = 3.71 \times 10^{-4}M$

$[Zn\text{-}Fer_I] = 1.57 \times 10^{-5}M$

$[Fer_{II}]^{free} = 7.15 \times 10^{-4}M$

$[Zn\text{-}Fer_{II}] = 2.86 \times 10^{-6}M$

Substituting these values into equation 3, the concentration of Zn^{2+}free calculates to be 1.804×10^{-7}M. Since the PGM assay involved a 100-fold dilution, the concentration of free Zn^{2+} in assay was 1.804×10^{-9}M. In

order to calculate the expected reactivation under these conditions, we applied the equation formulated by Ray (53) relating activity of PGM to amounts of Zn^{2+} and Mg^{2+} in the assay:

$$\frac{Vmg^{app} - V_{zn}{}^{app}}{Vmg,\ zn - V_{zn}{}^{app}} \quad 1 + \frac{[Zn^{2+}]^{free} \cdot K_D{}^{app\ PGM\text{-}Mg}}{[Mg^{2+}]^{free} \cdot K_D{}^{app\ PGM\text{-}Zn}} \qquad (7)$$

We then substituted the metal concentrations as determined for our experiment and the constants as determined by Ray (54):

$[Zn^{2+}]^{free} = 1.804 \times 10^{-9}M$

$[Mg^{2+}]^{free} = 1 \times 10^{-3}M$

$K_D{}^{app\ PGM\text{-}Mg} = 0.632 \times 10^{-5}M$ at pH 7.4

$K_D{}^{app\ PGM\text{-}Zn} = 4 \times 10^{-12}M$

$V^{app}\ mg = 100\%$ = relative percent PGM activity with Mg^{2+} alone

$V_{zn}{}^{app} = 0.3\%$ = relative percent PGM activity with Zn^{2+} alone

The quantity Vmg,zn represented the percent PGM activity possible when Zn-PGM was incubated with a particular level of ferritin which experimentally (Figure 4.3A, page 71) produced 50% PGM reactivation. On a theoretical basis, we calculated that this ferritin chelation could reduce the $[Zn^{2+}]^{free}$ to $1.804 \times 10^{-9}M$ and that the reactivation under this condition would be 25.6%. The observed reactivation of 50% is higher than this theoretical value, but considering the calculations involved, it was a reasonable estimate. This confirmed that even though

ferritin had a lower affinity for Zn^{2+} than PGM it still had the ability to chelate Zn^{2+} when it was present in molar excess over the amount of PGM.

D. DISCUSSION

The data presented here demonstrates that ferritin can protect alkaline phosphatase, Na^{+}-K^{+} ATPase and PGM against the inactivation by Be^{2+}, and also that it can reverse the Be^{2+} inhibition of these enzymes once it has taken place.

The reactivation of the inhibited Na^{+}-K^{+} ATPase and alkaline phosphatase was complete and immediate. Consistent with this, our unpublished observations showed that ferritin was also able to remove Be^{2+}once it had been bound to alkaline phosphatase or Na^{+}-K^{+} ATPase. In contrast, even the highest levels of ferritin did not completely protect PGM against the Be^{2+} inhibition, although the rate of inhibition was drastically reduced. Hashimoto et al. had shown that PGM was irreversibly and completely inhibited by micromolar concentrations of Be^{2+}. It was further shown that the only way to reactivate Be-PGM was to expose it to 4M urea which caused the complete release of Be^{2+} but only 56% recovery of the initial enzymatic activity (29). Thus, it was understandable that inhibition by Be^{2+} was not completely prevented by ferritin. Surprisingly, when PGM was completely inhibited by Be^{2+}, subsequent addition of increasing levels of ferritin resulted in as much as 50% return in PGM activity.

The fact that ferritin acted as a chelating agent for Be-PGM did not necessarily imply that ferritin had a greater affinity for Be^{2+}

than did PGM. Based on the high molar ratio of ferritin to PGM (up to 10 fold) it was more likely that ferritin had a lower affinity for Be^{2+} than PGM, and was able to chelate Be^{2+} by a mass action principle. Perhaps the same principle operates for reactivation of Zn-PGM, Cd-PGM or Cu-PGM by ferritin. The elegant quantitative studies by Ray (54) have described the relationships between PGM, Zn^{2+} and Mg^{2+}. These studies and the data in chapter III were useful in illustrating the theoretical reactivation of Zn-PGM by ferritin employing the known binding parameters. The affinity of PGM for Zn^{2+} ($^{Zn}K_D^{PGM} = 4 \times 10^{-12}M$) exceeds the affinity of ferritin for Zn^{2+} [$^{Zn}K_D^{Fer} = 5.26 \times 10^{-6}M$ (high affinity sites)] by 6 orders of magnitude. However, the excess ferritin seems to overcome this difference in affinity. Our calculation of the expected reactivation based on known binding parameters indicates that the chelation by ferritin was indeed responsible for the observed reactivation. We have shown that for 50% PGM reactivation, a 1000 fold excess of high affinity ferritin-Zn sites and a 2000 fold excess of low affinity ferritin-Zn sites is required over the amount of PGM-Zn sites.

Another factor which had the effect of increasing the ferritin reactivation of Zn-PGM was the presence of Mg^{2+} in the assay. Since Mg^{2+} competes with Zn^{2+} for binding to PGM, and since Mg^{2+} is the best metal ion activator for PGM, the net inhibition of Zn^{2+} is reduced in the presence of Mg^{2+}. In the presence of 1mM Mg^{++} employed during assay, equation 7 shows that the effective dissociation constant of Zn^{2+} for PGM is increased from $4 \times 10^{-12}M$ to as much as $4 \times 10^{-9}M$. In contrast to PGM, ferritin had no affinity for Mg^{2+}. This was ascertained directly

by Mg^{2+}-ferritin binding assay and indirectly by the inability of ferritin to remove Mg^{2+} from PGM in the activity assay. Thus Mg^{2+} present did not compete with Zn^{2+} binding to ferritin but did compete with Zn^{2+} binding to PGM. The combined effect of Mg^{2+} (10^3 fold reduced binding of Zn^{2+} to PGM) and molar excess of ferritin sites ($1\text{-}2 \times 10^3$ fold excess) allows us to reconcile the large difference in binding affinities of PGM and ferritin (10^6 fold difference in K_D) with the favored reactivation by ferritin.

Reactivation of Be-PGM by ferritin required a similar molar excess for 50% reactivation. Perhaps the affinities of PGM and ferritin for Be^{2+} are of a similar 10^6 fold difference. However, there are a number of reasons to believe that reactivation of Be-PGM by ferritin is unlike reactivation of other divalent metal complexes (Cd^{2+} and Zn^{2+}). For instance, far less ferritin sufficed to achieve 25% reactivation of the PGM complex with Be than with other metal ions. This can be attributed to the properties of Be-PGM binding or Be-ferritin binding. As shown in chapter III, Be^{2+} is tightly bound to ferritin and is not removed by dialyzing against metal free buffer. Thus, it is not surprising that the reactivation curve for Be would differ from metals for which the binding is readily reversible both to PGM and to ferritin. The fact that rate of transfer of Be^{2+} from PGM to ferritin is much slower than it is for other divalent metal ions further underscores the probable difference in the nature of Be^{2+} binding compared to binding of Zn^{2+}, Cd^{2+} or Cu^{2+}.

If the differences in kinetic or equilibrium measurements are due to Be-ferritin binding, they may be based on the site at which the

Be^{2+} is bound or the number of metal ions bound. In chapter III we suggested that the Zn^{2+} and Cd^{2+} most tightly bound to ferritin were bound to the protein shell and that the loosely bound Zn^{2+} or Cd^{2+} ions were bound to the iron core. Cu^{2+} ions seem to bind only to the protein shell. Thus, for Zn^{2+}, Cd^{2+} and Cu^{2+}, PGM reactivation was due primarily to sites on the ferritin protein shell and not sites on the ferritin iron core. For Be^{2+} the situation was different. It appeared that Be^{2+} was binding most tightly to the iron core and to a lesser extent to the protein shell. Ferritin bound at least 800 gram atoms of Be^{2+} compared to a maximum of $\sim$400 gram atoms for Zn^{2+} and lesser amounts for other metals (chapter III). Most of the Be^{2+} was tightly bound to the iron core with an affinity comparable to that of the other metals tested.

It was hypothesized in chapter III that this core binding was due to Be-phosphate formation. Apoferritin bound far less of Be (up to 160 gram atoms) and with a much lower affinity.

We have shown in this chapter that apoferritin did not protect Na^{+}-K^{+} ATPase or alkaline phosphatase from inactivation by Be^{2+}, while ferritin completely protected these enzymes at similar protein concentration. Thus, although we have demonstrated Be^{2+} binding to apoferritin (chapter III), this binding was not of sufficient tightness to protect the enzymes.

If indeed most of the Be^{2+} bound to ferritin is bound to the iron core, it is likely that a small amount is weakly bound to the protein shell in the same way that Be^{2+} is bound to be apoferritin.

When we added Be-ferritin or Be-apoferritin to PGM, similar rates of inactivation were seen. That inactivation occurred at all with Be-ferritin indicated that ferritin could donate Be^{2+} to PGM as well as accept Be^{2+} from PGM.

The Be^{2+} which is so transferred seems to be only that which is bound to the protein shell. The pores of access to the ferritin core are 10 Å in diameter (5). For PGM, it is unlikely that any significant cross sectional area will have a diameter of less than 10 Å (W. J. Ray, personal communication). Thus, it is clear that at least some of the Be^{2+} on ferritin is bound to the outer protein shell.

The evidence presented thus far leads us to two possible models for Be^{2+} binding to ferritin. In one model (Figure 4.6A) Be^{2+} is bound to both protein shell (loosely) and the iron core (tightly) with no exchange between the two sites. Alternatively, (Figure 4.6B) the same binding occurs, but an equilibrium exists between Be^{2+} bound to protein shell and to the iron core. Such an equilibrium would favor binding to the iron core. Consequently, no detectable amount of Be^{2+} was released by dialysis. In both models, Be^{2+} bound to the protein shell is available to enzymes such as PGM, but as implied in the equilibrium model, depletion of the Be^{2+} bound to the protein would result in Be^{2+} redistribution from the core to the protein shell to maintain the equilibrium. The data presented in Figure 4.5, page 75, seems to favor this possibility. At 3 fold molar excess of PGM over Be^{2+}, over 90% of Be^{2+} from ferritin was removed. However, the remaining 10% could not be removed by higher PGM concentration. This was similar

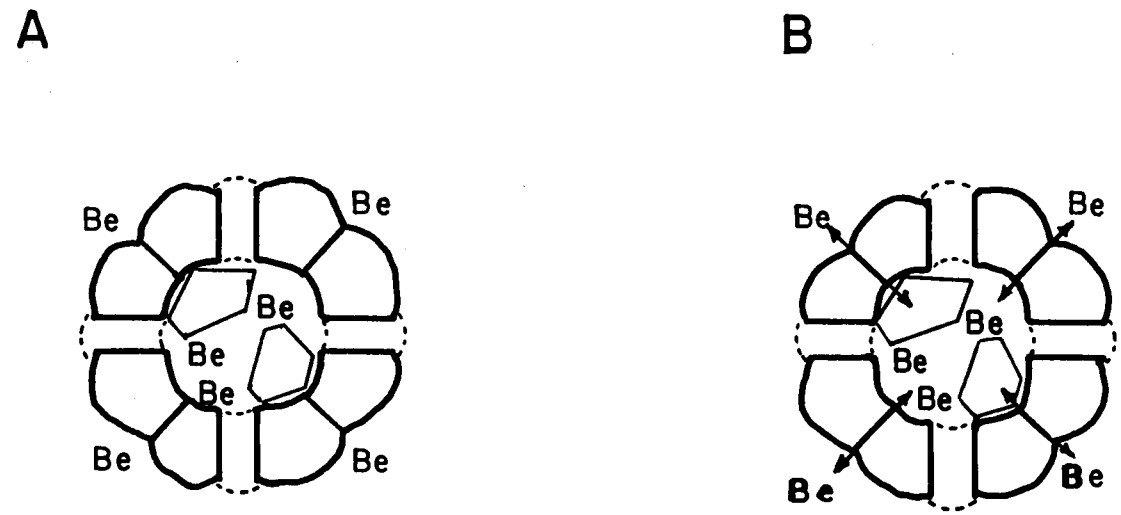


Figure 4.6. Two models for Be^{2+} binding to ferritin. In both diagrams shown above, Be^{2+} is bound in greatest amounts to the iron oxyhydroxide-phosphate core (irregular polygons) and in lesser amounts to the protein shell at regions of subunit contact. In (A) the access of Be^{2+} to the iron core is only through 10 Å pores in the protein shell. There is no interaction between Be^{2+} bound to the shell and the core in this model. In (B) there is an equilbrium between Be^{2+} bound to the protein shell and Be^{2+} bound to the iron core. Thus, access of Be^{2+} to the iron core might be both via the 10 Å pores and regions of subunit contact.

to the removal of Be^{2+} from ferritin by sulfosalicylic acid as shown in chapter III (Figure 3.12, page 54). Here again, about 10% of Be^{2+} remained bound to ferritin at molar excess of sulfosalicylic acid. Thus, Be^{2+} bound to the core of ferritin was just as accessible to PGM as it was to a small chelator which could penetrate the pores of the protein shell. This result strongly suggested a redistribution of bound Be^{2+} shown in the second model (Figure 4.6B). Based on the size of pores in the ferritin shell and the size of PGM as indicated above, it seems likely that Be^{2+} would have to be transferred from the iron core sites to external protein shell sites in order for PGM to chelate it. This would favor an equilibrium between the two types of sites.

It could be reasoned that PGM, although not penetrating the ferritin shell, might come close enough to the region of the 10 Å pore to extract Be^{2+} ions through the pore without a direct contact with the iron core. If this were the case, then the noninteracting sites model (Figure 4.6A) might be sufficient to explain the data. However, given different modes of extracting Be^{2+} from the ferritin shell and ferritin core, we should have seen a kinetic difference between apoferritin and ferritin donation of Be^{2+} to PGM. As is shown in Figure 4.4, page 74, the rates of transfer were indistiniquishable for Be-ferritin and Be-apoferritin. Thus, the equilibrium model of binding sites (Figure 4.6B) appears likely.

The ability of PGM to remove Be^{2+} from ferritin under these conditions does not rule out ferritin as a protective agent. It simply

requires that the amount of ferritin be in excess of the amount of PGM in order to make the binding of Be to ferritin favored over binding to PGM. The reactivation of Be-PGM (Figure 4.3D, page 71) illustrates this point. This appears to be the case *in vivo* also. We have found that ferritin is in a two-fold molar excess over PGM in mouse liver homogenates (unpublished data).

It can also be speculated that the mobilization of Be^{2+} observed here is very similar to that which occurs for iron. The binding of Be^{2+} to apoferritin is very similar to the binding of Zn^{2+}, Cd^{2+} and Cu^{2+} (chapter III). Treffry and Harrison (73) have shown that Zn^{2+} binding sites are actively involved in iron deposition and mobilization. Thus, it is reasonable that the mobilization of Be^{2+} from the iron core of ferritin to PGM occurs through a similar mechanism, even though the chemical nature of the Be^{2+} core complex is different from the iron core complex. If this is the case, it might make Be-ferritin binding a useful model for iron sequestration by ferritin.

In summary, our *in vitro* experiments show that ferritin can protect or reactivate critical metabolic enzymes from Be^{2+} inhibition. The reactivation of Be-PGM is similar to that of other PGM metal complexes in that a molar excess of ferritin is essential. However, the binding of Be^{2+} to ferritin is of a different nature, being most tightly bound to the iron core, and less tightly bound to the protein shell. The two types of sites appear to be in equilibrium with each other allowing for mobilization of core bound Be^{2+}. In light of the data presented here the Be-ferritin association may be a useful tool for

the study of iron sequestration by ferritin as well as being a protective agent in Be toxicity. It would also be of interest to examine whether binding of Be^{2+} to ferritin hinders the functions of storage, transport and detoxification of iron (27) in the same way that Zn^{2+} has been shown to inhibit these functions (43).

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