The bovine and chick vitamin D-induced intestinal calcium-binding proteins (CaBP) bind lead. Bovine CaBP binds 2 atoms of lead/molecule, and chick CaBP binds 4 atoms of lead/molecule and these values are identical to those for calcium binding. Calcium-displacement studies indicate significantly higher affinities for lead than for calcium for both proteins. All evidence indicates that lead is bound to the 4 high affinity calcium-binding sites on chick CaBP and to the corresponding 2 high affinity sites on bovine CaBP, and that binding of lead to sulfhydryl groups is, relatively, not significant. Calmodulin, troponin C, and oncomodulin also bind lead with high affinities and in preference to calcium, indicating that lead binding is a general property of proteins belonging to the troponin C superfamly of calcium-binding proteins.

The vitamin D-induced intestinal calcium-binding proteins (CaBP) represent the best defined molecular expression of the vitamin D endocrine system (1). Whereas these proteins designated as the calcium-binding domains on the basis of their precise function in this process remains unknown. Their amino acid sequence of the bovine CaBP (50000) is known (2), as is the crystal structure (3) and the solution conformation (4). This protein binds 2 Ca(II) atoms/molecule with an apparent intrinsic association constant (k\text{Ca}) of 4.3 x 10^4 M^-1 (5). Two sequence regions have tentatively been designated as the calcium-binding domains on the basis of the E-F hand concept (2, 3). In addition, structural changes induced by the binding of calcium result in a molecule which is functionally resistant to trypic digestion (6). The chick CaBP (M_\text{r} = 28000) binds 4 Ca(II) atoms/molecule with high affinity (k_\text{Ca} = 2 x 10^6 M^-1) (7). Both CaBPs bind several other cations, notably Sr(II), Ba(II), Cd(II), and the La(III) series elements (8, 9), in a fashion apparently related to their ionic radii relative to that of Ca(II). Recent reports linking intestinal lead absorption to dietary vitamin D, calcium, and phosphate status (10, 11), as well as the demonstration of a close relationship between lead absorption and intestinal CaBP content (11, 12), suggest the possible involvement of CaBP in intestinal lead transport.

The possibility of such involvement, together with the observation that rat intestinal CaBP binds lead (13), prompted the present studies in which we examine, in some detail, the lead-binding properties of the bovine and chick CaBPs, as well as several other high affinity Ca(II)-binding proteins.

**Experimental Procedures**

The vitamin D-induced chick and bovine CaBPs were purified as described previously (14). Bovine brain calmodulin was provided by Dr. Barry Levine, Oxford, England, bovine skeletal troponin C, by Dr. James Potter, University of Miami, Florida, and oncomodulin, by Dr. John MacManus, National Research Council, Canada. All proteins were subjected to a preliminary dialysis versus 3 x 1000 volumes of 1.0 mM Pipes, 150 mM KCl buffer, pH 6.5, at 4°C for 48 h. Equilibrium dialysis experiments were carried out in this same buffer containing added lead or calcium at 4°C for 48 h with moderate stirring. Dialysis sacs were prepared from Spectrapore 1 membrane tubing (National Scientific, Cleveland, Ohio) with a nominal M_c cut off of 8000. There was no detectable leakage of the proteins through this casing.

Calcium and lead concentrations were determined directly by atomic absorption spectrophotometry (Model 360 AAS, Perkin-Elmer, Norwalk, CT) in which concentrations of these elements permitted, or by specific activity measurements, where necessary. Stock lead and calcium solutions were prepared from analytical grade reagents and the concentrations determined by atomic absorption spectrophotometry relative to commercially prepared standards (Fisher).

Calcium and lead were purchased from New England Nuclear. Liquid scintillation counting and γ spectrometry were performed using a Beckman LS 200 and Beckman γ-500 counter, respectively. Protein concentrations were determined by amino acid compositional analysis on a Beckman 119Cl analyzer, as previously described (2). Cysteine groups on the chick CaBP were blocked by S-pyridylethylthiol following a modification of the method of Friedman et al. (15). Chick CaBP (2.5 mg lyophilized from water) was dialyzed versus 0.5 ml of 2 M N-ethylmorpholine acetate buffer, pH 8.0. One μl of β-mercaptoethanol was added and incubated at room temperature for 2 h, followed by 3 μl of 4-vinyl pyridine (Aldrich) for 90 min. S-Pyridylethylated CaBP was separated from reaction products on a 0.9 cm x 25-cm column of Sephadex G-25 (fine) equilibrated with 1.0 mM Pipes, 150 mM KCl buffer, pH 6.5, and stored frozen until use.

**Results and Discussion**

Direct analysis of lead binding to chick CaBP was first accomplished by equilibrium dialysis, using ^{203}Pb and Scatchard plot data representation. Chick CaBP (1.6 x 10^{-6} M, 0.5 ml) was dialyzed versus 50 ml of Pipes/KCl buffer mixture containing ^{203}Pb and stable Pb(II) at 1 x 10^{-4}, 5 x 10^{-4}, 2.5 x 10^{-5}, 0.9 x 10^{-5}, 4.6 x 10^{-6}, 2.0 x 10^{-6}, 7.0 x 10^{-6}, and 3.3 x 10^{-7} M. At 48 h, aliquots were removed from outside and inside the sacs for determination of, respectively, free and total Pb(II) and Ca(II).

The Scatchard plot of the Pb(II)-binding data (Fig. 1) clearly indicates binding of 3.7 mol of Pb(II)/mol of chick...
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1 to the equation of Bredderman and Wasserman (7).

$$k_{a}^{\text{Pb}} = -\frac{\alpha}{1 - \alpha} \frac{k_{a}^{\text{Ca}}(\text{Ca})_{F}}{(\text{Pb})_{F} \cdot n/\bar{V}}$$

(1)

The value obtained, by this method for the chick CaBP, was $k_{a}^{\text{Pb}} = 1.57 \times 10^{7} \pm 0.11 \times 10^{7} \text{M}^{-1}$, a value considerably greater than the reported $k_{a}$ for Ca(II) of $2 \times 10^{6} \text{M}^{-1}$.

Displacement of bound Pb(II) by Ca(II) was next examined for chick CaBP. Following sampling for the 209Pb-binding data, stable Ca(II) was added to each outside solution to a final concentration of $1 \times 10^{-4} \text{M}$ (equivalent to the highest Pb(II) concentration). Dialysis proceeded for an additional 48 h and 209Pb binding was again determined. The results (Fig. 1) clearly show 209Pb displacement, in all cases, by added Ca(II). In addition, while the entire binding curve is shifted, reflecting lower occupancy of sites by Pb(II), the apparent $k_{a}^{\text{Pb}}$ (-slope) for both the high and lower affinity sets of sites remain essentially unaltered, confirming the competitive nature of Ca(II) and Pb(II) binding.

Where estimation of $k_{a}^{\text{Pb}}$ is possible at single concentrations of Ca(II) and Pb(II) (above), corroboration of this value by multiple point analysis via a Ca(II)-displacement curve was accomplished for both the bovine and chick CaBPs. The displacement of 45Ca by Pb(II) at differing Pb(II) concentrations (from $5 \times 10^{-7} \text{M}$ to $1 \times 10^{-4} \text{M}$) at a single stable Ca(II) concentration ($1.1 \times 10^{-6} \text{M}$ for bovine and $1.5 \times 10^{-6} \text{M}$ for chick CaBP) was examined by equilibrium dialysis. In all cases, CaBPs were pre-loaded with Ca(II) and 45Ca prior to addition of Pb(II).

The plotted data (Fig. 2) show the Pb(II) concentrations necessary to displace 50% of the bound Ca(II), under conditions described above, to be $1.8 \times 10^{-6} \text{M}$ for the chick and $4.3 \times 10^{-6} \text{M}$ for the bovine CaBPs. At the known concentration of Pb(II) sufficient to displace 50% of the bound Ca(II), Equation 1 becomes simplified to the following.

$$k_{a}^{\text{Pb}} = k_{a}^{\text{Ca}}(\text{Ca})_{F} / (\text{Pb})_{F} \cdot n/\bar{V}$$

(2)

Computation of $k_{a}^{\text{Pb}}$ for the chick and bovine CaBPs yield values of $1.61 \times 10^{7} \text{M}^{-1}$ and $1.08 \times 10^{7} \text{M}^{-1}$, respectively, confirming that the actual affinities of both proteins are greater for Pb(II) than Ca(II).

Ca(II)- and Pb(II)-binding to the chick and bovine CaBPs were examined by equilibrium dialysis under several sets of conditions (Table I). As expected, native chick CaBP bound 3.6 mol of Ca(II)/mol of protein in the absence of Pb(II). In

CaBP with high affinity ($k_{a}^{\text{Pb}} = 1.1 \times 10^{6} \text{M}^{-1}$) and 1 or 2 additional moles of Pb(II) with $k_{a}^{\text{Pb}} = 7 \times 10^{4} \text{M}^{-1}$.

Since ambient Ca(II) concentrations, arising from the dialysis buffer and the chick CaBP, were determined to be relatively high ($6.25 \times 10^{-6} \text{M}$) at all concentrations of Pb(II), competition for binding sites between Pb(II) and Ca(II) could be significant. At this free Ca(II) concentration, the computed occupancy of Ca(II)-binding sites, by Ca(II) (in the absence of Pb(II) and assuming a $k_{a}$ for Ca(II) of $2 \times 10^{8} \text{M}^{-1}$) would be 3.7, effectively diminishing the experimentally determined apparent $k_{a}^{\text{Pb}}$ considerably.

Straightforward determination of $k_{a}^{\text{Pb}}$ by simple Scatchard plot representation was therefore precluded. Similarly, methodological difficulties obviated the use of EDTA- or EGTA-Ca(II) or -Pb(II) buffer systems in the presence of 2 soluble binders and 2 cations. As an approach to estimation of $k_{a}^{\text{Pb}}$, exclusive of possible Ca(II) binding, single point analysis at Pb(II) concentrations corresponding to the 4 high affinity Pb(II)-binding sites were made by applying the data from Fig.

**FIG. 1.** Scatchard plot representation of 209Pb equilibrium dialysis data covering the range of free Pb(II) concentrations (Pb)$_{F}$ from $1 \times 10^{-4}$ M to $3.3 \times 10^{-5}$ M. 0, no added Ca(II); O, 1 $\times 10^{-4}$ M added Ca(II).

**FIG. 2.** Displacement curves for the displacement of 45Ca by Pb(II) for the vitamin D-induced chick and bovine CaBPs. See text for details.
the presence of essentially equimolar concentrations of Ca(II) and Pb(II) (0.9-1 x 10^{-6} M), only 0.3 mol of Ca(II)/mol of protein remained bound, whereas an additional 3.7 mol of Pb(II)/mol of protein were bound, indicating a total binding capacity for Pb(II) and Ca(II) of 4.

The native bovine CaBP was also shown to bind 2 mol of Ca(II) or 2 mol of Pb(II)/mol of protein with a combined total binding capacity for Ca(II) and Pb(II) of 2 mol/mol of protein.

The implication of experiments thus far, based on similar stoichiometry of binding and mutual displacement, was that Pb(II) and Ca(II) compete for binding at the same set(s) of sites. However, the proclivity of lead to interact with sulfhydryl groups to form lead mercaptides raised the possibility of Pb(II) binding to protein SH groups (secondary sites) with concomitant stoichiometric allosteric release of Ca(II) from the Ca(II)-binding sites.

The significance of Pb(II)-sulfhydryl interaction was examined by equilibrium dialysis in the presence of 1 mM β-mercaptoethanol (13-fold molar excess over protein sulfhydryls) and equimolar concentrations of Pb(II) and Ca(II), as well as Ca(II) alone. Whereas Ca(II) binding to CaBP was not influenced by β-mercaptoethanol (Table I), Pb(II) binding was diminished somewhat. This reduction was not significant in terms of molar ratio of protein SH and β-mercaptoethanol, however, and may have been due to Pb(II)-mercaptoethanol interaction, effectively reducing the free Pb(II) concentration.

The involvement of protein sulfhydryl groups in Pb(II)-binding to CaBP was essentially eliminated by forming the S-β-pyridylethylcysteine derivative of chick CaBP and repeating the dialysis experiments. Pb(II) binding to the SH-blocked protein (100% of total cysteine recovered as S-β-pyridylethylcysteine-Cys by compositional analysis, based on 3 mol of cysteine/mol of protein) was unaltered. Binding of Ca(II) by S-β-pyridylethylcysteine-CaBP also was not significantly decreased (within the limits of experimental error for a single point determination), confirming the findings of Ingersoll and Wasserman (9) that sulfhydryl groups are not involved in Ca(II) binding. These results, together with those for Pb(II) binding to bovine CaBP (which contains no sulfhydryl groups) support the conclusion that Pb(II) and Ca(II) compete for the same set(s) of high affinity-binding sites.

Similarities in primary structure corresponding to the predicted Ca(II)-binding domains among members of the troponin C superfamily of calcium-binding proteins suggested that high affinity Pb(II)-binding may not be unique to the vitamin D-induced CaBPs. In order to test this possibility, Pb(II) displacement of 45Ca from bovine brain calmodulin and bovine skeletal troponin C was studied under identical conditions to those described for the vitamin D-induced CaBPs. Both proteins, pre-equilibrated with 45Ca (CaBP = 1.0 x 10^{-6} M), were dialyzed against varying concentrations of stable Pb(II), as before. The results (Fig. 3) clearly show that Ca(II) is displaced by Pb(II) from both proteins and that the concentration of Pb(II) required for 50% displacement of Ca(II) is of the same order of magnitude (~micromolar) as observed for the vitamin D-induced CaBPs. Computation of the affinity constants was complicated, in these cases, since troponin C is reported to contain 4 Ca(II)-binding sites each, with widely differing kCa (17) and, whereas calmodulin is known to contain 4 Ca(II)-binding sites in 2 sets, uncertainty exists as to the distribution of these sites and their kCa values (16). For purposes of comparison, however, it is evident from the results in Fig. 3 that the average kCa for both proteins.

### Table 1

| Sample            | Pb(II) | Ca(II) | Pb/ Ca | Pb Ca | Ca Pb | PbCa
|-------------------|--------|--------|--------|-------|-------|-------|
| Native chick CaBP | 1.0 x 10^{-6} | 5.0 x 10^{-6} | 3.6 | 2.0 | 1.5 | 0.2 |
| Native CaBP       | 1.0 x 10^{-6} | 2.0 x 10^{-6} | 3.7 | 2.0 | 1.5 | 0.2 |

### Diagram

**Fig. 3.** Displacement curves for the displacement of 45Ca by Pb(II) for calmodulin (1.4 x 10^{-6} M) and troponin C (0.5 x 10^{-6} M). See text for details.
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nature and geometry of Pb(II) binding to CaBPs, as well as any Pb(II)-induced structural changes, must await detailed solution conformational (NMR) or crystallographic studies.

The studies reported here conclusively demonstrate specific high affinity binding to several calcium-binding proteins and suggest it to be a general property of those proteins which constitute the troponin C superfamily of "E-F hand" type calcium-binding proteins. The physiological significance of the observations, especially related to lead toxicity, remain to be studied. However, these Pb(II)-protein interactions may well be of importance considering the broad role of free Ca(II) ion as an important second messenger and the association of this class of proteins with a variety of Ca(II)-mediated cellular processes.

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