The Binding of Calcium and Yttrium Ions to a Glycoprotein from Bovine Cortical Bone

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The binding of Ca^{2+} and Y^{3+} to an acidic glycoprotein from bovine cortical bone, bone sialoprotein, was determined from the titration curves at $I\ 0.2$ in the presence and absence of the cations. The binding of Y^{3+} was greater than that of Ca^{2+} . The value for the association constant, k , for the interaction with Y^{3+} increased with pH, from $\log k\ 2.93$ at pH 3.4 to $\log k\ 3.50$ at pH 4.4, and the number of binding sites/mol. increased from 4.6 at pH 3.4 to 9.1 at pH 4.4. It is proposed that the binding site consists of three carboxyl groups, but it is likely that the binding is a strong electrostatic interaction rather than a co-ordination linkage. A chondroitin sulphate-protein complex also extracted from bovine cortical bone interacted with Y^{3+} and Ca^{2+} to a similar extent as did bone sialoprotein. It is suggested that these materials are present in bone at the resting and resorbing surfaces and that they contribute to the deposition of yttrium, americium and plutonium at these sites.

Many of the metal ions that are either major products of nuclear fission or are actinide elements are deposited in the skeleton to a considerable extent after parenteral injection into rats (Hamilton, 1947, 1948). Several of these 'bone-seeking' elements have been studied to a considerable extent because of their high affinity for bone tissues and the concomitant hazard to health. Strontium uptake has been correlated with calcium metabolism (Copp, Axelrod & Hamilton, 1947), and radioautographic and microradiographic techniques have shown that strontium is deposited in the bone on areas of low calcification on both periosteal and endosteal surfaces at sites where bone growth was taking place (Jowsey, Owen & Vaughan, 1953; Owen, Jowsey & Vaughan, 1955). This pattern of strontium uptake is in accord with the suggestion that strontium isomorphously replaces calcium in the hydroxyapatite mineral lattice (Vaughan, 1956). Yttrium and americium, however, appear to be deposited on highly calcified surfaces, not covered by osteoid tissue, that are either resting or resorbing (Jowsey, Sissons & Vaughan, 1956; Herring, Vaughan & Williamson, 1962), and it has also been suggested that plutonium is found at similar sites rather than on surfaces where new bone is being laid down (Herring *et al.* 1962).

Because the resting and resorbing surfaces, at which yttrium, americium and plutonium are deposited, stain strongly in the periodate-Schiff reaction (Jowsey *et al.* 1956) and because this

staining procedure in connective tissues has been correlated with the content of carbohydrate-containing materials (Leblond, Glegg & Eidinger, 1957), Herring *et al.* (1962) suggested that the deposition of these elements in bone was related to the presence of such a material at the resting and resorbing surfaces. They suggested that the acidic glycoprotein (BSP†) that had been isolated from bovine cortical bone (Herring & Kent, 1961, 1963) might possibly play a part in the binding.

The binding of Ca^{2+} and Y^{3+} to BSP has been determined by measuring their ability to displace the titration curve of the carboxyl groups to a lower pH. The advantages of this method of measuring the binding are (i) that much smaller quantities of material than would be required by most other methods can be used (cf. Rosoff, Lewin, Hart, Williams & Lazlo, 1958) and (ii) that the type of interaction determined by displacement of the titration curves is a strong one, since competition with H^+ for carboxylate anions is occurring. The special disadvantage of the method is that it reveals only the number of such titratable groups involved: the actual number of metal ions bound to them can only be ascertained by making certain further assumptions, which have been later justified by their self-consistency.

MATERIALS AND METHODS

Preparation of BSP. BSP was extracted from bovine cortical bone with EDTA salts, and purified by the method of Herring (1964), and was from the same preparation as

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† Abbreviation: BSP, bone sialoprotein.

used in the preceding paper (Peacocke & Williams, 1967). For the conductivity titrations a different preparation, designated BSP5, was used. It was extracted and purified by the same method, but it had not been characterized physically. Hydrolysis of sialic acid from BSP was effected by heating at 80° for 1 hr. in 0.1 N-H₂SO₄.

Other materials. Poly-L-aspartic acid and the sodium salt of poly-L-glutamic acid were obtained from Pilot Chemicals (Watertown, Mass., U.S.A.) and *N*-acetylneuraminic acid was from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.). Bovine serum albumin was the crystalline protein obtained from Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex) and ribonuclease was the crystalline protein from Seravac Laboratories Ltd. (Maidenhead, Berks.).

Denaturation of ribonuclease. A solution of 8M-urea was acidified with acetic acid to pH 4 and then adjusted to pH 8 with aq. NH₃ (sp.gr. 0.88) to destroy any isocyanate ions. A solution of crystalline ribonuclease (100mg.) in 8M-urea (4ml.) was treated with excess of mercaptoethanol (0.5ml. of an aqueous 5% solution). Sufficient solid iodoacetic acid (0.3g.) to react with the reduced protein and with excess of mercaptoethanol was added, and the solution was left at room temperature for 10 min. It was then transferred to dialysis tubing and successively dialysed against distilled water (2l.) for 1 hr., a 0.5% solution of the disodium salt of EDTA for 6 hr., and two changes of 0.2M-NaCl for 24 hr. each.

Determination of concentrations of solutions. Solutions of BSP, BSP from which the sialic acid had been removed (de-sialated) and *N*-acetylneuraminic acid were analysed after titration as described in the preceding paper (Peacocke & Williams, 1967).

Solutions of bovine serum albumin, ribonuclease and denatured ribonuclease after titration were estimated by the biuret method (Gornall, Bardawill & David, 1949). Bovine serum albumin and ribonuclease solutions of known concentration in the range 0–5 mg. of protein/ml. were used as standards; the concentrations of solutions of denatured ribonuclease were determined by comparison with the ribonuclease calibration curve.

Titration procedures. Titrations were carried out at 25.00 ± 0.05° and *I* 0.2 as described in the preceding paper (Peacocke & Williams, 1967). Solutions of CaCl₂ were prepared by dissolving a weighed quantity of Ca(OH)₂ in a calculated excess of 0.1N-HCl and then neutralizing with 0.1N-NaOH, so that the total ionic strength was 0.2. The YCl₃ solutions of 100.2 were prepared similarly from spectrographically pure Y₂O₃ (Johnson, Matthey and Co. Ltd., Wembley, Middx.).

Titrations in the presence of Y³⁺ and Ca²⁺ were carried out on 5ml. of solution, made up from 1 ml. of the solution in 0.2M-NaCl of the material to be titrated and 4 ml. of the added YCl₃ or CaCl₂ solution. The pH of the added metal chloride solution was previously adjusted to within ± 0.1 pH unit of that of the 0.2M-NaCl against which the solution of macromolecule had been dialysed, in order that in all titrations of a particular material approximately the same concentration of free H⁺ ions should be present in the solutions before they were mixed in the titration cell. Solvent blanks were titrated immediately before the corresponding solution (see Peacocke & Williams, 1967); the solutions in these solvent-blank titrations differed from solutions (described above) in that 1 ml. of the dialysed

solution of BSP (etc.) was replaced by 1 ml. of the 0.2M-NaCl against which it had been dialysed.

Corrections for solvent blanks were always made and the titration curves were calculated in terms of g.equiv. of H⁺ bound (or dissociated)/g. of material.

Conductivity titrations. A 50mm-YCl₃ solution, pH 4.0, was added from an Agla syringe to 10ml. of a solution of BSP in 2mm-acetate buffer, pH 4.0, at 25° in a small conductivity cell. The conductivity of the solution throughout the titration was measured with a conductivity bridge (Cambridge Instrument Co.), and all experimental values of conductivity were corrected to the starting volume.

RESULTS

Extent of binding. The number of H⁺ ions bound that are measured in a titration experiment can be expressed as 1 - α, where α is the degree of dissociation. If metal ions (M) are present that bind to anions, such as the carboxylate anions (C⁻) of BSP, the species present in solution may be denoted as HC, C⁻ and CM, where CM is not meant to specify the stoichiometry of the binding, but represents those anions binding metal ions and not available to H⁺. For this case the apparent degree of dissociation, α', may be defined by the relations:

$$(1 - \alpha')[C_T] = [HC]$$

and

$$(\alpha' - \alpha_M)[C_T] = [C^-]$$

where $[C_T] = [HC] + [C^-] + [CM]$ and $\alpha_M = [CM]/[C_T]$, the fraction of the anions that bind metal ions.

Two methods were used to calculate α_M from the displacement of the titration curves in the presence of metal ions.

Method A (Kono & Ikegami, 1966). The titration of the anions in the absence of metal ions may be represented by:

$$pK_0 = pH - \log \frac{\alpha}{(1 - \alpha)} + F(\alpha, I, S) \quad (1)$$

where $F(\alpha, I, S)$ is a function of charge (α), ionic strength (*I*) and shape (*S*), if the only groups titrating over the pH range are the anions C⁻.

In the presence of the metal ions that bind to C⁻, with *I* and *S* unchanged, this becomes:

$$pK_0 = pH' - \log \frac{(\alpha' - \alpha_M)}{(1 - \alpha')} + F(\alpha' - \alpha_M, I, S) \quad (2)$$

The anions that have bound cations are assumed not to contribute to the charge on the macromolecule, so that each Ca²⁺ or Y³⁺ ion is regarded as binding to two or three carboxylate anions respectively.

Kono & Ikegami (1966) showed that α_M can be calculated graphically, without making any assumptions about *F*, from a plot of $T(\alpha)$ against α, and from values of the displacement, ΔpH, along

the pH axis caused by metal ions at given values of α , since:

$$\Delta\text{pH} = T(\alpha) - T(\alpha - a_M) \tag{3}$$

where $T(\alpha) = \text{pH} - \log[1/(1 - \alpha)]$, and can be derived from the titration in the absence of metal ions.

Method B. This is a modification of that of Gilbert & Myers (1960). The titration of the carboxyl groups in BSP is known to obey the relation:

$$\text{p}K_0 = \text{pH} - \log \frac{\alpha}{(1 - \alpha)} + A\alpha \tag{4}$$

where A is an empirical constant that can be calculated from Fig. 3 in the preceding paper (Peacocke & Williams, 1967). The degree of dissociation, α , is again assumed to be proportional to the net negative charge of the macromolecule.

In the presence of metal ions, as in eqn. (2):

$$\text{p}K_0 = \text{pH}' - \log \frac{(\alpha' - a_M)}{(1 - \alpha')} + A(\alpha' - \nu a_M) \tag{5}$$

where ν is the increase in net positive charge on each carboxyl group caused by the binding of a metal ion.

The difference, ΔpH , in pH between the titration curves in the presence and absence of metal ions at a given value of α ($=\alpha'$) is therefore, by eqns. (4) and (5):

$$\Delta\text{pH} = \log \frac{\alpha}{(\alpha - a_M)} - \nu A a_M \tag{6}$$

So, from ΔpH values at given values of α , by a method of successive approximation, it is again possible to calculate values of a_M . However, a value of ν has to be assumed in this treatment and for Y^{3+} binding there are two possible extremes: $\nu = 3$, if one Y^{3+} ion binds to each carboxylate anion

(the CY^{2+} model); or $\nu = 1$, if one Y^{3+} ion binds to three anions (the C_3Y model). On the latter basis, the calculated values of a_M should be the same as those obtained by method A: there is indeed good agreement (Table 1), the small observed differences resulting from deviations of the derivative plot from linearity (Fig. 3 in Peacocke & Williams, 1967).

Fig. 1. Effect of Ca^{2+} and Y^{3+} on the titration curve of BSP at 25° and $I\ 0.2$. All curves were fully reversible. The total salt composition at the start of each titration was: A, 0.2M-NaCl ; B, 40mm-CaCl_2 in 80mm-NaCl ; C, 0.5mm-YCl_3 in 197mm-NaCl ; D, 1.0mm-YCl_3 in 194mm-NaCl ; E, 3.25mm-YCl_3 in 180mm-NaCl ; F, 20mm-YCl_3 in 80mm-NaCl . (The titration curve in 0.1mm-YCl_3 in 199mm-NaCl is omitted.)

Table 1. Comparison of values of a_M calculated for the C_3Y model by methods A and B

pH and α refer to a titration curve of BSP in 3.25mm-YCl_3 in 180mm-NaCl . ΔpH is the difference in pH at constant α between this curve and the corresponding titration curve in 0.2M-NaCl . The temperature was 25° .

pH	ΔpH	Degree of dissociation of the carboxyl groups (α)	a_M	
			Method A	Method B ($\nu=3$)
4.38	1.32	0.87	0.49	0.49
4.25	1.25	0.85	0.48	0.46
4.14	1.16	0.81	0.45	0.42
4.03	1.07	0.77	0.44	0.38
3.92	0.98	0.72	0.36	0.35
3.80	0.90	0.66	0.32	0.31
3.67	0.83	0.60	0.28	0.28
3.41	0.69	0.49	0.21	0.22
3.27	0.53	0.41	0.15	0.16
3.10	0.40	0.34	0.11	0.11
2.96	0.34	0.29	0.08	0.09
2.68	0.22	0.21	0.05	0.05

Fig. 2. Variation in the fraction of carboxyl groups in BSP involved in binding Y^{3+} and Ca^{2+} , a_M , with pH, calculated (a) by method A, assuming each Ca^{2+} or Y^{3+} ion bound to two or three carboxylate anions respectively, and (b) by method B, assuming each Ca^{2+} ion ($\nu=2$) and each Y^{3+} ion ($\nu=3$) bound to one carboxylate anion. Total salt compositions: Δ , 20mM- YCl_3 in 80mM-NaCl; $\bullet$, 3.25mM- YCl_3 in 180mM-NaCl; $\square$, 1.0mM- YCl_3 in 194mM-NaCl; $\blacktriangle$, 0.5mM- YCl_3 in 197mM-NaCl; $\circ$, 0.1mM- YCl_3 in 199mM-NaCl; $\blacksquare$, 40mM- $CaCl_2$ in 80mM-NaCl.

Ca^{2+} and Y^{3+} displace the reversible titration curves of BSP (Fig. 1), and from these displacements a_M has been calculated (Fig. 2) as a function of pH by method A for the C_3Y model, each Ca^{2+} or Y^{3+} ion being assumed bound to two or three carboxylate anions respectively, and by method B for the CY^{2+} model, each Ca^{2+} ion ($\nu=2$) and Y^{3+} ion ($\nu=3$) being assumed to bind to one carboxylate anion. These two models should represent the two extremes that the binding can take.

Both calculations show that as many carboxyl groups in BSP bind Ca^{2+} in 40mM-calcium chloride as bind Y^{3+} in 0.1mM-yttrium chloride, so that Y^{3+} binds several hundred times as strongly as Ca^{2+} .

Binding curves of Y^{3+} to BSP. The relations (Fig. 3) between the number, r , of Y^{3+} ions bound/mol. of BSP and the concentration of free Y^{3+} (as $\log[Y^{3+}]_{free}$) at pH 3.4, 3.8, 4.0 and 4.4 were

Fig. 3. Binding curves of Y^{3+} to BSP: the variation of the number (r) of Y^{3+} ions bound/mol. of BSP with the logarithm of the free Y^{3+} concentration ($\log[Y^{3+}]_{free}$): (a) for the C_3Y model and (b) for the CY^{2+} model. $\circ$, pH 4.4; $\blacktriangle$, pH 4.0; Δ , pH 3.8; $\bullet$, pH 3.4. These are calculated from the results plotted in Fig. 2 as described in the text.

derived from the variation of a_M with pH by use of the following equations. For the C_3Y model:

$$r = \frac{47.8}{3} a_M \text{ and } [Y^{3+}]_{free} = \frac{V}{v} (C - \frac{1}{3} B a_M)$$

and for the CY^{2+} model:

$$r = 47.8 a_M \text{ and } [Y^{3+}]_{free} = \frac{V}{v} (C - B a_M)$$

where 47.8 is the total number of carboxyl groups/mol. (Peacocke & Williams, 1967), V is the initial volume of the titrated solution (5ml.), v is the volume of the solution at that pH, C is the initial total molar concentration of yttrium chloride and B is the initial concentration of BSP (equiv. of carboxyl groups/l.).

From the values of r and $[Y^{3+}]_{free}$ the total number, n , of binding sites/mol. and the association constant, k , for the binding were calculated from:

$$r = \frac{nk[Y^{3+}]_{free}}{1 + k[Y^{3+}]_{free}}$$

by plotting $1/r$ against $1/[Y^{3+}]_{free}$, when the slope is $1/kn$ and the intercept on the ordinate axis is $1/n$ (Fig. 4 and Table 2).

In Fig. 4 the points are omitted for 0.1mM-

Table 2. *Binding parameters for the interaction of Y^{3+} with BSP*

pH	C_3Y model			CY^{2+} model		
	Association constant ($\log k$)	Stability constant ($\log K$)	Maximum no. of binding sites/mol. (n)	Association constant ($\log k$)	Stability constant ($\log K$)	Maximum no. of binding sites/mol. (n)
3.4	2.93	3.25	4.6	2.93	3.20	6.4
3.8	3.17	3.46	6.9	3.15	3.43	8.8
4.0	3.24	3.70	8.3	3.23	3.49	10.1
4.4	3.50	3.68	9.1	3.46	3.78	11.1

Fig. 4. Plots of $1/r$ against $1/[\text{Y}^{3+}]_{\text{free}}$ for BSP: (a) for the C_3Y model and (b) for the CY^{2+} model. $\circ$, pH 4.4; $\triangle$, pH 3.8; $\bullet$, pH 3.4. The straight lines were calculated by the method of least mean squares.

yttrium chloride (total) because the errors are too large and for pH 4.4 in 20 mM yttrium chloride, because precipitation (of either hydrated yttrium oxide or possibly an yttrium-BSP complex) occurred.

Unlike the values of k in Table 2, a true stability constant for the binding of Y^{3+} to BSP should involve equilibrium between the cations and anions only, and should be independent of pH.

The true stability constants, K , are defined as:

$$K = \frac{[\text{CM}]}{[\text{C}^-][\text{M}]_{\text{free}}}$$

and are related to the association constants, k , by:

$$k = K \cdot \frac{[\text{C}^-]}{[\text{C}^-] + [\text{CM}]}$$

The values of K were obtained by plotting $1/R$ against $1/[\text{Y}^{3+}]_{\text{free}}$, these quantities being calculated from the same experimental results as in the determination of the binding curves, since $R = r/\alpha'$, where α' is the apparent degree of dissociation of the carboxyl groups at the corresponding point on the titration curve, and is the fraction of carboxyl groups not binding H^+ .

However, the calculated values of K (Table 2) still appear to vary with pH as much as k .

Each type of binding constant has particular merits. The value of the stability constants, K , represents a more accurate measure of the strength of the binding between the anions and cations and facilitates comparison with inorganic stability constants, but it is more usual to consider the binding equilibrium of biological macromolecules with small ions or molecules in terms of the association constants, k .

Titration of other macromolecules. To learn more about the nature of the binding between Y^{3+} or Ca^{2+} and BSP, a similar series of titrations were carried out on a variety of other macromolecules. No extensive calculations were made of the degree of binding, but the following empirical criterion was applied. The depression of pH, ΔpH , caused by ligand cations on the titration curve of a set of like carboxyl groups should be similar for a given value of a_{M} , irrespective of their number. Thus, if yttrium chloride solutions caused a smaller ΔpH in the carboxyl titration curve of a given molecule than in BSP, it was assumed that the extent of interaction of Y^{3+} with the carboxyl groups of the macromolecule was much less than with BSP.

All the macromolecular solutions titrated showed

a greater binding of Y^{3+} than of Ca^{2+} . The materials can be divided into two classes with respect to the ΔpH caused by the yttrium chloride solutions:

(i) ΔpH was of similar magnitude to that of BSP: BSP from which the sialic acid had been hydrolysed (Fig. 5), poly-L-glutamic acid, poly-L-aspartic acid and bone chondroitin sulphate-protein complex.

(ii) ΔpH was much smaller than with BSP: bovine serum albumin, ribonuclease and denatured ribonuclease (Fig. 6). The maximum measured extent, a_M , and number, r , of Y^{3+} ions (in 20mm- Y^{3+} chloride) bound to the three proteins has been estimated by method A (Table 3). The resulting value for bovine serum albumin is of the same order as that obtained by Rosoff *et al.* (1958), who found that $n=6$ at pH 5.3 by equilibrium dialysis.

N-Acetylneuraminic acid was also titrated in the presence of yttrium chloride and calcium chloride solutions (Fig. 7); because of the much lower pK_0 of these carboxyl groups, the results are not strictly comparable with the other materials. They are unusual in showing much the same ΔpH with

40mm-calcium chloride and with 20mm- Y^{3+} chloride, which indicates very little interaction with either cation above pH 3.5. The change of direction in the titration curve in 20mm- Y^{3+} chloride above pH 4.8 was due to precipitation of material from solution.

The change of electrical conductivity produced on addition of 50mm- Y^{3+} chloride, pH 4, to a solution of BSP in 2mm-acetate buffer, pH 4, has been compared with the results of addition of 50mm- Y^{3+} chloride to an equal volume of acetate buffer against which the sialoprotein solution had been dialysed (Fig. 8). The values of n calculated from two such experiments by the method used by Zubay & Doty (1958) are compared (Table 4) with n at the same pH calculated from pH titration. It is to be noted that these are not strictly comparable since a different preparation (BSP 5) was used in the conductivity titration and the total ionic strength was very different. The agreement between the two conductivity titrations is reasonable considering that very little material was available (13.1mg. and 4.9mg. in each conductivity titration respectively).

DISCUSSION

In the light of all the available evidence it seems most probable that the binding of Y^{3+} to BSP is best represented by the C_3Y model employed in

Fig. 5. Titration curves of de-sialated BSP at 25° and I 0.2. Total salt concentrations: ○, 0.2M-NaCl; ●, 40mM- $CaCl_2$ in 80mM-NaCl; △, 20mM- YCl_3 in 80mM-NaCl. Only points from forward titrations are shown, although all titrations were fully reversible.

Fig. 6. Part of the titration curve of denatured ribonuclease at 25° and I 0.2. The denaturation of the ribonuclease is described in the Materials and Methods section. Total salt concentrations: ○, 0.2M-NaCl; ●, 40mM- $CaCl_2$ in 80mM-NaCl; △, 20mM- YCl_3 in 80mM-NaCl. Only points from the forward titrations are shown, although all titrations were fully reversible.

Table 3. *Maximum measured binding of Y^{3+} to proteins*

The binding was calculated by the method A, from titrations in 200 mM-NaCl and in 20 mM- YCl_3 in 80 mM-NaCl. It is assumed in calculating the number of Y^{3+} ions bound/mol., r , that the number of titratable carboxyl groups/mol. in bovine serum albumin is 100 (Tanford, Swanson & Shore, 1955), in ribonuclease is 11 (Tanford & Hausenstein, 1956) and in denatured ribonuclease 19, assuming maximum reaction with iodoacetic acid.

	pH	Maximum measured value of a_M	Corresponding no. of Y^{3+} ions bound/mol. (r)
Bovine serum albumin	5.05	0.13	4.3
Ribonuclease	5.0	0.04	0.1
Denatured ribonuclease	4.9	0.06	0.4

Fig. 7. Titration curves of *N*-acetylneuraminic acid at 25° and *I*0.2. Total salt concentrations: —, 0.2M-NaCl; ---, 40mM- CaCl_2 in 80mM-NaCl; ·····, 20mM- YCl_3 in 80mM-NaCl. All titrations were reversible.

Fig. 8. Conductivity titration of BSP5: the conductivity changes on addition of 50mM- YCl_3 , pH 4.0, to (a) a solution of BSP5 (4.9mg.) in 10ml. of 2mM-acetate buffer, pH 4.0, (○) and (b) to 10ml. of 2mM-acetate buffer, pH 4.0 (●). All conductivities are corrected to the starting volume.

Table 4. *Maximum binding of Y^{3+} to BSP at pH 4 calculated from conductivity titrations*

The solvent in the conductivity titration of BSP5 was 2mM-acetate buffer; the pH titration is of BSP at *I*0.2, taken from Table 2 for the C_3Y model.

	Maximum no. of Y^{3+} ions bound/mol. (n)
Conductivity titration 1	14.8
Conductivity titration 2	17.5
pH titration	8.3

method A, i.e. that one Y^{3+} ion binds to a site consisting of three carboxyl groups.

Mandel & Leyte (1962) found that one Cu^{2+} ion binds to two carboxylate anions in polymethacrylic acid, and Mathews (1964) proposed that one $\text{Co}(\text{NH}_3)_6^{3+}$ ion is bound to chondroitin sulphate on a site consisting of three carboxyl groups. These results suggest that bi- and ter-valent cations bind to two or three singly charged anionic sites respectively on flexible polymers with a high density of carboxyl groups. Moreover a Y^{3+} ion linked to three carboxylate anions would be analogous to the structure proposed for the complex of La^{3+} with EDTA, $\text{La}(\text{EDTA})^-$, in which the cation is co-ordinated to three of the four carboxylate anions (Moeller, Moss & Marshall, 1955).

Experimental evidence in favour of the C_3Y model is provided by the values (Table 2) of the binding constants (k or K) and the maximum number of binding sites (n).

If the binding were of the type CY^{2+} , it would be reasonable to expect that the calculated maximum number of binding sites, n , would be equal or nearly equal to the number of carboxyl groups in the molecule. In fact, the values of n are only 20–25% of the total number of groups. The values of n calculated for the C_3Y model are of the same magnitude as those for the CY^{2+} model, but with the C_3Y model the number of carboxyl groups involved is

$3n$ and so approaches much more nearly the total number of carboxyl groups in the molecule. This favours the C_3Y model as the most rational description of the binding situation. That $3n$ still falls short of the total number of carboxyl groups/mol. is understandable since the spatial arrangement of some of the carboxyl groups might well not allow three of them to bind to one Y^{3+} ion.

Further, the calculated parameters for both the C_3Y and CY^{2+} models are almost the same. This suggests that they are both, in fact, equivalent, and this could only be so if, when the binding is of the type CY^{2+} , two other carboxyl groups are prohibited, presumably by electrostatic interaction, from participating in further binding for each Y^{3+} bound. Since the groups so prohibited would almost certainly be adjacent to the group binding the Y^{3+} , the CY^{2+} model is then indistinguishable in practice from the C_3Y model in which each Y^{3+} ion is directly linked to the three groups.

Further evidence for the C_3Y model is furnished by the titrations of the other macromolecules in the presence of Y^{3+} . Only those molecules with a high density of carboxyl groups show a strong interaction, whereas proteins with a small proportion of carboxyl groups, bovine serum albumin and ribonuclease, show weak binding. If the binding were of the form CY^{2+} , there is no reason why bovine serum albumin, for example, which has 100 titratable carboxyl groups/mol. (Tanford *et al.* 1955), should not interact with Y^{3+} as strongly as does BSP. Its failure to do so can only be explained if the binding is polyfunctional, and in the native proteins, with their lower proportion of carboxyl groups and their rigid tertiary structure, interaction between one Y^{3+} ion and three close carboxyl groups is sterically much less probable. Even destruction of the tertiary structure of ribonuclease by reduction of the disulphide bonds and converting the free thiol groups into cysteine carboxymethyl groups ($-S \cdot CH_2 \cdot CO_2H$) appeared to make little difference to the relative amount of binding.

The only evidence that favours binding of one Y^{3+} ion to only one carboxylate anion is that the values of the stability constants ($\log K$ 3.2–3.8; see Table 2) are very similar in magnitude to the first binding constant (K_1) for the binding of Y^{3+} to citric acid (H_4L):

where $\log K_1$ is 3.6 (Tomkins & Major, 1947). This would not be inconsistent with the C_3Y model if the bonds in the BSP complex are weaker than in the citrate complex and are electrostatic interactions rather than co-ordinate bonds, or if each Y^{3+} ion in the BSP complex is linked to only one of the three carboxylate anions at the binding site, but thereby

prohibits further participation of the two adjacent carboxyl groups.

Whether or not sialic acid groups participate in the binding is problematic. BSP from which the sialic acid has been hydrolysed appears to interact just as strongly as does BSP, and sialic acid itself appears not to interact much more strongly with Y^{3+} than Ca^{2+} and not very strongly with either, though this may be because it is a small molecule and consequently incapable of polyfunctional interactions. However, *N*-acetylneuraminic acid combines more weakly with H^+ ions than most carboxyl groups, having a much lower pK_0 , and may therefore interact more weakly with other cations. It is not clear from this titration evidence if in BSP the sialic acid carboxylate ions interact with Y^{3+} to a smaller extent than do the aspartyl and glutamyl carboxylate ions.

It is not surprising that the maximum binding values calculated by conductivity and pH titrations do not agree. Those Y^{3+} ions constrained by the BSP from conducting electricity to their full extent may not be bound sufficiently strongly to affect the H^+ equilibria. This suggests that the binding of Y^{3+} determined by pH titration represents only a part, albeit the strongest part, of a range of interactions of Y^{3+} and other cations with BSP.

Bone chondroitin sulphate-protein complex also appears to bind Y^{3+} to a similar extent as does BSP, and, although a complete study has not been made, the indications are that the interaction is much stronger than that measured between Y^{3+} and protein-free chondroitin sulphate from cartilage by Gilbert & Myers (1960). This difference may result from the nature of the associated protein, and preliminary experiments (G. M. Herring, personal communication) indicate that this polypeptide component is in fact part of a sialoprotein, probably BSP, that is associated with the chondroitin sulphate. This would agree with the suggestion (Anderson, 1962) that the structure of the chondroitin sulphate-protein complex in cartilage, proposed as chains of polysaccharide attached to an inner core of protein (Partridge, Davis & Adair, 1959, 1961), could be better explained if the inner core was a glyco- or sialo-protein rather than an ordinary protein.

Several workers have proposed that the inorganic part of bone, rather than any organic material, is responsible for the retention of yttrium and plutonium in bone. However, these experiments have been performed on synthetic hydroxyapatite (Neuman, Mulryan & Martin, 1960) or on bone that has been ashed or subjected to strong alkali treatment (Jowsey, Rowland & Marshall, 1958; Foreman, 1962), and such experiments are not necessarily applicable to bone *in vivo*. Further, comparison of the ability of these materials to bind yttrium and

plutonium with that of the organic fraction of bone, prepared by demineralizing the bone with EDTA, is not valid since this treatment will liberate BSP and chondroitin sulphate into solution (Herring & Kent, 1961, 1963).

The presence of BSP and also of chondroitin sulphate-protein complex in bone and the ability of these molecules to interact strongly with the highly charged ions of yttrium and also of thorium (Peacocke & Williams, 1966) will undoubtedly contribute to the retention of these elements in the bone matrix, provided that there is some mechanism for equilibration of the cations between the blood carrying them and the sites of these molecules in the bone, and provided that the interaction between the bone materials and Y^{3+} is sufficiently strong to remove the Y^{3+} from the material to which it is bound in the blood. Boocock & Popplewell (1966) have shown that plutonium in serum is carried by proteins and not by small diffusible molecules, although the binding constants to the inorganic anions in the blood are probably much higher. This would seem to indicate that these diffusible binding agents are present in too low a concentration to affect the distribution of the cations. If this is so, the results presented here would suggest that competition for Y^{3+} between BSP and any native serum protein would result in transfer of the Y^{3+} to BSP.

It is probable that BSP interacts as strongly with americium and plutonium as it does with yttrium and thorium respectively. Americium is chemically similar to yttrium and the lanthanides, and its similarity to them in the way it becomes distributed in bones and tissue after being taken into the body, either parenterally or into the bloodstream, has been noted (Hamilton, 1947; Taylor, 1962; Durbin, 1962; Herring *et al.* 1962). Thorium was suggested as tracer for plutonium that could be easily handled in the titration apparatus without any radiation hazard (D. M. Taylor, personal communication). The relative amounts of binding of Ca^{2+} on the one hand and Y^{3+} and thorium (Peacocke & Williams, 1966) on the other by BSP, combined with the strong reaction of this molecule in the periodate-Schiff stain, suggests an explanation of the difference in distribution in bone between Sr^{2+} at the growing surfaces and yttrium, americium and plutonium at those sites that stain strongly in the periodate-Schiff technique, namely the resting and resorbing surfaces.

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