The Binding of Hydrogen Ions to an Acidic Glycoprotein from Bovine Cortical Bone

By A. R. PEACOCKE AND P. A. WILLIAMS*

Nuffield Department of Clinical Biochemistry, Radcliffe Infirmary, Oxford

(Received 5 May 1967)

The H^+ ion dissociation of bone sialoprotein in 0.2M-sodium chloride at 25° was studied. The total content of carboxyl groups available for titration was calculated by comparing the titration curve with the titration curves of three model systems and by the use of analytical data. This comparison showed that 7.0 carboxyl groups/mol. do not participate in the titration, and it is proposed that these are aspartic acid or glutamic acid carboxyl groups present as amides; this is also indicated by titration of the sialoprotein after acid hydrolysis. The titration of carboxyl groups was found to agree well with the Linderstrøm-Lang equation for spherical macroions.

The organic matrix of compact bone tissue makes up 21% of the total weight; the other constituents are inorganic material (71%) and bound water (8%) (Eastoe & Eastoe, 1954). The main constituent of the organic matrix is insoluble collagen, but also present are materials that contain polysaccharide residues and these may make up as much as 10–11% of the organic materials (D. Olroyd & G. M. Herring, personal communication). Herring & Kent (1961, 1963) isolated four polysaccharide-containing fractions from bovine cortical bone by mild extraction at pH 7.9 with the sodium salts of EDTA. These were (a) a chondroitin sulphate fraction, (b) two glycoprotein fractions (previously named mucoproteins I and II) and (c) an acidic glycoprotein that had a high content of sialic acid and that was named bone sialoprotein. Both BSP† and the two glycoprotein fractions showed a strong reaction to the periodate-Schiff staining technique and it was suggested that they contributed to this reaction observed histologically in bone (Herring & Kent, 1963).

Complete analyses of BSP (Andrews & Herring, 1965; Andrews, Herring & Kent, 1967) show that, as well as a high content of sialic acid, BSP has an exceptionally high content of glutamic acid and aspartic acid residues in the protein moiety. From these analytical results the minimum molecular weight should be 23000 (Andrews & Herring, 1965), and examination in the analytical ultracentrifuge yielded a molecular weight of $25\,000 \pm 500$

for BSP extracted by EDTA, and of $21\,800 \pm 1600$ for BSP extracted by phosphate buffer (Williams & Peacocke, 1965). This latter preparation was thought to have been more degraded on account not only of its lower molecular weight but also of its wider distribution of sedimentation coefficient.

The structure proposed for BSP (Andrews & Herring, 1965; Andrews *et al.* 1967) is a single polypeptide chain to which all the carbohydrate residues are linked as a single highly branched moiety. The sialic acid groups are the terminal residues of this carbohydrate prosthetic group. This and the following paper (Williams & Peacocke, 1967) describe a study of the binding of H^+ and certain metal ions to BSP; a preliminary report of this work has been published elsewhere (Peacocke & Williams, 1966). The H^+ ion equilibria were investigated not only because BSP has an unusual structure for a biological polyelectrolyte, with some of the dissociating carboxyl groups clustered together on the carbohydrate prosthetic group, but also because such a study was a necessary basis for examining the binding of metal cations of radio-biological interest reported in the following paper (Williams & Peacocke, 1967).

MATERIALS AND METHODS

Preparation of BSP. BSP was prepared from bovine cortical bone by the simplified EDTA extraction procedure of Herring (1964) and is the material denoted BSP (EDTA) by Andrews & Herring (1965) and Williams & Peacocke (1965). It was isolated in co-operation with Dr G. M. Herring of the Medical Research Council Bone-Seeking Isotopes Research Unit, Churchill Hospital, Oxford.

* Present address: Department of Biochemistry and Soil Science, University College of North Wales, Bangor.

† Abbreviation: BSP, bone sialoprotein.

Sialic acid groups were hydrolysed from BSP by heating a solution in 0.1N-H₂SO₄ at 80° for 1 hr.

Other materials. Poly-L-glutamic acid as the sodium salt and poly-L-aspartic acid as the free acid were obtained from Pilot Chemicals (Watertown, Mass., U.S.A.); the latter was dissolved in 0.07N-HCl before dialysis. *N*-Acetylneuraminic acid was obtained from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.).

Determination of concentrations of solutions. The amount of BSP present in solutions from titration experiments was estimated by measuring the sialic acid content by the direct Ehrlich method (Werner & Odin, 1952); the percentage of sialic acid in the dry substance was taken as 20.7% (Andrews & Herring, 1965). Solutions of BSP from which the sialic acid residues had been hydrolysed were analysed by comparing the extinctions at 275m μ (Andrews, 1966). Solutions of *N*-acetylneuraminic acid were analysed by the thiobarbituric acid method (Warren, 1959).

Determination of titration curves. The material to be titrated was dissolved in 0.2M-NaCl so as to be within the range 7–30mm in carboxylic acid groups. These solutions (apart from the *N*-acetylneuraminic acid solutions) were dialysed against 0.2M-NaCl (250ml.) overnight, after which both the solution and the solvent against which it had been dialysed were kept separately at –15° until required.

HCl and NaOH were used as acid and alkali in all titrations and were made up to be 0.07N from standard ampoules (British Drug Houses Ltd., Poole, Dorset); these solutions were made 0.165M in NaCl, so that equal volumes of acid and alkali, when mixed, produced a neutral solution of 0.2M-NaCl. The starting volume of all titrated solutions was 5ml., made up of 1ml. of the dialysed solution of the macromolecular substance and 4ml. of the 0.2M-NaCl against which it had been dialysed. Immediately before titration of the macromolecule solution, 5ml. of the dialysed 0.2M-NaCl was titrated as a solvent blank.

All titrations were carried out at 25.00 $\pm$ 0.05°, under an atmosphere of N₂, which was passed over the solution after previously being washed with conc. NaOH and 0.2M-NaCl. Standard acid or alkali was added from an Agla micro-meter syringe fitted with a capillary glass tip (accuracy $\pm$ 0.0002ml.), and the pH of the solutions being titrated was measured to $\pm$ 0.001 pH unit with a screened glass electrode (type GHS33; Electronic Instruments Ltd.) and the calomel electrode connected to a Vibron electrometer and pH-measuring unit (Electronic Instruments Ltd.). This instrument was calibrated immediately before the solvent-blank titration and checked after the titration of the macromolecule solution with 0.05M-potassium hydrogen phthalate, pH4.005. The drift during the two titrations (about 3hr.) was within the range $\pm$ 0.005pH unit. The calibration was not altered between the solvent-blank and the solution titrations, as the accuracy of the experiment depended on both titrations being carried out at an identical setting of the instrument; for the same reason both titrations were carried out with the minimum possible time-interval between them.

Complete titration curves, both forward and back, over the range pH2.2–11 were determined in two separate experiments. The titration from neutral solution (i.e. at the pH of the solution after dialysis against 0.2M-NaCl) to pH2.2 and the back titration were calculated in g.equiv. of H⁺ bound/g. of solute and the forward and back titrations from neutral solution to pH11 were calculated in g.equiv.

of OH[–] bound/g. of solute ($\equiv$ g.equiv. of H⁺ dissociated/g. of solute). Solvent-blank corrections were calculated for all titrations.

RESULTS AND DISCUSSION

The complete titration curve of BSP in 0.2M-sodium chloride at 25° is shown in Fig. 1; the curve is drawn through points from the forward titrations and points from the back titrations are included to show the reversibility of the titration. The reversibility indicates that there were no irreversible structural changes, usually associated with denaturation, occurring between pH2.2 and pH11. Since there was no dependence of the titration on time, any changes of conformation that do occur must be instantaneous.

To calculate the binding of cations to the carboxyl groups of BSP (Williams & Peacocke, 1967) it is necessary to know the number of such groups involved in the titration. Since the sialic acid carboxyl groups have a much lower p*K* than do the carboxyl groups of aspartic acid and glutamic acid, it was not possible to titrate BSP to completion by pH2.2, and the number of carboxyl groups available for titration was estimated by comparison of the experimental titration curve with a theoretical titration curve constructed by reference to the titration curves of three model systems. The systems were solutions in 0.2M-sodium chloride of

Fig. 1. Titration curve of BSP in 0.2M-NaCl at 25°. O, Forward titrations; ●, back titrations. $\bar{Z}_H$ is the mean number of H⁺ ions dissociated/mol. from the acid end point of the titration.

poly-L-glutamic acid, poly-L-aspartic acid and *N*-acetylneuraminic acid, which were considered to be the best models for the glutamyl, aspartyl and sialic acid residues respectively in BSP. The titration curves of these model systems between pH 10 and pH 2.2 were combined in the proportions that the respective residues are present in BSP as determined by analysis (Andrews & Herring, 1965), and the resulting curve is compared with the experimental curve (Fig. 2). To facilitate comparison the ordinates of both curves were adjusted to coincide at pH 7.0, where the titration of carboxyl groups was

considered to start. The difference between the theoretical and the experimental curves indicates that 2.8×10^{-4} equiv./g. of BSP, or 7.0 carboxyl groups/mol. of mol.wt. 25 000, with *pK* about 5.3, do not participate in the titration. If it is assumed that 7.0 aspartic acid or glutamic acid residues are not titrating because they are in the amide form, the present result accords with those of Andrews (1966), who showed that there were not more than 10 amide groups/mol.

Further evidence in favour of this hypothesis comes from the titration curve of BSP from which the sialic acid has been hydrolysed (see Fig. 5 in Williams & Peacocke, 1967). Below pH 7.0, 21.0×10^{-4} g.equiv. of H^+ are bound/g. of de-sialated BSP. If the acid hydrolysis had removed only the sialic acid residues, leaving those aspartyl and glutamyl residues that titrate in native BSP (see Table 1) as the only other carboxyl groups, then 15.6×10^{-4} g.equiv. of H^+ should be bound/g. of de-sialated BSP. However, if there are 7.0 amide groups/mol. these would at least be partially hydrolysed under the acid conditions to free carboxyl groups. If this hydrolysis were complete then 1 g. of de-sialated material should bind 19.1×10^{-4} g.equiv. of H^+ to carboxyl groups, which is in much better agreement with the experimental value.

In determining the titration curve of *N*-acetylneuraminic acid, it was possible to titrate only 70% of the available carboxyl groups above pH 2.2 because of their low *pK* in this molecule. In constructing the theoretical titration curve down to pH 2.2, the sialic acid groups are therefore assumed to titrate only to this extent. Below pH 4 the experimental and theoretical curves are parallel (Fig. 2); this validates the assumption that all of the sialic acid groups in the molecule, as determined by analysis, are available for titration. The only discrepancy in the number of carboxyl groups available for titration and those present as determined by analysis appears therefore to be in the amino acid side-chain carboxyl groups. Hence,

Fig. 2. Comparison of the experimental titration curve of BSP in 0.2*M*-NaCl at 25° (----) with the theoretical titration curve (—). The construction of the theoretical curve is described in the text. The difference curve (○—○) indicates that 7.0 carboxyl groups/mol. with *pK* 5.3 do not combine with H^+ .

Table 1. Comparison of number of titratable groups with analytical results for BSP

Analytical results are taken from Andrews & Herring (1965). The content of titratable sialic acid carboxyl groups was estimated as described in the text.

pH range	Nature of groups titrating	Titration results		Analytical results (moles/25 000 g. of BSP)
		(g.equiv. of H^+ bound/g. of BSP)	(g.equiv. of H^+ bound/25 000 g. of BSP)	
<2.0– 7.0	Carboxyl	19.1×10^{-4}	16.6 sialic	16.6 sialic
			31.2 Asp + Glu	21.7 Glu
7.0– 9.0	Imidazole and α -amino	1.1×10^{-4}	2.8	16.5 Asp
9.0–11.0	Phenolic hydroxyl and ϵ -amino	1.4×10^{-4}	3.5	1.2 His
				2.2 Tyr
				2.7 Lys

when the total content of carboxyl groups in BSP was required for the study of cation binding, it was calculated as the sum of the sialic acid, glutamyl and aspartyl carboxyl groups as determined analytically by Andrews & Herring (1965), from which were subtracted the 7.0 groups/mol. that did not titrate and that were probably present as amides. On this basis the total content of carboxyl groups is 19.1×10^{-4} equiv./g. of BSP, or 47.8 groups/mol. On the assumption that the carboxyl groups start to titrate at pH 7.0, the position of maximum binding of H^+ was estimated, and the scale of $\bar{Z}_H$ in Fig. 1 was constructed from this point.

The number of H^+ ions bound/mol. between pH 7.0 and pH 9.0 may be taken as a measure of the number of imidazole and α -amino groups, and the number bound between pH 9.0 and pH 11.0 as the combined number of ϵ -amino and phenolic groups, and the results can be compared (Table 1) with the analysis of Andrews & Herring (1965). On this basis, the number of imidazole and α -amino groups that titrate agrees poorly with the analytical results, although the agreement would be better if Andrews (1966) had been able to detect a free α -amino group. On the same basis, one or two ϵ -amino or tyrosine groups would appear not to titrate, possibly because the titration was not taken to a sufficiently high pH. More probably, pH 9.0 is too arbitrary a point at which to divide the two sets of titratable non-carboxylic groups: in fact, these non-carboxylic groups total 6.3/mol. from the titration, which agrees well with the value of 6.1 from analysis (histidine + tyrosine + lysine).

For the titration of the carboxyl groups a derivative plot of $[pH - \log \alpha/(1 - \alpha)]$ against α (the

degree of dissociation of the carboxyl groups) and $\bar{Z}_H$ was made (Fig. 3). The linearity of the plot is in good agreement with the equation of Linderström-Lang (1924), namely:

$$pK_0 = pH - \log \frac{\alpha}{(1 - \alpha)} + 0.868w\bar{Z} \quad (1)$$

and suggests strongly that no major conformational change takes place in the BSP molecule over the range pH 2.2–7.0 since such changes normally result in a non-linear plot, as in the swelling of bovine serum albumin at low pH (Tanford, Swanson & Shore, 1955; Tanford, Buzzell, Rands & Swanson, 1955) or the helix-coil transition of poly-L-glutamic acid (Wada, 1960). From the slope of the least-mean-squares line (Fig. 3), w was determined as 0.048 and was also calculated by the theory of Linderström-Lang (1924) from:

$$w = \frac{e^2}{2DrkT} \left[1 - \frac{\kappa r}{(1 + \kappa a)} \right] \quad (2)$$

where κ is $(8\pi Ne^2 I / 10^3 D k T)^{1/2}$, e is the charge (e.s.u.) on the electron, N is Avogadro's number, k is Boltzmann's constant, D is the dielectric constant of water at 25°, T is 298° K, r is the radius of the BSP molecule (20.3 Å), a is the radius of nearest approach of an ion to BSP (22.8 Å) and I is the ionic strength of the solution (0.2). The radius of the BSP molecule (r) was calculated as described by Tanford (1962), on the assumption that it was spherical, that 0.2 g. of water is bound/g. of protein and that the partial specific volume of BSP is 0.64 ml./g. (Williams & Peacocke, 1965). The radius, a , of nearest approach of an ion to a macromolecule is taken (Tanford, 1962) as $r + 2.5$ Å. The value of w calculated from these equations is 0.056, in good agreement with the experimental value of 0.048. This agreement suggests that the assumptions made in the derivation of eqns. (1) and (2), namely that the charge is spherically distributed, is approximately valid for the glycoprotein molecule, and excludes the possibility that the carboxyl groups are in a linear array, which would lead to the different relations for flexible polyacids; presumably the chain is coiled up on itself so that the dissociating groups reside on the outer surface of a molecule of globular form. This would not conflict with the earlier physicochemical evidence on the shape of the molecule (Williams & Peacocke, 1965). The foregoing analysis of the number and nature of the groups in BSP dissociating H^+ ions has been applied to the study of its binding of metal ions as described in the following paper (Williams & Peacocke, 1967).

The authors acknowledge a Medical Research Council training award to P. A. W.

Fig. 3. Plot of $[pH - \log \alpha/(1 - \alpha)]$ versus α and $\bar{Z}_H$ for the carboxyl groups of BSP in 0.2 M-NaCl at 25°. The line was calculated by the method of least mean squares.

REFERENCES

- Andrews, A. T. de B. (1966). D. Phil. Thesis: University of Oxford.
- Andrews, A. T. de B. & Herring, G. M. (1965). *Biochim. biophys. Acta*, **101**, 239.
- Andrews, A. T. de B., Herring, G. M. & Kent, P. W. (1967). *Biochem. J.* **104**, 705.
- Eastoe, J. E. & Eastoe, B. (1954). *Biochem. J.* **57**, 453.
- Herring, G. M. (1964). *Proc. 1st Europ. Bone & Tooth Symp., Oxford*, p. 263. Oxford: Pergamon Press Ltd.
- Herring, G. M. & Kent, P. W. (1961). *Biochem. J.* **81**, 5P.
- Herring, G. M. & Kent, P. W. (1963). *Biochem. J.* **89**, 405.
- Linderstrøm-Lang, K. (1924). *C. R. Lab. Carlsberg, Sér. chim.*, **15**, no. 7.
- Peacocke, A. R. & Williams, P. A. (1966). *Nature, Lond.*, **211**, 1140.
- Tanford, C. (1962). *Advanc. Protein Chem.* **17**, 69.
- Tanford, C., Buzzell, J. G., Rands, D. G. & Swanson, S. A. (1955). *J. Amer. chem. Soc.* **77**, 6421.
- Tanford, C., Swanson, A. & Shore, W. S. (1955). *J. Amer. chem. Soc.* **77**, 6414.
- Wada, A. (1960). *Molec. Phys.* **3**, 409.
- Warren, L. (1959). *J. biol. Chem.* **234**, 1971.
- Werner, I. & Odin, L. (1952). *Acta Soc. med. upsalien.* **57**, 230.
- Williams, P. A. & Peacocke, A. R. (1965). *Biochim. biophys. Acta*, **33**, 294.
- Williams, P. A. & Peacocke, A. R. (1967). *Biochem. J.* **105**, 1177.