- Borst, P. & Slater, E. C. (1960). *Biochim. biophys. Acta*, **41**, 170.
- Buchanan, J. M. & Anfinsen, C. B. (1949). *J. biol. Chem.* **180**, 47.
- Bücher, T., Klingenberg, M. & Zebe, E. C. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, vol. 12, p. 153. London: Pergamon Press Ltd.
- Bursell, E. (1963). *J. Insect Physiol.* **9**, 439.
- Chance, B. & Connelly, C. M. (1957). *Nature, Lond.*, **179**, 1235.
- Chance, B. & Hagihara, B. (1961). *Symp. Intracellular Respiration: Phosphorylating and Non-phosphorylating Oxidation Reactions; Proc. 5th int. Congr. Biochem., Moscow*, vol. 5, p. 19. London: Pergamon Press Ltd.
- Chance, B. & Williams, G. R. (1954). *Fed. Proc.* **13**, 190.
- Chance, B. & Williams, G. R. (1955). *J. biol. Chem.* **217**, 383.
- Chappell, J. B. & Greville, G. D. (1961). *Nature, Lond.*, **190**, 502.
- Charles, R., Tager, J. M. & Slater, E. C. (1963). *Biochim. biophys. Acta*, **74**, 33.
- Cleland, K. W. & Slater, E. C. (1953). *Biochem. J.* **53**, 547.
- Darrow, R. A. & Colowick, S. P. (1961). In *Methods in Enzymology*, vol. 5, p. 226. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Fukuda, T. (1957). *Nature, Lond.*, **180**, 245.
- Gale, E. F. (1945). *Biochem. J.* **39**, 46.
- Graffin, A. L. & Ochoa, S. (1950). *Biochim. biophys. Acta*, **4**, 205.
- Gregg, C. T., Heisler, C. R. & Remmert, L. F. (1960). *Biochim. biophys. Acta*, **45**, 561.
- Gregg, C. T., Johnson, J. R., Heisler, C. R. & Remmert, L. F. (1964). *Biochim. biophys. Acta*, **82**, 343.
- Huiling, F. & Slater, E. C. (1961). *J. Biochem., Tokyo*, **49**, 493.
- Hunter, F. E. (1949). *J. biol. Chem.* **177**, 361.
- Hunter, F. E. (1951). In *Symposium on Phosphorus Metabolism*, vol. 1, p. 297. Ed. by McElroy, E. D. & Glass, B. Baltimore: The Johns Hopkins Press.
- Jones, E. A. & Gutfreund, H. (1961). *Biochem. J.* **79**, 608.
- Judah, J. D. (1951). *Biochem. J.* **49**, 271.
- Kilby, B. A. & Neville, E. (1957). *J. exp. Biol.* **34**, 276.
- Krebs, H. A. & Bellamy, D. (1960). *Biochem. J.* **75**, 523.
- Lardy, H. A., Johnson, D. & McMurray, W. C. (1958). *Arch. Biochem. Biophys.* **78**, 587.
- Levenbook, L. & Hollis, V. W. (1961). *J. Insect Physiol.* **6**, 52.
- Miller, A. (1956). In *Encyclopedia of Plant Physiology*, vol. 2, p. 573. Ed. by Ruhland, W. Berlin: Springer-Verlag.
- Ochoa, S. (1943). *J. biol. Chem.* **151**, 493.
- Ochoa, S. (1948). *J. biol. Chem.* **174**, 133.
- Ochoa, S., Mehler, A. H. & Kornberg, A. (1948). *J. biol. Chem.* **174**, 979.
- Price, G. M. (1961). *Biochem. J.* **80**, 420.
- Price, G. M. & Moriya, S. (1962). *Biochem. J.* **84**, 98P.
- Pucher, G. W., Abrahams, M. D. & Vickery, H. B. (1948). *J. biol. Chem.* **172**, 579.
- Remmert, L. R., Heisler, C. R. & Gregg, C. T. (1961). *Fed. Proc.* **20**, 50.
- Sacktor, B. & Cochran, D. G. (1958). *Arch. Biochem. Biophys.* **74**, 266.
- Siu, P. M. L. & Wood, H. G. (1962). *J. biol. Chem.* **237**, 3044.
- Slater, E. C. (1953). *Biochem. J.* **53**, 157.
- Slater, E. C. & Holton, F. A. (1953). *Biochem. J.* **55**, 530.
- Utter, M. F. & Keech, D. B. (1963). *J. biol. Chem.* **238**, 2603.
- Utter, M. F. & Kurahashi, K. (1954). *J. biol. Chem.* **207**, 787.
- Van den Bergh, S. G. (1962). *Respiration and Energy Production in the Flight Muscle of the Housefly Musca domestica L.* Amsterdam: Rototype.
- Van den Bergh, S. G. & Slater, E. C. (1962). *Biochem. J.* **82**, 362.
- Winteringham, F. P. W. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, vol. 12, p. 201. London: Pergamon Press Ltd.

Biochem. J. (1964), **93**, 136

The Use of a Gel-Diffusion Technique for the Determination of Metal Ion-Protein Association Constants, and its Application to the Zinc Ion-Collagen System

BY B. G. NEWSOM* AND I. G. F. GILBERT

Physics Department, Institute of Cancer Research, Royal Cancer Hospital, London, S.W. 3

(Received 16 January 1964)

The precise measurements needed for the determination of metal ion-protein association constants have usually been carried out in dilute aqueous solutions, and it is often not clear if the

* Present address: Division of Protein Chemistry, C.S.I.R.O., Victoria, Australia.

results can be applied to solid or gel-like protein systems. Bone, cartilage and arterial walls are obvious examples where metal ion deposition is both important and possibly more complex than would be shown by a study of the proteins in free solution. We have therefore attempted to deter-

mine association constants in gel systems, and in the present paper describe a technique based on radiotracer-diffusion measurements.

The possibility of investigating metal ion-protein interactions by measurements of the diffusion of metal ions in protein gels has been suggested by a number of theoretical studies of diffusion accompanied by reaction with the stationary phase, especially those of Hill (1928) and Hermans (1947). However, although a great number of gel-diffusion measurements have been carried out (reviewed by Newsom, 1963), the derivation of ion-binding data from such measurements appears not yet to have been attempted. For self-diffusion in a protein gel of a reversibly formed metal ion complex Fick's second law of diffusion takes the form (Crank, 1956):

$$\frac{\partial C}{\partial t} = \frac{D_g^0}{R+1} \cdot \frac{\partial^2 C}{\partial x^2} \quad (1)$$

where C is the total metal ion concentration, D_g^0 is the diffusion coefficient of the metal ion in the gel in the absence of binding, and R is the ratio of bound metal ion concentration to free metal ion concentration. The observed diffusion coefficient is thus given by:

$$D_g = D_g^0/(R+1). \quad (2)$$

Therefore at any given metal ion concentration R can be derived from the observed D_g , if D_g^0 can be otherwise obtained.

In the approach described below the self-diffusion coefficient of the metal ion in free solution of the same composition as that used to prepare the gel, D_0 , has been determined, and by working in sufficiently dilute gels it has been possible to derive the relationship between D_g^0 and D_0 both theoretically and also experimentally from measurements with suitable non-binding ions. The requirement that the gel should be dilute restricts the technique to the more readily gel-forming proteins, and collagen has therefore been chosen as a suitable protein for investigation by this method. Zinc ions, for which measurements are reported below, has proved to be reversibly bound by the protein, but the technique has the advantage that it may also be applied to obtain quantitative data for irreversible or relatively slow metal ion-protein interactions, if the appropriate diffusion equations (Crank, 1956) are used.

EXPERIMENTAL

Preparation of collagen. Collagen was obtained from rat skin by the isoionic heat-solubilization technique of Veis & Cohen (1954). This material forms rigid gels at low concentrations and has undergone only minimal and reversible degradation (Veis & Cohen, 1960). The preparation was

finally freeze-dried, and had a residual water content (determined by drying to constant weight at 108°) of 19.6%. In terms of dry weight the hydroxyproline content was $12.4 \pm 0.3\%$ (determined by the method of Neuman & Logan, 1950), and the tyrosine content 0.76% (determined by the method of Beavan & Holiday, 1952). These values are in good agreement with those given by Gross & Piez (1960) for rat-skin collagen, namely 12.4% of hydroxyproline and 0.65% of tyrosine. The ultraviolet absorption in acid solution was approximately constant between 250 and 280 m μ , and was similar to that described by Loofbourow, Gould & Sizer (1949). The same batch of material was used in all the work with $^{65}\text{Zn}^{2+}$ ions.

Preparation of gels. Weighed amounts of the freeze-dried collagen preparation (making a correction for the residual moisture content) were dissolved in 0.15 M-sodium acetate buffer, pH 6.5, with or without added ZnCl_2 , by heating to 40°, and poured into polythene tubes (0.6 cm. internal diam. $\times$ 3.5 cm. long), where the gel was allowed to set at 20°.

Radioisotopes. Solutions of $^{65}\text{ZnCl}_2$ (specific radioactivity 1 c/g. of Zn), $^{22}\text{NaCl}$ (specific radioactivity 1 c/g. of Na) and $^{137}\text{CsCl}$ (carrier free) were obtained from The Radiochemical Centre, Amersham, Bucks. All samples were assayed for γ -activity in 2½ in. or 5 in. NaI well-type scintillation counters. About 0.05 μC of isotope was used for each diffusion run.

Diffusion measurements (a) Gel diffusion. The method of Jakubovic, Hills & Kitchener (1958) was used. A 2 μl . droplet of buffer containing radiotracer was applied to the freshly sliced open end of a tube of gel, which was then placed upright in a closed vessel, containing buffer solution to maintain the humidity, in a thermostat at 20.0°. Diffusion was allowed to proceed for 3½–16 hr., after which the tubes were cut into 1 mm. sections with a hand microtome. The sections were weighed and small corrections made for variability in section size, and assayed for radioactivity. For these diffusion conditions the appropriate solution of the diffusion equation is (Crank, 1956, p. 11):

$$C = \frac{M}{(\pi D_g t)^{1/2}} \cdot \exp(-x^2/4D_g t) \quad (3)$$

where C is the concentration of diffusing material at a perpendicular distance x from the surface on which an amount M of diffusing substance was initially placed, and t is the time. For each of the experiments, which were carried out at least in duplicate, $\log C$ (expressed in terms of counts/sec./mg.) was plotted against x^2 and the diffusion coefficient derived from the slope which equals $-1/9.212 D_g t$. A series of such plots for the diffusion of $^{65}\text{Zn}^{2+}$ ions in the collagen gel is given in Fig. 1.

(b) Diffusion in free solution. Measurements of self-diffusion coefficients were made, in quadruplicate, by the capillary-tube method (Wang, 1952), with streamline stirring of the outer solution as recommended by Mills (1955). A closed glass tube (diam. 2.5 cm.) rotating at about 35 rev./min. was used as the stirrer, and the condition of streamline flow was established by observing the passage of dye solutions and of oil droplets over the tops of the capillary tubes with a travelling microscope.

Ultrafiltration. Measurements were carried out by the method of Wilkinson (1960), in which the pressure of a mercury column forces solution through the dialysis membrane. The collagen was necessarily studied below the

gel point, as a 0.4% solution, in acetate buffer of the same strength and pH as used in the gel-diffusion experiments.

Six successive samples of ultrafiltrate were collected, each corresponding to about 2% of the total radioactivity, and the fraction ultrafiltered calculated from the average of the last three samples by which time the ultrafiltrate had attained a constant specific radioactivity. This was carried out both in the presence and absence of collagen, thus automatically correcting for $^{65}\text{Zn}^{2+}$ ions taken up by the membrane. A small correction, about 0.4%, was made for the free volume fraction in the case of the collagen solution.

RESULTS AND CALCULATIONS

The self-diffusion coefficients of ^{65}Zn tracer in collagen gels containing 38.7 g. of collagen/l. and a range of concentrations of added zinc chloride are plotted as a function of total Zn^{2+} ion concentration in Fig. 2. Each point represents the mean of two separate determinations; the agreement between each pair varied from within 4% to better than 1%, with a mean difference of about 2%. Plots of $\log C$ against x^2 , from which the diffusion coefficients were calculated, are given, for one series of experiments, in Fig. 1.

The results of the free solution diffusion measurements are given in Table 1, for $^{65}\text{Zn}^{2+}$ ions in acetate buffer at two total Zn^{2+} ion concentrations. The influence of Zn^{2+} ion concentration on the diffusion coefficient within the experimental range is small, and interpolated values were used at the intermediate concentrations.

Self-diffusion coefficients of Na^+ and Cs^+ ions in free solution and in gels of different collagen concentration are plotted in Fig. 3. Results for two batches of collagen are shown in the Figure. All the experiments with $^{65}\text{Zn}^{2+}$ ions were carried out with the same batch (numbered III in Fig. 3).

Derivation of the degree of Zn^{2+} ion-collagen association. The calculation of the ratio, R , of bound Zn^{2+} ions to freely diffusible Zn^{2+} ions within the gel requires the diffusion constant, D_g^0 , of Zn^{2+} ions within the gel in the absence of binding. For the present system, consisting of a dilute gel of a flexible linear protein, it has been possible to derive D_g^0 from diffusion experiments with alkali-metal ions, which are assumed to be bound only negligibly by the collagen at the experimental ionic strengths.

A number of workers (Friedman & Kraemer, 1930; Jacobsen, 1959) have written general equations for the diffusion of ions in gels of the form used by White & Dorion (1961):

$$D_g^0 = D_0 VF/h \quad (4)$$

where D_0 is the diffusion coefficient of the ion in free solution in the absence of gelling agent, V is a free-volume factor, F is a correction for the drag of the walls of the capillary pores, and h is a tortuosity

Fig. 1. Diffusion of tracer $^{65}\text{Zn}^{2+}$ ions in collagen gels containing different total Zn^{2+} ion concentrations. Details are given in the text. Each line corresponds to a single run, and for the series illustrated the times allowed for diffusion were about equal (6×10^4 sec.). The total Zn^{2+} ion concentration of each run increases upwards, the actual concentrations being: A, radiotracer; B, 1 mM; C, 2.5 mM; D, 8 mM; E, 10 mM; F, 20 mM; G, 25 mM.

Fig. 2. Diffusion coefficients of tracer $^{65}\text{Zn}^{2+}$ ions in collagen gels as a function of total Zn^{2+} ion concentration. Details are given in the text.

Table 1. Free diffusion coefficients of $^{65}\text{Zn}^{2+}$ ions in 0.15M-sodium acetate buffer, pH 6.5

Details are given in the text.		
Concn. of Zn^{2+} ions	$10^6 D$ (cm. ² /sec.)	No. of determinations
Radiotracer	6.16 ± 0.17 (S.D.)	4
25 mM	5.81 ± 0.05 (S.D.)	4

factor [or, in the early theory of Friedman & Kraemer (1930), a viscosity factor]. Other theoretical models have been put forward, but that of Mackie & Meares (1955) has been criticized (Lagos & Kitchener, 1960) and is not verified by the results of the present work, and that put forward by Lauffer (1961) as an extension of the theory of Wang (1954) for the self-diffusion of water in solutions of globular proteins does not seem to be applicable to gels of fibrous proteins and also is not supported by the present results.

The factors in the general gel-diffusion equation are conveniently examined individually. As in the treatment by White & Dorion (1961) the factor V represents the fractional area across the gel that is available for diffusion of solutes, and thus equals the free volume fraction, $(1-\phi)$, where ϕ is the volume fraction occupied by the gelling agent. This may easily be seen by considering the (infinitesimal) volume swept out by an arbitrary plane through the gel when moved parallel to itself by an infinitesimal distance.

The drag factor, F , is given by the Faxen (1922) correction:

$$F = 1 - 2.104r/a + 2.09(r/a)^3 - \dots \quad (5)$$

and replaces the earlier, incorrect, Ladenburg expression which was used by Friedman & Kraemer (1930). In this equation a is the radius of the capillary pores in the gel, and r is the radius of the diffusing ion. The gels used in the present work are

both dilute and composed of long rod-like but perhaps flexible molecules, and the calculation of Ogston (1958) may be used for the pore size, which gives $1/a$ proportional to $\phi^{\frac{1}{2}}$. Thus the gel-diffusion equation may be written:

$$D_g^0 = D_0(1-\phi) (1 - 2.1Ar\phi^{\frac{1}{2}} + \dots)/h \quad (6)$$

where A is a constant of proportionality. In the present case we are concerned with gels made from highly purified polymers and there is no reason to include a viscosity factor in h . With h as a pure tortuosity factor, White & Dorion (1961) use the value $h = 1$, and this has been verified experimentally in the present system from the diffusion results with $^{22}\text{Na}^+$ and $^{137}\text{Cs}^+$ ions. By using the ionic radii of Nightingale (1959) theoretical curves have been drawn through the experimental data in Fig. 3, for an empirical value of A (which is nearly the same for both batches of collagen) and with $h = 1$. The excellent fit of the theoretical curves, demonstrating both a constant value of A and the value of h as unity, verify the equation in its applicability to the collagen gels used in the present experiments. The final gel-diffusion equation is thus:

$$D_g^0 = D_0(1-\phi) (1 - 2.1Ar\phi^{\frac{1}{2}} + \dots) \quad (7)$$

Eqn. (4) has been verified for polyacrylamide gels by White & Dorion (1961), who determined a for a number of solutes from this equation and independently from permeability measurements and found good agreement between the pore radii given by the two methods.

It is of interest to consider the size of the errors that may arise if this equation were not used in the interpretation of the results with Zn^{2+} ions. In the first place, complete neglect of the effect of the gel on the diffusion coefficient in the present experiments would cause an error of 19% in D_g^0 , and the assumption that the effect of the gel on the diffusion coefficient of free Zn^{2+} ions is the same as that on, say, Na^+ ions, would cause an error of no more than about 1%. These relatively small differences should be compared with the effect of binding on the diffusion coefficient which at the lowest Zn^{2+} ion concentration studied causes a decrease in D_g of about 70%.

The calculation of D_g^0 requires values to be assigned to A and r . The value of A follows from the experiments mentioned above with Na^+ and Cs^+ as the diffusing ions (Fig. 3), with the same batch of collagen as in the experiments with Zn^{2+} ions, and was found to be $56/\text{\AA}$.

In the Zn^{2+} ion-diffusion experiments the diffusing ions are mostly $\text{Zn}^{2+}(\text{aq.})$ and $\text{ZnO}_2\text{C}\cdot\text{CH}_3^+$, with smaller amounts of the diacetate complex $\text{Zn}(\text{O}_2\text{C}\cdot\text{CH}_3)_2$. The radius of the diacetate is calculated from X-ray-crystallographic data (Van Niekerk, Schoening & Talbot, 1953) to be about

Fig. 3. Diffusion coefficients of tracer $^{22}\text{Na}^+$ and $^{137}\text{Cs}^+$ ions in collagen gels of different protein concentration. The lines drawn are given by eqn. (7). Details are given in the text.

4.13 Å, and the hydrated Zn^{2+} ion has a radius of 4.30 Å (Nightingale, 1959). The monoacetate complex is presumably intermediate in size, and a value of 4.3 Å has been taken as the mean radius of the diffusing ionic species (in this range D_g^0 is little sensitive to variations in the diameter of the diffusing solute; a 10 % error in r would alter D_g^0 by only about 1.6 %). The values of D_g calculated in this way are plotted in Fig. 2 along with the observed gel-diffusion coefficient, D_g , as functions of total Zn^{2+} ion concentration.

From eqn. (2), the ratio of the bound Zn^{2+} ion concentration to the free Zn^{2+} ion concentration is given by:

$$R = D_g^0/D_g - 1 \quad (8)$$

and these ratios have been calculated from the data of Fig. 2 and Table 1, and are plotted in Fig. 4 as a function of the total bound Zn^{2+} ion concentration, m_t .

Derivation of association constants. The association of a metal ion and a protein may be described by the general equation given by Gurd & Wilcox (1956), which when only one species of bound metal ion is present in the system may be written in the form:

$$m_t = \sum_i m_i = \sum_i \frac{C_i k_{iM} \Gamma_M a_M}{1 + k_{iH} \Gamma_H a_H + k_{iM} \Gamma_M a_M} \quad (9)$$

where m_i is the concentration of metal ion bound at sites of the i th type, C_i is the total concentration of sites (free or occupied) of type i , k_{iH} and k_{iM} are the intrinsic association constants of the sites for H^+ and for metal ions respectively, a_M is the thermodynamic activity of the metal ions in solution and a_H the H^+ ion activity, and Γ_H and Γ_M represent the ratios of the activity coefficients of the protein without and with association with H^+ and with

metal ions respectively. Gurd & Wilcox (1956) also derive an expression which may be written:

$$\frac{m_i}{h_i} = \frac{k_{iM} \Gamma_M a_M}{k_{iH} \Gamma_H a_H} \quad (10)$$

in which h_i represents the concentration of H^+ ions bound to sites of the i th type, and hence deduce:

$$m_i = k_{iM} \Gamma_M a_M (C_i - m_i - h_i) \quad (11)$$

Hence:

$$R = \sum_i \frac{C_i k_{iM} \Gamma_M}{1 + k_{iH} \Gamma_H a_H + k_{iM} \Gamma_M a_M} \quad (12)$$

which may be written in the form:

$$R = \sum_i (k_{iM} \Gamma_M C_i - m_i k_{iM} \Gamma_M) / (1 + k_{iH} \Gamma_H a_H) \quad (13)$$

The factors Γ_H and Γ_M are interpreted in terms of electrostatic effects (Gurd & Wilcox, 1956). For collagen the average distance between binding sites, for example between the relatively abundant carboxyl side chains, is great enough for such electrostatic effects to fall off greatly with increasing ionic strength (Gilbert & Myers, 1960), and the simplifying assumption is made that electrostatic effects at the ionic strengths of the present investigation are negligible, i.e. $\Gamma_H = \Gamma_M = 1$. A similar conclusion was reached by Kenchington & Ward (1954) from potentiometric-titration experiments with normal and chemically modified gelatins at a range of ionic strengths. In any case the collagen is studied near the isoelectric point of undenatured collagen (Gilbert, 1960) and the proportion of amino acid residues associated with Zn^{2+} ions is very small. The binding equations thus take the simpler forms:

$$R = \sum_i C_i k_{iM} / (1 + k_{iH} a_H + k_{iM} a_M) \quad (14)$$

$$= \sum_i (k_{iM} C_i - m_i k_{iM}) / (1 + k_{iH} a_H) \quad (15)$$

If there is only one type of Zn^{2+} ion-binding sites these equations become:

$$R = C k_M / (1 + k_H a_H + k_M a_M) \quad (16)$$

$$= (k_M C - m k_M) / (1 + k_H a_H) \quad (17)$$

and a plot of R against m , both of which are known experimentally, should be linear, with intercept $k_M C / (1 + k_H a_H)$ and slope $-k_M / (1 + k_H a_H)$, from which C is readily determined. If k_H is known, high accuracy not always being necessary, k_M follows; otherwise a maximum value for k_M may be assigned. The data obtained, however, are sufficient in any case to describe the metal ion association at the experimental pH, and the constant

$$k_M / (1 + k_H a_H)$$

may be called the apparent association constant, k' , at the experimental pH.

Fig. 4. Ratio of bound Zn^{2+} to free Zn^{2+} ions in collagen gels of constant protein content, as a function of bound Zn^{2+} ions. Details are given in the text.

Table 2. *Association of Zn²⁺ ions and collagen: successive approximations*

Details are given in the text. 1 group/1000 amino acid residues is equivalent to $C = 0.422 \times 10^{-3}$ equiv./l.

$\log k_3$	$k_3 C_3$	$10^3 C_3$	$\log k_2$	$k_2 C_2$	$10^3 C_2$	$\log k_1$	$k_1 C_1$	$10^3 C_1$
	0	—	—	0	—	3.36	2.245	0.985
	0	—	1.98	0.64	6.67	3.57	1.605	0.429
	0	—	2.30	1.055	5.80	3.74	1.19	0.218
	0	—	2.19	1.055	6.80	3.74	1.19	0.218
$k_3 \rightarrow 0$	0.026	≥ 10	2.26	1.055	5.90	3.77	1.16	0.197
$k_3 \rightarrow 0$	0.080	$\geq 3; \leq \infty$	2.40	1.08	4.26	3.60	0.90	0.225
1.24 (mean)	0.145	8.3 (mean)	2.46	1.08	3.70	3.76	1.02	0.179
1.32 (mean)	0.18	8.6 (mean)	2.52	1.065	3.20	3.76	1.00	0.175
1.15 (mean)	0.18	13 (mean)	2.50	1.065	3.40	3.76	1.00	0.175

The plot (Fig. 4) of R against m_i is not linear, and shows at once that Zn²⁺ ions are bound to more than one type of site on the collagen molecule. A process of successive approximation has been used to obtain data for each type of binding site contributing to the experimental binding curve. The types of site were numbered in order of decreasing association constant. It is apparent that the first type of site makes its largest relative contribution to the observed degree of Zn²⁺ ion binding at the lower Zn²⁺ ion concentrations, that is as $m_i \rightarrow 0$. Hence, by using eqn. (17), a first approximation to $k_1/(1+k_{1H}a_H)$ and to C_1 was obtained from an asymptotic approximation to the curve of Fig. 4 near the R axis. The values obtained in this way are given in the first line of Table 2. From these values m was next calculated over the entire experimental range, and $\sum_2^n m_i$ was obtained by subtraction from the experimental values of m_i .

It was found that $\sum_2^n m_i/a_m$ gave an approximately

straight-line plot against $\sum_2^n m_i$; that is, n was taken

equal to 2, and values of $k_{2M}/(1+k_{2H}a_H)$ and C_2 were obtained as described above. These values are given in the second line of Table 2, and from them m_2 was calculated over the entire experimental range, subtracted from m_i , and the difference was treated as a second approximation to m_1 and plotted in the same way as above. The second approximations to $k_{1M}/(1+k_{1H}a_H)$ and C_1 are given in the second line of Table 2. This process was repeated until the values derived in five successive steps converged (Table 2), when it was necessary to introduce a third binding site, relatively most prominent at the higher Zn²⁺ ion concentrations, to represent the data fully. This necessitated changes in the constants for the first binding site, and the whole process was repeated, yielding the values given in the sixth line of Table 2. With further repetition of the process satisfactory convergence of the data was obtained by the ninth successive approximation, as shown in Table 2. It

Fig. 5. Final approximations to the linear contributions to Fig. 4, each corresponding to one of the three classes of binding site. Details are given in the text.

is a consequence of this method of calculation that the middle terms, which rely on all the experimental points, are more accurately given than the first and third terms in the calculation, of which greatest weight is given to the points at either end of the plot of R against m_i . The individual m_i of the final approximations are plotted in Fig. 5, against the corresponding m_i/a_m values, and the fit of the complete theoretical curve to the experimental results is shown in Fig. 4. The calculated constants are given in Table 3; these are mean values, and are slightly different from the 'best-fit' values of Table 2.

Influence of the buffering anion on the observed constants. The Zn²⁺ ions are to some extent in the form of complexes with the acetate of the buffer solution used, and from the data of Cannan & Kibrick (1938) the ratio:

$$[\text{Zn}^{2+}]/([\text{Zn}^{2+}] + [\text{ZnO}_2\text{C}\cdot\text{CH}_3^+]) = 0.38$$

(neglecting higher complexes). To convert the observed constants then to solutions free from buffering anions the $\log k_i$ values should all be slightly increased, but no correction has been made since the $\text{ZnO}_2\text{C}\cdot\text{CH}_3^+$ complex itself presumably

Table 3. *Apparent association constants of Zn^{2+} ions and collagen, and concentrations of binding sites*

The values apply at 20°, in 0.15M-sodium acetate buffer, pH 6.5. The concentrations, C_i , of binding sites are given in residues/1000 amino acid residues, which is equivalent to moles/91930 g. of collagen. For site 2 the values were obtained from an unweighted least-squares plot of the relevant final data shown in Fig. 5 and the errors are standard deviations. For site 1 the product $k'_1 C_1$ has an error similar to that of $k'_2 C_2$ but the individual values are less well defined and the errors given represent the extreme ranges of values derived graphically. The error given for k'_3 is also a graphical range.

Site (i)	$\log k'_i$	C_i
1	3.92 ± 0.40	0.40 ± 0.29
2	2.55 ± 0.05	7.26 ± 1.09
3	0.71 ± 0.22	70 (assumed)

has some affinity for the protein sites, and a correction using the above ratio (an increase of 0.4 in $\log k_i$) is likely to be too large.

Comparison of the results with those of ultrafiltration experiments. As a check of the gel-diffusion calculations the binding of Zn^{2+} ions by collagen was investigated by the completely independent technique of ultrafiltration. This technique requires the use of collagen solutions of much lower protein concentration than those used in the gel-diffusion experiments, and measurements were restricted to the lowest (radiotracer) $^{65}Zn^{2+}$ ion concentration, where the degree of binding is maximal, to permit sufficient accuracy.

For a final collagen concentration of 4.85 g./l., the ratio of total $^{65}Zn^{2+}$ to free $^{65}Zn^{2+}$ ions was calculated as described above to be 1.298, and hence $R = 0.298$. At this tracer $^{65}Zn^{2+}$ ion concentration it follows from eqn. (15) that R is proportional to the collagen concentration and hence is calculated from the gel-diffusion measurements to be 0.282 at the collagen concentration of the ultrafiltration experiments. The ultrafiltration technique thus gives results in nearly exact agreement with those obtained from the gel-diffusion measurements.

DISCUSSION

It is convenient to consider each class of Zn^{2+} ion-binding site separately.

Site 1. The concentration of this site is very low, and corresponds to about 0.4 site/1000 amino acid residues, or to an equiv.wt. of about 220 000. This is of a similar order of magnitude to the molecular weights of soluble collagen (345 000) and of heat-denatured collagen (138 000) (Boedtker & Doty, 1956), which suggests that the binding site may involve end groups of the collagen chains. In view

of the evidence that the 'parent gelatins' (Scatchard, Oncley, Williams & Brown, 1944) are multi-chain structures (Courts & Stainsby, 1958; Courts, 1959; Pouradier, 1958) it is possible that the binding may be due to chelation of Zn^{2+} ions between amino acid end groups of two or three cross-linked chains, which would be expected to provide an especially favourable configuration.

If this site were due to chelation between two or more carboxyl groups, an enhanced binding of other metal ions, in particular of Ca^{2+} ions, would be expected at low total metal ion concentrations. However, diffusion experiments with $^{47}Ca^{2+}$ ions of high specific radioactivity (Newsom, 1963) do not show a degree of binding greater than that of relatively high concentrations of Ca^{2+} ions by the free carboxyl groups of collagen (Gilbert, 1960), and it may be concluded that this first site is not predominantly carboxyl in nature. If the site is in fact an end grouping it would appear then to involve the N-terminal residues.

An alternative possibility is that this first site represents a sub-class of the second series of sites (discussed below), owing to one imidazole site on each chain being in a favourable position for chelate formation with an adjacent group. Chelation between carboxyl and imidazole groups of adjacent amino acid residues has been suggested to account for the enhanced binding of Zn^{2+} ions by serum albumin at low total Zn^{2+} ion concentrations (Rao & Lal, 1958*a, b*). However, this apparently requires the formation of an at least 11-membered chelate ring, which certainly in smaller molecules would seem to have little intrinsic stability (Martell & Calvin, 1952).

Site 2. This site, which corresponds to an equiv.-wt. of 12 600 or 7.3 equiv./1000 amino acid residues, has a Zn^{2+} ion-binding constant close to that expected for the imidazole side chains of the histidine residues. The apparent $\log k'$ (2.55) may be compared with a $\log k_1$ value of 2.58 for imidazole (Edsall, Felsenfeld, Goodman & Gurd, 1954) at a similar ionic strength and temperature, and of 2.82 at 0° for the binding of Zn^{2+} ions at the imidazole groups of serum albumin (Gurd & Goodman, 1952). The content of the second site appears to be somewhat higher than the histidine content of rat-skin collagen, namely 5.1 residues/1000 amino acid residues, found by Gross & Piez (1960). This may partly be due to computational error, but may indicate binding of Zn^{2+} ions to other amino acid side chains, present in lower concentrations, e.g. of methionine or hydroxyllysine.

Site 3. The third class of sites has a $\log k$ value similar to that for the association of Zn^{2+} ions with acetate, for which $\log k_1$ is 1.03 (Cannan & Kibrick, 1938), and may be tentatively identified with the carboxyl side chains of the aspartate and glutamate

residues. The concentration of these sites is less well defined than of the other two binding sites but is not incompatible with the free carboxyl content of 70 groups/1000 amino acid residues (calculated as aspartate + glutamate amide links) of rat-skin collagen given by the amino acid analyses of Gross & Piez (1960). Substitution of this value for C_3 leads to $\log k_3$ 0.71, a little lower than the acetate association constant, as expected owing to competition with the acetate buffer (discussed above). At the experimental pH the carboxyl groups are nearly all ionized and no correction need be made for competition between Zn^{2+} and H^+ ions.

SUMMARY

1. The self-diffusion coefficients of Na^+ , Cs^+ and Zn^{2+} ions over a range of concentrations have been measured in dilute collagen gels and in free solution, in the same conditions of pH and ionic strength.

2. The results with Na^+ and Cs^+ ions support a porous-network model of the collagen gel, and assuming that alkali-metal ions are scarcely bound by the protein in the experimental conditions it is possible to estimate the ratio of protein-bound to free Zn^{2+} ions from the diffusion measurements.

3. The results are in accordance with there being three classes of Zn^{2+} ion-binding site on the collagen molecule.

4. The strongest binding site has $\log k'$ 3.92, and corresponds to about 0.4 equiv./1000 amino acid residues.

5. The second class of binding sites has $\log k'$ 2.55 and corresponds to 7.3 equiv./1000 residues, and is mainly attributed to imidazole side chains.

6. The third class of sites is tentatively identified with free carboxyl groups.

7. The degree of Zn^{2+} ion binding at radiotracer concentrations given by the diffusion method has been confirmed by an ultrafiltration study.

We thank Professor W. V. Mayneord for his interest and encouragement, and Dr W. Anderson for helpful advice.

REFERENCES

- Beavan, G. H. & Holiday, E. R. (1952). *Advanc. Protein Chem.* **7**, 319.
- Boedtker, H. & Doty, P. (1956). *J. Amer. chem. Soc.* **78**, 4267.
- Cannan, R. K. & Kibrick, A. (1938). *J. Amer. chem. Soc.* **60**, 2314.
- Courts, A. (1959). *Biochem. J.* **73**, 596.
- Courts, A. & Stainsby, G. (1958). In *Recent Advances in Gelatin and Glue Research*, p. 100. Ed. by Stainsby, G. London: Pergamon Press Ltd.
- Crank, J. (1956). *The Mathematics of Diffusion*. Oxford: The Clarendon Press.
- Edsall, J. T., Felsenfeld, A., Goodman, D. S. & Gurd, F. R. N. (1954). *J. Amer. chem. Soc.* **76**, 3054.
- Faxen, H. (1922). *Ann. Phys., Lpz.*, **68**, 89.
- Friedman, L. & Kraemer, E. O. (1930). *J. Amer. chem. Soc.* **52**, 1295.
- Gilbert, I. G. F. (1960). *Biochim. biophys. Acta*, **40**, 156.
- Gilbert, I. G. F. & Myers, N. A. (1960). *Biochim. biophys. Acta*, **42**, 469.
- Gross, J. & Piez, K. A. (1960). In *Calcification in Biological Systems*, p. 395. Ed. by Sognnaes, R. F. Washington, D.C.: American Association for the Advancement of Science.
- Gurd, F. R. N. & Goodman, D. S. (1952). *J. Amer. chem. Soc.* **74**, 670.
- Gurd, F. R. N. & Wilcox, P. E. (1956). *Advanc. Protein Chem.* **11**, 311.
- Hermans, J. J. (1947). *J. Colloid Sci.* **2**, 387.
- Hill, A. V. (1928). *Proc. Roy. Soc. B*, **104**, 39.
- Jacobsen, H. (1959). Ph.D. Dissertation: Brooklyn Polytechnic Institute.
- Jakubovic, A. O., Hills, A. J. & Kitchener, J. A. (1958). *J. Chim. phys.* **55**, 263.
- Kennington, A. W. & Ward, A. G. (1954). *Biochem. J.* **58**, 202.
- Lagos, A. E. & Kitchener, J. A. (1960). *Trans. Faraday Soc.* **56**, 1245.
- Lauffer, M. A. (1961). *Biophys. J.* **1**, 205.
- Loofbourn, J. R., Gould, B. S. & Sizer, I. W. (1949). *Arch. Biochem.* **22**, 407.
- Mackie, J. S. & Meares, P. (1955). *Proc. Roy. Soc. A*, **232**, 498.
- Martell, A. E. & Calvin, M. (1952). *Chemistry of the Metal Chelate Compounds*, p. 139. New York: Prentice-Hall.
- Mills, R. (1955). *J. Amer. chem. Soc.* **77**, 6116.
- Neuman, R. E. & Logan, M. A. (1950). *J. biol. Chem.* **184**, 299.
- Newsom, B. G. (1963). Ph.D. Thesis: University of London.
- Nightingale, E. R. (1959). *J. phys. Chem.* **63**, 1381.
- Ogston, A. G. (1958). *Trans. Faraday Soc.* **54**, 1754.
- Pouradier, J. (1958). In *Recent Advances in Gelatin and Glue Research*, p. 265. Ed. by Stainsby, G. London: Pergamon Press Ltd.
- Rao, M. S. N. & Lal, H. (1958a). *J. Amer. chem. Soc.* **80**, 3222.
- Rao, M. S. N. & Lal, H. (1958b). *J. Amer. chem. Soc.* **80**, 3226.
- Scatchard, G., Oncley, J. L., Williams, J. W. & Brown, A. (1944). *J. Amer. chem. Soc.* **66**, 1980.
- Van Niekerk, J. N., Schoening, F. R. L. & Talbot, J. H. (1953). *Acta cryst., Camb.*, **6**, 720.
- Veis, A. & Cohen, J. (1954). *J. Amer. chem. Soc.* **76**, 2476.
- Veis, A. & Cohen, J. (1960). *Nature, Lond.*, **186**, 720.
- Wang, J. H. (1952). *J. Amer. chem. Soc.* **74**, 1182.
- Wang, J. H. (1954). *J. Amer. chem. Soc.* **76**, 4755.
- White, M. L. & Dorion, G. H. (1961). *J. Polym. Sci.* **55**, 731.
- Wilkinson, R. H. (1960). *J. clin. Path.* **13**, 268.