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MICROELECTROPHORETIC STUDIES OF SOLUBLE COLLAGEN

I. G. F. GILBERT

Physics Department, Institute of Cancer Research, Royal Cancer Hospital, London (Great Britain)

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SUMMARY

The electrophoretic mobility of soluble collagen of rat tail tendon, adsorbed on oil droplets, has been measured in a range of salt solutions between pH 1 and pH 12, maintained at constant ionic strength. Specific effects of the lower valent buffer ions used appear small and the corresponding mobilities lie on a smooth curve. These mobilities parallel the hydrogen ion binding expected from the amino acid composition, with a long isoelectric zone in the range pH 7.7 $\pm$ 0.8, in agreement with reported isoelectric points of native collagen.

Addition of the divalent cations: Pb^{++}, Cu^{++}, Ca^{++}, Mg^{++}, or of the anions: citrate, phosphate, pyrophosphate, oxalate, at constant ionic strength shifts the mobility, in order of decreasing effect, to more positive or negative values respectively, with consequent shortening and raising or lowering of the isoelectric zone. These specific effects are attributed to binding of these ions by the protein, and the quantitative interpretation is discussed.

INTRODUCTION

As part of a programme of investigation into the sites and modes of deposition of radioisotopes in bone, an electrophoretic study has been made of the interactions of collagen, in defined conditions, with a range of anions and cations. Difficulties of purification have necessitated the preparation of collagen from a source other than bone; for this purpose rat tail tendon has been used owing to its almost complete solubility in mildly acid solutions.

Electrophoretic mobility measurements of soluble collagen in free solution are considerably restricted in range by difficulties due to its extremely high viscosity and ready precipitation[1-3]. BROWN AND KELLY[1] confined their mobility determinations to the pH range 3.55–4.86; TOMLIN AND TURNER[2] to pH 3.2; and BENSUSAN AND HOYT[3] to a single pH of 8.3 in a stabilising buffer solution. On the other hand microscopic electrophoresis, in which soluble material is adsorbed on small particles, allows rapid measurements in extremely dilute solutions, with consequent minimisation of effects of viscosity and coagulation. Among the collagens this technique has only been applied previously to ground native hide collagen[4,5]. Its application in the present work to soluble collagen, allows the conditions of electrophoresis to be extended to include a wider range of pH and electrolyte solutions than is otherwise attainable for this material.

EXPERIMENTAL

The microelectrophoresis cell (Fig. 1) comprised a glass capillary U-tube, with ground and polished flats in the observation zone, mounted vertically, with silver-silver chloride electrodes sealed in ground glass cones, and held in corresponding sockets.

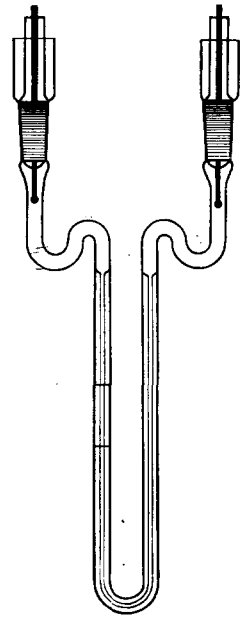

Fig. 1. Microelectrophoresis cell.

Diffusion of products of electrolysis from the electrode compartments was hindered by double bends between the electrodes and the capillary. Observation was through a horizontal microscope, with a × 20 objective and × 10 eyepiece with graticule, calibrated against a stage micrometer. Illumination was through the substage condenser, in contact with the capillary, and at as wide an aperture as possible to minimise the depth of focus. Particles were observed in the central plane of the capillary at a distance of 0.146 the bore diameter from the front, to eliminate the effect of electro-osmotic flow[6], using the previously calibrated fine adjustment of the microscope. The standard correction of HENRY[7] was applied. In measuring the bore diameter it was found advantageous to fill the capillary with a liquid (*e.g.* carbon tetrachloride) of a refractive index near that of the glass, rather than with water, to minimise errors due to optical distortion.

A power pack was used to provide a stable potential, usually of about 110 V. The field strength was calculated from the applied voltage and the resistance of the cell when containing solutions of known conductance; direct calculation from the dimensions of the cell gave a similar but less accurate result. Particle movements were timed to 0.1 sec, with current reversal between measurements, and the mean calculated from at least twenty observations. Most measurements were made between 19° and 21° and were corrected to 20° for viscosity changes by Stokes' Law. The viscosity of the suspensions and collagen solutions used was within 1 % of that of pure water.

The protein was adsorbed on paraffin oil droplets of 1–3 μ diameter, prepared in suspension by a steam-jet method similar to that of DOUGLAS[8] but using superheated steam. Mobilities were measured at surface saturation as shown by the values remaining constant on increasing the protein concentration.

Soluble collagen was prepared from rat tail tendon (of the "August" or "Marshall"-strain, 120–150 g weight), by dissolution in 0.01 *M* acetic acid, followed by purification by a method similar to that of NODA[9]. The solution was filtered through a No. 1 sinter, precipitated, firstly with 5 % NaCl solution, and secondly and thirdly by dialysis against repeated changes of 0.02 *M* NaH_2PO_4; and each time washed and redissolved. The final precipitate was washed 3 times with 0.1 *M* NaCl and with water and redissolved in 0.01 *M* acetic acid. Stock solutions were preserved at 4°, or quickly frozen at — 12°. This material on dialysis against phosphate buffer gave a flocculent mass of the characteristic needle-like crystals.

The final suspensions for electrophoretic measurement, were all of 0.10 ionic strength, and buffered with appropriate mixtures of NaCl, HCl, Na acetate, glycine, Tris (tris (hydroxymethyl) methylamine) and NaOH. The effects of higher valent ions were studied in some instances using their own buffer capacity and maintaining the ionic strength at 0.10 with NaCl. The pH of all solutions was determined immediately before measurement, and for the weaker buffer solutions after the measurements also.

RESULTS

Mobility and pH

The graph (Fig. 2) shows the mobilities of two batches of soluble collagen. Above pH 11.8 mobilities were irreproducible probably due to partial degradation. Both batches have almost identical mobilities except below pH 3, where there is a difference of about 6 %. Specific effects of the lower valent buffer ions used appear small; in

regions of overlap there is little difference in mobility, and a smooth curve, without breaks, may be drawn through all the results.

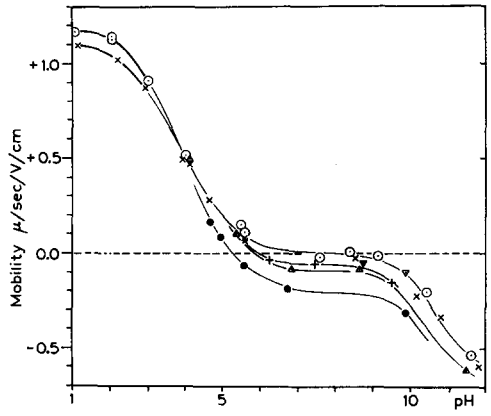

Fig. 2. Microelectrophoretic mobility of adsorbed soluble collagen: Ionic strength = 0.10, 2 batches (⊙) and (×), in the presence of 0.01 *M* phosphate △—△; citrate; (●); +—+ pyrophosphate; ▽—▽ oxalate.

The isoelectric zone is long and flat, between pH 7 and 8.5. The isoelectric point is consequently very sensitive to even small effects of added ions, and is best represented as pH 7.7 ± 0.8.

The influence of di- and tri-valent anions

Mobilities were also determined in solutions containing citrate, phosphate, pyrophosphate, and oxalate anions, all 0.01 *M*, with additional NaCl or appropriate monovalent buffer to bring the ionic strength to 0.10. The results are plotted in Fig. 2. Addition of these anions caused negative shifts in mobility of up to about $0.1 \cdot 10^{-4}$ cm^2 sec^{-1} V^{-1}; in order of decreasing effectiveness:

Citrate > phosphate > pyrophosphate > oxalate; oxalate having very little effect. Correspondingly the isoelectric point was shifted to considerably more acid values. In solutions of the same concentration the isoelectric points were: citrate, 5.3 ± 0.1; phosphate, 5.8 ± 0.2; pyrophosphate, about 5.9.

In the case of citrate, mobilities were determined at a range of concentrations at constant pH (6.7) and the same ionic strength (0.10), and are plotted in Fig. 3. The mobility increment is almost linear at lower citrate concentrations but decreases at higher values.

The influence of divalent cations

Similar determinations were made of the effect of added divalent metal ions (Mg^{++}, Ca^{++}, Cu^{++}, all 0.01 *M* and Pb^{++}, 0.005 *M*) on the mobility, in buffer solutions chosen to minimise complex formation with the other ions present. The only buffer used in the presence of Pb^{++} and Ca^{++} was HCl–NaCl, and hydrolysis restricted the experimental range to below pH 5. The alkaline buffers used for solutions of Mg^{++} and Ca^{++} were Tris and OH^-, in conditions of negligible complex formation or hydrolysis.

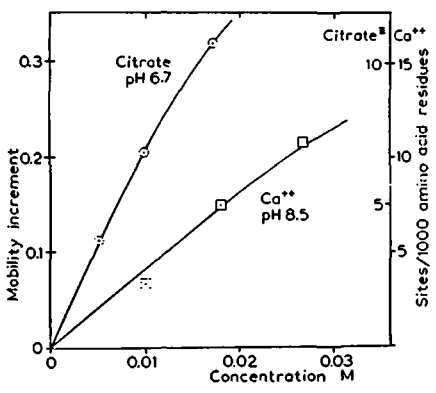

Fig. 3. Mobility increment (positive for Ca^{++}, negative for citrate) against concentration at constant pH and Ionic strength = 0.10.

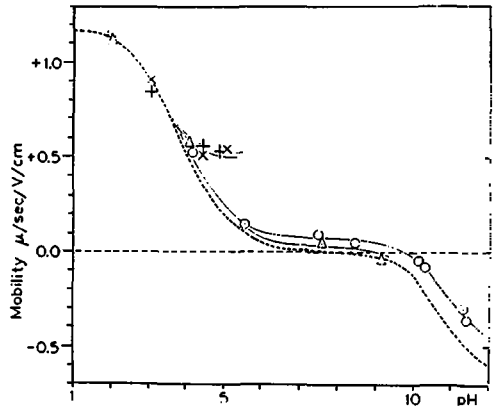

Fig. 4. ···, microelectrophoretic mobility of adsorbed soluble collagen: Ionic strength = 0.10; ⊙—⊙, in the presence of 0.01 *M* Ca^{++}; A···A, Mg^{++} 0.01 *M*; —·—, Cu^{++} 0.01 *M*; +---+, 0.005 *M* Pb^{++}.

The results are plotted in Fig. 4. Above pH 3, with the possible exception of Mg^{++}, the mobilities are all shifted to more positive values. The effects of Pb^{++} and Cu^{++} are sharp and pronounced, in spite of the relatively short pH range, whilst the effect of Ca^{++} appears to reach a plateau at pH 6–7 and then to increase only gradually. The isoelectric point is correspondingly shifted to higher values, and in the 0.01 *M* Ca^{++} solution used is at pH 9.7 ± 0.2. Results for a sequence of calcium concentrations at constant pH (8.5) and ionic strength are plotted in Fig. 3. The mobility increment is almost linear with Ca^{++} concentration up to about 0.03 *M*.

DISCUSSION

The similarity of the mobilities of free and adsorbed proteins is well established in many instances[10], and it is of interest to compare the present measurements as far as possible with those in free solution. Published mobilities of soluble collagen are at scattered pIIs, and large differences of temperature or of the buffer solutions used make precise comparison difficult[2,3,11] although no large discrepancies are apparent. The most extensive and also the most comparable determinations are those of BROWN AND KELLY[1] for rat skin soluble collagen, which presumably has an amino acid composition similar to rat tail tendon[13], in acetate buffers between pH 3.55 and 4.86. These results are at a lower temperature (10°) and ionic strength (0.05) than the present series, but parallel them. Moreover their single point at an ionic strength of 0.10, at pH 4.12, agrees with the present value when corrected for viscosity changes to the lower temperature ($0.35 \cdot 10^{-4}$ cm^2 sec^{-1} V^{-1}).

The isoelectric point of the present results (pH 7.7 ± 0.8) can be compared with those of insoluble collagens. Particular interest attaches to the agreement with values determined microelectrophoretically for untreated hide collagen of pH 7[4] and pII 7.8[5]. Similar values have in fact been found by a variety of methods: pH 7.0[16]; pH 7.6–7.8[15]; and pH 7.55–8.15[17]. A much closer relationship between soluble and insoluble collagens is therefore shown than indicated by the previous determination of

the isoelectric point of soluble collagen by BROWN AND KELLY, who extrapolated their mobility determinations linearly, to obtain an isoelectric point of pH 5.8. Neglect of the flattening-off of the mobility curve has thus caused an error of about 2 pH units.

In solutions of constant ionic strength and in the absence of other ionic interactions, the mobility of many adsorbed proteins is proportional to the net protein charge due to equilibria with hydrogen ions[10,11]. This accords with the simple Smoluchowski equation, which is applicable to non-conducting spheres the size of the oil droplets of the present study[19]. However, the work of BULL[11] suggests that owing to the possibility of preferred orientations on adsorption the proportionality constant may vary with the protein. For soluble collagen in the absence of the higher valent ions the pH-mobility curve (Fig. 2) parallels closely the net charge caused by titration of the amino acid residues present[13]. This parallelism is shown very clearly by comparison with the hydrogen ion binding curve at ionic strength 0.5 of hide collagen[18], which has a similar amino acid content. Differences of H^+ binding by collagen in 0.1 *M* and 0.5 *M* salt solutions are scarcely detectable[12], and the agreement with the present work is shown quantitatively by the apparent p*K* values. BOWES AND KENTEN[18] obtained a p*K* for the carboxyl groups, titrated from about pH 5.5 downwards, of 3.5, whilst that shown by the "half titration point" of the present results is 3.7. The same flat region between pH 5.5 and pH 9.5 is again indicated by both techniques. This agreement together with the lack of specific effects on the mobility of the lower valent buffer ions used, shows that these ions are bound only slightly, if at all, to the protein.

In contrast to the small effects of the lower valent ions, certain of the higher valent ions studied have considerable effects on the mobility. The specificity of these effects suggests that they are due to binding of the ions at sites on the protein. If the proportionality between charge and mobility still applies it is possible to calculate the number of ions of a given charge, required to be bound to cause the observed mobility shifts. The proportionality constant is conveniently derived from the mobility increment due to titration of the carboxyl groups, of which the amino acid and probable amide link content[13] show there to be 83 titratable groups for each thousand amino acid residues. Increasing displacement of the mobility curves thus corresponds to increasing degree of binding, in which order the anions are:

Oxalate < pyrophosphate < phosphate < citrate; and the cations:

$$Mg^{++} < Ca^{++} < Pb^{++} \approx Cu^{++}$$

For citrate and calcium ions the numerical results are plotted on the right hand ordinate of Fig. 3.

The decreasing binding of the divalent cations shown at lower pHs indicates that carboxyl or imidazole sites are involved. Binding of lead at imidazole sites is supported by work by other techniques with insoluble collagen[20] and gelatin[22]. Binding of calcium at carboxyl sites is supported by work on hide collagen[21], although the experimental conditions were not well defined, which showed a large amount of binding at high calcium concentrations, decreasing greatly below pH 3.5. If all carboxyl groups are accessible the results of Fig. 3 yield an intrinsic association constant, ($\log K_{Ca} = 0.57$), very close to that of an isolated carboxyl group in, for example, acetic acid[23].

The sites of anion binding presumably involve cationic amino or guanidinium groups, and the pH effects are probably caused by varying ionization of the small ion rather than of the protein.

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