[13] W. F. H. M. MOMMAERTS, *Arkiv Kemi Mineral Geol.*, 19A (1945) No 17.
[14] H. PORTZEHL, G. SCHRAMM AND H. H. WEBER, *Z. Naturforsch.*, 5b (1950) 61.
[15] R. G. PARRISH AND W. F. H. M. MOMMAERTS, *J. Biol. Chem.*, 209 (1954) 901.
[16] M. DUBUISSON, A. DISTECHE AND A. DEBOT, *Biochim. Biophys. Acta*, 6 (1950) 97.
[17] H. A. MCKENZIE, S. ROSE AND M. B. SMITH, unpublished.
[18] P. H. VON HIPPEL, H. K. SCHACHMAN, PEARL APPEL AND M. F. MORALES, *Biochim. Biophys. Acta*, 28 (1958) 504.
[19] W. F. H. M. MOMMAERTS AND B. B. ALDRICH, *Biochim. Biophys. Acta*, 28 (1958) 627.
[20] H. A. MCKENZIE AND R. G. WAKE, *Aust. J. Chem.* 12 (1959) 712.
[21] A. G. OGSTON AND J. M. A. TILLEY, *Biochem. J.*, 59 (1955) 644.
[22] A. G. OGSTON AND M. P. TOMBS, *Biochem. J.*, 66 (1957) 399.
[23] H. A. MCKENZIE AND M. B. SMITH, *Adelaide Meeting A.N.Z.A.A.S.*, August, 1958.
[24] T. C. TSAO, *Biochim. Biophys. Acta*, 11 (1953) 368.
[25] G. A. GILBERT, *Discussions Faraday Soc.*, 20 (1955) 68.
[26] M. DUBUISSON, *Arch. Intern. Physiol.*, 51 (1941) 133.
[27] E. MIHALYI, *Enzymologia*, 14 (1950) 224.

Biochim. Biophys. Acta, 40 (1960) 141–149

LIGHT SCATTERING IN SOLUTIONS OF NATIVE AND GUANIDINATED RABBIT TROPOMYOSIN

CYRIL M. KAY* AND KENNETH BAILEY
Department of Biochemistry, University of Cambridge, Cambridge (Great Britain)

(Received August 10th, 1959)

SUMMARY

1. The fundamental molecular weight of rabbit tropomyosin has been established by light scattering as 54,050 ± 1000 at pH 2 and 52,600 ± 1000 at pH 12.

2. Light scattering measurements have been carried out in the same solvent media as the earlier osmotic pressure work. The aggregation of tropomyosin particles as the ionic strength is diminished is clearly brought out by this technique, the molecular weight increasing from 52,900 at I = 1.1 to 101,000 at I = 0.1. These values are about 20 % lower than the figures deduced from osmotic pressure and are probably more reliable in view of the lower protein concentrations used.

3. Light scattering measurements have been extended to the ionic strength range below 0.1 where aggregation is most pronounced. The data suggest that up to the hexamer stage the polymerization process is end-to-end.

4. The preparation and characterization of a guanidinated derivative of rabbit tropomyosin by reaction with S-methylisothiourea are described.

INTRODUCTION

Previous investigations have shown that the particle weight of rabbit tropomyosin, owing to its reversible polymerization and depolymerization, is a sensitive function

* Present address: Department of Biochemistry, University of Alberta, Edmonton, Alberta (Canada).

of the ionic strength of the dispersing buffer. BAILEY, GUTFREUND AND OGSTON[1] estimated the molecular weight by sedimentation-diffusion and osmotic pressure measurements as 90,000 for a solution of pH 6.5 and ionic strength 0.27. TSAO, BAILEY AND ADAIR[2], using the osmotic pressure technique, found that only in strongly depolymerizing media such as 6.7 *M* urea and 0.01 *N* HCl, could the monomeric molecular weight of 53,000 be obtained. Measurements in salt solutions of ionic strengths from 0.1 to 1.1 at pH 6.5 showed that the average molecular weight varied from 135,000 to 65,000, diminishing with increasing salt concentration. DOTY AND SANDERS[3], in a light scattering study, duplicated the experimental conditions of TSAO *et al.* and found that at ionic strength 0.1 the particle weight was 100,000, decreasing to 55,000 at ionic strength 1.1. These values are about 20 % lower than the corresponding values derived from osmotic pressure and the difference is well outside the range of experimental error of the two methods. In order to clarify this apparent discrepancy it was felt desirable to repeat the light scattering work and to extend the measurements to the ionic strength range below 0.1 where aggregation is most pronounced. In this region of higher molecular weights, light scattering is vastly superior to osmotic pressure, since the intensity of scattering goes up with increasing molecular weight whereas osmotic pressure goes down.

The second aspect of this study is concerned with the preparation and properties of guanidinated rabbit tropomyosin. One of the major analytical differences between rabbit tropomyosin and *Pinna nobilis* tropomyosin is the replacement of the lysine residues of the former by arginine in the latter. It has been suggested that this amino acid difference may be a reflection of physico-chemical differences between the two proteins[4, 5], since rabbit tropomyosin is of the water-soluble variety and tends to aggregate in the absence of salt ions while *Pinna* is of the less soluble globulin type and shows no polymerizing tendency. In order to test this suggestion the lysyl residues of rabbit tropomyosin were converted to homoarginine ones by reaction with S-methylisothiourea. Some of the physico-chemical properties of the resulting guanidinated derivative are reported in this paper.

METHODS AND MATERIALS

Preparation of native protein samples

The isolation of rabbit tropomyosin followed the procedure of BAILEY[6], with isoelectric precipitation of the protein from the muscle extract at pH 4.6 to 4.8, and subsequent salting out in the 41 to 70 % $(NH_4)_2SO_4$ saturation range. Purification was achieved by reprecipitation of the protein and resalting out with $(NH_4)_2SO_4$. It was then stored at 0° as an $(NH_4)_2SO_4$ paste. Prior to use, the paste was dissolved in and dialyzed against water until free of $(NH_4)_2SO_4$, and then equilibrated against several changes of the outer liquid to be used in light scattering measurements. Protein concentrations were determined by either the micro-Kjeldahl method, taking 16.7 % as the nitrogen content of the protein[6] or interferometrically, using a value of 0.188 for its refractive increment (see below).

Guanidination of rabbit tropomyosin

A modified form of the procedure of ROCHE, MORGUE AND BARET[7] was used. A three-times crystallized preparation was dissolved and precipitated at pH 5 with

0.1 *M* acetate buffer, brought to pH 9 with *N* NaOH and cooled in ice. 10 ml (approx. 0.17 g protein) were then mixed with 0.7 g S-methylisothiourea (cyanamid). 1 ml of concentrated NH_3 was now added to bring the pH to 10. A control without addition of the urea derivative was treated similarly and both were left for 2 days at 4°. Each was then dialyzed against several changes of a solution containing 16 g $(NH_4)_2SO_4$ in 0.01 *M* acetate buffer pH 5.4. The control sample precipitated as a mixture of amorphous and crystalline material showing that the alkali treatment in itself had caused no extensive denaturation, while the treated portion was entirely amorphous.

Estimation of guanidino groups

The SAKAGUCHI method[8] as modified by CHERVENKA AND WILCOX[9] was applied after hydrolysis of the protein for 6 h with 6*N* HCl at 100°. Both with edestin and with untreated rabbit tropomyosin the method gave values rather higher than those obtained by the STEIN-MOORE method, *e.g.*, the SAKAGUCHI method gave values of 17.6 and 9.2 g arginine/100 g protein for edestin and tropomyosin as compared with the STEIN-MOORE values of 16.8 and 7.8 respectively. The number of lysine groups remaining unsubstituted could, however, be determined directly by preparing the DNP-protein, hydrolyzing and estimating the DNP-lysine after extraction into ether as the ε-DNP-α-methoxycarbonyl derivative[4].

Extent of guanidination

The percentage of guanidino groups calculated as arginine after correction for overestimation by the SAKAGUCHI method was 25.4, corresponding to 146 residues/10^5 g protein. In fully guanidinated tropomyosin, allowing for the increase of molecular weight by addition of amidine groups, there should be 105 homoarginine and 40 arginine residues. The SAKAGUCHI method thus indicates that guanidination was complete, but the direct estimation of lysine by the FDNB method revealed a lysine content of 1.9 % corresponding to 13 residues/10^5 g protein. Taken together, however, the two methods do suggest a high degree of substitution, *i.e.*, at least 132 out of a possible total of 145 residues/10^5 g protein contain guanidino groups.

Optical measurements

Some of the light scattering measurements at 90° at a wavelength of 546 mμ were carried out in an apparatus described by GORING AND JOHNSON[10]; others were performed with a Brice-Phoenix photometer, using an opal glass primary standard for the determination of absolute turbidities[11]. Solutions of tropomyosin which had been previously clarified by initial high speed centrifugation (20,000 × *g*) for 30 min were filtered either through collodion membranes or millipore filters of pore size 0.45 μ prior to the measurements. Similarly determined turbidities on the pure solvents were subtracted from the readings obtained with tropomyosin solutions. Scattering dissymmetries at either 60° and 120° or 45° and 135° were determined concurrently with the 90° intensities. Depolarization was found to be negligible; hence no depolarization correction was applied.

The refractive index increment, dn/dc, of both native and guanidinated rabbit tropomyosin was determined in a Zeiss interferometer using white light ($\lambda = 5600$ Å) to label the zero-order band. These measurements were corrected to 5461 Å using the dispersion equation of PERLMANN AND LONGSWORTH[12]. The value of dn/dc for both

proteins was indistinguishable being 0.188 at $\lambda = 5461$ A and 25°; this yielded a value of $3.95^{\circ}\ 10^{-6}$ for the Debye factor, H, in the light scattering equation.

Weight-average molecular weights were calculated by use of the well-known equation for small symmetrical scatterers[13]:

$$\frac{Hc}{\tau} = \frac{1}{M} + 2Bc \qquad (1)$$

A modified form of this equation was used in the case of those solutions exhibiting appreciable angular dissymmetries of scattering due to the phenomenon of intraparticle interference[14] viz:

$$\frac{Hc}{\tau_{corr.}} = \frac{1}{M} + 2Bc \qquad (2)$$

where $\tau_{corr.} = \tau \cdot P(90)^{-1}$ and P(90) is a particle scattering factor accounting for the decrease of scattering due to intraparticle interference and derivable from the dissymmetry ratio Z. Dissymmetry ratios were also used to evaluate molecular lengths in accordance with the graphic solutions given by DOTY AND STEINER[15] and JOHNSON[16].

RESULTS AND DISCUSSION

Molecular weight of the fundamental monomer

In order to establish the monomeric molecular weight by light scattering, experiments were carried out in two media known to favor depolymerization: acid at pH 2 and alkali at pH 12. The data, plotted in the conventional manner as the dependence of the turbidity function Hc/τ on c, are shown in Fig. 1. The observed dissymmetries of scattering were 1.04 and 1.05 in acid and alkali respectively. Since these values are well within experimental error no correction was applied to the observed turbidities. The extrapolated values of Hc/τ, $1.85 \cdot 10^{-5}$ at pH 2 and $1.92 \cdot 10^{-5}$ at pH 12, correspond to molecular weights of 54,050 ± 1000 and 52,600 ± 1000 respectively. These values are in good agreement with the molecular weight of 53,000 found by osmotic pressure for rabbit tropomyosin in 0.01 N HCl and in 6.7 M urea[2].

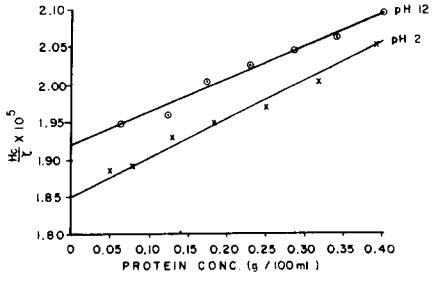

Fig. 1. A plot of Hc/τ as a function of concentration for rabbit tropomyosin: ×, in acid at pH 2; ⊙, in alkali at pH 12.

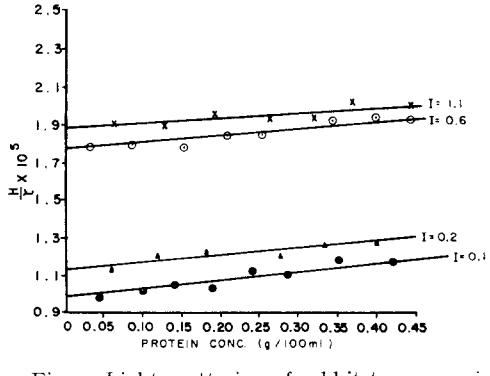

Fig. 2. Light scattering of rabbit tropomyosin in NaCl–phosphate buffer solutions. Solvents: (a) ●, 0.06 M phosphate buffer, pH 6.5, I = 0.1; (b) ▲, 0.06 M phosphate–0.1 M NaCl, pH 6.5, I = 0.2; (c) ⊙, 0.06 M phosphate–0.5 M NaCl, pH 6.5, I = 0.6; (d) ×, 0.06 M phosphate–1.0 M NaCl, pH 6.5, I = 1.1.

Aggregation of rabbit tropomyosin

The first group of experiments were carried out in the same solvent media as used in the osmotic pressure work. Accordingly, the protein was dialyzed against NaCl solutions of varying concentration, each containing 0.06 *M* phosphate buffer, pH 6.5. Representative experiments at ionic strengths 0.1, 0.2, 0.6 and 1.1 are shown in Fig. 2 and a summary of all the results is given in Table I.

Since the observed dissymmetries of scattering did not differ significantly from unity, the fundamental DEBYE equation was used directly in the calculation of molecular weights. The aggregation of tropomyosin particles as the ionic strength is diminished is clearly brought out by this technique, the molecular weight increasing from 52,900 to 100,500 over the ionic strength range covered. It is also to be noted that these values, which substantiate those reported by DOTY AND SANDERS, are about 20 % lower than the values obtained by osmotic pressure under comparable conditions. However, greater reliability should be placed on the light scattering measurements in view of their accessibility to a protein concentration range 10 times smaller than that attainable with osmotic pressure (0.05 % as compared with 0.5 %); hence with light scattering less uncertainty is associated with the extrapolated ordinate intercepts.

TABLE I

AGGREGATION OF RABBIT TROPOMYOSIN OVER THE IONIC STRENGTH RANGE 0.1 TO 1.1

Solvent system	*pH*	*Ionic strength*	*Scattering dissymmetry Z*	$\left(\frac{Hc}{\tau}\right)_{c \to 0}$	$M_{light\ scattering}$ *(This study)*	$M_{o.p.}$ (TSAO *et al.*)	$M_{light\ scattering}$ (DOTY AND SANDERS)
0.06*M* phosphate	6.5	0.1	1.06	0.99	101,000	135,000	100,000
0.06*M* phosphate–0.1 *M* NaCl	6.5	0.2	1.03	1.13	88,500	111,000	
0.06*M* phosphate–0.5 *M* NaCl	6.5	0.6	1.05	1.78	56,200	67,000	
0.06*M* phosphate–1.0 *M* NaCl	6.5	1.1	1.01	1.89	52,900	64,500	55,000

In order further to explore the aggregation phenomenon, scattering-concentration dependencies were determined for rabbit tropomyosin at ionic strengths 0.04, 0.02 and 0.01. All three solutions exhibited appreciable dissymmetry of scattering as is shown in Fig. 3. Since the dissymmetries showed no perceptible dependence on protein concentration they were averaged at the different concentrations. These average values were then used to calculate molecular lengths for tropomyosin, assuming different possible particle shapes, by means of the graphs given by DOTY AND STEINER[15]; the results are collected in Table II.

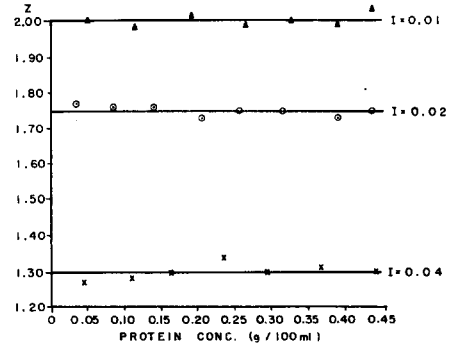

Fig. 3. A plot of the dissymmetry function *Z versus* protein concentration for rabbit tropomyosin in phosphate buffer of pH 6.5 and ionic strengths 0.01 (▲), 0.02 (⊙) and 0.04 (×).

TABLE II

MOLECULAR LENGTH OF RABBIT TROPOMYOSIN FOR DIFFERENT POSSIBLE PARTICLE SHAPES

Solvent system	$Z = \frac{I_{45}}{I_{135}}$	*Rod-shaped model (Length, Å)*	*Random monodispersed coil (Length, Å)*	*Polydispersed coil**
Phosphate buffer (pH 6.5, I = 0.04)	1.30	1305	898	877
Phosphate buffer (pH 6.5, I = 0.02)	1.75	2040	1408	1306
Phosphate buffer (pH 6.5, I = 0.01)	2.00	2448	1632	1510

* Root mean square of distance between ends of coil, Å.

Of the 3 possible particle shapes, the rod-model is the most probable for rabbit tropomyosin since recent optical rotatory dispersion measurements suggest that the greater part of its molecule is folded in the α-helical form[17]. Therefore in all further discussions, we shall consider the rabbit tropomyosin molecule as a rod-shaped entity.

Because of the appreciable angular dissymmetry of scattering shown by the tropomyosin solutions, it was necessary to multiply the turbidity values, as obtained by measuring the scattered light at 90°, by the reciprocal of the particle scattering factor, $P(90)^{-1}$. From the DOTY-STEINER graphs the factors for correcting the turbidity for rods of the above specified lengths are 1.27, 1.68 and 1.88 (corresponding to ionic strengths 0.04, 0.02 and 0.01 respectively). The corrected turbidity values were then used in equation (2) to evaluate weight-average molecular weights. The values of $Hc/\tau_{corr.}$ are plotted as a function of c in Fig. 4, where the best straight lines have been drawn by the method of least squares. The limiting ordinate intercepts and their corresponding molecular weights are listed in Table III.

Taking the molecular weight of the monomer as 53,000 and its length as 400 Å^2, the above data suggest that the aggregation process has extended to the hexamer level over the ionic strength range 1.1 to 0.01. Furthermore the aggregation appears to be end-to-end at least to the hexamer stage in view of the essential constancy of the ratio molecular weight/length (M/l). If the aggregation were side-by-side the ratio of M/l would have increased six-fold over this ionic strength range. Thus these data reaffirm an earlier conclusion[18] that the polymerization appears to be electrostatic in origin and essentially linear, at least in the early stages.

TABLE III

AGGREGATION OF RABBIT TROPOMYOSIN OVER THE IONIC STRENGTH RANGE 0.04 TO 0.01

Solvent system	$\frac{Hc}{\tau_{corr.}} \cdot 10^6$	M	*Length, l (Å)*	$\frac{M}{l}$
Phosphate–KCl buffer (pH 6.5, I = 1.1)		52,900	400	132
Phosphate buffer (pH 6.5, I = 0.04)	0.48	154,300	1305	118
Phosphate buffer (pH 6.5, I = 0.02)	3.83	261,100	2040	128
Phosphate buffer (pH 6.5, I = 0.01)	3.18	317,600	2448	129

Physico-chemical properties of guanidinated rabbit tropomyosin

The frequent observation that protein derivatives are less soluble than the parent protein[19] is borne out by guanidinated tropomyosin. The treated material was amorphous and soluble only in concentrated urea solution or in acid at pH 2; it was quite insoluble in neutral solution even with added salt. Of interest in this connection are

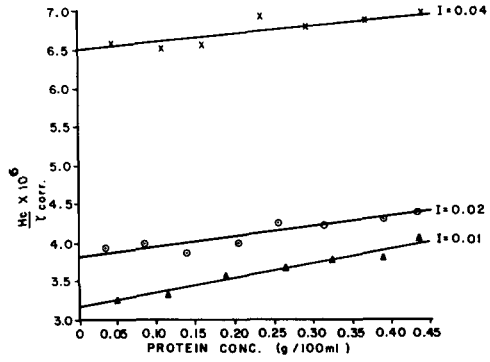

Fig. 4. $Hc/\tau_{corr.}$ *versus* c data for rabbit tropomyosin in phosphate buffer of pH 6.5 and ionic strengths 0.01 (▲), 0.02 (⊙) and 0.04 (×).

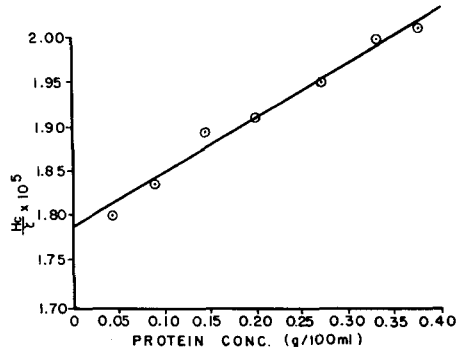

Fig. 5. A plot of Hc/τ *versus* c for guanidinated rabbit tropomyosin in acid at pH 2.

the findings of TAN AND TSAO[20] who, after treating rabbit tropomyosin with O-methylisourea, studied the effect of varying degrees of guanidination on solubility and polymerizability. The protein remained reasonably soluble in salt solutions when 20–40 % of the ε-amino groups were substituted, but with further substitution a marked decrease of solubility and salting out occurred between ionic strength 0.02 and 0.04.

Sedimentation measurements were carried out in a Spinco model E analytical ultracentrifuge. A single symmetrical peak indicated that the derivative was homogeneous at pH 2 and ionic strength of 0.10. The calculated $s_{20,w}$ of 3 S agrees very well with that of native rabbit tropomyosin.

The results of a scattering-concentration dependency study on the guanidinated protein in acid at pH 2 are summarized in Fig. 5. A molecular weight of 55,900 ± 1000 was calculated from the limiting ordinate intercept of $1.79 \cdot 10^{-5}$. This molecular weight is greater by about 1850 than that obtained for the native protein in the same solvent system (see Fig. 1) and corresponds to the addition of some 44 amidine groups. Although little quantitative significance should be attached to this figure in view of the smallness of the change relative to the experimental error associated with the method, the observed variation is in the right direction. It is also to be noted that the slope of the above plot, $d/dc\ (Hc/\tau)$, which is proportional to the interaction constant B, has a value of $6 \cdot 10^{-3}$ as compared to $5 \cdot 10^{-3}$ for the native protein in the same medium. This virtual identity of slope in the 2 cases suggests that the guanidination process has produced no drastic change in the charge pattern of the protein.

ACKNOWLEDGEMENTS

One of the authors (C. M. K.) would like to express his gratitude to Dr. P. JOHNSON of the Department of Colloid Science for making available the facilities of his laboratory and for helpful advice and to the Life Insurance Medical Research Fund for a grant which enabled the purchase of a Brice-Phoenix light scattering photometer. Thanks are also due to the American Cyanamid Company of Great Britain for a gift of S-methylisothiourea.

This investigation was carried out while one of the authors (C.M.K.) was holder of a Life Insurance Medical Research Fund Postdoctoral Fellowship, 1956–1957.

REFERENCES

1 K. BAILEY, H. GUTFREUND AND A. G. OGSTON, *Biochem. J.*, 43 (1948) 279.
2 T. C. TSAO, K. BAILEY AND G. S. ADAIR, *Biochem. J.*, 49 (1951) 27.
3 P. M. DOTY AND J. SANDERS, cited by K. BAILEY in H. NEURATH AND K. BAILEY, *The Proteins*, Vol. II, Part B, Academic Press, Inc., New York, 1954, p. 1001.
4 K. BAILEY, *Biochim. Biophys. Acta*, 24 (1957) 612.
5 K. BAILEY AND J. C. RUEGG, *Biochim. Biophys. Acta*, 38 (1960) 239.
6 K. BAILEY, *Biochem. J.*, 43 (1948) 271.
7 J. ROCHE, M. MORGUE AND R. BARET, *Bull. soc. chim. biol.*, 36 (1954) 85.
8 A. J. P. MARTIN AND R. L. M. SYNGE, *Advances in Protein Chem.*, 2 (1945) 57.
9 C. H. CHERVENKA AND P. E. WILCOX, *J. Biol. Chem.*, 222 (1956) 635.
10 D. A. I. GORING AND P. JOHNSON, *J. Chem. Soc.*, (1952) 33.
11 B. A. BRICE, M. HALWER AND R. SPEISER, *J. Opt. Soc. Am.*, 40 (1950) 768.
12 G. E. PERLMANN AND L. G. LONGSWORTH, *J. Am. Chem. Soc.*, 70 (1948) 2719.
13 P. DEBYE, *J. Appl. Phys.*, 15 (1944) 338.
14 P. DOTY AND J. T. EDSALL, *Advances in Protein Chem.*, 6 (1951) 35.
15 P. DOTY AND R. F. STEINER, *J. Chem. Phys.*, 18 (1950) 1211.
16 P. JOHNSON, unpublished work.
17 C. COHEN AND ANDREW G. SZENT-GYÖRGYI, *J. Am. Chem. Soc.*, 79 (1957) 248.
18 T. C. TSAO AND K. BAILEY, *Discussions Faraday Soc.*, 13 (1953) 145.
19 W. L. HUGHES, JR., H. A. SAROFF AND A. L. CARNEY, *J. Am. Chem. Soc.*, 71 (1949) 2470.
20 P. H. TAN AND T. C. TSAO, *Sci. Sinica, (Peking)*, 6 (1957) 1049.

Biochim. Biophys. Acta, 40 (1960) 149–156

MICROELECTROPHORETIC STUDIES OF SOLUBLE COLLAGEN

I. G. F. GILBERT

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

(Received August 12th, 1959)

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.