region alkaline to the isoelectric point but acid to the isoelectric point there is disagreement, the mobilities of the adsorbed protein being too low in the more acid region. It is evident that the mobilities of the protein adsorbed on Nujol would be still lower and those for B.S.A. adsorbed on glass particles would be less than those of Nujol. It is our view that the negative charge on the underlying surface upon which protein has been adsorbed serves to neutralize a substantial part of the positive charge of the adsorbed protein in the region acid to the isoelectric point and in the case of glass, the roughness of the surface is also of importance in reducing the mobilities at any *p*H.

We are of the opinion that it is a mistake to expect quantitative agreement between the electrophoretic mobilities of adsorbed and of dissolved protein even after correction has been made for the difference in particle size. The adsorbed protein molecules have, no doubt, been oriented and or distorted in various ways and, in general, represents a very complex situation. Electrophoresis of adsorbed protein has an inherent interest of its own and can reveal changes in the adsorbed molecules which are difficult to detect by other techniques.

Acknowledgment.—Financial support for this research was provided by the National Science Foundation for which we wish to express our thanks.

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[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT, STATE UNIVERSITY OF IOWA]

Electrophoresis of Ribonuclease: Isoelectric Points on Various Adsorbents

BY LEWIS B. BARNETT[1] AND HENRY B. BULL

RECEIVED FEBRUARY 18, 1959

The isoelectric point of ribonuclease adsorbed on glass, Dowex 50 cation-exchange resin, Dowex 2 anion-exchange resin, paraffin and Nujol has been determined in various buffer systems at an ionic strength of 0.05 and at 25°. Comparison of these data with the isoelectric points obtained by the moving boundary (Tiselius) method indicates significant shifts after adsorption. These shifts depend upon the buffer system employed and the adsorbing medium.

Various investigators have noted the similarity between the mobilities of dissolved and adsorbed proteins. Abramson, Moyer and Gorin[2] cite studies on horse serum albumin[3-5] and horse pseudoglobulin.[4,6] Ribonuclease and bovine fibrinogen have been investigated as adsorbed proteins by Douglas and Shaw[7] and the similarity of isoelectric points noted between adsorbed and dissolved molecules. Bull[8] has concluded that the effective electrophoretic radii of adsorbed protein molecules are very much larger than the radii of the protein molecules in solution. One would therefore expect the mobilities of dissolved and adsorbed proteins to be different, as a general rule.

It is the purpose of the paper to study the isoelectric points of adsorbed ribonuclease as a function of the adsorbing medium and ionic environment and compare the findings with the isoelectric points of dissolved ribonuclease.

Methods

Ribonuclease.—The crystalline ribonuclease was obtained from Armour and Co. (Lot No. 381-059). This protein was rendered isoionic by passage of a solution through an ion-exchange column of the Dintzis type.[9] Concentrations were determined by drying in a vacuum oven at 105°.

Electrophoretic Mobilities.—Electrophoretic measurements of the particles were conducted in a microelectrophoretic cell supplied by Arthur H. Thomas and Co. (See Bull[8] for a more complete description of the apparatus.) The cell was mounted laterally. Komagatas' correction was applied to find the correct position for observing mobilities.[10] Mobilities were taken at room temperature and corrected to 25° by multiplying by the ratio of the viscosity of water at the two temperatures. Conductances were measured at room temperature. The *p*H was determined with a Beckman Model GS *p*H meter. Concentration *vs.* mobility curves were plotted for each set of conditions and the concentration at which maximum adsorption occurred was used to obtain mobilities of adsorbed ribonuclease. (This protein concentration for maximum adsorption is listed with each adsorbing medium.)

Glass.—Powdered Pyrex glass in a standard suspension was used as a stock solution of particles. The diameters of the particles were about 2 μ. Maximum adsorption occurred at 2.55 mg. ribonuclease/g. glass. Solutions were equilibrated overnight.

Dowex Resins.—Dowex 50-X12 cation-exchange resin (Lot No. 3648-16) and Dowex 2-X10 anion-exchange resin (Lot No. 4034-34) of 200-400 mesh were obtained from the Dow Chemical Company. The Dowex 50 and Dowex 2 resins were of low porosity and medium porosity, respectively.

The resins were washed thoroughly and used in the salt form. The dry particles were powdered with a mortar and pestle and fractionated by sedimentation, discarding the larger pieces. The diameters of final particles were about 5 μ. The concentration of the final suspension was determined by drying in a vacuum oven at 105°. After equilibration of the resin and protein over-night, maximum adsorption occurred at 0.50 mg. and 94 mg. ribonuclease/mg. dry resin for Dowex 50 and Dowex 2, respectively. (The latter adsorption was measured at *p*H 7.76, at which *p*H both the protein and anion-exchange resin are positively charged.) Experiments have shown that the conductance of the resin particles is greater than that of 0.05 *M* NaCl.[11] This observation introduces a serious reservation concerning the electrophoresis of resin particles with and without a cover of protein. The difficulty arises in two ways: (1) Since the conductance of the particle is greater than that of the solution of 0.05 *M* NaCl, the current density through the particle will be greater than that through the solution and the

(1) Predoctoral Fellow of the United States Public Health Service.

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(3) H. A. Abramson, *J. Gen. Physiol.*, **15**, 575 (1932).

(4) L. S. Moyer, *J. Biol. Chem.*, **122**, 641 (1938).

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(8) H. B. Bull, THIS JOURNAL, **80**, 1901 (1958).

(9) H. M. Dintzis, Ph.D. Thesis, Harvard University, 1952.

(10) S. Komagata, Researches Electrotech Lab. Tokyo No. 348, 1933.

(11) H. B. Bull, unpublished data.

potential gradient in the solution in the immediate vicinity of the particle will be less than that calculated. Accordingly, the mobility of the particle will be less than it would be for a non-conducting particle. (2) The second difficulty has to do with electrophoresis through the particle which will contribute an unknown velocity vector to the particle.

Paraffin.—White, filtered paraffin (hard) was purchased from the Fisher Scientific Co. (Lot No. 761367). The melting point was 68–70°. In order to remove contaminating substances, the paraffin was purified by melting in the presence of concentrated NaOH and washing with H_2O until all of the base had been removed. Solidification followed each washing. Five grams of paraffin was melted by heating in a water-bath and to it was added about 100 ml. of boiling water. The suspension was shaken for 20–30 minutes (until solidification of most of the paraffin had occurred) and then cooled with shaking under tap water. The large particles of paraffin were removed from the surface. This suspension was used as a stock solution within 30 minutes. Solutions were equilibrated for 2 hr. Maximum adsorption occurred at 0.003 mg. ribonuclease and 60 mg. ribonuclease/10 ml. of paraffin suspension in NaOH–HOAc and Michaelis buffers, respectively. Adsorption in tris buffer was very similar to that in the Michaelis buffer.

Nujol.—Nujol was of the commercial variety manufactured by Plough, Inc. To 100 ml. of H_2O was added 10 ml. of Nujol, and the resulting mixture was shaken vigorously for 15–20 minutes. The top layer of Nujol was removed and the suspension was used as a stock solution within a few minutes. Protein and Nujol solutions were equilibrated for 2 hr. Maximum adsorption was found to be at 6 mg. ribonuclease/10 ml. of Nujol suspension.

Buffers.—Acetate buffer (NaOAc), tris buffer and Michaelis buffer[12] have been used at an ionic strength $\Gamma/2$ of 0.5.

Results

Shown in Fig. 1 are the electrophoretic mobilities of glass, Dowex-50 resin and paraffin coated with ribonuclease in acetate buffer, $\Gamma/2 = 0.05$. The

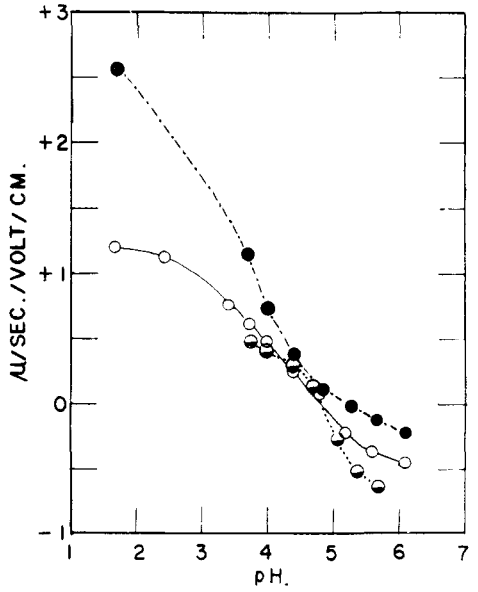

Fig. 1.—Electrophoretic mobilites as a function of pH at an ionic strength of 0.05 in acetate buffer (NaCl plus HCl at pH values below 3.5) and at 25°. Ribonuclease adsorbed on: ●, paraffin; ○, Dowex-50 resin; ◒, glass.

observed isoelectric points are 4.85, 4.85 and 5.22, respectively. The data of Fig. 2 are the microelectrophoresis studies of ribonuclease adsorbed on Dowex-2 resin and paraffin in tris buffer, $\Gamma/2 = 0.05$. The isoelectric points are at 8.54 and 6.0,

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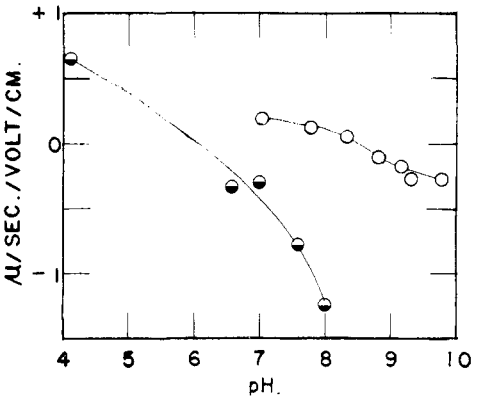

Fig. 2.—Electrophoretic mobilities as a function of pH at an ionic strength of 0.05 in tris buffer and at 25°. Ribonuclease adsorbed on: ◒, paraffin; ○, Dowex-2 resin.

respectively. Figure 3 contains the mobilities of ribonuclease adsorbed on Nujol and paraffin in Michaelis buffer, $\Gamma/2 = 0.05$, showing isoelectric points of 7.95 and above 9.1, respectively. The dashed line is from the work of Douglas and Shaw[7] for ribonuclease adsorbed in Nujol at 25° in Michaelis buffer, $\Gamma/2 = 0.05$. This isoelectric

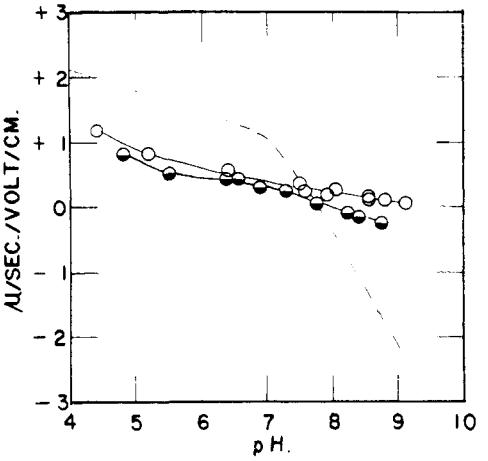

Fig. 3.—Electrophoretic mobilities as a function of pH at an ionic strength of 0.05 in Michaelis buffer and at 25°. Ribonuclease adsorbed on: ○, paraffin; ◒, Nujol. Dashed line is from Douglas and Shaw[7] for ribonuclease adsorbed on Nujol.

point is 7.8. The extreme difference in mobilities at pH's on either side of the isoelectric point may be due to different preparations of Nujol. (Douglas and Shaw used a protein concentration of 0.005% while maximum adsorption did not occur for us until the ribonuclease concentration was at 0.04%.)

Discussion

The similarity of the mobilities between adsorbed and dissolved horse serum albumin and horse pseudoglobulin may be explained by a preferential orientation of the protein on the charged surface, decreasing the charge density of the protein.[8] The data of Douglas and Shaw[7] for bovine fibrinogen and ribonuclease, adsorbed on Nujol particles, are subject to additional interpretations.

Although bovine fibrinogen adsorbed and dissolved resulted in an identical isoelectric point examination of the two curves shows that the two mobilities differ significantly at other *p*H's. The comparison of the isoelectric points of adsorbed and dissolved proteins will be discussed later. In the case of ribonuclease the two systems were studied in different buffers; acetate and phosphate for the dissolved protein[13] and Michaelis buffer for the adsorbed protein. Such a comparison of isoelectric points in the presence of varying anions is unwise.

Egg albumin has been studied adsorbed on various particles[1,14] and its isoelectric point (4.82) is consistantly higher than that of the dissolved protein (4.55).[5] Bull[8] has noted that the isoelectric point of Pyrex glass covered with bovine serum albumin was 4.88, compared to the value of 4.52 for the dissolved protein under similar conditions.[15] Insulin also exhibits a shift in its isoelectric point upon adsorption.[16]

Table I contains the isoelectric points (pI) which have been obtained for ribonuclease along with the method, type of buffer and ionic strength. The

TABLE I

SUMMARY OF ISOELECTRIC POINTS OF RIBONUCLEASE

Type of electrophoresis	Buffer	$\Gamma/2$	pI	Ref.
Tiselius	Na_2HPO_4–NaH_2PO_4	0.055	7.8	13
	Borate	.03	9.5	13
	Na veronal	.01	9.45	18
	Na glycine	.01	9.45	18
	Na_2HPO_4–NaH_2PO_4	.055	7.8	18
	NaOAc	.05	>6.0	[a]
	Tris	.05	8.95	[a]
	Tris	.01	9.36	[a]
	KCl	.05	9.10	[a]
	KCl	.01	9.42	[a]
Paper	Na_2HPO_4–NaH_2PO_4	.055	7.6	17
	Na_2HPO_4–NaH_2PO_4	.36	5.9	17
	H_3PO_4–NaOH	.2	6.4	17
	NH_3–HOAc	.2	9.1	17
Micro- (adsorbed on)				
Nujol	Michaelis	.05	7.8	7
Nujol	Michaelis	.05	7.95	[b]
Paraffin	Michaelis	.05	>9.10	[b]
Paraffin	Tris	.05	6.00	[b]
Paraffin	NaOAc	.05	5.22	[b]
Glass	NaOAc	.05	4.85	[b]
Dowex 50	NaOAc	.05	4.85	[b]
Dowex 2	Tris	.05	8.54	[b]

[a] Unpublished results from this Laboratory. [b] This paper.

paper electrophoresis results are included for completeness and will not be discussed. In sodium acetate buffer, Tiselius moving boundary electrophoresis indicates a pI above 6, while microelectrophoresis of both glass, Dowex-50 ion-exchange resin and paraffin coated with ribonuclease yields pI's of 4.85, 4.85 and 5.30, respectively. Electrophoresis of ribonuclease adsorbed on paraffin and Dowex-2 ion-exchange resin results in pI's of 6.00 and 8.54, respectively. Under similar conditions the dissolved protein exhibits a pI of 8.95. Therefore it appears that the pI of ribonuclease adsorbed on various particles is shifted from that of the dissolved protein.

The comparison of the isoelectric points of ribonuclease dissolved in phosphate buffer and adsorbed on Nujol in Michaelis buffer by Douglas and Shaw seems inappropriate since the results in Table I indicate a dramatic effect of buffer ions on the isoelectric points of both the adsorbed and dissolved protein. It would be more realistic to compare the results for adsorbed protein in Michaelis buffer with the moving boundary studies of Alberty, *et al.*,[18] in Na veronal. Since the pI's for ribonuclease in tris, KCl and Na veronal buffer are nearly the same at $\Gamma/2 = 0.01$, we should not be too far off in assuming that the pI of the protein in Na veronal will be about 9 at $\Gamma/2 = 0.05$. The isoelectric point of 7.9 on Nujol is a significant shift from this. The results of ribonuclease adsorbed on paraffin are quite unexpected and may be caused by a configuration change of the molecule, such as a spreading of the protein at the paraffin surface.

Several explanations may be postulated to explain the difference in isoelectric points between dissolved and adsorbed protein.

(1) As suggested by Bull,[8] the adsorbed protein molecules may be oriented or distorted at the solid–water interface. Such an orientation or distortion might be associated with significant electrostatic interaction between the charge on the adsorbed protein and the underlying surface and result in a decrease in the isoelectric point of protein adsorbed on a positively charged particle whereas a negative particle would increase the isoelectric point of the adsorbed protein. The data for ribonuclease adsorbed on Pyrex glass, on Dowex-50 resin, on Nujol and on paraffin (in tris buffers) are consistent with the idea of electrostatic interaction between the adsorbed protein and the adsorbing surface. However, the results obtained with ribonuclease adsorbed on Dowex-2 resin and on paraffin (in Michaelis buffers) as well as those with egg albumin and with bovine serum albumin adsorbed on Pyrex glass are contrary to the notion of electrostatic interaction.

(2) Close packing of the protein molecules might result in a significant shift in the pK of the ionizing groups. One would expect the resulting shift in the isoelectric point to be toward neutrality. Such a shift has in fact been observed for bovine serum albumin, egg albumin and ribonuclease in all buffers except acetate. Three adsorbing particles in acetate buffer all result in a shift of the isoelectric point of ribonuclease to around 5. (In tris the pI of the protein adsorbed on paraffin is changed from 8.95 past neutrality to 6.00.)

(3) The protein might undergo extreme structural changes, resulting perhaps in a denatured form.

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This could cause a vastly different array of charges on the surface of the molecule thereby altering the isoelectric point. Hartman *et al.*,[19] postulate such a phenomenon in connection with the formation of an inactive form of trypsin upon adsorption onto Pyrex glass. Inactivation is accompanied with a change of the isoelectric point from 10.8 for the dissolved protein to 7. However, ribonuclease is active in the adsorbed state.[20]

(19) R. S. Hartman, J. B. Bateman and H. E. Edelhoch, THIS JOURNAL, **75**, 5748 (1953).

(20) H. B. Bull and L. B. Barnett, in preparation.

It is concluded that certain combinations of phenomena such as those described, perhaps coupled with other similar effects, might well explain the entire array of microelectrophoretic data. That in some instances the isoelectric point of a dissolved and adsorbed protein is the same is quite plausible.

Acknowledgments.—We wish to express our thanks to the United States Public Health Service for their Research Fellowship and to the National Science Foundation for financial support.

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[CONTRIBUTION FROM THE DIVISION OF NUCLEOPROTEIN CHEMISTRY OF THE SLOAN-KETTERING DIVISION OF CORNELL UNIVERSITY MEDICAL COLLEGE]

Shear Degradation of Deoxyribonucleic Acid[1]

BY LIEBE F. CAVALIERI AND BARBARA H. ROSENBERG

RECEIVED DECEMBER 1, 1958

The shear forces developed by a simple glass atomizer have been used to cleave DNA molecules. Light scattering, sedimentation and viscosity measurements show that the cleaved molecules are stiff chains and that no detectable denaturation has occurred. The results show that the fragments result from double-chain scissions. The extrapolation of the zero concentration lines of the light scattering curves is discussed and it is suggested that the error of the weight-average molecular weight obtained is small.

In physical and biological studies on deoxyribonucleic acid (DNA) it is frequently desirable to have at hand a means of decreasing its molecular weight in a known and simple manner. While enzymatic digestion, ionizing radiation and sonication[2] appear to be useful tools for this purpose, the first two have serious disadvantages, namely, the relatively large number of single-chain breaks as opposed to double-chain scissions, and the occurrence of denaturation.

In this paper, we describe a simple method for degrading DNA by mechanical shear.[3] No free radicals are formed and double-chain scission is the only observable type of fracture. No detectable denaturation occurs. A unique characteristic of this method is that the cleavage is non-random; larger molecules are more readily cut than smaller ones, resulting in decreased polydispersity.

Experimental

Apparatus and Procedure.—The apparatus consists of an all-glass atomizer of the type used for spraying paper chromatograms.[4] The diameter of the central capillary and the width of the annular space were 0.15 mm. each. The results were found to depend slightly on these dimensions as well as on the solid angle of the air cone, which was about 20°. The atomizer was modified so that the velocity of the liquid stream issuing from the central capillary and the velocity of the surrounding air stream were controlled by separate air (or nitrogen) pressures; it was convenient to use for all runs a nitrogen pressure of 5 lb./in.^{2} to produce the liquid stream. The applied pressure for the air stream ranged from 7 to 117 cm. of mercury. The spray was collected in a Hoffman bulb, which was in turn connected to the liquid chamber of the atomizer. After a spraying, the nitrogen pressure was released and the stopcock connecting the Hoffman bulb to the liquid chamber of the atomizer was opened, thus permitting recycling in simple fashion.

Sedimentation.—Sedimentation velocity measurements were carried out using a Spinco Model E Ultracentrifuge equipped with ultraviolet optics. The DNA concentration was 0.03 mg./ml., in 0.2 *M* salt. Sedimentation distribution curves were calculated using the method of Schumaker and Schachman.[5]

Light Scattering and Viscosity.—The details of these measurements have been given elsewhere.[6]

DNA Samples.—The DNA used in this study was extracted from *Diplococcus pneumoniae* and deproteinized by the method of Kay, Simmons and Dounce,[7] Emmanuel and Chaikoff[8] or Sevag. In spite of the fact that the initial sedimentation distributions differ somewhat depending on the method of isolation of the DNA, the sheared DNA's showed essentially the same distributions for comparable shear rates. The data given in this paper were obtained with DNA isolated according to Kay, *et al.* Calf thymus DNA behaved similarly.

Results and Discussion

Variables.—The DNA and salt concentrations, temperature, liquid velocity and air pressure were all examined for their effects on the cleavage products. The range of DNA concentration was 0.03–0.8 mg./ml., the salt concentration range was 0.002–0.2 *M*, the temperature range was 0–60° and the liquid velocity was varied 50-fold. In no case did these variations change the nature of the products, as shown by physical measurements. However, the pressure of the air stream is critical. The higher the air pressure, the greater the shear gradient and the lower the molecular weight of the cleaved DNA; furthermore, fewer passages through

(1) This investigation was supported in part by funds from the National Cancer Institute, National Institutes of Health, Public Health Service (Grant CY-3190) and from the Atomic Energy Commission (Contract No. AT(30-1)-910).

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