[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE UNIVERSITY OF DELAWARE]

The Structure and Constitution of a Mucus. I. The Polyelectrolyte Nature of Mucus Solutions Derived from *Busycon canaliculatum*

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The evidence from viscosity measurements, light scattering experiments and ultraviolet spectral studies suggests strongly that Busycon mucus is a polyelectrolyte of very high molecular weight. The properties characteristic of mucus may be directly correlated with a spherically-shaped molecule of polyelectrolyte nature. Selected model synthetic polyelectrolytes were shown to have physical properties similar to mucus.

Mucus substances are among the fundamental polymeric materials found in the animal body. Their wide distribution in such diverse materials as gastric mucin,[2] cartilage,[3] tumor fluids,[4] collagen,[5] blood group substances[6] and bone tissue[7] points to the possibility that a large number of biochemically important reactions may occur in its presence. Thus, an understanding of the structure of mucus and its solution behavior is one of the primary problems in biochemistry. Previous workers have recognized that a mucus is basically constituted of a mucoprotein–mucopolysaccharide complex and considerable emphasis in these studies has been given to the polyhexoseamine nature of the polysaccharide moiety.[1-9]

Ronkin[10a] in a description of the properties of this (fresh) mucus[10b] summarized its outstanding features as an ability to bind large quantities of water, a fiber forming quality and an "elastic recoil" quality which is characterized by the unwinding tendency of the mucus after being stirred in a given direction with a glass rod. These properties and others with which this report is concerned have been studied by means of viscosity, light scattering and ultraviolet spectral measurements as part of an investigation into the structure and constitution of the mucus.

The mucus was obtained from the hypobronchial gland of the large marine snail, *Busycon Canaliculatum L.*[11] This source has the advantage of being readily available, and relatively free from contaminating factors so that the mucus could be studied in its natural state. Moreover the choice of such a comparatively simple species as the source of the mucus for investigation avoids many of the problems arising from the presence of accompanying secretions and difficultly separable enzymes often found in other mucous materials.

(1) Taken in part from the dissertation of V. E. Shashoua submitted in partial fulfillment of the Ph.D. at the University of Delaware.

(2) M. L. Wolfrom and F. A. H. Rice, THIS JOURNAL, **69**, 1833 (1947).

(3) K. Meyer and E. M. Smyth, *J. Biol. Chem.*, **119**, 507 (1937).

(4) K. Meyer, *Physiol. Rev.*, **27**, 335 (1947).

(5) J. H. Highberger, J. Gross and F. O. Schmitt, *Proc. Nat. Acad. Sci.*, **37**, 286 (1951).

(6) E. Kabat, "The Harvey Lectures," Vol. 46, Academic Press, Inc., New York, N. Y., 1951, p. 252.

(7) P. A. Levene, "Hexoseamines and Mucoproteins," Longmans Green & Co., London, 1925.

(8) K. Meyer, *Advances in Protein Chem.*, **2**, 249 (1945).

(9) K. Meyer, *Ann. Conf. Protein Metabolism, Bull. Biol. Research*, Rutgers Univ., **9**, 64 (1953).

(10) (a) R. R. Ronkin, *Arch. Biochem. Biophys.*, **56**, No. 1, 76 (1955); (b) where not otherwise specified in this manuscript, the term mucus will be used here to designate Busycon mucus.

(11) This animal is available during most of the year from Woods Hole, Massachusetts.

The freshly extracted Busycon mucus has a viscosity of 60–100 centipoises in 0.5 *N* NaCl at 30° for a solution containing as low as 0.3% solids. It is a colorless, slimy solution which exhibits a strong Tyndall effect. Microscopic examination, after removal of cellular debris by centrifugation, shows some filamentous aggregates, which are readily homogenized into fine particles by stirring under high shear. The homogenized sample shows no structural features in an optical microscope, but exhibits the presence of fine particles in rapid "Brownian motion" when examined in a dark-field microscope. A study of a homogenized sample in an electron microscope showed the presence of primary particles of about 200–300 Å. in diameter which were occasionally aggregated into large, 1000 Å. particles.

Experimental

Extraction of the Mucus.—The fresh hypobronchial glands were isolated according to Ronkin's method,[12] and then extracted with various salt solutions. In a typical preparation, glands from thirty snails were allowed to stand for two hours in an ice-cooled 0.5 *N* sodium chloride solution. This ensures the complete hydration of the mucous glands and initiates the evolution of mucus. The product is rolled off as a viscous, gelatinous strand onto an applicator stick and deposited in an ice-cooled beaker. With intermittent addition of saline solvent and gentle agitation it was possible to extract 200–300 cc. of mucus during a 2–4 hour period. The fresh mucus was homogenized by means of a magnetic stirrer for 2 hours and the cellular debris removed by two half-hour centrifugations at 2600 r.p.m. The product solution, characterized by a blue opalescence at this point, was further purified by dialysis against distilled water to remove the salt. This was carried out with the best results at about 4° in the presence of a few drops of toluene acting as an antibacterial agent. It required from 2–4 days of continuous dialysis to obtain a relatively salt free preparation.

Viscosity Studies.—The viscosities were measured in an Ostwald–Fenske viscometer,[13] using 10-ml. portions of mucus. The viscometer was placed in a water-bath at 30 ± 0.02° and the flow time of the solution was measured by means of a stopwatch. Three readings were taken at each dilution and the average flow time was used in the calculation. The dilutions were performed by removing increments from the viscometer by means of a syringe and replacing them with the corresponding amounts of solvent. The solutions were stirred while in the viscometer by blowing a gentle stream of nitrogen through the capillary end of the viscometer. The viscosity measurements in buffered solutions were made on mucus originally extracted with buffers prepared from admixture of 0.1 *M* sodium citrate and 0.2 *M* Na_2HPO_4 solutions. In all cases the mucus solutions were behaving as Newtonian fluids, *i.e.*, the viscosity followed a linear relationship with respect to rate of shear. Assurance of this behavior was obtained by following the procedure described by Ronkin.[10]

(12) R. R. Ronkin, *Biol. Bull.*, **103**, 296 (1952).

(13) M. R. Cannon and M. R. Fenske, *Ind. Eng. Chem., Anal. Ed.*, **10**, 297 (1938).

Light Scattering Studies.—Two methods of mucus purification were used in the light scattering experiments. The first was a rigorous filtration procedure through a series of sintered glass filters of coarse, medium and fine porosity followed by a series of Seitz filters of pore sizes 5 and 1 μ. The results from this method demonstrated that the observed particle sizes were not due to traces of bacteria and cellular debris. The second method was simply a centrifugation at 16,000 r.p.m. in a "Sorval" centrifuge for two half-hour periods. This method was found to give the particle size results similar to those obtained by the rather involved filtration process and was adopted, therefore, in all subsequent light scattering studies.

The Aminco light scattering photometer most often was used in the particle size determinations by the "angular dissymetry" method.[14] The measurements were carried out in a semi-octagonal cell with a capacity of 30 ml. In each experiment, the glassware and the cell used were first cleaned with distilled water and then with hot acetone vapor in order to ensure the complete removal of dust particles. The outer optical surfaces of the cell were wiped clean with a 1% detergent solution before use.

The Brice–Phoenix instrument[15] was used for the molecular weight determinations by the turbidity method.[27] The value of dn/dc, the refractive index gradient, was determined by using a differential refractometer according to Brice's method.[16]

For each mucus solution, a number of readings were taken at angles of 0, 45, 90 and 135° to the incident light beam. The measurements were first carried out on the pure solvent and then on the mucus sample. The results from the 90 and 0° measurements were used to calculate the 90° turbidity and the data at 45 and 135° were used occasionally to calculate particle size by the "angular dissymetry" method. All the measurements were carried out using an hydrogen source that was filtered to give a monochromatic beam at λ 5360 Å.

Ultraviolet Spectral Studies.—The ultraviolet spectra were obtained by using a "Carey" Spectrophotometer (Model XX). The determinations were carried out in 1 cm. quartz cells, on concentrations ranging from 0.025 to 0.15%. The spectra were found to be particularly susceptible to traces of iron impurities, so that care was taken to avoid contamination of the mucus during its isolation; see later discussion on this point.

Results and Discussion

Extraction of Mucus.—In its natural state Busycon mucus is secreted into sea water to function principally as a lubricant in a high ionic strength medium. This suggested that there may be optimum conditions (pH, ionic strength, etc.) for the evolution of mucus. An investigation of this possibility is summarized in Table I.

It is seen that the largest quantity of mucus was evolved at a pH of 7.2, where the condition of the hypobronchial gland was soft, but that there was no evolution of mucus at pH 3, where the gland became stiff. This condition was found to be irreversible since no mucus was evolved when the glands from pH 3 were removed and allowed to stand for 1 hour at pH 8. Each extraction in experiments 1–6 was repeated in the presence of 0.5 N NaCl, but no changes from the tabulated observations were noted. Thus, the optimum yield of mucus appeared to be somewhere between pH 7.2 and 8.0, near the isoelectric point at pH 7.7 (see ultraviolet spectral studies).

It seems clear from experiments 7–22 that the presence of ions was essential for the extraction of mucus since none was evolved in distilled water;

TABLE I

EXTRACTION OF MUCUS IN VARIOUS MEDIA

Exp. no.	Medium	Condition of gland	Relative[a] yield of mucus
1	Phosphate–citrate buffer pH 3.0	Stiff	0
2	Phosphate–citrate buffer pH 4.0	Stiff	0
3	Phosphate–citrate buffer pH 6.0	Stiff	1
4	Phosphate–citrate buffer pH 6.6	Soft	2
5	Phosphate–citrate buffer pH 7.2	Soft	4
6	Phosphate–citrate buffer pH 8.0	Soft	3
7	Distilled water	Soft	0
8	0.5 M NaCl	Soft	4
9	.5 M LiCl	Soft	3
10	.5 M KCl	Soft	4
11	.5 M NH_4Cl	Soft	4
12	.5 M $(NH_4)_2SO_4$	Soft	4
13	.5 M $(NH_4)_2Ac$	Soft	4
14	.5 M NaAc	Soft	4
15	.5 M $BaCl_2$	Soft; mucus bearing layer became detached and readily removable from remainder of gland tissue	0
16	.5 M $CaCl_2$		0
17	Satd. $(NH_4)_2(COO)_2$		0
18	Satd. $Na_2(COO)_2$		0
19	0.5 M KCNS	Soft	3
20	.5 M $CuCl_2$	Stiff and coiled	0
21	.5 M $FeCl_2$	Stiff and coiled	0
22	.5 M $Al_2(SO_4)_3$	Stiff and coiled	0

[a] Note: the highest relative yield is represented by the largest number.

moreover, the ions examined seemed to be quite selective in this regard. Mucus was copiously secreted in the presence of monovalent cations and, with the exception of the oxalate anion, there appeared to be no influence by either univalent or divalent anions. All the salts of divalent and trivalent cations were found to inhibit the evolution of mucus, and with the exception of Ba^{++} and Ca^{++} this was always accompanied by a stiffening and coiling of the gland. In the case of $CuCl_2$ and $FeCl_3$ there was complete absorption of the salts from solution by the hypobronchial gland. This was so pronounced that even a single gland absorbed the total contents of 30 cc. of 0.5 M $FeCl_3$ at the mucus-bearing layer of the gland. The behavior of $BaCl_2$, $CaCl_2$, $(NH_4)_2(COO)_2$ and $Na_2(COO)_2$ solutions was quite different from all the others. Here the glands remained soft and all the mucus-bearing layer became completely detached from the other tissues of the gland.[17] It would appear that there is a special affinity for calcium, barium and oxalate ions. A study of the ash content of mucus supports[18] this observation, where it is found that Ca^{++} ions form an integral part of the structure of mucus. The above results serve to illustrate that the evolution of mucus is markedly influenced by both pH and type of ions, and point to the possible presence of specific sites in mucus which are selective to Ca^{++} and Ba^{++} ions.

Viscosity Studies.—Many investigations into the viscosity behavior of biologically active substances related to mucus have been carried out. Fuoss[19] and Katchalsky[20] have pointed out the similarity between polyelectrolytes and such materials as chondroitin sulfuric acid and hyaluronic acid.

(14) G. Oster, *Anal. Chem.*, **25**, 1165 (1953); see also reference 26.

(15) (a) Brice–Phoenix Precision Instrument Co., 3805 N. Fifth St., Philadelphia 40, Pennsylvania; (b) B. A. Brice, M. Halwer and R. J. Speiser, *J. Opt. Soc. Am.*, **40**, 768 (1950).

(16) B. A. Brice and M. Halwer, *ibid.*, **41**, 1033 (1951).

(17) For a detailed description of the cell structure of the Busycon mucus gland see ref. 12.

(18) Results being prepared for publication.

(19) R. M. Fuoss, *Disc. Faraday Soc.*, **11**, 125 (1951).

(20) A. Katchalsky, *J. Polymer Sci.*, **12**, 159 (1954).

Heidelberger and Kendall[21] have reported on the polyelectrolyte behavior of polysaccharides. In Busycon Mucus, viscosity studies demonstrate that polyelectrolyte behavior also can be exhibited by the total protein–carbohydrate complex. Figures 1 and 2 show plots of the viscosity data according to the Kraemer[22] and Huggins[23] equations.

In Fig. 1 both η_{sp}/C and log η_r/C *vs.* C plots give the characteristic non-linear curves associated with polyelectrolyte behavior in water solutions. Figure 2 shows a plot of the viscosity data using 0.5 N NaCl as the solvent. Here, again, non-linear plots were obtained. This is perhaps not unexpected since mucus is a complex mixture containing a protein and a polysaccharide and, therefore, the behavior of a polyampholyte might be anticipated. Wall[24] has claimed that the Huggins equation

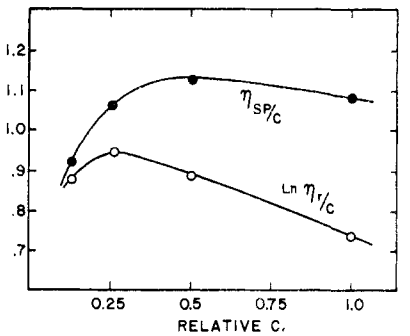

Fig. 1.—Viscosity measurements in distilled water.

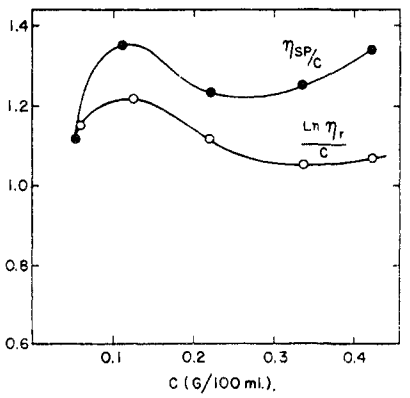

Fig. 2.—Viscosity measurements in 0.5 N NaCl.

holds only for polyelectrolytes when the counterion binding is kept relatively constant on dilution. Thus, the above experiments, in addition to demonstrating polyelectrolyte behavior, indicate also that 0.5 N NaCl does not keep the counterion binding constant. This was anticipated by the extraction studies on Busycon mucus which establish a specific affinity for calcium ions, and from the inorganic analysis which confirm the presence[18] of large amounts of Ca^{++}. Thus, it was to be expected that viscosity measurements in the presence of an excess of only the native calcium ion would give straight line plots for the Huggins equation. The reasoning behind this expectation was based on the assumption that no ionic interchange and no change in the counterion binding could occur in a medium of relatively high Ca^{++} concentration. Table II summarizes the results of such experiments and Fig. 3 is a plot of these results according to the Huggins equation where

$$[\eta] + K[\eta]^2C = \eta_{sp}/C \qquad (1)$$

the various symbols having the same significance as employed in references 22 and 23.

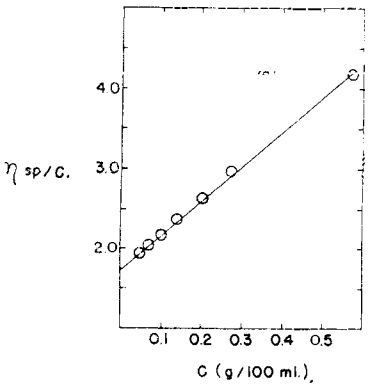

Fig. 3.—Intrinsic viscosity measurement in 0.5 M $CaCl_2$.

TABLE II

VISCOSITY OF MUCUS IN 0.5 M $CaCl_2$

Concn. (g./100 ml.)	Flow time, sec.	η_r	η_{sp}/C
0	83.8	1.00	..
0.57	282.5	3.37	4.16
.285	154.9	1.850	2.98
.200	127.3	1.520	2.61
.140	111.2	1.328	2.35
.098	101.4	1.211	2.16
.068	95.3	1.138	2.02
.048	91.5	1.093	1.93

Extrapolation of the linear plot in Fig. 3 to zero concentration gave an intrinsic viscosity of 1.73. Clearly, then, the viscosity data indicate that mucus is a polyampholyte of very high molecular weight, containing calcium ion as an essential constituent of its total structure.

Further evidence for the polyelectrolyte behavior of mucus was obtained from viscosity measurements at different pH values. Figure 4 shows a plot of the specific viscosity against pH. Similar to the results from the extraction studies, there appeared to be a maximum of specific viscosity between pH 7.2 and 8.2 which is in the region of the isoelectric point (pH 7.7). At a pH greater than 10.8, a precipitate was formed so that no measurements could be made. Intrinsic viscosity, $[\eta]$, measurements on mucus in phosphate (0.2 M Na_2HPO_4) citrate (0.1 M) buffers showed that straight line plots, were only obtained near the isoelectric point. Table III shows some of the data obtained in these studies. Apparently, the value of the intrinsic viscosity, or the "molecular volume," is dependent on the pH at which the measurements were made, again confirming the polyelectrolyte character of mucus.

The polyelectrolyte behavior of mucus is further

(21) M. Heidelberger and F. Kendall, *J. Biol. Chem.*, **95**, 127 (1932).

(22) E. O. Kraemer, *Ind. Eng. Chem.*, **30**, 1200 (1940).

(23) M. L. Huggins, THIS JOURNAL, **64**, 277 (1942).

(24) F. T. Wall and J. W. Drenan, *J. Polymer Sci.*, **7**, 83 (1951).

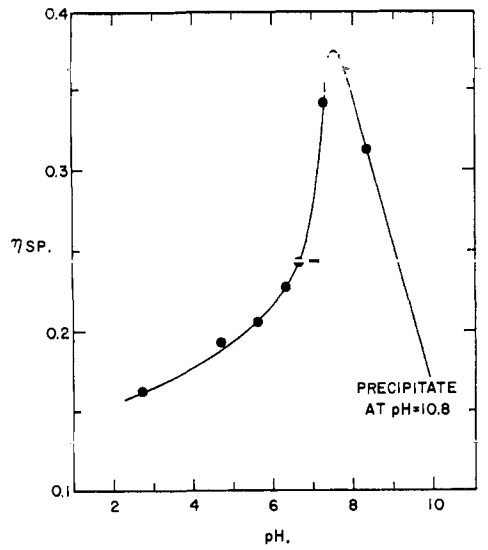

Fig. 4.—Effect of hydrogen ion concentration on the η_{sp} of mucus. Concentration in the buffer solution is 0.18%.

TABLE III
VISCOSITY DATA FOR MUCUS

pH 7.2				pH 8.0			
C	η_{sp}/C	$\sqrt{C}$	C/η_{sp}	C	η_{sp}/C	$\sqrt{C}$	C/η_{sp}
0.202	2.66	0.45	0.376	0.286	1.95	0.535	0.514
.162	2.40	.39	.417	.200	1.87	.513	.535
.120	2.18	.359	.459	.160	1.79	.40	.560
.103	2.04	.32	.490	.128	1.72	.358	.582
.052	1.91	.228	.524	.051	1.48	.226	.675
.026	1.71	.161	.586				
	$[\eta]$ = 1.46				$[\eta]$ = 1.27		

emphasized by the fact that the Fuoss–Strauss equation[25] can be applied to the viscosity measurements, where A and B are constants. Figure 5 represents the linear plot observed when the data of Table III are applied in eq. 2.

$$\frac{\eta_{sp}}{C} = \frac{A}{1 + B\sqrt{C}} \qquad (2)$$

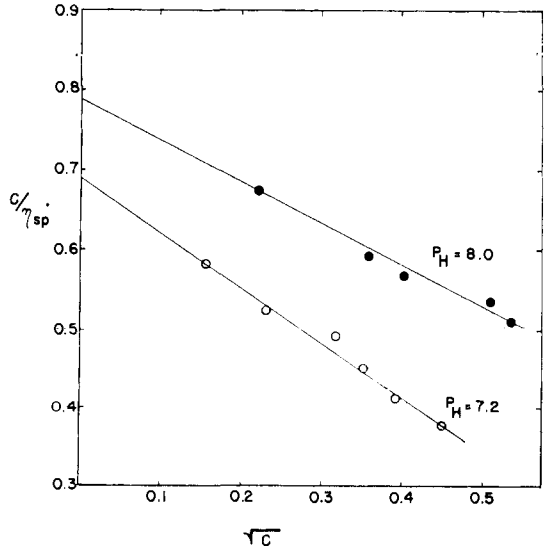

Fig. 5.—Application of the Fuoss–Strauss equation to mucus.

(25) R. H. Fuoss and U. P. Strauss, *Ann. N. Y. Acad. Sci.*, **51**, 836 (1949).

The viscosity results again illustrate the dependence of molecular weights on the hydrogen ion concentration and provide further proof of the polyelectrolyte nature of mucus.

Light Scattering Studies.—The light scattering measurements on Busycon mucus were used for a threefold purpose: namely, for the determination of particle size and possibly particle shape by the angular dissymmetry method of Debye[26] for a more precise determination of the molecular weight by the 90° turbidity method[27] and finally for obtaining additional intelligence on the polyelectrolyte nature of mucus.

Debye's method for obtaining the particle size from the disymmetry coefficient essentially measures the particle volume and is eminently suitable for biological systems where very small amounts of material are available. The method demands no knowledge of polymer density, polymer refractive index, absolute solution concentration and solution refractive index and, in fact, requires only a knowledge of the solvent refractive index and the wave length of the monochromatic source used. The theory and the practical applications of this method have been discussed at length by Oster[14] and Flory.[28]

The scattered intensities at 45 and 135° of the 5360 Å. incident beam were used to calculate the P_θ value for each of the angles according to

$$P_\theta = \left(\frac{R_\theta}{R_0}\right)_M - \left(\frac{R_\theta}{R_0}\right)_S \qquad (3)$$

where R_0 = the intensity reading at 0°, R_θ = the intensity reading at the angle θ and the subscripts M and S denote the values obtained for the mucus solution and pure solvent, respectively. The quantity Q corresponded to the ratio $P_{45°}/P_{135°}$. For each mucus solution studied in this fashion the value of Q was obtained for each of a series of concentrations. A plot of $1/Q$ *vs.* the relative concentration when extrapolated to infinite dilution afforded an estimate of Q_0. Table IV and Fig. 6 show some representative results obtained.

By means of electron micrograph studies it was found that the mucus particles were not rod-shaped but rather approximated either a spherical or a

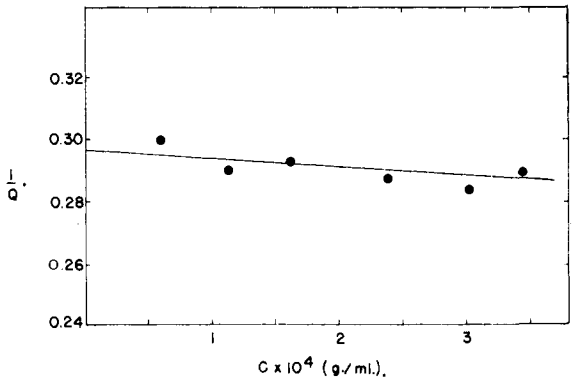

Fig. 6.—Angular disymmetry measurements on mucus; $1/Q = P_{135°}/P_{45°}$.

(26) P. P. Debye, Ph.D. Thesis, Cornell University, 1945.
(27) P. Debye, *J. Phys. Colloid Chem.*, **51**, 18 (1947).
(28) P. J. Flory, "Principles of Polymer Chemistry," Cornell University Press, New York, N. Y., 1953.

TABLE IV

ANGULAR DISYMMETRY MEASUREMENTS ON MUCUS

$C \times 10^4$, g./ml.	$P_{45°}$	$P_{135°}$	Q	$1/Q$
3.56	0.7630	0.221	3.452	0.290
3.02	.6745	.1913	3.525	.284
2.38	.5218	.1502	3.474	.288
1.60	.3412	.1000	3.412	.293
1.12	.2411	.0699	3.449	.290
0.59	.1200	.0360	3.354	.300

random coil model. Having established that mucus is of polyelectrolyte nature it readily may be inferred that molecular shape changes must be expected to occur in its solutions in the absence of added electrolyte. For a polymer of spherical dimensions these shape changes would of necessity be confined to a swelling and deswelling, *i.e.*, changes in spherical radius for which light scattering theory is quite rigorously observed. However, considering a random coil model as typical of many synthetic polyelectrolytes, both Fuoss[19] and Katchalsky[20] have presented evidence to the effect that a fundamental shape change to a rod form occurs at high dilution when the counter ion binding is not kept constant. Consequently, the light scattering data would not be consistent with either model, random coil or rod.

Referring again to Fig. 6, it is seen that the plot of $1/Q$ *vs.* concentration is linear even in the absence of added electrolyte. These results support the view that no shape changes occur with dilution and, therefore, the mucus particles must be spherically shaped. Disymmetry calculations from 45 and 135° readings were thus carried out using the familiar theory[26]

$$P_\theta = \frac{3}{X^3}(\sin X - X \cos X)^2 \qquad (4)$$

where $X = 2\pi D/\lambda \sin \theta/2$, D = the particle diameter assuming spherical dimensions, and λ = the wave length of the incident beam *in vacuo*. Standard curves depicting the values of Q as a function of D/λ were computed from eq. 4. These were

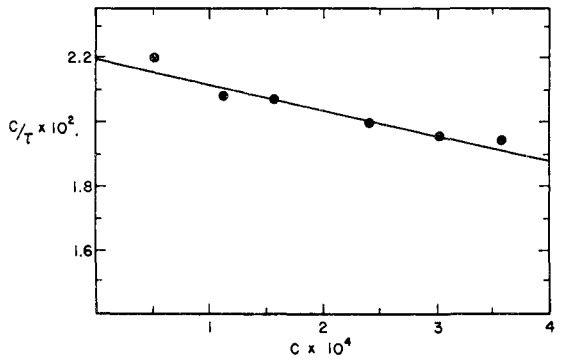

Fig. 7.—Determination of the molecular weight of mucus by 90° turbidity measurements.

then used as references for determining the particle diameters of the mucus particles as listed in Table V. Variation in particle size is seen to be only slightly sensitive to solvent; apparently swelling changes do not occur readily within the small range of solvent properties studied.

TABLE V

PARTICLE SIZE DETERMINATIONS IN DIFFERENT SOLVENTS

Sample no.	Solvent	Particle, size, Å.
1	H_2O	1890
2	N,N-Dimethylformamide/H_2O(2/1)	2250
3	0.5 *N* NaCl	1760
4	H_2O	2230
4A	1.8% NaCl	2290
4B	2.36% NaCl	2270
4C	7.08% NaCl	2380
4C (after standing 85 hr.)	7.08% NaCl	1940

Molecular Weights from Light Scattering Data.—Utilizing relations developed by Debye,[29] weight average molecular weights could be computed from 90° turbidity data

$$H\frac{C}{\tau} = \frac{1}{\bar{M}_w} + 2BC \qquad (5)$$

$$H = \frac{32\pi^3}{3\bar{N}_0}\frac{n_0^2}{4}\left(\frac{dn}{dc}\right)^2 \qquad (6)$$

where

τ = the absolute turbidity
$\bar{M}_w$ = weight average molecular weight
$\bar{N}_0$ = Avogadro's number
n_0 = refractive index of solvent
λ = wave length of the incident beam
C = concentration
B = a constant
$\frac{dn}{dc}$ = refractive index gradient

A typical plot of C/τ *vs.* C (see for example Fig. 7), was extrapolated to infinite dilution to give C/τ^0 values equal to the reciprocal of the molecular weight in accordance with eq. 6. These data were corrected for the destructive interference caused by the greater forward than backward scattering of light by the procedure of Debye and co-workers.[30] Table VI summarizes the turbidity measurements and Table VII lists the molecular weight results and the correction factor $I_{90°}$ used in the molecular weight calculations in accordance with

$$H\frac{C}{\tau_0} = \frac{1}{\bar{M}_w I_{90°}} \qquad (7)$$

TABLE VI

MOLECULAR WEIGHTS FROM TURBIDITY MEASUREMENTS

Mucus sample I			Mucus sample II			Mucopolysaccharide fraction		
$C \times 10^4$	$\tau \times 10^3$	$C/\tau \times 10^2$	$C \times 10^4$	$\tau \times 10^2$	$C/\tau \times 10^2$	$C \times 10^3$	$\tau \times 10^3$	C/τ
0.59	2.67	2.210	2.98	1.100	2.710	4.08	0.57	7.16
1.12	5.35	2.093	5.63	2.157	2.611	7.80	0.67	11.63
1.6	7.68	2.083	8.01	3.286	2.436	11.18	1.18	10.03
2.38	11.81	2.01	10.14	4.209	2.409	14.88	1.15	12.94
3.02	15.26	1.973	12.07	5.354	2.254			
3.5	18.12	1.975	13.83	6.353	2.176			

Table VII also shows the molecular weight obtained for a sample of the polysaccharide fraction of the mucus, isolated according to the procedure of Bacila and Ronkin.[31]

It will be noted that the mucopolysaccharide–mucoprotein complex has an average molecular

(29) P. Debye, *J. Appl. Phys.*, **15**, 338 (1944).

(30) P. Debye, L. Bewilogua and E. Ehrhardt, *Z. Physik.*, **30**, 84 (1929).

(31) M. Bacila and R. R. Ronkin, *Biol. Bull.*, **103**, 296 (1952).

TABLE VII

MOLECULAR WEIGHT DATA

Sample no.	Composition	M_w	dn/dc	$I_{90°}$
1	Mucus	3.5×10^7	0.1559	0.487
2	Mucus	2.7×10^7	.1559	0.487
3	Polysaccharide	2.5×10^6	.0272	...

weight of 3.1×10^7 as compared with 2.5×10^6 for the mucopolysaccharide fraction. Since Busycon mucus is approximately composed of 50% mucopolysaccharide[18] it follows that both the mucoprotein and mucopolysaccharide are extremely high molecular weight materials. The molecular weight obtained for the polysaccharide fraction bears comparison with the values reported for hyaluronic acid isolated from sundry sources. For example, Blumberg and Oster[32] give a value of 8×10^6 for spherical particles of 2100 Å. diameter for hyaluronic acid isolated from umbilical cord and Rowen, Brunish and Bishop[33] report 1.27×10^6 for hyaluronic acid from vitreous humor. The diameter of the spherical particles of dried Busycon mucus is 208 Å. as calculated from the molecular weight and density of the dried sample according to

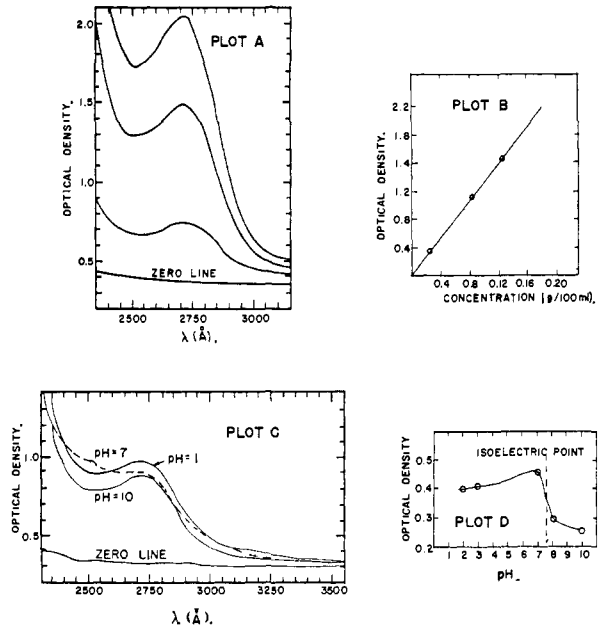

Fig. 8.—Ultraviolet spectral studies on mucus.

$$\overline{M}_w = \frac{4}{3}\pi r^3 \rho N_0 \qquad (8)$$

This result is quite close to the primary particle size observed in the electron micrographs. A comparison of this result with the particle size from the angular disymmetry measurements shows that there is an 9–10 fold increase in size or about 800–1000 fold increase in particle volume as a result of swelling by the solvent. The high degree of swelling accounts for the viscosity properties of mucus and provides a physical basis for the observed solvent-holding power of mucus.

(32) B. S. Blumberg and G. Oster, *Science*, **120**, 432 (1954).

(33) J. W. Rowen, R. Brunish and F. W. Bishop, *Biochem. & Biophys. Acta*, **19**, 480 (1956).

The negative slope of the C/T *vs.* C plot (see Fig. 7, *e.g.*) is contrary to the prediction of the eq. 5 from which it is derived and with experimental results usually obtained for standard non-ionic polymers. It is, however, quite characteristic of polyelectrolytes[34] and must be considered as an observation confirming the postulated polyelectrolyte nature of Busycon mucus.

Ultraviolet Spectral Studies.—Mucus solutions were found to have a distinct absorption band in the region of 250–300 mμ with a maximum at 272 mμ. This band was characterized as due solely to the protein fraction in mucus, since the isolated carbohydrate fraction did not exhibit this characteristic. Figure 8 shows some of the typical ultraviolet absorption properties of mucus, including a Beers–Lambert law plot B at 272 mμ, by means of which the concentration of mucus solutions may be determined. Plot 8c illustrates the sensitivity of spectral absorption with *p*H. This suggests that some new structural feature is (reversibly) produced in the low *p*H region. The variation of absorption at 272 mμ with *p*H, in fact, can be used to determine the isoelectric range of mucus as shown by plot 8D.

It has been reported by Sinsheimer, *et al.*,[35] that only the three amino acids phenylalanine, tyrosine and tryptophan have absorption spectra in the

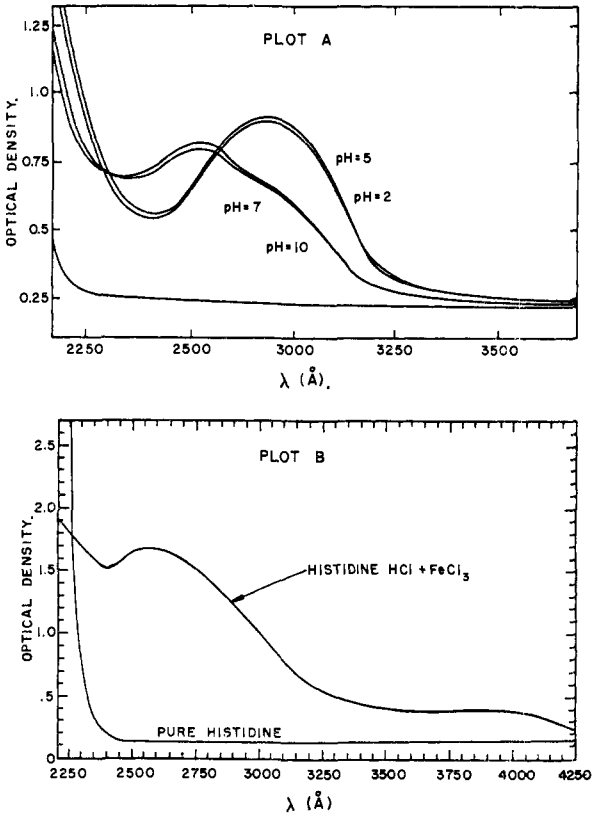

Fig. 9.—Comparison of the anomalous ultraviolet spectrum of mucus with that of the histidine hydrochloride–ferric chloride complex.

(34) P. Doty and J. T. Edsall, *Advances in Protein Chem.*, **6**, 37 (1951).

(35) R. L. Sinsheimer, J. F. Scott and J. R. Loofbourow, *J. Biol. Chem.*, **187**, 313 (1950).

ultraviolet range. It was also noted by Lerner[36] and Ferand[37] that proteins and polypeptides containing these amino acids have ultraviolet absorption in the 270–280 mμ range. The spectral properties of mucus may thus be taken to support the identification of two of the amino acid constituents, namely, phenylalanine and tyrosine.[38] The latter, it can be demonstrated, accounts very well for the observed spectral shifts with change in hydrogen ion on the basis of the familiar bathochromic displacement on conversion of phenols to phenolic anions. This type of behavior is strongly reminiscent of polyelectrolytes in general as characterized in the investigations of Katchalsky and Miller on synthetic polyelectrolytes such as polyvinylpyridine[39] and Butler, Conway and Janers[40] on natural polyelectrolytes of the nature of desoxyribonucleic acid.

Spectral Anomalies.—In several instances an anomalous spectrum was observed for a mucus sample. Instead of an absorption maximum at 272 mμ, a strong band appeared at 250 mμ, *p*H 7. This behavior could be found in both the freshly extracted as well as by the thoroughly dialyzed anomaly. It was suspected that some contamination had occurred which was not removable by dialysis.[41] Figure 9, Plot A illustrates that a substantial shift in λ_{max} from 250 to 268 mμ takes place in going from *p*H 7 to 5. The literature[42] reveals similar observations have been made in the instance of histidine samples contaminated by ferric ions. These experiments were repeated with pure histidine with and without added ferric chloride all shown in Fig. 9, plot B. It may be contended on this basis that a trace of ferric ion bound to the histidine constituent of the mucoprotein moiety of mucus was responsible for the spectral anomaly.

Acknowledgment.—We are obliged to Professor R. R. Ronkin of the Department of Biological Sciences of the University of Delaware and Dr. P. Hamilton of the A. I. du Pont Institute of Wilmington, Delaware, for their helpful discussions and valuable suggestions.

(36) A. B. Lerner and C. P. Barnum, *Arch. Biochem.*, **10**, 417 (1946).

(37) K. Ferand, M. S. Dunn and J. Kaplan, *J. Biol. Chem.*, **112**, 323 (1936).

(38) Results to be discussed in a future publication from these laboratories.

(39) A. Katchalsky and I. R. Miller, *J. Polymer Sci.*, **13**, 57 (1954).

(40) J. A. V. Butler, B. E. Conway and D. W. F. Janers, *Trans. Faraday Soc.*, **50**, 612 (1954).

(41) Subsequently, on reviewing the history of the anomalous samples, it was realized that contamination by traces of iron was indeed possible.

(42) F. Ellinger, *Biochem. Z.*, **215**, 279 (1929); **248**, 436 (1932).

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The Condensation of Nitromethane with D-Erythrose, D-Arabinose, D-Mannose and D-*glycero*-D-*gala*-Heptose in Aqueous Alkali

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The reaction of nitromethane with aldose sugars in aqueous alkali has been studied in the sequence of condensations necessary to convert D-erythrose to D-*erythro*-L-*manno*-octose by the nitromethane synthesis. In each instance, the less soluble of the pair of epimeric nitroalcohols produced was isolated without difficulty. In the reactions with D-mannose and D-*glycero*-D-*gala*-heptose, the deoxynitroalditols crystallized directly from the aqueous, alkaline reaction mixtures.

The condensation of nitromethane with aldose sugars usually has been carried out with sodium methoxide in absolute methanol, from which system the sodium salts of the deoxynitroalditols normally precipitate as the condensation reaction progresses.[1] In a previous communication[2] we reported the condensation of nitromethane with D-arabinose in aqueous sodium hydroxide solution to give, after hydrolysis of the intermediate 1-deoxy-1-nitro-D-mannitol, a 22% yield of D-mannose as the phenylhydrazone. The aqueous reaction conditions now have been extended to D-erythrose, D-mannose and D-*glycero*-D-*gala*-heptose to obtain, respectively, 1-deoxy-1-nitro-D-arabitol, 1-deoxy-1-nitro-D-*glycero*-D-*gala*-heptitol and 1-deoxy-1-nitro-D-*erythro*-L-*manno*-octitol.

In a single experiment with D-erythrose, nitromethane and aqueous sodium hydroxide, 1-deoxy-1-nitro-D-arabitol was isolated in only 7% yield. In view of the results obtained with aldoses of greater chain length, it is believed that the isolation by crystallization of the deoxynitropentitol was inhibited in this instance by the presence of a considerable amount of unchanged D-erythrose. D-Arabinose in aqueous, alkaline nitromethane, under the best of a variety of reaction conditions, provided crude 1-deoxy-1-nitro-D-mannitol in 17% yield, and the unchanged starting sugar was recovered in 40% crude yield. Of most interest in the present work were the condensations of D-mannose and D-*glycero*-D-*gala*-heptose with nitromethane. Here, the 1-deoxy-1-nitro-D-*glycero*-D-*gala*-heptitol and 1-deoxy-1-nitro-D-*erythro*-L-*manno*-octitol crystallized directly from the aqueous, alkaline reaction mixtures in 11 and 25% yield, respectively, and further amounts of the deoxynitroalditols were obtained from the filtrates.

The present work, taken with previous observations on the nitromethane–aldose condensation reaction,[1,2] leads us to the following conclusions. In homogeneous alkaline solution, in either methanol or water, the aldoses, nitromethane and the deoxynitroalditols establish an equilibrium. The equilibrium may be displaced in favor of the deoxynitroalditols either by their precipitation as alkali

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