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

The Structure and Constitution of Mucus Substances. II. The Chemical Constitution of Busycon Mucus

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The amino acid constitution of the protein portion of Busycon mucus has been elaborated. Ornithine was found to be most unusual among the seventeen amino acids present. Glucosamine and galactosamine have been identified by chromatographic means. The mole ratio of acidic to basic amino acids was established to be almost three. A relatively large amount of calcium was noted among the several metal ions shown to be present in the purified residue after dialysis and lyophilization. A sulfur constituent has been shown to be entirely in the form of ionic sulfate which is labilized without either acid or base. A structural hypothesis portraying Busycon mucus as a polyhexoseammonium sulfate linked to an acidic protein moiety through calcium ions appears to correlate all the available data and to be consistent with certain specific tests which have been contrived. The possible generality of this type of structure and constitutional similarities between Busycon mucus and other mucus substances are also considered. Synthetic analogs have been prepared in the interest of demonstrating how the structural features account for some of the physical properties of Busycon mucus.

In a previous paper[2] the physical properties of a mucus derived from the large marine snail, *Busycon Canaliculatum L.*, were shown to be due to its polyelectrolyte nature. It was found that this mucus is a high molecular weight polymer (about 3×10^7) consisting of a polysaccharide (mol. wt. approx. 2.5×10^6) complexed with a protein and that this combination behaved as a swollen, spherical molecule in solution. Our objective in the present undertaking was to determine the chemical structures responsible for this behavior, the mode of linkage of the polysaccharide to the protein, and to compare the mucus derived from this relatively simple source with the various mucus substances which are to be found in a large number of biological fluids.

Experimental

Preparation of the Samples.—The mucus was extracted by Ronkin's[3] method according to the procedure outlined in reference 2. The inorganic analyses were carried out on samples extracted with either 0.5 *N* sodium chloride or 0.5 *M* ammonium acetate. All the preparations were centrifuged in a Sorvall centrifuge to obtain cell-free extracts before further examination. The analyses on the dry mucus were performed on lyophilized material. No attempt was made to remove the last traces of water usually associated with freeze-dried substances. The lyophilized mucus was always obtained as a fluffy white fibrous product.

Analyses of the Inorganic Ash.—The ash content was determined by pyrolysis of the dried mucus in silica crucibles at 900° for 3 hr. in a furnace. The emission spectrum of the ash was analyzed by means of a Bausch and Lomb spectrograph and the elements were identified by their spectral bands and lines according to published data.[4] The quantitative analyses for sodium and calcium were performed on solutions of the ash in dilute sulfuric acid (*ca.* 0.1 *N*) using flame photometry. For this a hydrogen–oxygen flame was used in a Beckman D. U. apparatus and spectral lines at 422 and 589 mμ were examined for sodium and calcium, respectively. The analytical procedure was similar to that described by Hawk, Oser and Summerson[5] and the Beckman Flame Photometry manual.[6] In each determination stock solutions of calcium sulfate and sodium sulfate were prepared as controls and compared to the unknown. The concentration of the standards was adjusted by dilution until two solutions encompassing the unknown were obtained. In each case an effort was made to be as similar in concentration to the test sample as possible.

Fractionation of the Mucus.—The following is a typical fractionation procedure, adapted from the method of Bacila and Ronkin,[7] for the gross fractionation of this mucus into its component polysaccharide and protein fragments. A 100-ml. solution of the mucus (about 0.2 g. in 0.5 *N* NaCl at *p*H 7) was stirred with 100 ml. of chloroform for 16 hr. at room temperature. The resultant milky product was then centrifuged (10 min. at 2600 r.p.m.) to give a clear aqueous layer above a clear chloroform layer with the denatured protein as a precipitate at the interface. Next, the aqueous layer was isolated and the fractionation continued with a fresh quantity of chloroform; the process was repeated until there was no further precipitate at the interface (generally four such treatments were sufficient). The combined protein fractions were then filtered, washed with distilled water to remove any adhering carbohydrate fraction and then dialyzed for three days against a continuous flow of distilled water to remove salts. The product was next lyophilized to give a white fibrous protein (approx. 40 mg.). The carbohydrate fraction remained in the aqueous layer. It was purified by dialysis for three days and lyophilized to give a white fibrous product (approx. 30 mg.). The carbohydrate fraction gave a positive Molisch and a negative biuret test, while the protein fraction gave the opposite results. The biuret and Molisch tests were carried out according to the methods described in Hawk, Oser and Summerson.[5]

A study of the conditions for mucus fractionation by the chloroform technique showed that an electrolyte was necessary in order to cause the separation of the polysaccharide–protein complex; a dialyzed sample gave no precipitate even on prolonged stirring with chloroform. Furthermore, the type of salt in the aqueous phase did not appear to be critical; for example, NH_4Cl, NH_4Ac, $(NH_4)_2SO_4$ were as effective as NaCl. It seemed very clear that the protein was denatured by the chloroform only in the presence of an electrolyte.

Hydrolysis of the Mucus.—The mucus was hydrolyzed into its component amino acids and carbohydrates with 6 *N* HCl at 120°. The method consisted of heating 20–40 mg. of mucus in a sealed tube with 20 ml. of the acid for 48 hr. When the polysaccharide–protein complex was hydrolyzed, a black precipitate was obtained together with a brown solution of the amino acids, whereas the isolated protein gave clear yellow solutions and no precipitate. The products of hydrolysis were isolated and purified according to the method described by Block, Durrum and Zweig.[8] The amino acids and carbohydrates were then either dissolved in a 10% solution of 2-propanol in water for paper chromatographic studies or in a citrate buffer for ion-exchange chromatographic analysis.

Table I summarizes the results of a study of a number of different hydrolytic conditions. It was found that the use

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

(2) H. Kwart and V. E. Shashoua, THIS JOURNAL, **80**, 2230 (1958).

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

(4) W. R. Brode, "Chemical Spectroscopy," John Wiley and Sons, Inc., New York, N. Y., 1943.

(5) P. B. Hawk, B. L. Oser and W. H. Summerson, "Practical Physiological Chemistry," 13th Ed., 1954, p. 653.

(6) Beckman Instruction Manual 334-A for Model DU and B Flame Spectrophotometers, Will Corp., Rochester, N. Y., 1954.

(7) M. Bacila and R. R. Ronkin, *Biol. Bull.*, **103**, 296 (1952); see also E. A. Kabat, "Blood Group Substances," Academic Press, New York, N. Y., 1956, p. 130.

(8) R. J. Block, E. L. Durrum and A. Zweig, "Paper Chromatography and Paper Electrophoresis," Academic Press, Inc., New York, N. Y., 1955.

of sulfuric acid as the reagent for hydrolysis gave poor results in spite of the fact that the mucus completely dissolved in the acid. Alkaline hydrolysis with barium hydroxide also failed to give good results.

The isolated carbohydrate fraction was hydrolyzed with 4 *N* HCl for four hours on a boiling water-bath and examined by paper and ion exchange chromatography for its components.

TABLE I

HYDROLYSIS OF MUCUS AT 120°

Solvent	Hydrolysis time (hr.)	Results
4 *N* H_2SO_4	24	Valine
6 *N* H_2SO_4	48	Alanine
10% $Ba(OH)_2$	48	Aspartic acid, glucose, alanine
6 *N* HCl	24	17 amino acids[b] and glucosamine
6 *N* HCl	48	17 amino acids[b] and glucosamine
4 *N* HCl[a]	24	17 amino acids[b]

[a] This analysis was performed on the isolated mucoprotein fraction. [b] See Table IX for details.

Paper Chromatographic Analysis.—The qualitative composition of the hydrolyzates of the mucus and the isolated mucoprotein fraction was determined by descending paper chromatography. The techniques employed were essentially those described in Block, Durrum and Zweig.[8] Three separate solvent systems were used to analyze and compare the unknown mixtures with standards on Whatman #1 papers. These were the phenol–water (4/1) mixture of Consden, Gordon and Martin[9] using sodium cyanide to evolve hydrogen cyanide for stabilization of the phenol; the lutidine–collidine–water mixture with 2% added diethylamine of Dent[10] and the recently described solvent of Connell and Haines,[11] *n*-propanol–phosphate buffer [80/20 (0.03 *M* *p*H 6.7)]. This latter solvent has the advantage that traces of salts do not hinder the motion of the amino acids. In the use of the propanol solvent, the papers were first thoroughly soaked with the buffer and dried before placement of the amino acids and the unknown mixtures. All the constituents of the solvents were obtained from Eastman Organic Chemicals Co., with the exception of the phenol which was obtained from Mallinkrodt Chemical Co.

Table II shows the R_F values for the standard amino acids using the 1-propanol–phosphate buffer together with the specific colors of the spots obtained immediately after spraying with ninhydrin reagent (0.1% in acetone) and drying (1–2 min. at 80°). These colors present an additional means of identification of the amino acids. The R_F values for the other two solvents used in this work have been published elsewhere.[9]

Ion-exchange Chromatography.—The quantitative analysis of the protein was carried out by ion-exchange chromatography using the techniques of Moore and Stein[12] as modified by Hamilton and Anderson.[13] A 10-mg. sample was used for each analysis on a column (100 cm. × 1 cm.) of Dowex 50 × 8 resin. The acidic and the neutral amino acids were determined in a separate experiment from the basic amino acids using a buffer sequence of 0.1 *M* *p*H 3 citrate at 40° followed by 0.1 *M* *p*H 4.5 citrate as shown in Fig. 2. The basic amino acids were eluted with a series of three buffers: 0.1 *M* citrate *p*H 3.83, 0.1 *M* phosphate containing 0.01% Triton X-155 and 1.5% benzyl alcohol at 25°, 0.1 *M* citrate *p* 3.41 (see Fig. 1). The tyrosine content of the phenylalanine–tyrosine mixture was estimated by the method of Thomas.[14] The proline and ornithine were analyzed by the method of Chinard.[15]

Degradation of Chitosan.—Chitosan (essentially a poly-2-aminoglucose from hydrolyzed crab shells)[16] was degraded into a water-soluble fraction for use in studies on synthetic models of mucus. The method consisted of soaking 20-g. samples of chitosan in 500 ml. of hydrochloric acid at *p*H 3 for different times and temperatures. The change in the intrinsic viscosity of the isolated product in 0.25 *N* NaCl with time was used as the criterion of the degradation efficiency. Table III shows the results obtained. It was found that the most effective means of obtaining a low viscosity product was a 3 hr. treatment at 100°. This material was then neutralized with an excess of NH_4OH, dialyzed for three days and the precipitate and solution in the dialysis bag lyophilized. This preparation was used in the synthetic model studies.

TABLE II

R_F VALUES OF THE STANDARD AMINO ACIDS WITH 1-PROPANOL–PHOSPHATE BUFFER (0.03 *M* *p*H 6.7)

Amino acid	R_F value	Ninhydrin color
α-Alanine	0.34	Violet
Arginine	.07	Violet
Aspargine	.12	Brown
Aspartic acid	.07	Green
L-Glutamic acid	.09	Violet
Glutamine	.16	Pink
Glycine	.21	Red
Histidine hydrochloride	.08	Red
Hydroxylysine	.05	Violet
L-Hydroxyproline	.25	Red
L-Isoleucine	.56	Violet
L-Leucine	.60	Violet
L-Lysine hydrochloride	.56	Violet
L-Methionine	.47	Pink
L-Phenylalanine	.54	Gray
L-Proline	.40	Yellow
DL-Serine	.18	Gray
D-Threonine	.24	Gray
Tyrosine	.65	Violet
DL-Valine	.46	Pink

TABLE III

DEGRADATION STUDIES ON CHITOSAN

Temp. = 22°		Temp. = 100°	
Hydrolysis time, hr.	$[\eta]$	Hydrolysis time, hr.	$[\eta]$
0	2.55	0	2.55
24	2.08	3	0.98
120	1.96		

Results and Discussion

Freshly extracted Busycon mucus solutions (0.2% to 0.5% solids) contain a substantial amount of inorganic matter bound to an organic fragment. The ratio of organic to inorganic content appears to depend upon the extent of dialysis to which the sample is subjected. Table IV summarizes a study of the solids and ash content of mucus. It is seen that the undialyzed samples had as much as 12% inorganic material while the completely dialyzed samples contained only 5% inorganic ash.

TABLE IV

Expt.	Extracting solvent	Mucus solids content, %	Ash content, %
1	0.5 *N* NaCl	0.25[a]	
2	.5 *N* NaCl	.40[a]	11.9
3	.5 *M* $(NH_4)_2Ac$	.69	4.85
4	.5 *M* $(NH_4)_2Ac$	1.7	4.6

[a] The solids content of these samples was determined as the difference in the solids content of the extracting solvent and the mucus solution.

Composition of the Inorganic Portion of the Mucus.—The elements present in the ash from

(9) R. Consden, A. H. Gordon and A. J. P. Martin, *Biochem. J.*, **38**, 224 (1944).

(10) C. E. Dent, *ibid.*, **43**, 169 (1948).

(11) G. E. Connell and C. S. Haines, *Nature (London)*, **177**, 344 (1956).

(12) S. S. Moore and W. H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).

(13) P. Hamilton and R. A. Anderson, *ibid.*, **213**, 249 (1955).

(14) L. E. Thomas, *Arch. Biochem.*, **5**, 175 (1944).

(15) F. P. Chinard, *J. Biol. Chem.*, **199**, 91 (1952).

(16) P. F. Viktrov and I. M. Malofis, *C. A.*, **38**, 874 (1944).

mucus samples before and after dialysis were determined by emission spectroscopy. Table V summarizes the results. No search in the spectral region of the heavy metals was carried out for the undialyzed sample. The significant fact in Table V is the presence of sodium and calcium in major amounts: the other elements can be accounted for by the equilibration of the mucus with sea water in the live animal.

TABLE V

ELEMENTS IN THE ASH OF MUCUS[a]

Before dialysis			After dialysis		
Major	Minor	Trace	Major	Minor	Trace
Na	Sr	Ba	Na	Mg	Ba
Ca	Li		Ca	Si	Sr
				Cu	Ag
				Al	
				Fe	

[a] Minor = less than 1000 p.p.m. based on the ash; trace = less than 250 p.p.m. based on the ash.

Composition of the Organic Portion of Mucus.—Busycon mucus was fractionated by Bacila and Ronkin[7] into a protein fraction, which gave a positive biuret test and a negative Molisch test, and a polysaccharide fraction which gave the opposite results. In an attempt to determine the ratio of the polysaccharide to the protein, a mucus sample was extracted with ammonium acetate and divided into two portions. The first portion was dialyzed, lyophilized, weighed and ashed to give a result of 5% ash content. The second sample was fractionated into its component polysaccharide and protein, then each portion was dialyzed, lyophilized and ashed separately. The protein fraction was found to give no weighable ash, indicating that the inorganic portion of the mucus is more tightly associated with the carbohydrate fraction which had an ash content of 12%. This result gives a carbohydrate to protein ratio of about 40/60 for the complex, which agrees well with the value of 43 to 57 obtained by direct weighing of the fractions.

Properties of the Polysaccharide Fraction.—The freshly separated polysaccharide fraction was found to be acidic in nature, giving a solution of *p*H 4.5–5.9 in 0.5 *N* NaCl. Moreover, samples purified by dialysis and lyophilization were found to retain their solubility in water and to give acid solutions of *p*H 5.9. The solution had a blue opalescent appearance and turned colorless upon the addition of alkali. This is indicative of a change in particle size with *p*H and may be due to changes in degree of association of the molecules.

TABLE VI

ANALYSIS FOR Na^+ AND Ca^{++}

Sample no.	Extracting solvent	Amount of mucus, ml.	Treatment	Analysis, g. × 10^6 Ca^{++}	Na^+	Amount of ash, g. × 10^6	Mole ratio Na^+/Ca^{++}
1	0.5 *N* NaCl	0.0537 g.[a]	Not dialyzed	550	..	6.4	..
2	.5 *M* $CaAc_2$	..	Not dialyzed	..	140	..	..
3	.5 *N* NH_4Ac	50	Not dialyzed	466	3726	21	14
3A	.5 *N* NH_4Ac	50	Dialyzed	224	126	2.	1
4	.5 *N* NH_4Ac	30	Not dialyzed	625	2270	9.5	63.5
4A	.5 *N* NH_4Ac	30	Dialyzed 8 hrs.	312	105	3.8	1.7
4B	.5 *N* NH_4Ac	30	Dialyzed 3 days	25	76	[b]	5.5

[a] The weight of the mucus was determined by a difference in the sample and solvent solids content. [b] The ash could not be accurately weighed.

A quantitative analysis of the sodium and calcium content of the ash from the mucus of 50 snails was carried out by flame photometry. The results of this study are shown in Table VI. It is seen that sample 1, extracted with 0.5 *N* NaCl contained calcium, while sample 2, extracted with 0.5 *M* $CaAc_2$, had a substantial amount of sodium. A study of the effect of dialysis on the change in the composition of the ash from mucus samples extracted with 0.5 *N* NH_4Ac, showed that the mole ratio of Na^+/Ca^{++} depended on the extent of dialysis. Thus in experiments 4, 4A and 4B the mole ratio of Na^+/Ca^{++} decreased from an initial value of 63.5 to 1.7 at an intermediate stage of dialysis and finally became 5.5 at the end of the dialysis. This was accompanied by a progressive decrease in the quantity of ash in the mucus (see Table VI). A similar result was obtained for experiments 3 and 3A. The significance of these changes will be discussed in the section on "structural hypothesis" to follow.

The addition of $BaCl_2$ to a solution acidified with HCl gives an immediate precipitate of $BaSO_4$ indicating the presence of sulfate[17] groups in the polymer. This was confirmed by ashing the carbohydrate fraction and observing the sulfate in the ash. Also a sulfur analysis (7.2%) gave additional proof.

Bacila and Ronkin[7] have shown that the carbohydrate fraction is based on galactosamine and glucosamine. Their observation has been confirmed here by means of paper and ion-exchange chromatographic studies on the hydrolyzates of both the total protein–carbohydrate complex and the isolated carbohydrate fraction. The hydrolyzates from the latter (prepared with 4 *N* HCl at 100° for 4 hr.) showed the presence of both 2-aminoglucose and 2-aminogalactose in almost equivalent amounts. Furthermore, there were no uronic acids present, confirming the infrared analysis (*vide infra*). On the basis of these and other general considerations it is possible to postulate a structure for the polysaccharide fraction in which aminohexose units may contain a labile sulfate residue[17] with either a calcium or a sodium contra-ion. The elemental analysis of the polysaccharide fraction (Table VII) seems

(17) This deduction is to be distinguished from sulfamate or sulfate ester residues which are only labilized by acid hydrolysis. This distinction was recognized, for example, by Wolfrom and co-workers[18] in formulating a structure of heparin.

(18) M. L. Wolfrom, D. L. Weisblat, J. V. Karabinos, W. H. McNeely and J. McLean, THIS JOURNAL, **65**, 2077 (1943); M. L. Wolfrom and R. Montgomery, *ibid.*, **72**, 2861 (1950); M. L. Wolfrom, R. Montgomery, J. V. Karabinso and P. Rathgeb, *ibid.*, **72**, 5796 (1950). See also J. E. Josephs, H. Bostrom and V. Mutt, *J. Biol. Chem.*, **183**, 607 (1950).

to fit fairly well to a structure in which approximately two thirds of the aminohexose units are sulfated and there are roughly one tenth as many calcium contra-ions as sodium.

This structure is admittedly only a rough estimate since it is based on analyses of different batches of the polysaccharide which, depending on the extent of dialysis, are known to have different inorganic content. The postulated structure is in some measure confirmed by infrared analysis. The absorption bands characteristic of $-NH_2$, $-SO-$, $-SO_2-$ and $-OH$ are found at 6.15, 8.15, 9.4 and 2.9 μ, respectively. The bands corresponding to carboxyl, carboxylate anion and $-CONH-$ are conspicuously absent from the spectrum of the carbohydrate fraction indicating that there is no possibility within the accuracy of the infrared analysis of having an N-acetylglucosamine or a galacturonic type of structure. Furthermore, it is interesting to note that the RNH_3^+ absorption is clearly evidenced by a band at 6.15 μ.[19] Therefore, we may conclude from this and from the absence of carbonyl absorption in the infrared spectrum of the polysaccharide that the acidic nature of this fraction, demonstrated earlier by *p*H measurement, is not due to a hexuronic acid component but rather to the ammonium ion substituent on the carbohydrate backbone.

Properties of the Protein Fraction.—The protein fraction is isolated by chloroform fractionation method (see Experimental Section) as a denatured white fibrous precipitate. It is insoluble in acids but quite soluble in alkalies indicating that the protein is predominantly acidic in character. In addition, it appeared to be quite free from any measurable quantity of inorganic ions, since there was no ash obtained on pyrolysis. All the ash was associated with the carbohydrate fraction. Table VII shows a quantitative analysis of the elements in the protein and polysaccharide fractions. The products appear to be quite distinct. The infrared spectrum of the protein confirmed its polypeptide structure, showing the presence of bands at 6.1, 6.5 and 2.95 μ corresponding to $-CONH_2-$, carbonyl and $-NH$ bands. The spectrum also illustrated that the protein fraction was quite free from any detectable carbohydrate fraction.

TABLE VII

ELEMENTAL ANALYSIS

	Polysaccharide fraction	Protein fraction
% C	23.4	47.9
% H	4.6	6.6
% N	4.86	11.3
% S	7.2	..
% Ash	12.2	0

Chromatographic Analysis.—The results from paper chromatographic studies on the hydrolyzates of the mucus and its isolated mucoprotein fraction indicated that qualitatively there could be at least ten and as many as seventeen amino acids plus glucosamine present in the approximately ten spots on the chromatograms. Table VIII shows an arrangement of the amino acids in groups of about the same R_F value in order of increasing R_F values as found for each of the three solvent systems examined.

(19) L. J. Bellamy, "The Infrared Spectra of Complex Nuclei," Methuen and Co., London, 1954, p. 20, has stated that a band at 6.02–6.21 μ is characteristic of the ammonium ion.

TABLE VIII

PAPER CHROMATOGRAPHY

AMINO ACIDS ANALYSIS BY DIFFERENT SOLVENTS[a]

Phenol–water (4/1) + HCN	Lutidine–collidine–water (1/1/1) + 2% Et_2NH	*n*-Propanol–phosphate buffer *p*H 6.7, 0.03 *M* (80/20)
Aspartic acid	Ornithine	Ornithine
	Lysine	
Glutamic acid	Arginine	Aspartic acid
		Histidine
Cystine	Aspartic acid	
D-Glucoseamine		Arginine
	Glutamic acid	Lysine
Serine		
	Glycine	Glutamic acid
Glycine		
Histidine	Serine	Serine
	Histidine	Glycine
Threonine	Alanine	Threonine
	Serine	
Tyrosine	Proline, threonine	Alanine
Alanine		
	Valine	Proline
Ornithine		
Leucine	Tyrosine	Valine
Valine		
Isoleucine	Leucine	Leucine
Phenylalanine	Isoleucine	Isoleucine
		Phenylalanine
Proline	Phenylalanine	
Arginine		Tyrosine
Lysine		

[a] The amino acids above are arranged in groups which appear as single spots on the chromatograms in order of their increasing R_F values.

Figures 1 and 2 depict the results of the ion-exchange chromatographic studies (on Dowex 50-X8 columns) for the protein hydrolyzates. The presence of seventeen different amino acids is quite evident from the diagrams. In Fig. 1 the use of a citrate buffer at *p*H 3.83 removed all the acidic and neutral amino acids after about twenty fractions so that the basic amino acids were easily separable. In Fig. 2 there remained one small unidentified peak. This and the ammonia "peak" were not taken into account in the calculation of the quantitative amino acid content of the protein. Table IX summarizes the results obtained. It is of interest to note that there are four basic amino acids; arginine, lysine, histidine and ornithine in the protein. The presence of ornithine is especially noteworthy. Moreover, the two acidic amino acids, aspartic acid and glutamic acid are present in quite large proportions so that the mole ratio of acidic to the basic amino acids in this protein is about 2.96 indicating a predominant polyacid character. This is quite in keeping with ease of solubility of the protein in dilute alkaline solutions.

Structural Hypothesis.—Any proposed structure for this mucus must contain four structural features. These are a polysaccharide fragment based on a hexosamine, a sulfate ion attached to it, a

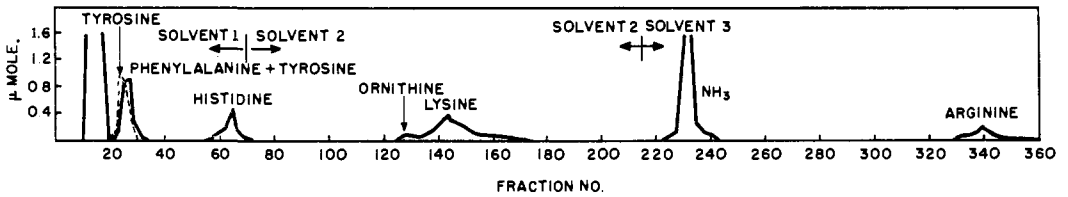

Fig. 1.—Ion-exchange chromatogram of the basic amino acids in the mucoprotein fraction; solvent 1 = 0.1 *M* citrate buffer, *p*H 3.83; solvent 2 = 0.1 *M* phosphate buffer; solvent 3 = 0.1 *M* citrate buffer, *p*H 3.41.

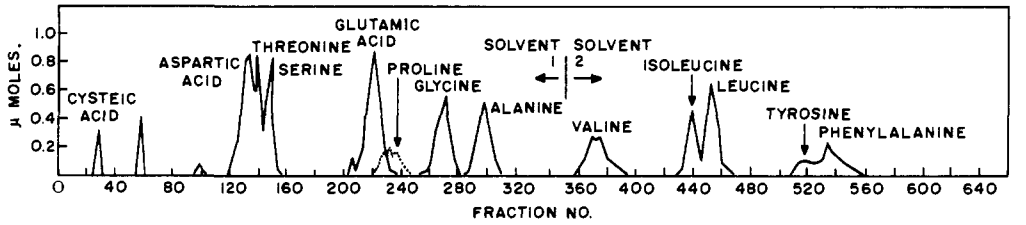

Fig. 2.—Ion-exchange chromatogram of the acidic and monoaminomonocarboxylic amino acids in the mucoprotein fraction; solvent 1 = 0.1 *M* citrate buffer, *p*H 3; solvent 2 = 0.1 *M* citrate buffer, *p*H 4.5.

protein fraction with a predominance of acidic groups and finally two inorganic ions Na^+ and Ca^{++}. If we consider the polysaccharide fraction, the sulfate ion has two favorable sites of attachment. The first is on the 2-position as an alkyl ammonium sulfate and the second is on the 6-position of the hexosamine as a sulfate ester. In chondroitin sulfate the sulfate was shown by Meyer[20] to be attached to the 6-position. However, in this mucus it seems more likely to be attached at the 2-position as an alkyl ammonium sulfate since here, unlike the case of chondroitin sulfate, the amino group is not acetylated,[21] as shown by the infrared evidence. The protein fraction, on the other hand, has a mole ratio of 2.96 for acidic to basic amino acids indicating that it may be considered as a polyacid. This is well supported by quantitative analysis and the fact that the protein, even in the denatured state, is soluble in 0.1 *N* alkali.

TABLE IX

QUANTITATIVE ANALYSIS OF THE PROTEIN FRACTION

Amino acid	μmoles	Weight, %
Cysteic acid	1.8	1.88
Aspartic acid	10.0	11.27
Threonine	5.9	11.23
Serine	5.0	9.09
Glutamic acid	12.0	15.28
Proline	2.70	2.69
Glycine	7.12	4.63
Alanine	7.23	5.57
Valine	6.00	6.08
Isoleucine	4.39	4.98
Leucine	8.13	9.22
Tyrosine	4.04	6.31
Phenylalanine	1.05	1.53
Histidine	2.28	3.41
Ornithine	0.30	0.35
Lysine	3.41	4.32
Arginine	1.42	2.14

One very attractive possibility that unites all the above structural elements is the assumption that the polysaccharide bisulfate fraction is complexed through the divalent calcium to the protein as shown in Fig. 3, the result being an ionically cross-linked polyelectrolyte. Thus the sulfate ion is most probably linked through the calcium ion to a carboxylic group from either a glutamic acid or an aspartic acid residue in the protein. The polysaccharide fraction has also a large number of sulfate groups in which sodium has replaced calcium as the other contra-ion, probably linked to the amino groups.

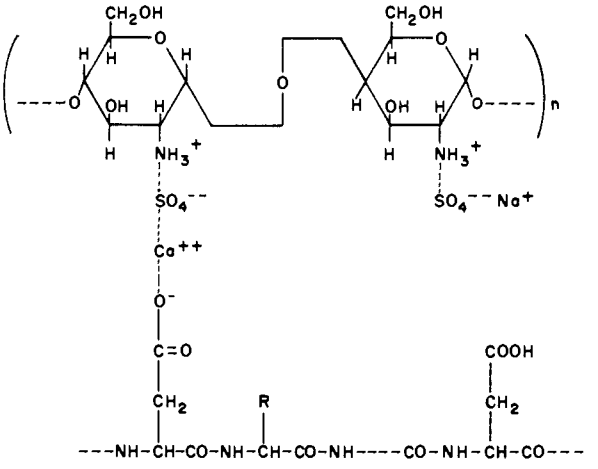

Fig. 3.—Structural hypothesis for Busycon Mucus; the α-D-(1 → 4)-linkage is assumed.

Evidence for the Structure.—The structure shown in Fig. 3 appears to agree quite well with the experimental evidence. It is of interest to list how some of the observations on this mucus are explained by such a structure.

(a) It is found that on fractionation all the inorganic ions remain associated with the polysaccharide fraction, since the protein shows no ash content. This is well-explained by the fact that the bond between sulfate and calcium is less susceptible to hydrolysis than a bond between the calcium and carboxylate ions.

(20) K. Meyer, *Disc. Faraday Soc.*, **13**, 271 (1953).

(21) The absence of acetylation of the amino function in heparin has been correlated[18] with a sulfamate linage.[17]

(b) It is found[8] that this mucus can be extracted by salt solutions containing any type of monovalent cationic salts, whereas no extraction is possible by salt solutions of divalent or polyvalent cations. This is explained by the fact that monovalent ions can exchange with the calcium and hence decrease the branched or cross-linked nature of the mucus to give more solubility; whereas divalent or polyvalent ions can only increase the degree of cross-linking by the same mechanism to make the mucus much less soluble.

(c) It is found that fractionation by the chloroform method is only possible when there is a univalent metal salt in the mucus solution, whereas no fractionation is possible in distilled water. This is explained by the fact that the protein, in an aqueous solution, is well bound to the solubilizing carbohydrate fraction. In an aqueous salt solution, however, ion exchange can take place to release the protein, which becomes denatured upon contact with chloroform.

A Test of the Structure.—One possible method for testing the structural hypothesis for mucus was obtained from the following experiments with sequestering reagents. It is known that disodioethylenediamine tetraacetate (Versene) complexes calcium. This suggested that, if Versene could be induced to complex with the calcium in the mucus, the weakly acidic protein would precipitate from a solution of even intermediate acid strength.

Two experiments were carried out in order to test this idea. The first consisted of stirring a freshly extracted mucus solution from 30 snails with a 5% Versene solution at a *p*H of 4.2, for 24 hr. followed by dialysis for three days against Versene solution and three days against distilled water. The dialysis was observed throughout its course, and it was not until the distilled water had removed a substantial amount of the Versene that a fibrous precipitate had formed in the dialysis bag. This was centrifuged from the mixture and found to give a positive biuret test. Moreover, this protein was not denatured. It was quite soluble in water and was only denatured when strong hydrochloric acid (6 *N*) was added. As a check for these observations the mucus sample was analyzed for sodium and calcium before and after the Versene treatment. It was found that over half the calcium had been removed in spite of the fact that the rate of complexing of Versene with calcium is very low at *p*H 4.2. Table X lists the results.

TABLE X

Expt. no.	Initial analysis, g. × 10³ Ca	Na	Final analysis, g. × 10⁴ Ca	Na
I	4.66	3.73	2.17	6.0
II	3.72	2.99	2.12	1.2

The second experiment was run at *p*H 8 in a similar manner to the first experiment. Here the dialysis was allowed to proceed for two days. No visible difference was noted in the mucus solution, but when a portion was acidified with 6 *N* HCl, a white precipitate of the protein appeared immediately.

Comparison of Busycon Mucus with Other Mucus Substances.—The essential points in the structure of Busycon mucus are a polysaccharide fragment linked to a protein through a calcium atom. One might ask as to how widely can this concept be applied? While a great deal of work had been done on the polysaccharide fragments of various mucus materials, comparatively little attention has been given to the protein fraction. In many cases no investigation of the inorganic cation constituents has been reported. Table XI lists literature data for three mucus materials in addition to Busycon mucus; cases in which the polysaccharide fragment has been identified for sulfur, hexosamine and the protein constitution has been examined for the mole ratio of acidic to basic amino acids.

The results in Table XI demonstrate a certain amount of generality. The acidic amino acids are present in approximately twice the molar proportion of the basic amino acids in organic constituents of compact bone,[22] human plasma mucus MP-I[23] and ovomucoid.[24]

TABLE XI

RELATION OF BUSYCON MUCUS TO OTHER MUCUS SUBSTANCES

Mucus source	Carbohydrate fraction composition	Protein fraction molar ratio of acidic/basic amino acids
Bone[22]	Galactosamine	2.32
	Glucosamine	
	Sulfur[a]	
Human plasma MP-I[23]	Hexoseamine	1.86
	Hexuronic acid	
	Sulfur[b]	
Ovomucoid[24]	Glucosamine[25]	1.87
Busycon mucus	Glucosamine	2.96
	Galactosamine	
	Sulfur[c]	

[a] Obtained after labilizing the sulfur by means of acid hydrolysis; probably in the form of sulfamate or sulfate ester. [b] The authors assumed the sulfur to be in the form of a sulfate ester. S. Schiller and F. K. Dewey[26] report that both hyaluronic acid and chondroitin sulfuric acid are present in human plasma. More recently Winzler[27] has noted that a sulfate free material can be obtained after treatment with an ion exchange resin. [c] Identified as ionic sulfate in this paper.

The results appear to indicate that Busycon mucus may possess certain important structural features in common with other mucus substances. The role of polyvalent inorganic ions such as calcium in the ionic linking of the mucopolysaccharide to the protein constituent of mucus clearly requires further attention as to its generality in other protein–mucopolysaccharide complexes.[28] Also, the observations by Grant[29] to the effect that intravenous injection of calcium ion inhibits the evolution of gastric mucus is certainly suggestive of the possibility that calcium ion may also be a significant factor in the constitution of mucus substances of importance in the human physiology.

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Synthetic Analogs of Busycon Mucus.—Broadly speaking a synthetic analog must be constituted of two acidic polyelectrolytes linked by a calcium ion. It should possess the viscosity properties of mucus as well as the unique characteristics[2] of "elastic recoil" and "stress-fibrilation." Two attempts were made to simulate this mucus behavior by reproducing synthetically these basic structural elements.

First, a 3% solution of polyacrylic acid ([η] 0.8) in water was mixed with calcium carbonate forming a product with 7.61 acrylic acid residues per calcium atom. The pertinent physical properties were qualitatively very similar to those of Busycon mucus. Moreover, it was found that addition of 0.5 *N* NaCl caused a decrease in these properties that was also reminiscent of Busycon mucus behavior.[2]

Finally, a somewhat closer analog was prepared from poly-2-aminoglucose bisulfate and polyacrylic acid. A solution of partially acid degraded chitosan was treated with an excess of sodium bisulfate, dialyzed and the volume subsequently adjusted to an 8% solution. This solution was stirred into an equal volume of 3% aqueous polyacrylic acid and followed by the addition of 2 ml. of 0.1 *N* $Ca(OH)_2$. The fresh mixture evidenced the typical behavior of Busycon mucus but with time further cross linking appears to have occurred with accompanying gelation.

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NEWARK, DELAWARE

COMMUNICATIONS TO THE EDITOR

A NEW SYNTHESIS OF IRONES

Sir:

Since the isolation of irone from orris root by Tiemann and Krüger[1] in 1893, various syntheses have been attempted. Studies of the structure of irone by Ruzicka and co-workers[2] in 1933 were followed in 1940 by their synthesis of 6-methylionones[2] later identified with the irones. In 1947 Naves and co-workers[3] reported a practical synthesis of 3-methylcitrals and 3-methyllinalool from which irone could be made. Another approach was made in 1952 by Grütter, Helg and Schinz[4] using thujacetone for the synthesis of 3-methylcitrals and their isomers. More recently the total synthesis of 5,6-dimethyl-5-hepten-2-one from methyl isopropenyl ketone, acetylene and diketene led to a marked improvement in the synthesis of irone in about eight steps.[5]

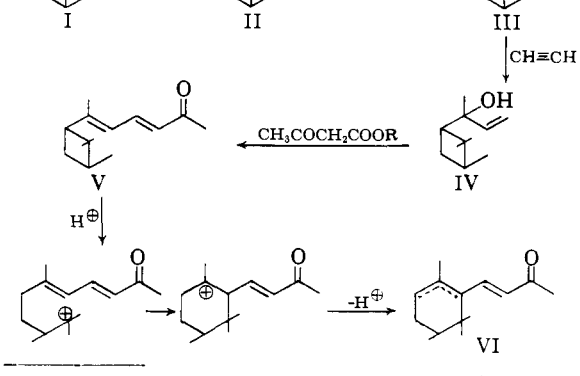
We wish to report a new synthesis of irones from α-pinene involving five steps: α-Pinene (I) was ozonized to yield 65–70% *cis*-pinonic aldehyde (II), b.p. 92° (1.5 mm.); $n^{20}D$ 1.4610; $\alpha^{25}D$ + 40°; purity 96% by oximation, and a small amount of pinonic acid.

Decarbonylation of (II) by means of palladium catalyst[6] at about 200° afforded the new ketone, pinonone, 1-acetyl-2,2,3-trimethylcyclobutane (III), (*cis* and *trans* mixture) in 80% yield, b.p. 53° (9 mm.); $n^{20}D$ 1.4410; semicarbazone of probable *cis*-isomer, m.p. 197–198°. (Found: C, 60.92; H, 9.84; N, 21.40); 2,4-dinitrophenylhydrazone, m.p. 201–202° (Found: C, 56.36; H, 6.38; N, 17.63).

Pinonone (III) and acetylene in the presence of sodium acetylide in liquid ammonia gave 3-(2,2,3-trimethylcyclobutyl)-but-1-yn-3-ol (IV) (*cis* and *trans* mixture) in almost quantitative yield, b.p. 74° (8 mm.); $n^{20}D$ 1.4667; $\alpha^{25}D$ −4°. *Anal.* Calcd. $C_{11}H_{18}O$: C, 79.46; H, 10.91. Found: C, 79.76; H, 11.02.

The butynol (IV) and ethyl acetoacetate[7] at 178–180° afforded cyclobutyl isomers of irone, 2-(2,2,3-trimethylcyclobutyl)-hepta-2,4-dien-6-one, referred to as cyclobutirones (V) in 65% yield, b.p. 95–105° (0.5 mm.); $n^{20}D$ 1.5080–1.5280. At least four main isomers were identified by gas–liquid partition chromatography. The most stable of the isomers was isolated from the mixture as the semicarbazone, m.p. 202–203° (ethanol). (Found: C, 67.92; H, 9.11; N, 15.87). 2,4-Dinitrophenylhydrazone, m.p. 198–199° (ethanol). (Found: C, 62.16; H, 6.62; N, 14.47).

The mixture of cyclobutirones (V) was rearranged to irone isomers (VI) by treatment for several hours with 85% phosphoric acid at 25° in over 50%



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