[CONTRIBUTION FROM THE RHEUMATIC FEVER RESEARCH INSTITUTE, NORTHWESTERN UNIVERSITY MEDICAL SCHOOL]

The Separation and Properties of a Mucoprotein from a Lymphatic Organ[1]

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A method for the separation of a high molecular weight mucoprotein from bovine palatine tonsils is given. Chemical and physical data, including sedimentation and diffusion coefficients, intrinsic viscosity, partial specific volume, isoelectric point, electrophoretic behavior, ultraviolet absorption characteristics, and nitrogen, phosphorus, hexose, hexosamine, hexuronic acid and lipid contents are reported.

Introduction

Little is known of the composition or properties of the proteins of lymphoid tissue. Nucleoproteins have been separated from the thymus[2-4] and characterized[5,6] as nucleohistones.

More recently Abrams and Cohen[7] reported electrophoretic and chemical analyses of the human tonsil and calf thymus extracts. Since these workers did not report carbohydrate analyses, no information is available regarding carbohydrate content of their extracts.

Several workers have isolated carbohydrate containing protein fractions from animal serum.[8-11] Mucoprotein I from human serum is the better characterized one due to the careful and complete work of Winzler and his associates.[11,12]

Mucoproteins have also been prepared from eggs,[13] gastric mucosa,[14] saliva[15] and urine.[16] Urinary mucoprotein and ovomucoid have been better characterized than the others due to the recent work of Tamm and Horsfall[16] and of Fredericq and Deutsch.[17]

The conditions of separation, the yield, and some chemical and physical properties of a mucoprotein isolated from bovine palatine tonsils will be discussed in this report.

Experimental

Bovine palatine tonsils were secured from freshly slaughtered animals.[18] After excision the glands were immersed immediately in cracked ice. The removal of muscle and external connective tissue was accomplished within a few hours after slaughter. Each bovine tonsil consists of two lobes of lymphoid tissue folded over a mucosal surface continuous with the pharyngeal mucosa. The tonsilar mucosa was exposed and scraped in order to remove mucus and any infected areas. The cleaned tonsil was then washed with cold 0.15 N NaCl, frozen and stored at $-56°$ until used.

Electrophoresis determinations were run in a conventional Tiselius assembly equipped with a Philpot–Svensson schlieren optical system.[19,20] Buffer compositions, ionic strength, pH and the potential gradient employed varied throughout this study and will be stated in each individual case. The experiments were performed in an all-glass cell assembly at a temperature of 0.4°. Conductivities were measured at 0.0°; the mobility values therefore obtain at 0.0°.[21] The conductivity of the buffer was used for mobility calculations. The values reported were calculated from tracings of enlargements of photographs of the descending patterns in the usual manner.[22]

Intrinsic viscosity measurements were made in 0.1 N sodium acetate (pH 7.3) at 37.0 ± 0.05° in a Fisher–Irany viscometer, according to methods discussed previously,[23] except that the mean velocity gradient $\bar{\beta}$ varied from 21 to 70 sec.$^{-1}$ in the present work.

Sedimentation constants were measured in the standard oil-turbine centrifuge in the laboratory of Prof. J. W. Williams at the University of Wisconsin. A speed of 50,400 r.p.m. was employed and the average temperature was 24°. Values of the sedimentation constant were determined from the slope of log x_i $vs.$ t_i^2, where x_i is the distance of the axis of rotation from the boundary and t_i^2 is the time of centrifugation corrected for the viscosity variation of the solvent medium with temperature. The sedimentation coefficients have been reduced to the value in water at 20° (s_{20w})[24,25] and are recorded in Svedberg units.

Diffusion measurements were made in the electrophoresis assembly described above. Duplicate determinations result from using both limbs of the cell. The boundaries were sharpened according to the method of Kahn and Polson.[26] The height and area, the second moment, the inflection point and the successive analysis methods were used for calculations.[27] The zero time correction suggested by Kahn and Polson[26] and by Longsworth[28] was applied to these measurements. The diffusion experiments were performed at 0.4°, corrected for temperature and viscosity effects, and reported as D_{20w} values according to the equation.[29]

$$D_{20w} = D_{exp} \frac{T_{293}}{T_{exp}} \left(\frac{\eta_{273°K}}{\eta_{293°K}}\right)_{H_2O} \left(\frac{\eta_{buffer}}{\eta_{H_2O}}\right)_{293°K} \quad (1)$$

Gosting and Morris[29] and Longsworth[30] have shown that these corrections are reasonably valid.

Partial specific volumes were calculated according to the method described by Kraemer[22] from a plot of w_1 $vs.$ V where w_1 is the weight fraction of protein and V the specific volume of the solution. The specific volumes were determined at

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(18) Considerable trauma of the gland occurs in the normal method of slaughter. The resulting contamination with blood serum proteins introduces problems which are largely avoided by the use of tonsils from kosher-slaughtered beef which are virtually free of blood. We wish to thank Dr. David Klein, of The Wilson Laboratories who suggested the use of these tonsils.

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(23) E. L. Hess and A. Coburn, *J. Gen. Physiol.*, **33**, 511 (1950).

(24) The s_{20w} values are corrected for the error in rotor temperature involved in earlier work with oil turbine centrifuges.[25]

(25) R. Cecil and A. G. Ogston, *Biochem. J.*, **44**, 33 (1949).

(26) D. S. Kahn and A. Polson, *J. Phys. Colloid Chem.*, **51**, 816 (1947).

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20° in redistilled water using a 5-ml. pycnometer. Protein concentrations used to calculate w_1 values were determined spectrophotometrically using the extinction coefficient at λ 280 mμ.

The extinction coefficient ($E^{1\%}_{\lambda=280}$) was determined with a Model DU Beckman spectrophotometer based on a sample dried at 105° *in vacuo*. A slit setting of 0.6 mm. was used in this determination. The extinction coefficient obtained by the purple color reaction with diphenylamine at 530 mμ was likewise measured on the Beckman instrument. The color reaction was performed according to the macro-procedure previously described.[31]

There is evidence that the absorption band at λ 530 mμ produced by heating the mucoprotein with the Dische diphenylamine reagent may be due to glycal type structures.[32] This analysis is based upon the purple color reaction[31] using the extinction coefficient for D-galactal $E^{1\%}_{530}$ 62.5 as a reference standard.

Chemical analyses were performed according to the procedures listed in Table I, and are based upon samples dried to constant weight at 105° *in vacuo*. Total lipid values were found by Soxhlet extraction procedures using 3:1 ethanol–ether mixture.

A Model G. Beckman *p*H meter calibrated daily, prior to use, against National Bureau of Standards recommended buffers[33,34] was used for all *p*H measurements.

TABLE I

CHARACTERIZATION DATA ON BTMI

s_{20w}	9.6S	0.15 *M* NaCl *p*H 5.4
D_{20w}	1.4×10^{-7} cm.² sec.$^{-1}$	0.15 *M* NaCl *p*H 5.4
V_{20w}	0.71	H_2O at 20° *p*H 5.4
pI_e	1.5 ± 0.1	$\mu = 0.05$
H	0.95 dl./g.	0.1 *M* NaOAc *p*H 7.3
M	570,000	Sedimentation and diffusion
f/f_0	2.82	Sedimentation and diffusion
u	-5.6×10^{-5} cm.² sec.$^{-1}$ volt^{-1}	Veronal μ 0.10 *p*H 8.6
u	-4.9×10^{-5}	Acetate μ 0.10 *p*H 4.7
$E^{1\%}_{280}$	6.2	H_2O *p*H 7.0
$E^{1\%}_{530}$	1.7	Purple color reaction with DPA[31]
Hexoses, %	11	Carbazole reagent[35]
	10	Anthrone reagent[36]
Hexoseamines, %	9.7	Morgan and Elson[37]
Hexuronic acid, %	None	Dische[38]
Pentoses, %	None	Dische[39]
Methyl pentoses, %	1.7	Dische[40]
Glycals, %	2	Purple color reaction[31,32]
Nitrogen, %	8.9	Nessler technique[41]
Phosphorus, %	0.055	Fiske–SubbaRow[42]
Lipid phosphorus, %	None	
Protein phosphorus, %	0.055	
Atoms *p*/molecule	10	
$SO_4^=$, %	None	Mease[43]
Lipid, %	5.8	Soxhlet extraction

(31) W. Ayala, L. V. Moore and E. L. Hess, *J. Clin. Invest.*, **30**, 781 (1951).

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(33) National Bureau of Standards Letter Circular 993, Aug. 10, 1950.

(34) For *p*H determinations < 5.5 the meter was calibrated against 0.05 *M* phthalate *p*H 4.01 at 25°. For *p*H determinations > 5.5 calibration was against 0.025 *M* phosphate *p*H 6.86 at 25°. The *p*H values < 2 are less certain and are based upon a calibration of the glass electrode at *p*H 2.15 with 0.01 *M* potassium tetroxalate.

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Fractionation.—All fractionation steps were carried out in a cold room (0–5°). The tonsils were homogenized in a Waring Blender at high speed for 15 seconds with cold 0.15 *N* NaCl. The time factor is critical in that excessive foaming and prolonged homogenization (30–45 sec.) will cause extensive denaturation, resulting in the insolubility of more than 75% of the otherwise soluble constituents. On the other hand, homogenization for less than 15 sec. results in poor yields. Foaming was reduced by inserting an empty centrifuge tube into the vortex of the solution during homogenization.

The homogenate was stirred for 30 minutes and then centrifuged in an SS-1 Sorvall angle-head centrifuge. Large batches of the homogenate were centrifuged after preliminary filtration through coarse gauze in a continuous-type Sharples Supercentrifuge at 40,000 g. The precipitate was again suspended in the same volume of cold 0.15 *N* NaCl as used previously, homogenized 10 sec. at high speed in the blender, stirred 30 minutes and centrifuged as before. The above operation was repeated a third time. The straw colored supernatants were combined and dialyzed against distilled H_2O.

According to Mirsky and Pollister[44] the soluble constituents represent the cytoplasmic elements of the cells. Additional extractions yielded further soluble materials up to approximately 7 g./100 g. tonsil for seven extractions, representing about 50% of the dry weight of the gland. The mucoprotein being considered in this report goes into solution almost completely in the first three extractions. An electrophoresis pattern of the extract is shown in Fig. 2A. Further details of the fractionation procedure are given in Fig. 1. Although the purified mucoprotein is quite soluble at *p*H values 3.7 and above, at two steps in the procedure it precipitated; once at *p*H 4.2 and again at *p*H 3.7. These precipitates seem to be complexes resulting from interaction of the mucoprotein with other constituents of the solution. The final step in the procedure involved extraction of the mucoprotein from an insoluble gel containing 20% lipids.

Data and Discussion

The characterization data are listed in Table I. The sedimentation coefficient has been measured at four concentrations, and exhibits pronounced concentration dependency as can be seen in Fig. 3. Part of the apparent concentration dependency exhibited in Fig. 3 may be due to the influence of electric charge on sedimentation behavior and part may be due to viscosity effects. For obvious reasons the sedimentation and diffusion experiments were not performed under isoelectric conditions. There is an uncertainty as to whether 0.15 *M* NaCl provides sufficient electrolyte at the *p*H at which the sedimentation experiments were performed to reduce the charge effect to insignificance.[22]

The importance of the viscosity of the solution in sedimentation studies has been discussed by Lauffer.[45] In our study, as can be seen in Fig. 3, the product $\eta_r s'$ is not invariant with concentration. Lauffer has also suggested[45] that $1/s$ is the function which should be extrapolated to infinite dilution. Our data plotted in this manner does not give a linear relationship, although the extrapolated value of s_{20w} thus obtained is identical with that shown on Fig. 3 and listed in Table I. With only four measurements of s_{20w} in a concentration-dependent system the extrapolated value must be considered tentative. A single symmetrical boundary was observed throughout the sedimentation studies.

The diffusion coefficient has been determined in duplicate experiments at a single concentration (0.36%). From observations of the rate of blurring

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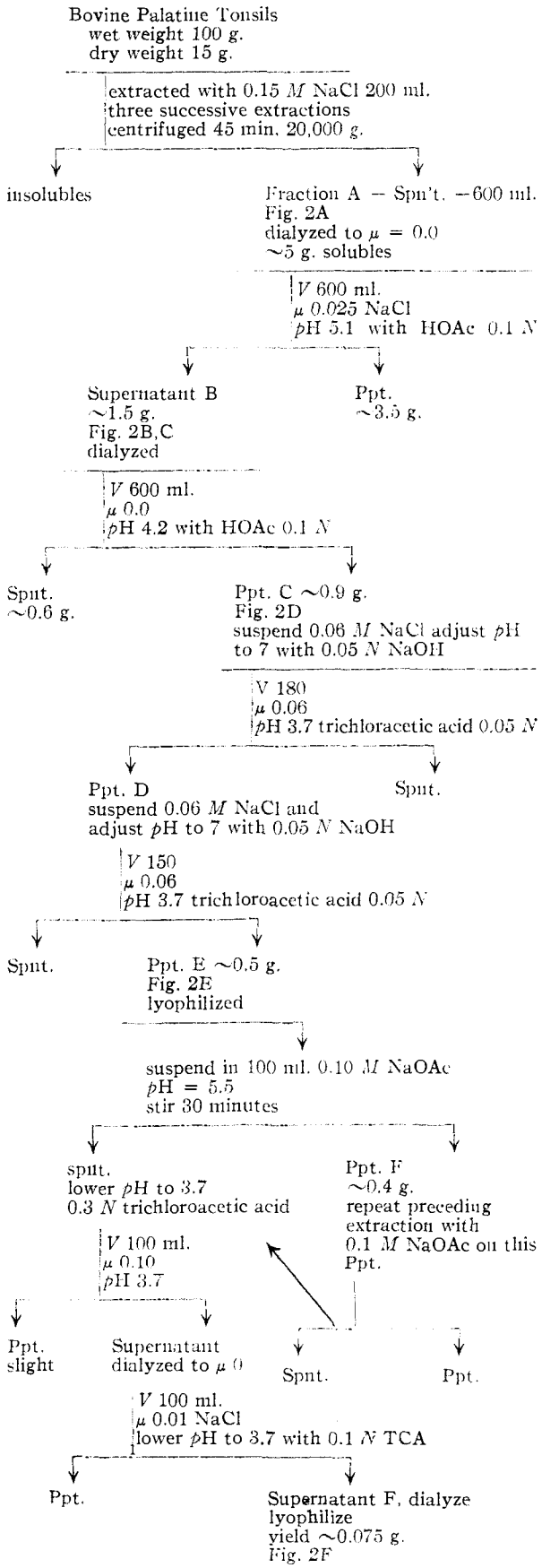

Fig. 1.—Schematic fractionation procedure for BTMI.

of the boundary at 0.4° the diffusion coefficients are calculated to be 0.50×10^{-7} cm.^{2} sec.$^{-1}$ by the inflection point method, 0.70×10^{-7} by the height and area method, 0.80×10^{-7} by the method of successive analysis, and 1.10×10^{-7} by the method of moments.

Normalization of the refractive index gradient–distance curve reveals skewness, probably indica-

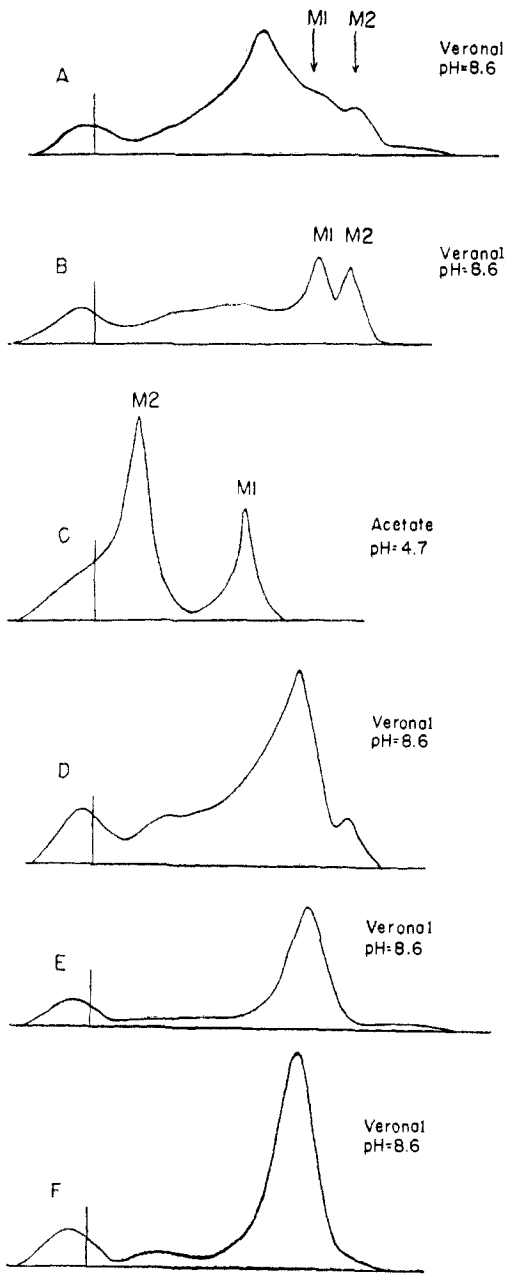

Fig. 2.—Electrophoretic patterns (descending) of various fractions of tonsil cytoplasm, enlarged 1.1×. All experiments, in μ 0.10 veronal buffers at pH 8.6 under a potential gradient 6.4–6.8 volt cm.$^{-1}$ for 120 minutes except for pattern C. C in μ 0.10 acetate at pH 4.7 and a potential gradient E 4.0 volt cm.$^{-1}$: A, supernatant A, concn. 1%; B, supernatant B, concn. 0.8%; C, supernatant B concn. 1.5%; D, precipitate C, concn. 1.5%; E, precipitate E, concn. 0.8%; F, supernatant F, concn. 1.5%.

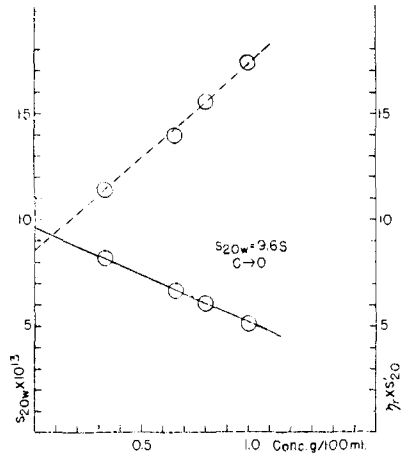

Fig. 3.—Concentration dependency of s_{20w} : ------, $\eta_r s'_{20}$ *vs.* C; ——, s_{20w} *vs.* C.

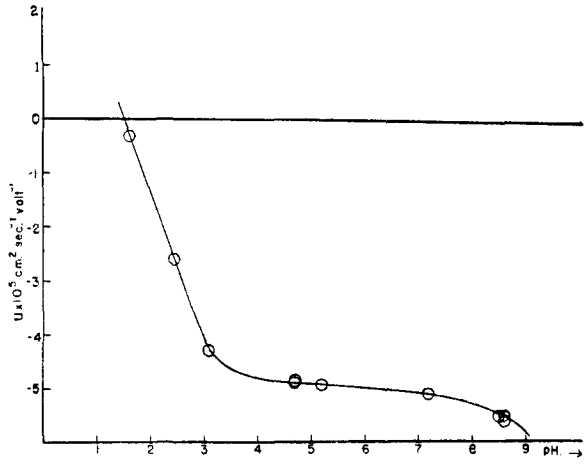

Fig. 4.—*p*H-Mobility relationship for BTMI: Mobilities calculated from descending patterns.

*p*H	Buffer	Conductivity, $\times10^{-3}$ ohm^{-1} cm.$^{-1}$	Ionic strength
1.6	0.03 *M* glycine Cl_3CCOOH	9.62	0.05
2.45	0.06 *M* glycine HCl	2.79	.025
3.05	0.1 *M* KH_2PO_4 0.007 *M* H_3PO_4	3.46	.10
4.7	0.08 *M* NaCl 0.02 *M* NaOAc HOAc	5.29	.10
5.15	0.08 *M* NaCl 0.02 *M* NaOAc HOAc	6.37	.10
7.2	0.04 *M* NaCl 0.06 *M* NaCac[a] 0.013 *M* HCac	4.33	.10
8.6	0.10 *M* NaV[b] 0.02 *M* HV	3.08	.10

[a] Cacodylate. [b] Diethylbarbiturate.

tive of concentration dependence of diffusion coefficients. The computations have been made by using properties of a Gaussian curve. The least certain portions of the curve are found at the maximum of the peak and at the base line. The inflection point method of calculation is therefore uncertain in that it is particularly sensitive to the height. The method of moments, on the other hand, weights heavily the portion of the curve where x_i is largest. We have chosen the average of the four methods as representing the best approximation. This leads to an uncorrected value $D_u = 0.78 \times 10^{-7}$ cm.^{2} sec.$^{-2}$. After correction, as indicated by equation (1), we have $D_{20,w} = 1.4 \times 10^{-7}$ cm.^{2} sec.$^{-1}$. This datum, when combined with other constants, gives a molecular weight of 570,000 for the mucoprotein.

The partial specific volume ($\bar{V} = 0.71$) is higher than values reported for other mucoproteins.[12,17] The partial specific volume of two separate preparations was $\bar{V} = 0.704$ and $\bar{V} = 0.714$, and is independent of concentration throughout the range 0.3 to 2.3% protein. Whereas the partial specific volume $\bar{V} = 0.71$ may well be correct, it can be shown that the lipid content, if assumed to be a contaminating lipoprotein, can affect the partial specific volume by three parts in the second decimal place. Likewise, the presence of water not removed at 105° *in vacuo* would raise the experimentally determined value. Since we are unable to account for more than 80% of the molecule in terms of carbohydrates, proteins and lipids it is reasonable to think that some of the remainder is water. Another possible explanation for the low nitrogen content of the fraction could be an unusual distribution of amino acid types. A preponderance of high molecular weight low nitrogen type amino acids such as tyrosine, cystine and leucine would lead to a low nitrogen value and allow as much as 70% of the molecule to be protein.

The *p*H mobility curve is shown in Fig. 4. It is unusual in that the isoelectric point in 0.05 ionic strength buffers is 1.5; however low isoelectric points have been reported for other mucoproteins.[10,11] The isoelectric point can be accounted for by postulating that the phosphorus is present in the form of hexose phosphates. Several hexose phosphates with $pK_1 < 1$ are known.[46] Phosphate analysis allows 10 phosphate groups per molecule of protein. The appearance of the curve in the *p*H region 1.5–4 can be attributed to the presence of cystine, which has a pK_2 2.1. The flatness of the curve between *p*H 4 and 8 suggests that very little histidine, aspartic and glutamic acids are present.

The intrinsic viscosity, H = 0.95 dl. g.$^{-1}$, is unusually large compared with most proteins. It is nearly five times the value reported for bovine fibrinogen,[23] generally considered to be the most asymmetric component in serum. The high intrinsic viscosity suggests that the mucoprotein molecules are probably heavily hydrated and asymmetric.

The frictional ratio $f/f_0 = 2.82$ determined from sedimentation and diffusion data likewise indicates pronounced asymmetry and hydration. If one assumes an unhydrated molecule with a prolate ellipsoidal shape the axial ratio can be calculated both from viscosity and from sedimentation diffusion data.[22,47] The values obtained from such calculations agree remarkably well, giving an

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$a/b = 44$ and $a/b = 45$, respectively. Such an axial ratio must be considered, however, only as the upper limit of asymmetry. Lauffer[48] has experimentally confirmed the validity of the Simha equation as applied to tobacco mosaic virus. His result hardly justifies an *a priori* assumption that the mucoprotein molecule has a prolate ellipsoidal shape or is unhydrated.

The molecular weight of approximately 570,000 obtained from sedimentation and diffusion data must be considered tentative due to the uncertainties with regard to s_{20w} and D_{20w} determinations.

An analysis of the electrophoretic pattern in Fig. 2F, assuming the same refractive indices for all components, shows that in addition to the main peak, 11% of other substances with mobilities ranging between zero and -5×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$ are present. The capacity of the mucoprotein to form complexes, as mentioned above, makes difficult the removal of all contaminants. In addition to the contaminants, there is the factor of spreading of the main peak itself. Our qualitative observations indicate that the bovine palatine tonsil mucoprotein will have a heterogeneity constant[49] greater than zero, and a definite $q(s)$ sedimentation distribution.[50,51]

Although essentially single boundaries are obtained both in sedimentation velocity experiments and from *p*H 2–8.6 in the electrophoretic work, spreading greater than can be attributed to diffusion is present.

The ultraviolet absorption spectrum is that of a typical protein with a maximum occurring at 277 mμ and a minimum at 253 mμ. The extinction coefficient $E^{1\%}_{\lambda=280} = 6.2$ is close to those reported for serum albumins.[52] Considering the low nitrogen (8.9%) content of the mucoprotein molecule, the ratio of combined tyrosine and tryptophan to other amino acids is probably twice that found in the serum albumins.

The analytical techniques available for quantitative determinations of the various carbohydrate constituents are rather unsatisfactory and at best give only a general idea of the carbohydrate contents. The interference by amino acids and sugars in the Elson and Morgan method has been discussed by Horowitz, Ikawa and Fling[53] and by Immers and Vasseur.[54]

Glycals and 2-deoxyhexoses do not react in the carbazole and anthrone analyses and therefore should if present be added to the hexose content of the molecule. Evidence for the presence of these substances is based on the diphenylamine reaction mentioned previously.

We have assumed that all of the substances extracted with 3:1 ethanol–ether using Soxhlet extraction procedures represent lipids. This assumption is of questionable validity as has been discussed by Wittcoff.[55] It is of interest that the extracted materials do not contain phosphorus and therefore are not phosphatides. A more adequate evaluation of the amount and nature of the lipid content of this protein is beyond the scope of the present report.

We have applied the term mucoprotein to our isolated fraction in accordance with terminology recommended by Meyer[56] who distinguishes mucoproteins from glycoproteins on the basis of the amino-sugar content. Inasmuch as BTMI contains 9.7% hexosamines it is classified as a mucoprotein. It is unlikely that BTMI is a constituent of the lymphocytes. Our evidence suggests that it comes from mucosal tissue present in considerable amounts in bovine palatine tonsils. Since this mucosal tissue is an integral part of the whole tonsil we have not attempted to separate it from the lymphoid tissue. Cytoplasmic extracts of both human tonsils and bovine thymus fail to indicate the presence of a component similar to BTMI. These glands contain very little mucosa compared to bovine palatine tonsils.

Most methods used for isolating mucoprotein fractions[8,9,13–15] involve rather drastic treatment; such as, extraction with hot carbonate, 90% phenol, 5% perchloric acid or heat coagulation. While mucoproteins appear to be unusually stable substances, alterations in physical and chemical properties are to be expected when drastic methods are employed. The conditions used for the separation of BTMI are mild in that they do not involve high salt concentrations, organic solvents or extreme *p*H values. For these reasons it seems probable that the somewhat unusual physical properties found in these studies are associated with the native state and are not the result of fractionation procedures.

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CHICAGO, ILLINOIS

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