It seems unlikely that these additional compounds have their origin in the tiny quantities of sulfobromophthalein-like impurities found in the dye solutions. Anyhow, free sulfobromophthalein and its major conjugates form the bulk of the dye appearing in the bile. Thus, the present investigation indicates that the small quantities of sulfobromophthalein-like impurities found in most commercial dye solutions for intravenous use do not interfere with the clinical use of the dye.

REFERENCES

(1) Krebs, J. S., and Bauer, R. W., *Am. J. Physiol.*, **194,** 37(1958).
(2) Combes, B., *J. Clin. Invest.*, **38,** 1426(1959).
(3) Javitt, N. B., Wheeler, H. O., Baker, K. J., Ramos, O. L., and Bradley, S. E., *ibid.*, **39,** 1570(1960).
(4) Barbier, F., and De Weerdt, G. A., Acta tertii conventus medicinae internae Hungarici, Budapest, Hungary, 1965.
(5) Baker, K. J., and Bradley, S. E., *J. Clin. Invest.*, **45,** 281(1966).
(6) Javitt, N. B., Third International meeting of the International Association for the Study of the Liver, Louvain, France, 1964.
(7) Javitt, N. B., *Am. J. Physiol.*, **208,** 555(1965).
(8) Javitt, N. B., *Gastroenterology*, **44,** 492(1963).
(9) Javitt, N. B., *Proc. Soc. Exptl. Biol. Med.*, **117,** 254 (1964).
(10) Grodsky, G. M., Carbone, J. V., and Fanska, R., *J. Clin. Invest.*, **38,** 1981(1959).
(11) Combes, B., and Stakelum, G. S., *ibid.*, **39,** 1214 (1960).
(12) *Ibid.*, **40,** 981(1961).
(13) Elewaut, A., Barbier, F., and Versieck, J., *Rev. Int. Hepat.*, **15,** 1045(1965).

Keyphrases

Sulfobromophthalein solutions—analysis
Impurities, sulfobromophthalein solution—identified
TLC—separation, identity
Colorimetric analysis
IR spectrophotometry—identity

Relationship Between Sputum Viscosity and Total Sialic Acid Content

By R. MUNIES, T. C. GRUBB, and R. E. CALIARI

Sputum samples collected from patients suffering from pulmonary diseases were hand homogenized, and the viscosity of the samples determined by measuring the flow time through a No. 100 Cannon-Fenske capillary viscometer at 37°. Total sialic acid content (free plus bound) was determined on an aliquot of each sample. In some patients producing an insufficient volume, samples from different patients were pooled and the data obtained treated separately. A sputum model was developed, and the viscosity and sialic acid content were determined in the same manner as the sputum. The data show a statistically significant linear relationship between viscosity and sialic acid concentration, until a point is reached where the acid content approaches a maximum and then plateaus, although the viscosity continues to increase.

Viscosity measurements of sputum have frequently been used for evaluating mucolytic or expectorant action of drugs. This measurement alone does not adequately describe the physicochemical properties of sputum. Measurements of adhesiveness and tensile strength of fibers should provide a more complete evaluation. However, reduction in viscosity is the prime objective to make the sputum more easily eliminated from the respiratory tract. Unfortunately "viscosity" is a term applied to homogeneous systems and is inappropriate for sputum, which is nonhomogeneous. Therefore, what is the appropriate measurement of viscosity to provide meaningful results?

Sputum viscosity has been measured in many different ways. Basch *et al.* (1) used a narrow glass tube and measured flow under pressure. However, Forbes and Wise (2) using the same technique could not obtain consistent readings on the same specimen. They, therefore, used a torque viscometer which measures viscosity by its dampening effect on the rotation of a metal cylinder suspended in it by a torsion wire. This instrument has limited use, because not less than 25 ml. is needed for each measurement and only fairly viscid samples can be measured accurately. In a study comparing the action of various expectorant drugs Simon and Harmon (3) blended the sputum specimens by vigorously stirring at

Received May 22, 1967, from the Vick Divisions Research & Development, Vick Chemical Company, Vick International Divisions of Richardson-Merrell Inc., Mount Vernon, NY 10553
Accepted for publication February 15, 1968.

constant speed and measuring the viscosity in an Ostwald viscometer. In this study it was necessary to dilute some samples with water and extrapolate the viscosity to zero dilution.

Since sputum is non-Newtonian, some investigators feel any measurement of viscosity must be made with an instrument which can measure and vary the velocity gradient and tangential stress separately (4). Rotational viscosimeters fill this need but also have their shortcomings. A cone and plate rotational viscosimeter has the disadvantage that the small quantity of sputum which can be measured may not be representative. In addition, lumps in the sample may not always fill the gap between the cone and vessel floor, thus the values of corresponding tangential stresses are not comparable.

All current methods appear to have some shortcomings, however, if a positive relationship between viscosity and a common ingredient of sputum can be established, chemical assay of this component may then avoid this dilemma.

It is generally accepted that glycoproteins are mainly responsible for the physical properties of mucous secretions and that the terminal unit of the carbohydrate prosthetic groups is in many instances sialic acid (5). The naturally occurring sialic acids are substituted neuraminic acid derivatives (*N*-acetyl, *N*-glycol, *N,O*,-diacetylneuraminic acid). However, in man only the *N*-acetylneuraminic acid is found.

Sialomucins are regular components of the mucoid material covering the epithelial surfaces of the respiratory, digestive, and urogenital tracts. Their relationship to the viscosity of mucous secretions has been alluded to by several investigators. Atassi *et al.* (6) reported that the bronchial secretions from patients with chronic bronchitis contained increased levels of sialic acid. Hoskins and Zamcheck (7) tested the serous saliva and mucoid saliva from one subject and found that the fucose and sialic acid content relative to hexosamine was less in the thinner material. Sialic acid was also more abundant in mucoid saliva than in gastric juice from the same subject. This work is supported by additional evidence in which relatively high ratios of concentrations of sialic acid to fucose were found in jelly from pseudomucinous ovarian cysts compared to that from pseudomyxomatous cysts (8). In the former, the cyst contains a viscoelastic gel. Gibbons (9) found a significant difference in sialic acid concentration between estrus and pregnancy samples of bovine cervical mucin. The thick plasto-elastic gel secreted during pregnancy had a higher sialic acid content than the thinner material secreted at estrus suggesting that the content of this acid is one of the factors affecting the viscosity of mucin.

The work cited above provides a theoretical framework for the hypothesis that there is a correlation between the viscosity of mucus secretions and the carbohydrate complex, namely the sialomucins synthesized and discharged from the mucus-secreting glands. The authors are unaware of any previous study with a sufficient number of samples to determine if a statistically significant positive correlation exists. Therefore, the purpose of this study was to quantitate both variables (viscosity and sialic acid) simultaneously to determine if this relationship for sputum does exist.

EXPERIMENTAL

Sample Collection—Sputum was obtained from patients with bronchiectasis, bronchial asthma, or pulmonary emphysema with the majority of samples from the latter two conditions.[1] No attempt was made to identify sample with disease.

The method used to obtain these samples was a modification of that described by Bickerman *et al.* (10). Essentially this consisted of having patients inhale a mixture of 0.2 ml. of 2.25% racemic epinephrine[2] and 1.0 ml. of 0.25% phenylephrine hydrochloride. Following this the patients inhaled a warm normal saline aerosol generated in a high flow nebulizer by means of an air pump. All of the material expectorated, during and for 10 min. following the aerosol inhalation, was collected and immediately frozen. Mucoid material recovered in this manner is reported to be from the tracheobronchial passageway (10).

Viscosity Determination—The frozen samples were brought to ambient temperature and passed through a Logeman hand homogenizer with the gap adjusted to the smallest permissible size. The homogenate was warmed to 37° and 8.0 ml. was transferred to a No. 100 Cannon-Fenske capillary viscometer positioned in a 37° constant temperature water bath, and allowed to equilibrate for 12–20 min. before the flow time was measured. The viscometer was washed in a chromic acid solution at the beginning of each day of use and rinsed with water and acetone and air-dried between each viscosity measurement. Viscosity calculations were based on a distilled water standard. Samples from different patients producing insufficient volume for viscosity measurements were pooled.

Sialic Acid Assay—The analytical procedure employed for determining total sialic acid content (free plus bound) measured as *N*-acetylneuraminic acid was that of Warren (11). The method is based upon periodate oxidation of sialic acids to form β-formylpyruvic acid, which is combined with thiobarbituric acid to form a chromophore that is extracted in cyclohexanone, and measured spectrophotometrically. Though there is some interference from 2-deoxyribose present in sputum, this can be readily compensated for as described by Warren.

[1] The authors are indebted to Dr. Hylan A. Bickerman for supplying all clinical material used in this study.
[2] Trademarked as Vaponephrine.

The above assay only measured free sialic acids. Therefore, 1.0 ml. of each sputum homogenate (pooled and unpooled) was diluted with 1.0 ml. of distilled water and heated at 80° in 2.0 ml. 0.2 *N* H_2SO_4 for 1 hr. in order to hydrolyze the bound acids. Then 0.2 ml. of the hydrolyzate, representing 0.05 ml. of sputum was assayed for total sialic acid.

Sputum Model—A partially purified mucoprotein preparation derived from commercial hog gastric mucin was prepared in the manner described by Sheffner (12). Colloidal suspensions ranging from 0.25–2.50% were prepared from this tan spongy material and their viscosity and sialic acid content determined.

RESULTS AND DISCUSSION

The authors are of the opinion that capillary viscometry is well suited to this investigation. If a relationship exists between viscosity and sialic acid content, it is a reasonable assumption that, although the viscosity values reported here may differ from the numerical values obtained when using a different measuring technique, the comparative relationship would still be valid. Even the best rotation viscometry remains a semiexact method for measuring the viscosity of sputum, and it is far from suitable for the determination of absolute values.

Although homogenization alters the integrity of mucoproteins and mucopolysaccharides, the writers concluded that homogenization would still be acceptable for investigating the relationship between viscosity and sialic acid content.

It was also concluded that though the patients had a variety of diseases, were on different drug therapy, or may have had slightly different concentrations of saline in their bronchial passages, it was unlikely that these factors could influence the interpretation of the results.

Unpooled Sputum—The sialic acid content reported is in μmole per 0.1 ml. of sputum.

Table I is a summary of the data obtained on unpooled sputum. The data graphically depicted in Fig. 1 show that there is a linear relationship between viscosity and sialic acid concentration until a point is reached where the acid content appears to approach a maximum and then plateaus, although the viscosity continues to increase. A linear relationship is evident until a viscosity of approximately 2 cps. is reached. This linearity is supported by a correlation coefficient of 0.979 obtained from samples 1–10 whereas a much lower coefficient is obtained by using the data from all the samples. Unfortunately only a few samples are on the plateau portion of the curve, so the maximum sialic acid value can only be estimated to be between 0.11 and 0.12 μmole.

TABLE I—UNPOOLED SPUTUM SAMPLES

Sample No.	Viscosity, cps.	μ moles, Sialic Acid/ 0.1 ml.
1	0.818	0.012
2	1.089	0.021
3	1.125	0.036
4	1.186	0.028
5	1.311	0.029
6	1.322	0.042
7	1.398	0.048
8	1.914	0.079
9	2.022	0.075
10	2.131	0.092
11	2.326	0.075
12	2.739	0.110
13	3.700	0.109
14	5.453	0.118

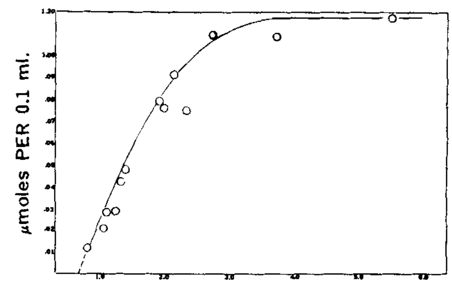

Fig. 1—Relationship between the viscosity and sialic acid concentration of unpooled sputum.

TABLE II—POOLED SPUTUM SAMPLES

Sample No.	Viscosity, cps.	μmoles, Sialic Acid/ 0.1 ml.
1	1.177	0.079
2	1.192	0.090
3	1.549	0.102
4	2.175	0.172
5	2.280	0.176
6	2.429	0.180
7	2.475	0.157
8	2.622	0.194
9	3.470	0.190
10	4.902	0.220
11	26.820	0.240

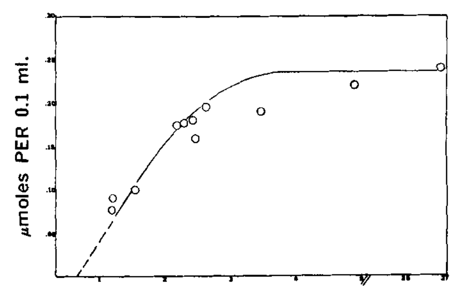

Fig. 2—Relationship between the viscosity and sialic acid concentration of pooled sputum.

It is significant that when the curve is extrapolated to the *X* axis, (that is, sialic acid equals 0.00 μ-moles), the interscept is 0.70 cps.—the viscosity of water at 37°.

Pooled Sputum—There was a greater percentage of high viscosity sputums in the pooled specimens (Table II) than in the unpooled. Figure 2 shows that the relationship between viscosity and sialic acid is the same for pooled and unpooled sputum. A sample with an extremely high viscosity (26.82 cps.) is five and one-half times greater than its nearest neighbor, yet there is a difference of only 0.02 μmole in sialic acid content. Though there is only one sample on this high end of the viscosity scale, it does emphasize the leveling of the acid concentration with a concomitant rise in viscosity above

TABLE III—SPUTUM MODEL

% Muco-protein	Viscosity, cps.	μmoles, Sialic Acid/ 0.1 ml.
0.25	0.94	0.007
0.50	1.26	0.014
0.75	1.66	0.023
1.00	2.22	0.028
1.50	3.00	0.043
2.00	4.00	0.055
2.50	5.00	0.069

2 cps. The correlation coefficient for the lower portion of the curve is 0.988, again indicating a linear relationship. When the curve is extrapolated to the X axis, it too intersects at 0.70 cps.

The major difference between the pooled and unpooled data is the magnitude of the sialic acid concentration relative to the same viscosity. Though the shapes of the curves are quite similar, the sialic acid content of the pooled samples is higher, with the plateau value approximately double that of the unpooled.

Sputum Model—The authors developed a simulated biological model in which some of the variables could be controlled. Gastric mucin was first selected because it represents a crude source of mucoprotein; however, the sialic acid content of the material available was quite low, and the viscosity of a standard suspension was difficult to duplicate on a day-to-day basis. A partially purified mucoprotein substrate which met the practical conditions needed for the model was used (12). The data are tabulated in Table III.

Because this model is a series of uniform aqueous suspensions, the curve has been drawn so that it is forced to intersect the X axis (sialic acid = 0.000) at the viscosity of water at 37°. The similarity of this curve (Fig. 3) to the two mucous curves is evident. Although the sputum model does not closely simulate natural mucus because it utilizes a lyophilized, partially purified mucoprotein, which disperses readily in water, it lends considerable weight to the relationship under study.

While it is premature to attempt to quantitate the viscosity of sputum in terms of sialic acid content, it appears that within limits and with continuing research this will be possible. The plateau seen

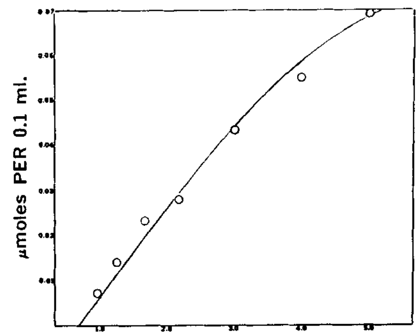

Fig. 3—Relationship between the viscosity and sialic acid concentration of sputum model.

in all three sets of data suggest, as expected, that there are other factors besides sialic acid affecting the viscosity of sputum.

REFERENCES

(1) Basch, F. P., Hollinger, P., and Poncher, H. G., *Am. J. Dis. Child* **62**, 1149(1941).
(2) Forbes, J., and Wise, L., *Lancet*, **273**, 767(1957).
(3) Simon, S. W., and Harmon, G. A., *J. Allergy*, **32**, 493(1961).
(4) Nicholas, R., *Med. Thorac.*, **21**, 223(1964).
(5) Gottschalk, A., *Ann. N. Y. Acad. Sci.*, **106**, 168 (1963).
(6) Atassi, M., Barker, S. A., Houghton, L. E., and Stacy, M., *Clin. Chem. Acta*, **4**, 741(1959).
(7) Hoskins, L. C., and Zamcheck, N., *Ann. N. Y. Acad. Sci.*, **106**, 767(1963).
(8) Tappan, V., and Zalor, V., *ibid.*, **106**, 722(1959).
(9) Gibbons, R. A., *Biochem. J.*, **73**, 209(1959).
(10) Bickerman, H. A., Sproul, E. E., and Baroch, A. L., *Dis. Chest.*, **33**, 347(1958).
(11) Warren, L., *J. Biol. Chem.*, **243**, 1971(1959).
(12) Sheffner, A. L., *Ann. N. Y. Acad. Sci.*, **106**, 298(1963).

Keyphrases

Sputum viscosity
Sialic acid content—sputum viscosity
Capillary viscosimeter—analysis
Colorimetric analysis