determined by their ability to maintain their position on a rotating rod.

(*d*) The most pronounced prolongation of hexobarbital sleep time in mice was produced by GPIB. Less impressive, but nonetheless significant, was the sleep potentiating effect of APIM. In equimolar concentrations neither PIA nor BPIP exhibited any influence on barbiturate depression.

(*e*) At comparatively high doses, the aldehydes were frequently observed to produce lacrimation, salivation, and flushing of the paw pads and ears, observations that prompted examination of the drugs for possible cholinomimetic activities.

(*f*) The estimated order of decreasing activity as lacrimatory stimulants in rats was APIM, BPIP (PIA), GPIB. This action was blocked by atropine premedication.

(*g*) None of the aldehydes provoked chromodacryorrhea in rats.

(*h*) PIA and BPIP and GPIB evoked short-lived tonic contractions in isolated guinea pig ileum that could be blocked by atropine. APIM produced only mild gut relaxation. There appears to be little relationship between the aldehydes' spasmogenic properties and their ability to stimulate lacrimation.

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Keyphrases

Phthalimidoaldehydes—synthesis, pharmacology
LD_{50} values—phthalimidoaldehydes
Locomotor activity—depressed
Hexobarbital sleep—prolonged
Lacrimatory responses—increased
Spasmogenic activity—evaluated

Thin-Layer Chromatography and IR Spectrophotometry of Commercial Sodium Sulfobromophthalein Solutions

By F. BARBIER and G. A. DEWEERDT

Techniques and results of analysis of some commercially available sulfobromophthalein (phenoltetrabromphthalein disulfonate) solutions by means of TLC and IR spectrophotometry are reported. The IR spectra of sulfobromophthalein and sulfobromophthalein-like compounds are presented and discussed. Three of the additional fractions found could be identified as phenoltetrabromphthalein and its mono- and trisulfonate derivatives. These impurities probably are due to imperfections in the synthesis of sulfobromophthalein. With but one exception they represent less than 1 percent of the total absorbance at 578 mμ. Such low concentrations do not interfere with the clinical use of sulfobromophthalein.

DESPITE THE WIDE use and established value of sulfobromophthalein (phenoltetrabromphthalein disulfonate) in the diagnosis of liver disease, little information is available on the composition of the dye solutions for intravenous use (1–5). The purpose of this study was to analyze some sulfobromophthalein solutions, commercially available in Belgium, with the aid of thin-layer chromatography (TLC) and IR spectrophotometry.

Received January 6, 1967, from the Policlinic of Internal Medicine, University of Ghent, Gent, Belgium.
Accepted for publication October 12, 1967.

METHODS

Sulfobromophthalein solutions for intravenous use and marketed by the following laboratories were analyzed: Vitarine Co., lot 9364; E. Merck, lots R493, R879, R959, and T959; Hynson, Westcott and Dunning, lots P_{23}, R_{96}, T_{47}, and U_{28}; and Simes, lot 3E29.

Thin-layer Chromatography—TLC was carried

out on chromatoplates (20 × 20 cm.) prepared by the technique of Stahl (thickness of the absorptive layer of Silica Gel G, Merck, 250 μ) with two solvent systems: butanol–acetic acid–water (30:7.5:12.5 v/v) and butanol–propionic acid–water (30:20:7.5 v/v). When the solvent front reached a height of 10 cm., the plate was removed, dried in air, and developed in ammonia vapors. The fraction corresponding with phenoltetrabromphthalein disulfonate was located with bromsulfalein Fuka. [The other reference compounds (phenoltetrabromphthalein and its mono- and tetrasulfonate derivatives) were kindly provided by N. B. Javitt, New York, N.Y.] All these compounds develop a purple color on alkalization. The fractions obtained were scraped off the plate, eluted with 0.1 *N* NaOH, and centrifuged. The absorptions in the visible range of the spectrum (wavelength scan from 320 to 750 mμ) of each fraction were scanned on a Beckman DB recording spectrophotometer. The percentage of each fraction was estimated from its absorbance at 578 mμ.

IR Spectrophotometry—All IR spectra were obtained with a Perkin-Elmer NaCl Infracord using the potassium bromide pellet technique.

Prior to preparative TLC of the fast-running fractions, RI and RII, 400 ml. of sulfobromophthalein solution (Simes) was alkalized with 1 Gm. of KOH and extracted twice with butanol. To this extract HBr was added till the purple color disappeared. Next the extract was washed twice with distilled water, evaporated *in vacuo* until saturation, and chromatographed on MN Silica Gel N-HR/UV 254 (Macherey, Nagel and Co.) absorptive layers of 500-μ thickness. Fraction RI was isolated with butanol–acetic acid–water (12:4.5:5.5 v/v) and fraction RII with hexane–butanol (20:1 v/v). Both fractions were visualized under UV light (wavelength of 254 mμ), scraped off the plate, and eluted with ethanol.

Sufficient amounts of pure fraction SII were obtained from lot P23 of Hynson, Westcott and Dunning and lot 3E29 of Simes. The dye solutions were chromatographed on MN Silica Gel N-HR with *tert*-butanol–water–butanol (32:10:5 v/v), visualized with KOH spray, scraped off the plate, eluted with water, concentrated *in vacuo*, and finally filtrated on Sephadex G 25.

RESULTS

Commercial sulfobromophthalein solutions always proved to be separable into several fractions by TLC (Table I). After alkalization, a total of eight additional purple fractions was demonstrated. In both chromatographic systems the R_f of the fastest running fraction (RII) corresponds with phenoltetrabromphthalein, and that of the other fast-running fraction (RI) with phenoltetrabromphthalein monosulfonate. No fraction corresponding with phenoltetrabromphthalein tetrasulfonate was found.

Table II shows the percentages of the fractions found in the sulfobromophthalein solutions. With the exception of the sulfobromophthalein, Simes lot 3E29, the additional fractions always represent less than 1% of the total absorbance at 578 mμ.

The visible spectra of sulfobromophthalein and all additional fractions were indistinguishable; so this technique is unable to characterize the multiple fractions eluted from the chromatogram. Fortunately IR spectrophotometry gives more gratifying results.

The phthalein compounds studied differ by the number of Br atoms linked to the phthalide group, and the number and the localization of the sulfonate substituents on the hydroxyphenyl groups (Fig. 1).

Table I—Sulfobromophthalein-Like Fractions of Commercial Sulfobromophthalein Solutions Separated by TLC

	Phenol-tetra-brom-phthalein	Phenol-tetra-brom-phthalein Mono-sulfonate	Phenol-tetra-brom-phthalein Tetra-sulfonate	Mfr. A[a] R959	Mfr. A[a] R879	Mfr. A[a] R493	Mfr. A[a] T959	Mfr. B[b] 9364	Mfr. C[c] 3E29	Mfr. D[d] P 23	Mfr. D[d] R 96	Mfr. D[d] T 47	Mfr. D[d] U 28
RII	+	...	...	...	...	...	...	...	+	...	...	...	+
RI	...	+	...	+	+	+	+	+	+	+	+	+	+
Sulfobromo-phthalein	...	...	...	+	+	+	+	+	+	+	+	+	+
SI	...	...	...	...	...	...	...	...	+	+	...	...	+
SII	...	...	...	...	...	...	...	...	...	+	+	+	+
SIII	...	...	...	+	+	+	+	...	+	+	+	+	+
SIV	...	...	...	...	...	...	...	...	...	+	...	...	...
SV	...	...	...	...	...	...	...	...	+	+	...	...	...
SVI	...	...	...	...	...	...	...	...	...	+	...	...	...
SVII	...	...	+	...	...	...	...	...	...	...	...	...	...
No. of fractions				3	3	3	3	2	6	8	4	4	6

[a] Merck. [b] Vitarine. [c] Simes. [d] Hynson, Westcott & Dunning.

Table II—Percentage of Sulfobromophthalein-Like Fractions Found in Commercial Sulfobromophthalein Solutions

	Mfr. A[a] R959	Mfr. A[a] R879	Mfr. A[a] R493	Mfr. A[a] T959	Mfr. B[b] 9364	Mfr. C[c] 3E29	Mfr. D[d] P23	Mfr. D[d] R96	Mfr. D[d] T47	Mfr. D[d] U28
RII	...	...	...	...	...	0.0723	...	...	...	0.0009
RI	0.0012	0.0007	0.0008	0.0020	0.0026	3.8718	0.0618	0.0683	0.0223	0.5762
Sulfobromophthalein	99.9558	99.9990	99.9990	99.9956	99.9974	95.9318	99.9054	99.9280	99.9741	99.4203
SI	...	...	...	...	...	0.0445	0.0054	...	...	0.0009
SII	...	...	...	...	...	...	0.0032	0.0027	0.0009	0.0007
SIII	0.0030	0.0003	0.0002	0.0024	...	0.0796	0.0205	0.0010	0.0027	0.0010
SIV	...	...	...	...	...	...	0.0016	...	...	...
SV	...	...	...	...	...	Traces	0.0017	...	...	...
SVI	...	...	...	...	...	...	0.0004	...	...	...

[a] Merck. [b] Vitarine. [c] Simes. [d] Hynson, Westcott & Dunning.

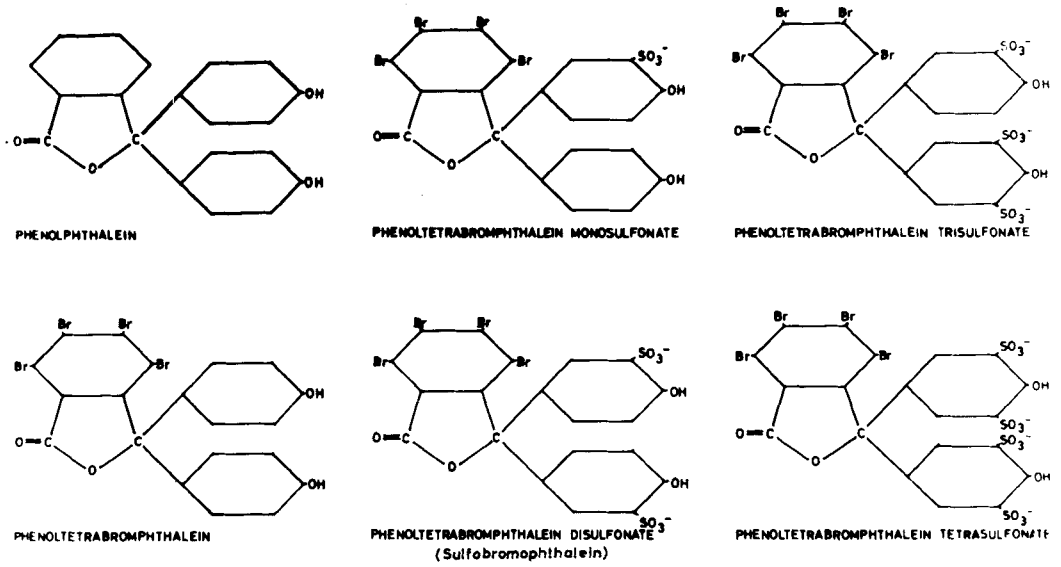

Fig. 1—Structural formulas of some of the phthalein dyes examined.

Phenolphthalein (Table III)—The band at 5.7 μ can be assigned to the —C=O group in the phthalide nucleus. This absorbance is present in all compounds examined and sufficiently individualized to provide reliable measurements of the concentration of the compound in the KBr pellet. Aromatic ring vibrations: in most cases three peaks are found (6.2, 6.6, and 6.9 μ); a fourth peak at 6.85 μ is perhaps due to the aromatic ring in the phthalide group. The strong 12-μ band arises from the adjacent pairs of H atoms on the phthalide group.

Phenoltetrabromphthalein (Fig. 2, Table III)—The halogenation has the following effects on the IR spectrum: absence of the band at 13.2 μ, and the mass increase induces a lowered vibrational frequency of the aromatic ring of the phthalide group and hence shifts its absorption from 6.85 μ toward longer wavelengths (7.4 μ).

Phenoltetrabromphthalein Monosulfonate (Fig. 2, Table III)—The introduction of one sulfonate substituent results in the following changes: (*a*) the band at 9.7 μ, which can be assigned to the sulfonate substituent, is very well individualized and provides a simple means of estimating the number of sulfonate substituents on comparing to the absorbance at 5.7 μ. (*b*) The site of the new substituent causes the loss of one adjacent pair of H atoms (decrease at 12 μ) which is replaced by one isolated H atom (weak absorption at 11 μ). (*c*) The mass increase in one hydroxyphenyl group lowers its frequency and shifts the aromatic ring absorption toward longer wavelengths. As only one group is involved, a reduplication of these bands results (6.6 and 6.7 μ; 6.9 and 7.1 μ).

Phenoltetrabromphthalein Disulfonate (Sulfobromophthalein) (Fig. 3, Table III)—The introduction of a second SO_3^- substituent restores the symmetry of the hydroxyphenyl groups. Hence, the reduplication of the aromatic ring peaks disappears and an absorption is now found only at 6.7 and 7.1 μ; the SO_3^- band at 9.7 μ doubles in comparison to the absorbance at 5.7 μ, and another adjacent H pair is replaced by an isolated H atom (further decrease at 12 μ, increase at 11 μ).

Phenoltetrabromphthalein Tetrasulfonate (Fig. 3, Table III)—Two more SO_3^- substitutents are introduced, resulting in a more pronounced shift of the aromatic ring peaks toward 6.9 μ (and perhaps 7.4 μ?); once more a doubled absorbance at 9.7 μ, four SO_3^- substituents now being present in one molecule; and since all adjacent H atoms are eliminated, the absorption at 12 μ disappears while the intensity of the band at 11 μ is much enhanced.

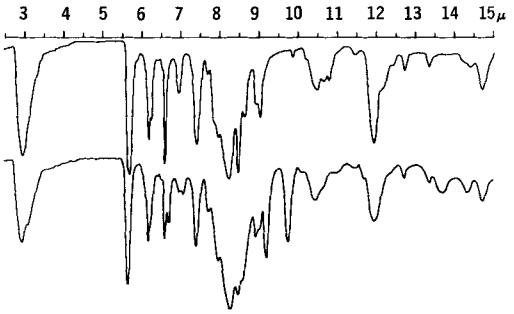

Fig. 2—Infrared spectra of phenoltetrabromphthalein (top curve), and phenoltetrabromphthalein monosulfonate (bottom curve).

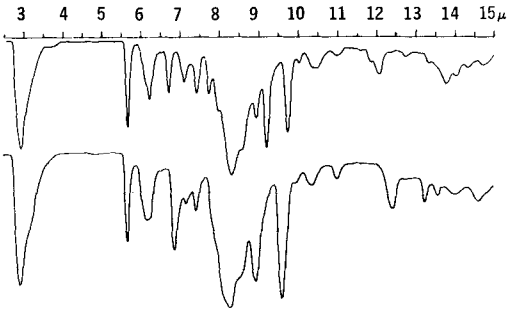

Fig. 3—Infrared spectra of phenoltetrabromphthalein disulfonate (sulfobromophthalein) (top curve) and phenoltetrabromphthalein tetrasulfonate (bottom curve).

TABLE III—MEAN INFRARED ABSORPTION BANDS OF SULFOBROMOPHTHALEIN-LIKE COMPOUNDS

	Phenolphthalein			Phenoltetrabromphthalein			Phenoltetrabromphthalein Monosulfonate			Phenoltetrabromphthalein Disulfonate			Phenoltetrabromphthalein Tetrasulfonate			Fraction RII			Fraction RI			Fraction Sulfobromophthalein			Fraction SIII		
	μ	cm.$^{-1}$	I	μ	cm.$^{-1}$	I	μ	cm.$^{-1}$	I	μ	cm.$^{-1}$	I	μ	cm.$^{-1}$	I	μ	cm.$^{-1}$	I	μ	cm.$^{-1}$	I	μ	cm.$^{-1}$	I	μ	cm.$^{-1}$	I
—O—	3.0	3350	5	3.0	3350	6	2.95	3400	5	2.9	3450	6	2.9	3450	6	2.95	3400	8	2.95	3400	6	2.95	3450	10	2.95	3400	7
—C=O	5.75	1740	*10*	5.7	1760	*10*	5.65	1770	*7*	5.65	1770	*6*	5.65	1770	*4*	5.72	1750	*10*	5.65	1770	*7*	5.65	1770	*8*	5.65	1770	*4*
	6.2	1620	5	6.2	1620	6	6.2	1620	4	6.2	1620	3	6.15	1630	2	6.2	1620	6	6.2	1620	5	6.2	1620	5	6.15	1625	sh
													6.25	1600	2										6.25	1600	7
	6.6	1520	8	6.6	1520	8	6.6	1515	4					...		6.6	1520	6	6.6	1515	4						
							6.7	1495	3	6.7	1490	3							6.7	1495	3	6.75	1480	5	6.75	1480	5
													6.85	1460	4										6.85	1460	5
	6.9	1440	4	6.9	1440	3	6.9	1440	2							6.9	1440	4	6.9	1440	3						
							7.1	1410	2	7.1	1410	2	7.15	1395	2				7.1	1410	3	7.15	1405	3	7.15	1400	sh
	6.85	1465	3																								
		...		7.4	1350	6	7.4	1350	5	7.4	1350	3	7.4	1355	2	7.4	1350	5	7.4	1350	5	7.4	1350	3	7.4	1350	sh
													8.9	1120	7										9.0	1115	8
—SO_3^-		...			...		9.15	1090	6	9.15	1090	7		...			...		9.2	1085	6	9.2	1085	7	9.15	1090	sh
		...			...		9.7	1030	*4*	9.7	1030	*6*	9.55	1045	*8*		...		9.7	1030	*4*	9.75	1025	*6*	9.7	1030	*7*
H		...			...		11.0	9.10	1	11.0	910	1	11.0	910	1		...		11.0	910	1	11.0	910	1	11.0	910	sh
H H	11.9	838	4	11.9	840	6	12.0	835	3	12.0	835	2		...		11.9	840	4	12.0	835	3	12.0	835	2	12.0	830	sh
H H H H	13.2	758	3		...			...			...			...			...			...			...			...	

Fraction RII (Table III)—The very low concentration of this compound resulted in tedious isolation and difficult purification. Two spectra were obtained but the purification was still incomplete. Nevertheless some conclusions were possible: (*a*) the phthalide group is present as a Br derivative (7.4 μ instead of 6.85 μ). A four-Br derivative is most likely, as the corresponding H atoms would give supplementary bands in the region 11–13 μ. (*b*) The hydroxyphenyl groups are symmetrical (6.6 and 6.9 μ); neither presence of SO_3^- groups (no absorption at 9.7 μ) nor of other substituents (strong band at 11.9 μ, no band at 11 μ). (*c*) The region 12–15 μ is identical with that of phenoltetrabromphthalein. Therefore it is concluded that compound RII is identical with phenoltetrabromphthalein.

Fraction RI (Table III)—A tetrabromphthalide group is present (7.4 μ). Asymmetry of the hydroxyphenyl groups (6.6 and 6.7 μ; 6.9 and 7.1 μ) related to the presence of one SO_3^- substituent (absorption at 9.7 μ) in one molecule (ratio of the absorbance at 5.65 μ to the absorbance at 9.7 μ) and a weak absorption at 11 μ suggest the replacement of one adjacent H pair by an isolated free H atom. The region 12–15 μ is identical with that of phenoltetrabromphthalein monosulfonate. It is concluded that fraction RI is identical with phenoltetrabromphthalein monosulfonate.

Fraction Sulfobromophthalein (Table III)—A tetrabromphthalide group is present (7.4 μ); hydroxyphenyl groups are symmetrical but shifted (6.75 and 7.15 μ); two SO_3^- substituents are present (ratio of the absorbance at 9.7 μ to the absorbance at 5.7 μ); adjacent pairs of H atoms are diminished but still present (12 μ); isolated free H atoms are present, indicating a substitution in the hydroxyphenyl groups (11.0 μ). The region 12–15 μ is identical with that of sulfobromophthalein. The conclusion is that fraction sulfobromophthalein is identical with phenoltetrabromphthalein disulfonate.

Fraction SIII (Table III)—The ratio of the absorbance at 9.7 μ (SO_3^- group) to the absorbance at 5.65 μ argues for the presence of three sulfonate substituents in one molecule. The number of Br atoms in the phthalide group cannot be clearly stated, as a (inconstant) band at 7.2 μ (NH_4^+?) obscures the region of 7.4 μ. The most probable number is four, as there is a little shoulder appearing at 7.4 μ. The band at 11 μ (isolated H atom in the hydroxyphenyl ring) is stronger, and the band at 12 μ (adjacent free H atoms) is weaker as compared to sulfobromophthalein. This argues for the presence of only two adjacent free H atoms in one molecule.

Phthalide groups with 2 adjacent H atoms as found in 3,6-dibromo-, 3,4-dibromo-, and 3,6-dichloroderivatives absorb at 11.6 μ (unpublished data). The absorbance of this band from the spectrum of fraction SIII suggests that the adjacent H atoms are situated on one of the hydroxyphenyl groups, which obviously contains only one of the sulfonate substituents. A similar type of hydroxyphenyl group is found in sulfobromophthalein and results in absorptions at 6.7 and 7.1 μ (shifted aromatic ring vibrations) and at 9.15 μ (SO_3^- substituent).

As a matter of course the other hydroxyphenyl group contains the two remaining sulfonate substituents and two free isolated H atoms. The latter type of hydroxyphenyl group is found in the tetrasulfonate derivative and results in absorptions at 6.85 and 7.15 μ (aromatic ring) and at 8.9 μ (SO_3^- substituent).

In fraction SII, the corresponding bands are found at 6.75 and 6.85 μ (aromatic ring); 7.1 and 7.15 μ, obscured (aromatic ring); 9.0 and 9.15 μ (SO_3^- group). This configuration is very well explained by the presence of both kinds of hydroxyphenyl groups in one molecule. Therefore this is most probably phenol (tetra ?) bromphthalein trisulfonate.

Fractions SI, SII, SIV, SV, and SVI were not obtained in sufficient amounts for IR analysis. Finally, the stability of aqueous solutions of phenoltetrabromphthalein and its mono-, di-, and tetrasulfonate derivatives was investigated. Chromatograms developed 1, 2, 3, and 4 months after preparing the solution revealed no additional fractions. Chromatograms of an aqueous solution of sulfobromophthalein (50 mg./ml.) autoclaved at 120° during 20 min. showed only one band, comparable in R_f to the standard dye.

DISCUSSION

TLC having a greater resolving power than paper and column chromatography successfully separates several sulfobromophthalein-like impurities in commercial sulfobromophthalein solutions. The chemical structure of two of the additional fractions was defined by IR spectrophotometry as phenoltetrabromphthalein and phenoltetrabromphthalein monosulfonate, whereas the third fraction has been tentatively identified as phenoltetrabromphthalein trisulfonate.

These investigations suggest that the additional fractions are due to imperfections in the synthesis of sulfobromophthalein. Because these impurities represent less than 1% of the total absorbance at 578 mμ in all but one of the sulfobromophthalein solutions for intravenous use examined, their influence on the disappearance rate of the dye from plasma must be negligible. Of paramount importance is the possible influence of these impurities on the number and composition of the sulfobromophthalein conjugates found in blood and bile. Indeed, Javitt (6, 7) demonstrated that phenolsulfodibromphthalein and phenolsulfotribromphthalein (8) are recovered mostly in bile, primarly as a glucuronide, and that conjugation of phenoltetrabromphthalein monosulfonate occurs mainly with glutathione although a glucuronide conjugate has also been found.

Phenoltetrabromphthalein tetrasulfonate and phenol 3,6-dibromphthalein disulfonate (9), on the other hand, formed no conjugates. At the present time no data are available about the fate of injected phenoltetrabromphthalein trisulfonate. Based on a considerable body of evidence (1–3, 10–12), it is now known that at least three of the four major ninhydrin-positive sulfobromophthalein compounds appearing in the bile consist of cleavage products and isomers of a conjugate of sulfobromophthalein and glutathione. In contrast to paper and column chromatography, TLC separates trace amounts of one to three additional sulfobromophthalein-like compounds in bile and blood of most patients (13).

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.

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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.