

# Rheological assessment of mucolytic agents on sputum of chronic bronchitics

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*In vitro* and *in vivo* studies were made on the sputum of chronic bronchitics using a Ferranti-Shirley cone and plate viscometer and a new stress relaxation method. The latter utilized a modified chemical balance and permitted study at very low shear rates. The results from simpler apparatus correlated well with the data obtained with the cone and plate viscometer. Significant reduction in four chosen parameters of viscosity occurred *in vitro* with *N*-acetylcysteine and with ascorbic acid-hydrogen peroxide-cupric ion complex (A.A.H.Cu.), but not with 4-molar urea. *In vivo* studies showed that, using freshly prepared A.A.H.Cu in an efficient hospital nebulizer, a statistically significant reduction in the four viscosity parameters occurred with subjective ease of expectoration. Nebulization of A.A.H.Cu performed with a simpler domestic-type nebulizer did not produce satisfactory reduction of viscosity or ease of expectoration. Rheological methods previously employed to study mucus and mucolytic agent activity are briefly reviewed.

The difficulty of expectorating excessive thick mucus formed in the bronchial tree in chronic bronchitics needs no emphasis. During infected phases, the purulent, coloured sputum is less viscid and appropriate antibiotic treatment may return the secretion to a white mucoid consistency of great viscosity. White mucoid sputum of non-infected phases is chemically heterogeneous, with mucoproteins and mucopolysaccharides both represented. Chemical constituents of mucus have been the subject of further research in recent years (Brogan, 1959; Gernez-Rieux, Biserte, Havez, Voisin, Roussel, and Degand, 1966; Waldron-Edward and Skoryna, 1966). The diverse nature of mucolytic agents perhaps reflects this chemical heterogeneity. Agents studied in the past include detergents (Palmer, 1957), hyaluronidase (McClellan and Hale, 1940), deoxyribonuclease (Armstrong and White, 1950), trypsin (Limber, Reiser, Roettig, and Curtis, 1952; Gernez-Rieux, Biserte, Havez, Voisin, Roussel, and Degand, 1964), chymotrypsin (Robinson, Woolley, and Altounyan, 1958), *N*-acetylcysteine (Webb, 1962; Sheffner, 1963; Sheffner, Medler, Jacobs, and Sarett, 1964; Hirsch and Kory, 1967) and related compounds, Martin, Causey, Sheffner, Wheeler and Corrigan, 1967), 4-molar urea (Waldron-Edward and Skoryna, 1966; Lieberman,

1967), ascorbic acid (Robertson, Ropes, and Bauer, 1941; Hale, 1944), and ascorbic acid-hydrogen peroxide-cupric ion system (Palmer, 1961).

Trypsin, chymotrypsin, and *N*-acetylcysteine have the disadvantages that they are expensive, and can be local irritants, giving bronchospasm and further wheezing (Bernstein and Ausdenmoore, 1964).

Although saturated urea solutions make many mucopolysaccharide systems more soluble, it has been found clinically that they have very little effect in reducing sputum viscosity, and furthermore an appreciable amount of urea may be absorbed into the blood-stream.

Of the remaining agents we have examined, the ascorbic acid-hydrogen peroxide-cupric ion system ('Ascoxal', Astra-Hewlett, referred to as A.A.H.Cu hereafter) has the advantages of being relatively innocuous when nebulized, non-irritant, and fairly cheap. This is a depolymerizing agent of muco-polysaccharide macromolecules.

The physical characteristics of sputum, obtained from patients suffering from chronic bronchitis, were examined by the Ferranti-Shirley cone and plate viscometer, and also by a new method which assesses stress relaxation [see glossary, p. 712]. The former instrument enables small amounts of non-ideal (or non-Newtonian) fluids [see glossary] to be studied for change in viscosity and elasticity under conditions of uni-

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formly changing shear rate. As such a method does not subject the sputum to forces comparable with those met in the bronchial tree, a new stress relaxation method has been introduced, whereby sputum elasticity is compared without subjecting it to strenuous forces.

The effect of the agents *in vitro* was determined by adding them to expectorated sputum, collected from bronchitic patients not being treated with mucolytic agents. The effect of nebulized A.A.H.Cu and water was then determined in patients by comparing sputum viscosity collected before and after nebulization treatment.

#### MATERIALS AND METHODS

##### IN VITRO STUDIES

*Examination of sputum with the Ferranti-Shirley viscometer* A general discussion of the underlying principles and operation of the Ferranti-Shirley viscometer (Fig. 1) is given by McKennell (1960).

One-millilitre aliquots of sputum were placed on the fixed plate and, after careful setting of the cone-to-plate gap, the cone was made to revolve uni-

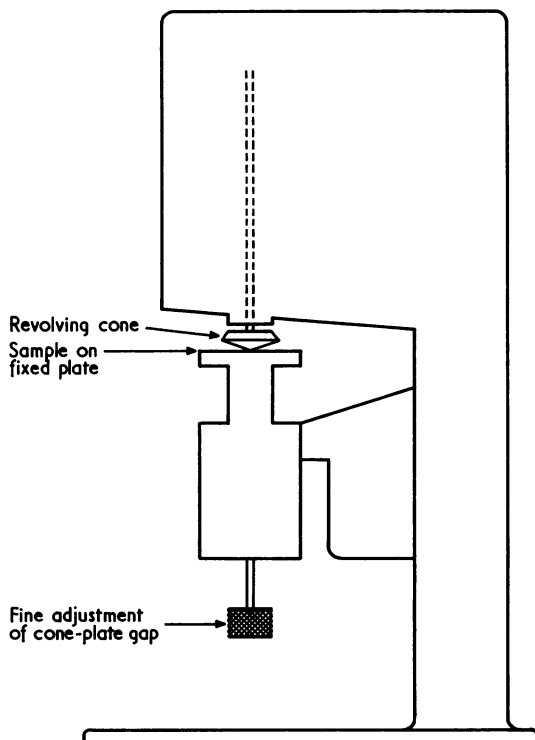


FIG. 1. Simplified diagram of a Ferranti-Shirley cone and plate viscometer.

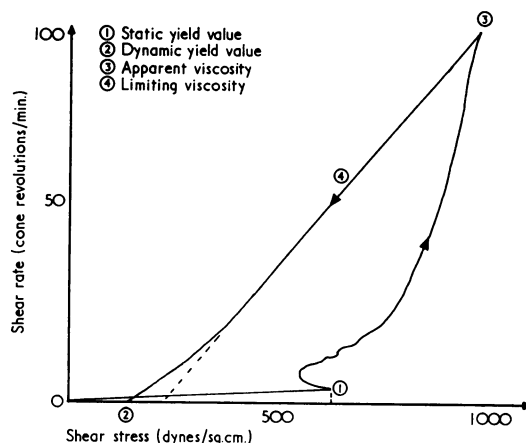


FIG. 2. Shear stress/shear rate relationship for mucoid sputum, obtained with the Ferranti-Shirley viscometer.

formly from rest to a maximum of 100 r.p.m. in 120 seconds. Having reached its maximum revolutions, the cone would then slow down uniformly over a further 120 seconds, thus giving two lines on the graph (see Fig. 2). The choice of a total operating time of 240 seconds represented a compromise which took account of the difficulties introduced by inertia if the cone was accelerating and decelerating too rapidly, and evaporation if the process was too slow.

Samples of sputum, as far as possible free of saliva, were collected from chronic bronchitic patients in a non-infected stage (that is, when the sputum was mucoid), and studied within two hours of collection. The patients were aged between 41 and 68 years, with a history of chronic bronchitis. All had daily cough and sputum production present for at least one year, as well as some emphysema (as evidenced by over-expansion of the lungs and radiographic changes) and asthma (*i.e.*, paroxysms of wheezing).

Freezing and homogenization of sputum were not performed so as to avoid breakdown of structure. Since at preliminary operating temperatures of 37° C., considerable thickening occurred due to evaporation, all studies were performed at 25° C. ± 0.1° C., taking precautions with a water gasket to avoid evaporation. A cone diameter of 70 mm. and angle 0.0062 radians were found to be suitable for a sputum volume of 1.0 ml.

The alteration of shear stress with changing shear rate (cone revolutions) for sputum is seen in Figure 2. The method provided four useful parameters measured from the graph, *viz.*, static and dynamic yield values, and apparent and limiting viscosities [for definitions, see glossary].

The trace in Fig. 2, covering the entire time scale of 240 sec., shows that sputum viscosity is greatly dependent on shear rate (no straight line relationship) and that sputum molecules are broken down under shear (*viz.*, ascending and descending lines of the

TABLE I  
CHANGES IN FOUR VISCOSITY PARAMETERS IN VITRO EFFECTED BY THE MUCOLYTIC AGENTS STUDIED

Agent <sup>1</sup>	Static Yield Value	Dynamic Yield Value	Apparent Viscosity	Limiting Viscosity	No. of Experiments
Water	73.8 ± 14.8	75.0 ± 12.6	77.9 ± 12.4	77.2 ± 12.6	10
A.A.H.Cu	44.4 ± 11.1	43.9 ± 16.5	39.0 ± 14.6	39.1 ± 13.7	10
	0.01 > P > 0.001	0.01 > P > 0.001	0.01 > P > 0.001	0.01 > P > 0.001	
Acetylcysteine	46.9 ± 10.1	41.4 ± 15.9	37.4 ± 8.2	45.6 ± 10.1	6
	0.02 > P > 0.01	0.01 > P > 0.001	0.01 > P > 0.001	0.01 > P > 0.001	
A.A.H.Cu with sod. edetate	55.7 ± 9.1	56.9 ± 6.4	63.0 ± 8.0	60.0 ± 11.5	4
0.005% CuSO <sub>4</sub>	80.4 ± 13.2	85.9 ± 23.7	81.9 ± 9.2	80.3 ± 10.0	4
4-M urea	80.9 ± 5.5	66.7 ± 11.7	78.2 ± 8.2	79.8 ± 6.0	4

<sup>1</sup> Contact time 20 minutes.  
The results are expressed as a % of original viscosity values, with standard deviations.

graph are different, a steeper slope indicating 'thinner' sputum). This indicates the serious limitation of single point determinations. A second measurement on the same sample gave a straight line trace following the approximate course of the previous down curve, and even after three hours no return to the original loop occurred. This means that sputum was broken down irreversibly under shear, indicating the absence of thixotropic properties [see glossary].

In a series of experiments 4.0-ml. samples of sputum were added with gentle mixing to 1.0 ml. of distilled water, or fresh A.A.H.Cu solution (75 mg. granules in 1 ml. water), or 20% *N*-acetylcysteine, or 4-molar urea, and 20 minutes later small portions were examined rheologically (Table I). In supplementary experiments the influence of contact time of *N*-acetylcysteine or A.A.H.Cu with sputum was investigated. The effects of the following factors on the four rheological parameters were also noted: (i) chelating the Cu<sup>++</sup> present in the preparation with excess sodium edetate, prior to mixing with sputum, and (ii) the sole introduction of 0.005% copper sulphate (Tables I and II).

TABLE II

INFLUENCE OF CONTACT TIME IN VITRO ON VISCOSITY

Fresh Mucolytic Agent Used	Contact Time (min.)			
	15	30	45	60
<i>N</i> -Acetylcysteine (20% w/v)	49.0	23.5	14.9	< 1
A.A.H.Cu (100 mg./ml. water)	72.8	30.3	28.5	26.1

The means of the four chosen viscosity parameters are expressed as a % of the original means.

#### Examination of sputum by a stress relaxation method

Four-millilitre samples of sputum were treated for 20 minutes *in vitro* with 1 ml. of water or fresh A.A.H.Cu (75 mg. granules per ml. water) and placed in a glass container (4 × 2.5 × 0.5 cm.), into which was immersed a glass plate (22 × 10 mm.) suspended from one arm of an electrical, damped balance (Fig. 3). The swing of the central pointer could be read off the scale at intervals of time, following the addition of 100 mg. to the other pan. The whole apparatus was housed in a temperature-controlled cabinet at 25° C. The stress relaxation values (arbitrary units

equivalent to the scale reading) at 60 seconds after the addition of the 100 mg. weight were found convenient for comparison.

The method afforded a simple comparative study on the effect of mucolytic agents on stress relaxation, a suitable parameter of elasticity [see glossary]. As the shear rates were very low, material was not broken down.

#### IN VIVO NEBULIZATION STUDIES

*Ferranti-Shirley cone and plate viscometer* Sputum samples were collected for three to four hours from 10 subjects, before these patients were treated with (1) water or (2) A.A.H.Cu (75 mg. granules per 1 ml. water) for 20 minutes, using the Collison nebulizer apparatus. Sputum was then collected for a further 30 minutes, enabling a comparison of viscosity to be made of samples before and after treatment. A 30-minute collecting period produced sufficient sputum volume to allow several recordings to be made, and ensured that the sputum collected did not just represent sputum well up the bronchial tree. Each patient received both treatments, but on different days.

A further series of six subjects was studied in a similar fashion where the patients inhaled one or other of the above agents for 20 minutes using a simple glass nebulizer.

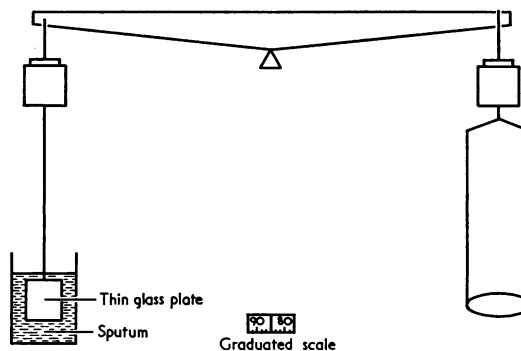


FIG. 3. Simplified diagram of the modified electrical damped balance used for the stress relaxation method.

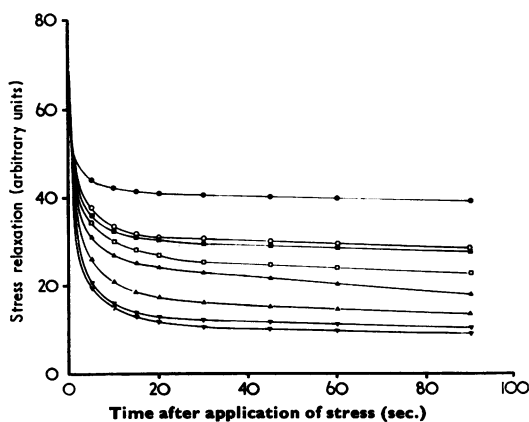


FIG. 4. Influence of contact time of A.A.H.Cu on stress relaxation of mucoid sputum: ● undiluted sputum; ○ sputum + water (control). Contact with A.A.H.Cu: ■ 5 min., □ 10 min., ▲ 15 min., △ 20 min., ▼ 30 min., ▽ 40 min.

**Stress relaxation studies** Similarly, sputum collected before and after nebulization treatment of the patient with water or A.A.H.Cu was examined by the simple method outlined earlier. Again each patient had both treatments, although on different days.

## RESULTS

### IN VITRO WORK

**Studies using the Ferranti-Shirley cone and plate viscometer** From Table I it will be seen that both *N*-acetylcysteine and A.A.H.Cu reduced sputum viscosity greatly, but that chelation of  $\text{Cu}^{++}$  impaired A.A.H.Cu mucolytic activity. By contrast, neither 4-molar urea nor 0.005% copper sulphate had mucolytic properties.

Table II shows that viscosity reduction was not an immediate process and that contact time should be defined.

**Stress relaxation studies** The effect on sputum stress relaxation of A.A.H.Cu and water *in vitro*, with time, is represented in Figure 4.

It will again be seen that mucolytic activity is not an instantaneous process, and therefore all readings have been made arbitrarily at 20 minutes. Table III compares stress relaxation values before and after treatment, *in vitro*, with A.A.H.Cu and water.

Significant reduction in muco-elastic properties, correlating with the cone and plate data, is shown.

### IN VIVO WORK

**Studies using the Ferranti-Shirley cone and plate viscometer**

(a) Collison nebulizer method (Table IV): Statistically significant reduction in all four parameters of viscosity occurred with A.A.H.Cu compared with water.

All the patients experienced subjective ease of expectoration even after nebulization with water, but 8 out of 10 patients thought that the benefit was greater with the mucolytic agent.

(b) Simple hand-operated nebulizer method (Table V): Although physical measurements suggested some reduction in viscosity, whatever parameter was used, the changes were small and could well have been due to chance. This finding was supported by the patients' own impressions. Only two had a preference for the mucolytic agent, the remaining eight expressing no preference.

TABLE III

COMPARISON OF STRESS RELAXATION VALUES OF MUCOID SPUTUM, 60 SECONDS AFTER APPLICATION OF STRESS, WITH STANDARD DEVIATIONS, FOLLOWING *IN VITRO* TREATMENT

Agent <sup>1</sup>	Stress Relaxation at 60 Seconds as % of Pretreatment Value
A.A.H.Cu .. .. .	45.7 ± 11.1
Water .. .. .	77.5 ± 12.8
P .. .. .	0.01 > P > 0.001 (n = 20)

<sup>1</sup> Contact time 20 minutes.

TABLE IV

CHANGES IN THE FOUR VISCOSITY PARAMETERS, AS MEASURED BY FERRANTI-SHIRLEY VISCOMETER AFTER NEBULIZATION WITH THE COLLISON APPARATUS

Mucolytic Agent	Static Yield Value	Dynamic Yield Value	Apparent Viscosity	Limiting Viscosity	No. of Subjects
A.A.H.Cu	66.6 ± 11.9	69.7 ± 11.3	67.2 ± 12.2	67.3 ± 12.6	10
Water ..	78.7 ± 11.4	82.7 ± 11.0	78.8 ± 8.3	79.8 ± 10.7	10
P value	0.05-0.02	0.05-0.02	0.05-0.02	0.05-0.02	

The results in Tables IV and V are expressed as a % of original viscosity values, with standard deviations.

TABLE V

CHANGES IN THE FOUR VISCOSITY PARAMETERS AS MEASURED BY FERRANTI-SHIRLEY VISCOMETER, AFTER NEBULIZATION WITH A SIMPLE HAND-OPERATED NEBULIZER

Mucolytic Agent	Static Yield Value	Dynamic Yield Value	Apparent Viscosity	Limiting Viscosity	No. of Subjects
A.A.H.Cu	87.3 ± 16.4	94.4 ± 26.1	87.2 ± 15.8	86.1 ± 16.8	6
Water ..	94.9 ± 7.7	98.0 ± 9.9	93.0 ± 14.8	88.1 ± 16.5	6

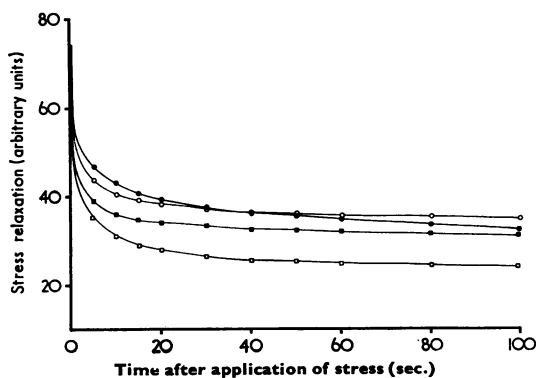


FIG. 5. Effect of nebulized A.A.H.Cu and water on stress relaxation of expectorated mucoid sputum in a patient. ○ prior to water nebulization. ■ after water nebulization. ● prior to A.A.H.Cu nebulization. □ after A.A.H.Cu nebulization.

**Stress relaxation studies** Figure 5 represents the reduction in stress relaxation of sputum, produced by Collison nebulized A.A.H.Cu and water in an individual patient.

The stress relaxation of the expectorated sputum was less after A.A.H.Cu nebulization than after water nebulization (Table VI).

TABLE VI

COMPARISON OF STRESS RELAXATION VALUES OF MUCOID SPUTUM 60 SECONDS AFTER APPLICATION OF STRESS, WITH STANDARD DEVIATIONS FOLLOWING NEBULIZATION TREATMENT

Agent <sup>1</sup>	Stress Relaxation at 60 Seconds, as % of Pretreatment Values
A.A.H.Cu .. ..	70.8 ± 9.3
Water .. ..	83.4 ± 10.4
P .. ..	0.05 > P > 0.02 (n=12)

<sup>1</sup> Collison apparatus, over 20 minutes.

The reduction in sputum stress relaxation, although still statistically significant, is much less compared with the *in vitro* results.

#### DISCUSSION

The earliest studies on mucus viscosity employed methods based on Ostwald's capillary flow viscometer (Robertson, Ropes, and Bauer, 1940; McClean and Hale, 1941), a technique, however, which should be confined to simple fluids obeying the laws of physics, *i.e.*, Ideal or Newtonian fluids. Usually, relative viscosity was measured against water. Later, viscometers based on timing the fall of a steel ball under gravity through mucus were used (Armstrong and White, 1950). These methods

only give a single point value for viscosity, and do not take into account the fact that viscosity may alter with shear rate: we do not think that elasticity can be studied successfully by these means.

The method of determining the force required to push a perforated disc through a column of sputum was used by Elmes and White (1954), Hirsch, Kory, and Hamilton (1966), and Hirsch and Kory (1967). This enables static yield values to be obtained, *i.e.*, minimum force required to produce flow.

Palmer (1957) made subjective and objective clinical studies of patients receiving nebulized water and detergent (Alevaire), sodium bicarbonate, and sodium chloride. The sputum was studied using a cone and plate viscometer, which reflected the deflection on to a circular scale for a given rotation speed. He concluded that the benefit to patients stemmed from rehydration of partly dried mucus. Later Palmer (1961) demonstrated the effects of A.A.H.Cu, *in vitro* and *in vivo*, again using a simple cone and plate viscometer with single point measurements of viscosity.

Mucolytic properties of *N*-acetylcysteine were reported by Sheffner in 1963 and Sheffner *et al.* (1964) using a viscometer tube and a Brookfield rotational viscometer. The *in vitro* effects of various mucolytic agents, including cysteine, *N*-acetylcysteine, lysozyme, trypsin, and pronase, were studied by Gernez-Rieux *et al.* (1964) using rotational methods.

Carson, Goldhamer, and Weinberg (1966) studied properties of cat mucus by determining the velocity of steel balls, moving on through mucus in an electromagnetic field, generated from probes fixed at critical distances from the trachea. They also noted that if mucus was made too thin then cephalad flow was impaired, *i.e.*, ciliary beating became ineffective.

The Ferranti-Shirley viscometer has been used to study mammalian mucus behaviour by Miller and Goldfarb (1965) in their preliminary studies prior to designing a simulated cilia apparatus using a vibrating spring.

*In vitro* studies on mucoid sputum from chronic bronchitics using (1) 20% *N*-acetylcysteine and (2) ascorbic acid-hydrogen peroxide-Cu solution reduced the four chosen viscosity parameters (static yield value, dynamic yield value, apparent viscosity, and limiting viscosity) by at least half (see Table I). The reduction of viscosity obtained with 4-molar urea or 0.005% copper sulphate solution, however, was not of statistical significance.

As noted by previous workers, both the ascorbic acid-hydrogen peroxide-Cu<sup>++</sup> solution and *N*-acetylcysteine had to be used immediately on preparation to obtain best results. Complete chelation by sodium edetate of Cu<sup>++</sup> present in the preparation, before the addition of mucus, reduced *in vitro* mucolytic activity but did not abolish it. The catalytic influence of Cu<sup>++</sup> on ascorbic acid-induced biological reductions has been examined by Barron, DeMeio, and Klemperer (1936), Dekker and Dickinson (1940), Meiklejohn and Stewart (1941), and Matsumura and Pigman (1965).

Unlike Miller and Goldfarb (1965) we could not detect thixotropic properties in bronchial mucus (*i.e.*, reversibility to original structure). Elmes and White (1954) also found no thixotropic behaviour.

The *in vivo* studies on chronic bronchitics using ascorbic acid-hydrogen peroxide-Cu<sup>++</sup>, nebulized by an efficient hospital nebulizer (Collison type), revealed a much smaller but useful reduction in the four viscosity parameters compared with the *in vitro* figures (see Table IV). All patients experienced some ease of expectoration even after the Collison-type nebulization with water, but 8 out of 10 patients thought that the benefit was greater with the mucolytic agent.

When using a simple hand-operated nebulizer no significant reduction in viscosity was obtained with ascorbic acid-hydrogen peroxide-Cu<sup>++</sup> or water, indicating unsatisfactory nebulization. Here, 2 out of 10 patients considered their expectoration to be easier with the mucolytic agent.

Complete loss of viscosity could render the passage of mucus by the bronchial cilia impossible, but insufficient viscosity reduction occurs with nebulization methods for this to be a risk, unless mucolytic agents are employed in bronchial lavage. A discussion and warning on this latter technique is given by Webb (1962).

The simple technique, involving a modified chemical balance, allows sputum to be examined rheologically in its natural state. Comparison of the stress relaxation values obtained correlate well with the results of the Ferranti-Shirley viscometer.

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## GLOSSARY

*Viscosity* (poise) =  $\frac{\text{shear stress (dynes per sq. cm.)}}{\text{shear rate (sec.}^{-1}\text{)}}$

*Relative viscosity* The viscosity of a fluid expressed relative to water

*Ideal (or Newtonian) Fluid* A fluid obeying all the rules of physics. For such fluids the rate of shear is in proportion to the applied stress. Most biological fluids are Non-ideal (or Non-Newtonian) and there is no direct proportion between rate of shear and applied stress

*Static yield value* Minimum force (dynes per sq. cm.) required to cause flow

*Dynamic yield value* Minimum force (dynes per sq. cm.) required to cause flow after the shearing process

*Apparent viscosity* Ratio of shear stress to shear rate at maximum shear rate, expressed in poise

*Limiting viscosity* Reciprocal of slope of linear portion of the graph. expressed in poise (limiting and apparent viscosity will be identical for ideal fluids)

*Stress relaxation* = A complex property involving viscosity and elasticity

*(Muco)elasticity* A muco-elastic material on being made to flow has innate ability to resume its previous position or shape

*Thixotropic property* Time-dependent, reversible loss of consistency accompanying the application of shear

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