VARIATION IN THE COMPOSITION OF SPUTUM IN CHRONIC CHEST DISEASES

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THE physiological secretions of the human respiratory tract are altered in quantity and possibly character in diseases of the airways such as chronic bronchitis and asthma (M.R.C. Committee on the Aetiology of Chronic Bronchitis (1965); Bukantz and Berns, 1958). Since excessive secretion of mucus is one of the principle features of these diseases, investigation of the way in which the mucus differs from that of normal bronchial secretion might illuminate the mechanism of the underlying morbid changes. Normal bronchial secretion, however, is not easy to obtain because intubation in itself causes inflammation (Havez, Roussell, Degand and Biserte, 1967) but sputum, the pathological counterpart of bronchial secretion, is readily available for study.

Since the original investigations of Muller (1896), many workers have attempted to elucidate the composition of the mucous ground substance of sputum but their investigations have largely been concerned with the high-molecular-weight components (Brogan, 1960; Masson, Heremans and Prignot, 1965). The ground substance of sputum, however, is a gel and this state consists of a continuous and a disperse phase. If sputum is centrifuged at high ***g***, it is possible to separate partially the continuous phase from the disperse phase (M. Campbell, personal communication). The supernatant consists of pure continuous phase and thus may be studied separately. Here, the use of this method in the investigation of the variation in sputum composition of 2 patients is described.

MATERIALS AND METHODS

Collection of sputum.—Two patients were selected for study, one of whom was suffering from chronic obstructive bronchitis and the other from continuous asthma. These diagnoses conformed to the criteria of the M.R.C. Committee on the Aetiology of Chronic Bronchitis (1965) and to the classification of asthma by Turner-Warwick (1966). Sputum was obtained from these patients at weekly intervals over a period of 10 weeks and each collection was carried out over 24 hr. The patients were instructed to expectorate only after coughing and not to spit into the containers so as to avoid contamination of the samples with saliva as far as possible. The specimens were examined in the laboratory within 2 hr. of the 24 hr. collection period.

Analytical methods.—Na^+ and K^+ estimations were carried out by flame photometry using an EEL flame photometer (Evans Electroselenium Ltd., Halstead, Essex). pH was measured with a SNF23/B flow electrode in conjunction with a Vibret pH Meter (Electronic Instruments Limited, Surrey), and calculated as H^+ concentration when the data from each patient were compared. PO''_4 concentrations were determined by Bartlett's (1959) modification of the Fiske-Subbarow method. Cl' was estimated by the method of Baar (1962) and carbohydrate by Hewitt's (1958) modification of the anthrone method with galactose as the standard. Total protein estimations were carried out by a biuret method described by Itzhaki and Gill (1964) with human serum albumin as the standard.

Separation of the continuous phase of sputum.—Each sputum sample was weighed and drained on muslin in a glass funnel after which the retained mucus was centrifuged at 3000 × ***g*** for 30 min. to remove air bubbles. A sample was then centrifuged at 118,000 × ***g*** at 4°

for 4 hr. in the SW39 rotor of a model L2 Spinco ultra-centrifuge (Beckman Instruments Inc., California, U.S.A.). The supernatant (continuous phase) was aspirated from the disperse phase and weighed. The disperse phase was lyophilized and weighed.

Ultrasonic homogenization of sputum.—Some 5–10 ml. of the whole sputum was transferred into the transducer cup of a Mullard ultrasonic generator with a maximum power output of 500 w and a frequency of 20 KHz/sec. The titanium probe had a diameter ratio of 1 : 3. The cup and contents were cooled in iced water to below 10° after which the power output was increased to above the cavitation threshold and homogenization allowed to proceed for one min. The sample was again cooled down to below 10° and this cycle was repeated 4 times. Throughout the 5 cycles of homogenization, the temperature of the specimen was not allowed to rise above 15°.

Electrophoresis.—The relative proportions of the high-molecular-weight components of the continuous phase and of the homogenized sputum were determined by electrophoresis on 10 × 2·5 cm. cellulose acetate strips (Millipore Filter Corporation, Watertown, Mass. U.S.A.). 10–15 μl of fluid was applied in duplicate to the strips. Larger volumes than normally recommended (Kohn, 1958) were used in the present investigation because the concentration of high-molecular-weight components in the continuous phase and in the homogenized sputum was less than that normally encountered in plasma. Electrophoresis was carried out at a current density of 0·6 mA./cm. width for 1 hr. in a barbiturate buffer of pH 8·6, I = 0·7. The strips were dried at 105° for 10 min. and were stained for 10 min. in 0·2 per cent Ponceau S in 3 per cent w/v trichloracetic acid. The strips were then washed in 3 changes of 5 per cent v/v acetic acid for 5 min. and cleared in a 1 : 2 v/v mixture of cyclohexanone and alcohol. The relative proportions of the high-molecular-weight components were estimated by densitometry (Kipp and Zonen Densitometer, Delft, Holland). Integration under the area of each peak was carried out automatically during each scanning process and a correction was made for varying band width. The linear relationship between protein concentration and the density of staining by Ponceau S, described by Ritts and Ondrick (1964), was confirmed with human serum albumin.

The reliability of this method of estimation was checked by cutting uncleared duplicate electrophoretic strips into their various bands, eluting the stain with 2·5 ml. of 0·2 N sodium hydroxide and adding 0·1 ml. of glacial acetic acid to each solution. The optical density of the resulting solutions was measured at 530 mμ on a S.P. 500 spectrophotometer (Unicam Instruments Ltd., Cambridge). No significant difference ($P > 0·2$) was found between the average proportions of the albumin component (*vide infra*) of the continuous phase of sputum when series of 10 duplicate estimations by the 2 methods were compared. Differences were found, however, between the average proportions of other high-molecular-weight components when determined by the 2 methods.

Lysozyme activity.—Immediately after electrophoresis of a sample of continuous phase or homogenized total sputum, a narrow longitudinal strip of the cellulose acetate membrane was cut off and stained in Ponceau S for 2 min. This served to mark the positions of the 3 main bands. The zones from the unstained portion of the strip which contained protein were placed on an agar plate the surface of which had been inoculated with *Micrococcus lysodeikticus*. Cellulose acetate strip not containing protein was also placed on the plate to act as control. The plate was incubated at 37° for 16 hr. by which time the presence of lysozyme in any of the bands was revealed as a zone of growth inhibition in the lawn of organisms.

Immunoelectrophoresis.—Immunoelectrophoresis was carried out by a modification of the method of Scheidegger (1955) using 1 per cent Agarose (Seravac Laboratories, Berkshire, England), in a barbiturate buffer pH 8·6, I = 0·05. The electrophoresis was carried out at 1·1 mA./cm. width for 110 min. at room temperature. The precipitation pattern was then developed with rabbit anti-human serum (Hyland Laboratory Products, Norfolk, England) by incubating the plate at 37° for 48 hr.

RESULTS

Variation in the proportion and in the ionic composition of the continuous phase of the sputum gel

The weight of sputum and the proportion that separated as continuous phase in the ultracentrifuge varied from specimen to specimen in each patient and were

unrelated. Similar specimen to specimen variations in ionic composition and pH were found in the continuous phase. With one exception these were unrelated to the weight of sputum and to the proportion that separated as continuous phase. In the patient with chronic bronchitis, there was a correlation ($P < 0{\cdot}05$) between the Na^+ concentration of the continuous phase and the weight of the sputum.

In spite of considerable individual variation, the average proportion of continuous phase was greater in the asthematic patient than in the bronchitic patient ($P < 0{\cdot}02$). Similarly, the average Cl′ concentration of the continuous phase of the asthmatic patient was greater than that of the bronchitic patient ($P < 0{\cdot}001$) but the average K^+ concentration was greater in the bronchitic than in the asthmatic patient ($P < 0{\cdot}01$). No significant differences in the averages of the total sputum weight or in the averages of the H^+, Na^+ and PO''_4 concentrations of the continuous phase were found between the 2 patients (Table I).

TABLE I.—*Comparison of the Mucous Ground Substance of Sputum from 2 Patients over a Period of 10 Weeks Showing the Variation in the Weight of Sputum and Proportion that Separated as Continuous Phase at 118,000 × g and the variation in pH and Ionic Composition of the Continuous Phase*

	Asthmatic patient		Bronchitic patient	
	Average value	Limits of determined values	Average value	Limits of determined values
Wt. of sputum (g.)	67	40–85	68	36–107
Per cent continuous phase	77	55–83	57	27–77
pH	6·6	5·4–7·6	7·5	6·3–7·9
Na^+ (m-equiv./l)	100	85–115	100	87–120
K^+ (m-equiv./l)	23	20–28	27	23–35
Cl′ (m-equiv./l)	100	89–106	88	78–97
PO''_4 (mg./100 ml.)	37	24–63	38	24–55

Separation of the high-molecular-weight components of the continuous phase of sputum

Electrophoresis on cellulose acetate strip showed that the high-molecular-weight components of the continuous phase of sputum were resolved in the barbiturate buffer into 3 main groups of components (Fig. 1a). The band migrating with the highest mobility towards the anode (A band) consisted largely of a single component, negative in reaction to Schiff's reagent, which was shown to be identical with human serum albumin on immunoelectrophoresis (Fig. 2). This major component was sometimes preceded by an unidentified minor component.

The band migrating towards the cathode (L band) consisted of a single sharp peak (Fig. 1a). This was also found to be negative to Schiff's reagent and possessed lysozyme activity which was not found in any of the other bands.

The high-molecular-weight components which remained near the origin were called the M complex. This wide band consisted of several components and gave a positive reaction with Schiff's reagent, suggesting that it contained the glycoproteins of the continuous phase. Two main components of the band could be distinguished in most of the specimens from the asthmatic patient (I and II, Fig. 1b). Two minor components giving sharp peaks near the origin on the anodal side of the scanning pattern could be seen on some occasions and were found to be caused by freezing and thawing of the continuous phase specimens (Fig. 1c). They were probably due to denaturation of the glycoprotein complexes.

For purposes of comparison, the total area under all the peaks was estimated and the proportion of each of the components (A, M and L) was calculated as a percentage of the total area (Table II).

Distribution of the high-molecular-weight components between the phases of the sputum gel of the 2 patients

The concentration of carbohydrate, precipitated by 3 volumes of ethanol, and that of total protein in the continuous phase were estimated and compared with the corresponding values determined on total homogenized sputum (Table II). These values were found to vary between weekly specimens from each patient over the period of study. The average concentrations of protein, both in the continuous phase and in the total sputum, did not differ between the patients and were not related to either the proportion of albumin in the continuous phase or that in the total sputum. The average concentration of precipitable carbohydrate was significantly greater in the continuous phase of the bronchitic patient as compared with the asthmatic patient ($P < 0.05$).

In both patients between 30–40 per cent of the precipitable carbohydrate and between 60–65 per cent of the total protein were found on average in the continuous phase of the sputum. The ratios of average protein to carbohydrate concentration in the continuous phase were 4·92 in the sputum of the asthmatic and 3·00 in that of the bronchitic. The average relative concentrations of protein and carbohydrate in the disperse phase were calculated from the average concentrations in the continuous phase and total sputum (Table II) and from the average proportion of continuous phase (Table I). These relative concentrations were 1950 mg./100 ml. of protein and 1100 mg./100 ml. of carbohydrate in the disperse phase of the asthmatic sputum and 1200 mg./100 ml. of protein and 755 mg./100 ml. of carbohydrate in that of the bronchitic.

EXPLANATION OF PLATES

FIG. 1.(a)—Electrophoresis pattern on cellulose acetate strip of the continuous phase of a specimen of sputum from the asthmatic patient showing the A, M and L bands corresponding to the albumin, glycoprotein and lysozyme components.

Electrophoresis was carried out for 60 min. at 0·6 mA./cm. width in a barbiturate buffer, pH 8·6 and I = 0·70, and the strip was stained with Ponceau S.

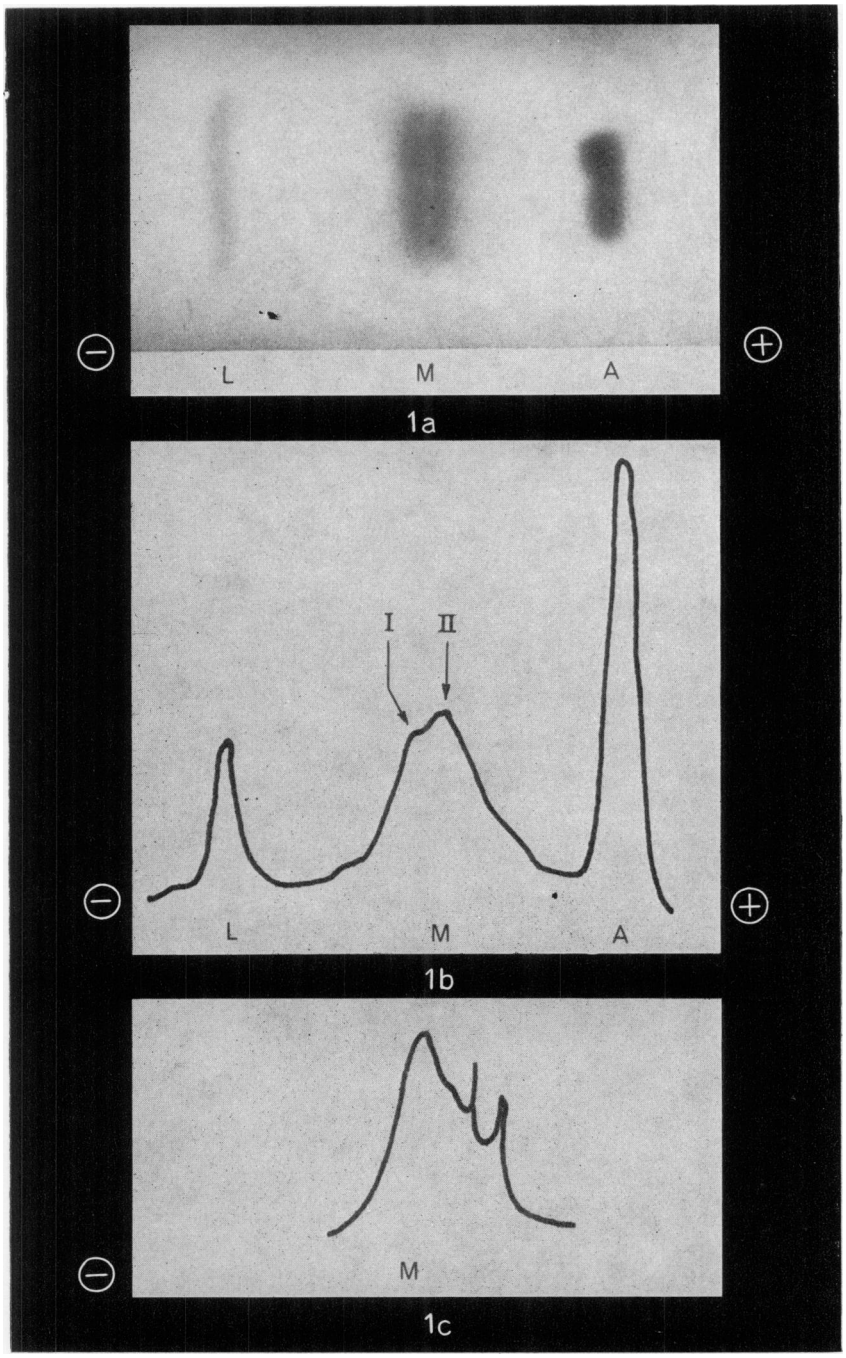
(b).—Densitometer scan of the electrophoretic strip, shown in (a), illustrating the resolution of the M band into two components.

(c).—Densitometer scan of the M band of a sample of the continuous phase of a specimen of sputum from the bronchitic patient; the sample was subjected to several cycles of freezing and thawing. This treatment gave rise to the appearance of two minor components on the anodal side of the M band.

FIG. 2.—Immunoelectrophoresis of the continuous phase of specimens of sputum from the asthmatic and bronchitic patients showing the identity with serum albumin of the bands moving with the highest mobility towards the anode. The precipitation patterns also show the greater variety of plasma proteins present in the continuous phase of the asthmatic as compared with that of the bronchitic sputum.

Electrophoresis was carried for 110 min. at 1·1 mA./cm. width in a barbiturate buffer, pH 8·6 and I = 0·05, using 1 per cent Agarose.

I = Human serum albumin, 0·1 per cent in 0·9 per cent NaCl. II = Continuous phase of sputum. III = Normal human serum, diluted 1/5 with 0·9 per cent NaCl. IV = Rabbit anti-human serum.



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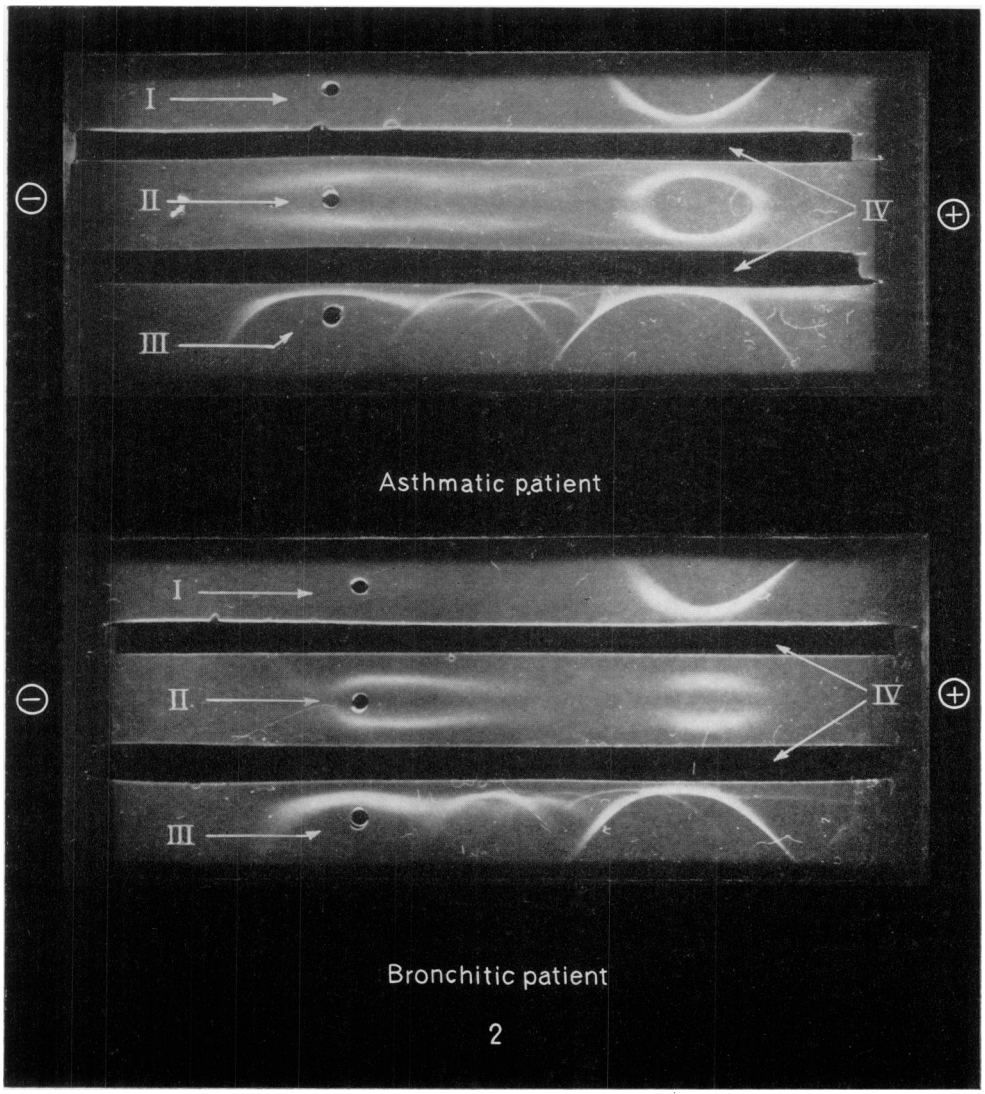

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TABLE II.—*Comparison of the Variation in the Distribution of High-molecular-weight Components Between the Phases of Sputum from 2 Patients over a Period of 10 Weeks*

	Asthmatic patient				Bronchitic patient			
	Continuous phase		Total sputum		Continuous phase		Total sputum	
	Average value	Limits of determined values	Average value	Limits of determined values	Average value	Limits of determined values	Average value	Limits of determined values
Precipitable carbohydrate (mg./100 ml. galactose)	106	86–145	337	181–500	171	90–265	422	219–800
Total protein (mg./100 ml. albumin)	522	385–635	851	560–1125	515	410–630	815	460–1145
Per cent A component	30	16–44	19	10–21	< 5	trace–9	< 5	trace–6
Per cent M component	57	45–70	69	68–71	72	60–80	82	75–92
Per cent L component	13	6–18	12	8–22	25	19–31	16	8–21

Estimations were carried out on total sputum, homogenized by ultra-sound, and on the continuous phase, separated by ultracentrifugation. The A, M and L components are the albumin, glycoprotein and lysozyme bands separated by electrophoresis.

Variation in the proportions of the high-molecular-weight components in the sputum of the 2 patients

Marked differences in the proportions of the individual high-molecular-weight components of the sputum were found between the 2 patients. The average proportion of the albumin (A) component in the bronchitic patient was less than 5 per cent both in the continuous phase and in the total sputum in contrast with the asthmatic patient in whom the continuous phase and total sputum yielded an average albumin proportion of 30 and 19 per cent of the high-molecular-weight components.

The ultrasonically homogenized sputum always contained less albumin than that found in the continuous phase in contrast with the consistently higher concentrations of the glycoprotein (M) complex found in homogenized sputum as compared with the continuous phase (Table II). The lowest proportion of albumin in the asthmatic was found during a remission of his illness brought about by a change in steroid therapy. In addition to the differences in the proportions of the albumin component between the 2 patients, the continuous phase of the asthmatic sputum contained a greater variety of plasma proteins than that found in the bronchitic sputum (Fig. 2).

A greater average proportion of the glycoprotein (M) complex was found in the continuous phase and total sputum of the bronchitic patient as compared with the asthmatic patient. These results reflect the difference found in the precipitable carbohydrates between the 2 patients. The proportion of lysozyme (L) component of the continuous phase of the bronchitic patient was on average nearly twice that of the corresponding phase of the sputum of the asthmatic patient.

DISCUSSION

The ultracentrifugation of sputum provides a convenient method of separating out the continuous phase of the mucous gel. It preserves the concentration of the components and does not cause their denaturation. This method has the further advantage that relatively small volumes of material in the range of 3–5 ml. can be examined. Previously reported methods of separating sputum into a sol phase and a gel phase involve freezing and thawing the sputum followed by dialysis against water and low speed centrifugation (Biserte, Havez and Cuvelier, 1963; Masson *et al.*, 1965), but such techniques do not preserve components in their original concentration. The present study has also shown that freezing and thawing of the continuous phase of the mucous gel tends to denature the glycoprotein complexes.

Investigation of 2 patients over a period of 10 weeks has shown that the proportion of the continuous phase of sputum varied considerably from week to week in each patient. The cause of this variation remains uncertain. It may have been due to the drying of the mucus in the airway during a variable time before expectoration. With one exception, however, variation in concentration of ions (Na^+, K^+, Cl' and PO''_4) in the continuous phase was unrelated either to variation in proportion of the continuous phase or to the amount of sputum produced during each 24 hr. period. It would seem, therefore, that changes in proportion of the continuous phase and in the concentration of ions may be related to the variation in glandular and goblet cell activity in response to inflammatory stimuli rather than to drying of the mucus in the airway.

The average value of the Na^+ concentrations in the continuous phase of both patients was less than that of normal plasma and the K^+ concentration was higher than that of plasma but less than that normally found within cells; both were present in higher concentrations than those reported by Girnez-Rieux, Biserte, Havez, Voisin and Cuvelier (1963) for whole sputum. PO''_4 concentrations found in the present investigation were similar to those normally found in plasma. The average K^+ concentration was higher in the patient with chronic bronchitis than in the patient with asthma even though the asthmatic patient was receiving steroid therapy. This suggests greater glandular activity in the chronic bronchitic patient than in the asthmatic patient as high secretory K^+ levels have been related to elevated exocrine activity in some subjects (Gordon and Cage, 1966).

The high-molecular-weight components of the continuous phase could be resolved into 3 main electrophoretic fractions. The A component was composed largely of serum albumin, thus confirming previous work in which the presence of this protein was demonstrated in sputum (Brogan, 1960; Havez *et al.*, 1967). The L component was largely composed of lysozyme and this enzyme has also previously been identified in sputum (Lorenz, Korst, Simpson and Musser, 1957; Masson *et al.*, 1965). The third fraction, the M complex, was heterogenous and composed mostly of glycoproteins. It is of interest that 2 variable minor components of this complex were related to freezing and thawing of the continuous phase which suggests that this procedure denatures glycoproteins. The glycoprotein complex was made up of at least 2 components but the resolution of this fraction was not described in the present investigation as the complex is at present under detailed investigation.

The distribution of the high-molecular-weight complexes between the phases of the sputum gel was measured by comparing the total protein and precipitable

carbohydrate in the continuous phase and in the total homogenized sputum. This distribution varied considerably during the period of study in each patient but, on average, between 30–40 per cent of the precipitable carbohydrate and between 60–65 per cent of the total protein were found in the continuous phase of the sputum in both patients. In neither patient, however, was the amount of protein related to the proportion of albumin in the sputum. The ratio of protein to carbohydrate concentration in the continuous phase and the relative concentrations of protein and carbohydrate in the disperse phase were greater in the sputum of the asthmatic than in that of the bronchitic patient.

A marked difference in the proportions of the principal high-molecular-weight components in the sputum was found between the 2 patients. An average of 30 per cent of the high-molecular-weight components of the continuous phase of the sputum from the asthmatic patient consisted of serum albumin whereas albumin comprised less than 5 per cent of the high-molecular-weight components of the sputum from the chronic bronchitic patient. It is of interest that the lowest concentration of albumin was found in the continuous phase and total sputum of the asthmatic patient at a time when a change in steroid therapy brought about clinical improvement. The relative concentration of albumin was always less in the total sputum than in the continuous phase suggesting that the bulk of this plasma protein was present in the continuous rather than in the disperse phase of the gel.

The complex nature of sputum makes it difficult to study the composition of this material with precision and to relate the results obtained to the morbid condition under examination. In any specimen, some salivary contamination is almost inevitable and this can be eliminated only by obtaining sputum by bronchoscopy which is rarely justifiable. Reid (1967), however, has shown by a comparison of salivary and sputum neuraminic acid levels that, for practical purposes, salivary contamination of sputum is not a problem. Wide weekly variations in composition and in the physical state of the mucous ground substance, as assessed by the proportion separating as continuous phase in the ultracentrifuge, were found in the sputum of each of the patients in the present investigation. In mucoviscoidosis, considerable variation in the physical state of sputum is brought about by changes in pH and ionic composition (Tappan and Zaler, 1963). No relation, however, was found in the present study between the physical state of the sputum and its pH or ionic composition. This suggests that the physical nature of the sputum gel in asthma and chronic bronchitis is not dependent on variation within physiological limits in pH or ionic composition.

Two types of secretion have been distinguished in sputum by McCarthy and Reid (1964), an " active " secretion from the glandular elements of the epithelium and a " passive " secretion or transudate through the lining of the airways. In the present investigation, the ratios of protein to carbohydrate concentration suggest that the transudate type of secretion predominated in the sputum of the asthmatic whereas the glandular type of secretion was the main element in that of the bronchitic patient. This would imply that the sputum of the asthmatic patient had more in common with an inflammatory exudate than that of the chronic bronchitic and this hypothesis is supported by the finding of a greater proportion of serum albumin and a greater variety of plasma proteins in the asthmatic as compared with the bronchitic sputum.

Mucoid impaction of the bronchi has been described post-mortem in patients

who have died from status asthmaticus (Bukantz and Berns, 1958). Although the sputum of the asthmatic patient in the present study was less viscid than that of the chronic bronchitic, as judged by the proportion separating at the continuous phase, the relative concentration of high-molecular-weight components in the disperse phase of the sputum of the asthmatic patient was the greater of the two. Rapid absorption of water, therefore, would tend to produce a more highly viscid gel in the asthmatic than in the bronchitic patient. The capacity of the asthmatic sputum to produce a highly tenacious gel, when the water in the continuous phase is removed, may account for the finding of plugs of inspissated mucus in the bronchi of the patients whose asthma has had a fatal outcome.

SUMMARY

The composition of sputum from an asthmatic and from a chronic bronchitic patient has been examined using ultracentrifugation and ultrasonic homogenization. Considerable variation was found in the proportion and in the ionic composition of the continuous phase of the sputum from each patient over 10 weeks but in neither was the physical state of the mucus related to its pH or ionic composition.

Serum albumin, lysozyme and a glycoprotein complex were the principal high-molecular-weight components indentified in the sputum of both patients. The proportion of albumin in the sputum of the asthmatic was greater than in that of the bronchitic and this protein was mostly present in the continuous phase. The ratio of protein to carbohydrate in the continuous phase, the variety of plasma proteins in this phase and the relative concentrations of protein and carbohydrate in the disperse phase were all greater in the asthmatic than in the bronchitic sputum. It was suggested that the sputum from the asthmatic patient had more in common with an inflammatory exudate than that from the chronic bronchitic.

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