THE HIGH MOLECULAR WEIGHT COMPONENTS OF SPUTUM

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THE healthy human bronchial tree is estimated by Policard and Galy (1945) to produce about 100 ml. of mucus in 24 hr., which is normally swallowed together with the secretions of the upper respiratory tract and bucco-pharynx. In certain respiratory diseases, notably chronic bronchitis and asthma, this mechanism becomes deranged and an alteration in the character of the secretion occurs together with a tendency towards excessive formation. This excessive and altered secretion is of significance in chronic bronchitis, as it causes obstruction of the smaller air-passages (Oswald, 1958). Similarly, the obstructive role of the viscid mucus in intrinsic bronchial asthma is a factor of considerable importance in the aetiology of this syndrome (Bukantz, 1958). A prominent clinical feature of chronic bronchitis and asthma is the expectoration of the intra-bronchial accumulation of mucus as sputum. Despite the likely role played by the mucous coagulum of sputum in such chronic respiratory diseases, relatively little work has been carried out on the ground substance of sputum since the investigations of Muller (1896 ; 1901).

In a recent paper (Brogan, 1959), the carbohydrate complexes of pooled sputum were examined. Two mucopolysaccharide fractions, similar in type to the blood-group substances, and a mucoprotein, which had some properties in common with the urinary mucoprotein described by Tamm and Horsfall (1952), were identified. Similar findings on the mucopolysaccharide fraction of sputum were obtained previously by Bukantz and Berns (1958) who also showed, serologically, that this fraction possessed blood-group activity. Both Bukantz and Berns (1958) and Brogan (1959) isolated the carbohydrate complexes of sputum by digestion with pepsin of sputum which had been precipitated with ethanol or dried with acetone. Although digestion with a proteolytic enzyme is an adequate method of isolating the carbohydrate complexes, which amount to less than 50 per cent of the total solids of pooled sputum, such a method decomposes all the protein complexes.

An investigation was therefore carried out with the object of ascertaining the nature of the other high-molecular-weight components of sputum. The purpose of this work was to attempt to elucidate further the nature of the gel composing the mucous ground-substance of sputum. Methods of rendering the mucous ground-substance of sputum soluble in water by shaking with ballotini or by the high-speed stirring technique, described by Marmion, Curtain and Pye (1953), were found to be unsatisfactory because foaming of the mucin occurred together with considerable denaturation of the components in the air-liquid interface of the foam. A means was sought, therefore, by which mechanical

shock could be applied to the mucous coagulum of sputum without causing it to foam. Ultrasonic vibration was found to fulfil this requirement.

The present paper describes a method of homogenizing sputum, using ultrasonic vibration, which renders the high-molecular-weight complexes, forming the coagulum, soluble in water whilst largely preserving the identity of the major components.

MATERIALS AND METHODS

Sputum.—Sputum was collected from patients who were suffering from long-standing respiratory diseases such as chronic bronchitis and asthma. Only specimens which would be assessed clinically as " mucoid " or faintly " mucopurulent " (Chamberlain, 1957), were retained for use. Each mucous coagulum was inspected and visible foreign bodies were removed. The mucous coagula were washed in several changes of water and were pooled together in a beaker. The volume of the pooled mucin was measured before transferring it to the metal cup of the ultra-sonic vibrator.

Analytical methods.—The methods of estimating total nitrogen, organic and inorganic phosphorus, reducing sugars, amino sugars, and pH have been described previously and were used without change. Similarly, the methods used for the paper partition chromatography and for the colorimetric and spectrophotometric measurements were the same as those used in the previous work (Brogan, 1959).

Electrophoresis.—Tiselius electrophoresis was carried out with a Perkin-Elmer apparatus.

Paper electrophoresis was carried out by a method closely similar to that described by Franglen (1953). Platinum electrodes were used in the electrophoresis apparatus and 0·05 M-sodium diethylbarbiturate-HCl buffer, pH 8·2, was employed. The electrophoresis was carried out for 12 hr. at a p.d. of 120 v. using Whatman No. 100 paper. Electrophoreses of sputum and of ultrasonically homogenized sputum were carried out simultaneously with that of normal human serum. Bromophenol blue was used to stain the bands.

Immuno-electrophoresis was carried out by a method based on that of Grabar and Williams (1955). The agar gel plates were prepared by mixing equal volumes of filtered 3 per cent agar and 0·1 M-sodium diethylbarbiturate-HCl buffer, pH 8·2, thus giving a final agar concentration of 1·5 per cent, the ionic strength of the block being 0·05. The dimensions of the agar slabs were 17 × 8 × 0·5 cm. and the wells for the antigens were each 2 × 0·3 cm., and were set with their inner edges approximately 1 cm. from the central channel. The potential gradient across the gel was 4 v. cm.$^{-1}$ and the electrophoresis was carried out for 5 hr. Rabbit anti-human serum was used as indicator in the central channel and normal human serum, as a 10 per cent (v/v) solution in 0·05 M-sodium diethylbarbiturate-HCl buffer, pH 8·2, was used as the control antigen. After the electrophoretic runs, the agar slabs were kept at room temperature in an atmosphere saturated with water vapour so that precipitin band formation could take place.

Ultra-sonic homogenization of sputum.—The following procedure proved most satisfactory for homogenizing a pool of sputum. Sputum (50 ml.) was poured into the metal cup of a Mullard Ultra-sonic Generator, E 6590 A, and the cup and its contents were allowed to come to 0°. The sputum was then subjected to ultra-sonic vibration at 20 k. cyc./sec. for 2 hr. A shorter period of time than 2 hr. always resulted in incomplete dissolution of the coagulum. The resulting solution, which had a greyish opalescence, was centrifuged at 0° at 1000 ***g*** to remove insoluble material. The supernatant solution was dialysed for 17 days against water at 0° and was then centrifuged at 15,000 ***g*** to remove a small amount of insoluble material. The resulting solution was then dried from the frozen state. A voluminous white powder (625 mg.) resulted.

The combined residues from the ultra-sonic homogenization were then washed several times with water and dried *in vacuo*. A grey-white powder (55 mg.) resulted. Microscopy showed that this residue was largely organised, consisting of the remains of leucocytes, epithelial cells and bacteria. There was also some amorphous basophilic material present.

The total yield of material, computed on a w/v basis, was 1·36 per cent of the parent sputum, being made up of 1·25 per cent of water-soluble high-molecular-weight complexes and 0·11 per cent of insoluble residue. This compares with an overall yield of 1·5 per cent for acetone-dried sputum (Brogan, 1959).

RESULTS

Properties of the ultra-sonically homogenized sputum.

Chemical properties.—The ultra-sonically homogenized sputum dissolved in water to give a solution of neutral pH. Addition of dil. HCl to pH 6·0 caused precipitation but continued addition of acid to 0·5 N-HCl caused the precipitate to re-dissolve. If NaCl was added to the solution to I = 0·005, precipitation did not occur with dil. HCl until pH 4·6 was reached. No precipitation occurred when NaCl was added, to a concentration of 0·58 M-NaCl, to a neutral aqueous solution of the ultrasonically homogenized sputum, but precipitation occurred when equal volumes of reagents that normally precipitate proteins were added to such solutions.

TABLE I.—*Comparison of the Composition of Ultra-sonically Homogenized Sputum and Acetone-dried Sputum.*

Analyses of the preparations were carried out on samples which were dried at 56° for 1 hr. The analytical results are expressed as a percentage of this dry weight. For methods of analysis, see text.

	Ultra-sonically homogenized sputum	Acetone-dried sputum
Yield (per cent)*	1·25	1·50
Total N (per cent)	8·8	10·4
Amino sugars (per cent)	9·3	10·8
Reducing sugars (per cent)	17·4	18·7
Total P (per cent)	0·08	0·62
Inorganic P (per cent)	0·08	—
Organic P (per cent)	Nil	—
Ratio amino sugars/total N	1·1	1·0

* The percentage yield was computed on a w/v basis with the original sputum.

In Table I, the analysis of ultra-sonically homogenized sputum is compared with that of acetone-dried sputum. It may be seen that the reducing-sugar content and that the ratio of amino sugars : total nitrogen are comparable with those of acetone-dried sputum. The total phosphorus content of the ultra-sonically homogenized sputum, however, is lower than that of acetone-dried sputum, and is made up wholly of inorganic phosphorus.

Physical properties.—In Fig. 1, the ultra-violet absorption spectrum of a 0·025 per cent solution of the ultra-sonically homogenized sputum in 0·85 per cent NaCl is compared with that of a comparable solution of the mucoprotein prepared from acetone-dried sputum (Brogan, 1959). This latter complex contained 0·74 per cent of organic phosphorus. It may be seen that the λ_{max} for the ultra-sonically homogenized sputum is 278 mμ compared with the λ_{max} for the sputum mucoprotein, which is 259 mμ. The total nitrogen contents of the ultra-sonically homogenized sputum and of the mucoprotein prepared from acetone-dried sputum are 8·8 per cent and 8·7 per cent respectively. The ultra-violet absorption spectrum together with the absence of organic phosphorus in this complex suggests that this preparation of the high-molecular-weight components of sputum probably does not contain nucleic acids. This is in contrast with the sputum mucoprotein prepared from acetone-dried sputum, as both the ultra-

violet absorption spectrum of this complex and its content of organic phosphorus indicate that the substance contains nucleic acids.

Tiselius electrophoresis of the ultra-sonically homogenized sputum was carried out in a 0·02 M-Na_2HPO_4-NaH_2PO_4 buffer, pH 7·4, containing 0·15 M-NaCl, for 75 min. at 105 v and 20mA. In Fig. 2, the ascending electrophoretic pattern of the ultra-sonically homogenized sputum is compared with that of normal human serum, the electrophoresis of which was carried out under the same conditions. It may be seen that the ultra-sonically homogenized sputum is composed of five components. The two lesser components, 1 and 5, account for approximately 13 per cent and 10 per cent of the total complex respectively. Components 2 and 3 together account for approximately 43 per cent of the complex, and com-

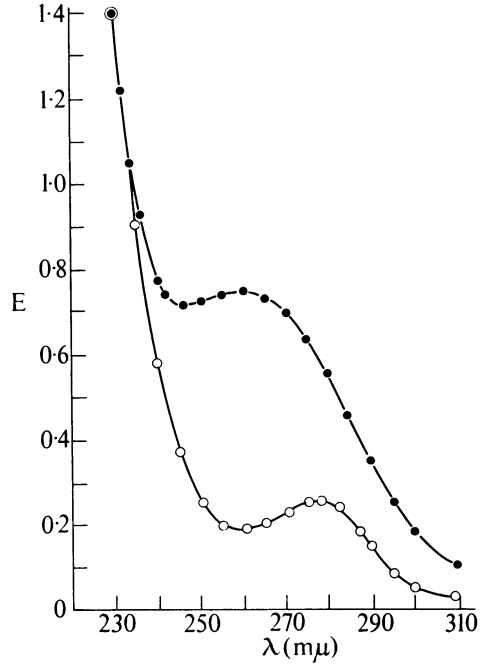

FIG. 1.—Ultraviolet-absorption-spectra curves of 0·025 per cent solutions of ultrasonically homogenized sputum (○) and of the sputum mucoprotein (●), prepared by digesting acetone-dried sputum with pepsin. Both complexes were dissolved in aq. 0·85 per cent NaCl. The λ_{max} for the ultrasonically homogenized sputum was 278 mμ and the λ_{max} for the sputum mucoprotein was 259 mμ.

ponent 4 for approximately 34 per cent of the complex. These figures are calculated by the method of Tiselius and Kabat (1939). It may be seen from Fig. 2 that component 4 of the ultra-sonically homogenized sputum migrates at the same speed as normal human serum-albumin.

Paper electrophoresis of the ultra-sonically homogenized sputum was carried out on a 1 per cent solution of the material in 0·05 M-sodium diethylbarbiturate buffer, pH 8·2, for 12 hr. at a p.d. of 120 v. Paper electrophoresis of normal human serum was carried out simultaneously as a control. The ultra-sonically homogenized sputum was resolved into two bands, one of which moved at the

same speed as the albumin of the human serum. The other band remained at the origin. It is the experience of the author that mucopolysaccharides and proteins conjugated with polysaccharides, such as the mucoproteins which can be isolated

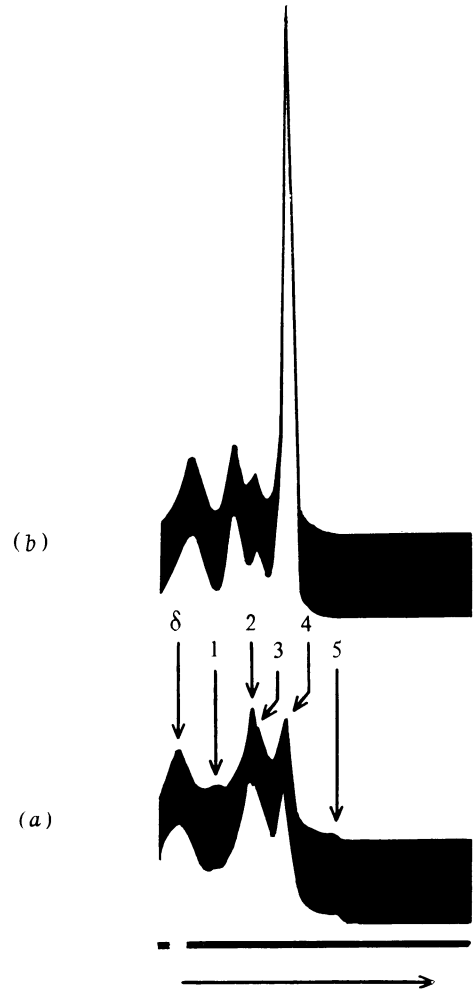

FIG. 2.—(*a*) Ascending Tiselius electrophoretic pattern of ultrasonically homogenized sputum showing the 5 components of this complex. (*b*) Ascending Tiselius electrophoretic pattern of normal human serum. Both the electrophoretic runs were carried out in 0·02M-Na_2HPO_4-NaH_2PO_4 buffer, pH 7·4, containing 0·15M-NaCl, at 100 v. and 20 mA. Both patterns were scanned at 75 min. The concentration of the ultrasonically homogenized sputum was 2 per cent (w/v) and that of the normal human serum was 25 per cent by vol. in the above phosphate buffer, the solution being dialysed against the buffer for 24 hr.

from sputum and urine, do not migrate on paper under the influence of an electric field. It may be suggested, therefore, that the component that migrates at the same speed as human serum albumin behaves as a protein in so far as it is able to move freely in an electric field on paper.

Paper electrophoresis of non-homogenized sputum, from the same pool as that from which the ultra-sonically homogenized sputum was prepared, was also carried out under the conditions described above. The purpose of this run was to ascertain whether the fraction which is present in ultra-sonically homogenized sputum and moves at the same speed as human serum albumin would be free to migrate in an electric field on paper from the native sputum coagulum. Only one band at the origin, however, was obtained : no change was observed if the time of electrophoresis was extended to 48 hr. This fraction, therefore, is

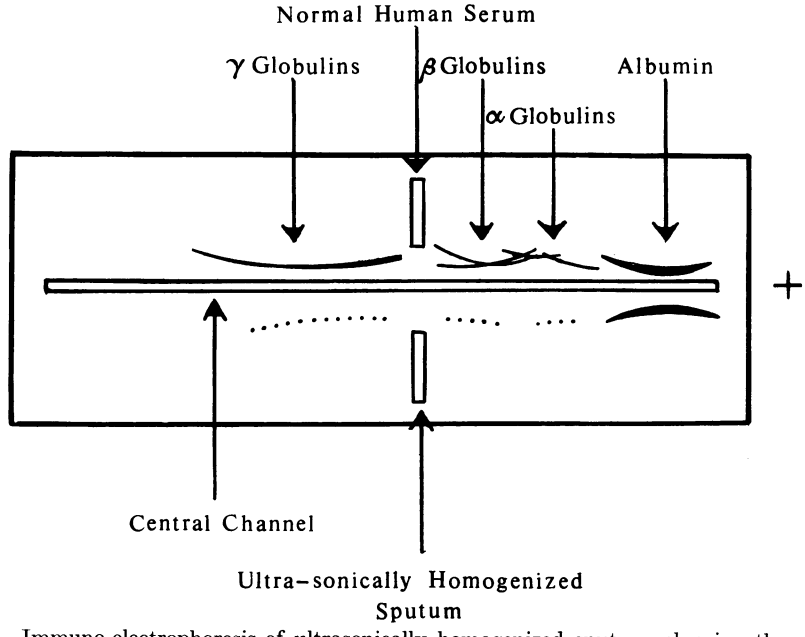

FIG. 3.—Immuno-electrophoresis of ultrasonically homogenized sputum, showing the precipitin zone corresponding to human serum albumin. The dotted lines indicate the faint precipitin zones, corresponding to the α, β and γ globulins which appeared after the agar-gel slab had been allowed to stand for 3 days at room temperature. The immuno-electrophoresis was carried out for 5 hr. at a potential gradient of 4 v cm.$^{-1}$. Normal human serum was used as control antigen and rabbit anti-serum to normal human serum was used, in the central channel, as indicator of precipitin formation.

apparently in a state in the parent sputum coagulum in which it is precluded from free movement in an electric field.

Immuno-electrophoresis was carried out on a 1 per cent solution of the ultra-sonically homogenized sputum in 0·05 M-sodium diethylbarbiturate buffer, pH 8·2. Normal human serum, diluted to a concentration of 10 per cent with the same buffer was used as a control and rabbit anti-human serum was used as an indicator of precipitin formation. The run was carried out for 5 hr. Fig. 3 gives the results of the immuno-electrophoresis. An intense precipitin zone was formed against the anti-serum by a fraction of the ultra-sonically homogenized sputum. This precipitin zone corresponded in position to that formed by the albumin of the human serum control. Faint precipitin zones corresponding to the α, β and γ globulins were formed but these did not appear until the gel had been kept

at room temperature for several days. It is thus likely that the component of ultra-sonically homogenized sputum that migrates at the same speed as human serum albumin, both in the Tiselius apparatus and in the paper electrophoresis apparatus, is composed largely of human serum albumin.

Partial Separation of the Carbohydrate Components of Ultra-sonically Homogenized Sputum

As the mucoprotein component of sputum was known to be insoluble at pH 3·3, this property was used to effect the separation of the mucoprotein complex of the ultra-sonically homogenized sputum from the mucopolysaccharide fraction in the manner described below. This method, however, was able to achieve only partial separation of these carbohydrate components from the albumin complex of the ultra-sonically homogenized sputum.

Ultra-sonically homogenized sputum (250 mg.) was dissolved in 50 ml. of water, NaCl was added to I = 0·005 and the solution was adjusted to pH 4·6 with 0·05 N-HCl. The addition of NaCl to I = 0·005 was a necessary step as pan-precipitation of all the components at pH 6·0 was liable to take place if 0·05 N-HCl were added to salt-free solutions of the ultra-sonically homogenized sputum. The resulting precipitate was centrifuged, suspended in 40 ml. of water and 0·05 N-NaOH added drop-wise until the precipitate dissolved, the resulting solution being pH 7·4.

Further 0·5 N-HCl was added to the supernatant solution, which largely contained the mucopolysaccharide fraction, until pH 1·8 was reached. No further precipitation occurred. The solution was neutralized with 0·5 N-NaOH, dialysed for 7 days against water at 0° and dried from the frozen state. A voluminous white powder A (56 mg.) resulted.

NaCl was added to the solution of the precipitate to I = 0·02 and the solution was adjusted to pH 1·8 with 0·5 N-HCl. The resulting precipitate was allowed to settle overnight and was then centrifuged. It is of interest that the supernatant of this stage contained most of the albumin fraction of the ultra-sonically homogenized sputum, the preliminary additiou of NaCl to I = 0·02 apparently encouraging this partial separation. The centrifuged precipitate was resuspended in 20 ml. of water and 0·05 N-NaOH was added drop-wise to dissolve the suspension, the resulting solution being pH 7·4. 0·05 N-HCl was added to this solution until pH 3·0 was reached ; the resulting precipitate, which was largely composed of the mucoprotein component of the ultra-sonically homogenized sputum, was allowed to stand overnight. The precipitate was then centrifuged, redissolved in 20 ml. of water by the drop-wise addition of 0·05 N-NaOH to pH 7·4, and the resulting solution was dialysed against water at 0° for 7 days and then dried from the frozen state yielding a voluminous white powder B (39 mg.).

Tiselius electrophoresis of the mucopolysaccharide fraction A showed that the main component corresponded to component 2 (Fig. 2) of the Tiselius pattern of the ultra-sonically homogenized sputum, and that it was non-homogeneous. Paper chromatography of a 0·5 N-HCl hydrolysate of this fraction revealed that amino sugars, galactose, fucose and traces of mannose were present. This is in contrast with the sputum mucopolysaccharide, isolated by peptic digestion of acetone-dried sputum, as no trace of mannose was found in this substance. (Brogan, 1959).

Tiselius electrophoresis of the mucoprotein fraction B showed that the main component corresponded to component 3 (Fig. 2) of the Tiselius pattern of the ultra-sonically homogenized sputum. The electrophoresis also showed that the main component was essentially homogeneous, in contrast with that of the mucopolysaccharide fraction A. Paper chromatography of a 0·5 N-HCl hydrolysate of the mucoprotein fraction B showed that it contained amino sugars, galactose, mannose and fucose. The electrophoretic patterns of both fractions A and B showed that each contained some of the albumin fraction of the parent ultra-sonically homogenized sputum.

DISCUSSION

The mucous ground-substance of sputum may be regarded as a gel, consisting of a discontinuous phase which, to all intents, is insoluble in water, and a continuous phase consisting of water, dissolved salts and possibly colloids in solution. The method of ultra-sonically homogenizing this gel very probably depended on the breaking down of the water-insoluble complex, forming the discontinuous phase, by mechanical shock. This procedure also may have resulted in some denaturation of the components forming the gel. The method, nevertheless, made possible the conversion of the sputum coagulum into a water-soluble state. The percentage dry-weight of the colloids of this solution together with the insoluble and largely organized residue was, in total, comparable with the precentage dry-weight obtained by drying sputum with acetone, especially when the fact is taken into account that the ultra-sonically homogenized preparation was salt-free in contrast with acetone-dried sputum.

The ultra-violet absorption spectrum of the ultra-sonically homogenized sputum together with the absence of organic phosphorus in this substance suggests that nucleic acids were very possibly absent from this preparation. This is in contrast with the mucoprotein, prepared from acteone-dried sputum by enzymic digestion, which had a relatively high organic phosphorus content and which had an ultra-violet absorption spectrum suggestive of the presence of nucleic acids (Brogan, 1959). Their presence, in this complex, may well have been an artefact of the method of preparation, and it may be suggested that nucleic acids very probably do not play a role in the structure of the gel forming the mucous ground-substance of sputum. This view finds support in the work of Armstrong and White (1950), who showed that fibres giving a positive Feulgen reaction were present in " purulent " sputum but absent from the ground substance of " mucoid " sputum, only the deoxyribonucleic acids of the cell nuclei of the latter type of sputum giving a positive Feulgen reaction. This work has since been confirmed by White, Elmes and Walsh, (1954), using phase contrast and electron microscopy. These workers also showed that incubation of the mucus from " mucoid " sputum with deoxyribonuclease and Mg^{2+} had no effect on the fibre structure of " mucoid " sputum.

Tiselius electrophoresis of the ultra-sonically homogenized sputum showed that at least five components were present. One of the components, accounting for approximately 34 per cent of the total colloids, was identified with human serum albumin. This component was not free, in the original sputum, to migrate in an electric field on paper, whereas it moved freely on paper when the sputum was ultra-sonically homogenized. This albumin fraction, in the native state, is therefore probably bound in complex formation possibly with one or

other or both of the carbohydrate complexes, to form the insoluble discontinuous phase of the sputum gel. This finding is in accordance with the work of Warfvinge (1956), who has investigated, by paper electrophoresis, the sputa of a large series of patients with various lung and bronchial diseases. He reports that only a faint and sometimes totally absent fraction of protein character, which moved on paper at the same speed as human serum albumin, was normally found in the specimens of sputum. In one case, a patient suffering from the comparatively rare bronchiolar carcinoma (pulmonary adenomatosis), an intensely staining band moving at the same speed as human serum albumin was found on examination of the sputum by paper electrophoresis. This finding was so unusual that Warfvinge (1956) suggested that it might be of use in the laboratory diagnosis of bronchiolar carcinoma.

Separation of the carbohydrate components of the ultra-sonically homogenized sputum showed that these fractions corresponded with the other two major components of the parent ultra-sonically homogenized sputum, which together accounted for approximately 43 per cent of the total colloids. The main component of the mucopolysaccharide fraction was electrophoretically non-homogeneous in contrast with that of the mucoprotein fraction, which was essentially homogeneous. The acid hydrolysate of the mucopolysaccharide fraction contained traces of mannose in addition to amino sugars, galactose and fucose. In this respect, this fraction differed from the mucopolysaccharides prepared by peptic digestion of acetone-dried sputum. The presence of mannose in this fraction may be an indication of the destructive effect of ultra-sonic vibration on macromolecules, as the mannose is likely to have originated from the mucoprotein fraction of the sputum. It is of interest that Bukantz and Berns (1958) also found mannose in the mucopolysaccharide fraction they prepared from sputum, and it is noteworthy that these workers used prolonged periods of incubation with pepsin to isolate their preparations from ethanol-precipitated sputum. In most other respects, both the mucopolysaccharide fraction and the mucoprotein fraction resembled the corresponding complexes isolated previously from acetone-dried sputum (Brogan 1959). As the method of separation was unable to free either of the fractions from the albumin component of the parent ultra-sonically homogenized sputum, it was not possible to compare the analyses of these fractions with those of the preparations from acetone-dried sputum.

The other two components of ultra-sonically homogenized sputum, together accounting for approximately 23 per cent of the total colloids, were not identified. The mobility of one component, in the Tiselius apparatus, exceeded that of human serum albumin and the mobility of the other lay between that of the β globulin and that of the γ globulin fractions of human serum. In addition to these minor components, immuno-electrophoresis showed that traces of α, β and γ globulins were also present.

Since a feature of respiratory diseases, such as chronic bronchitis and asthma, is an alteration in the nature as well as the amount of bronchial secretion such that it is expectorated as sputum (Oswald, 1958), the following mechanism of change from physiological bronchial secretion to pathological sputum may be suggested. In the present state of knowledge, it is not possible to establish the composition of normal bronchial secretion, as any intubation procedure is likely, in its own right, to produce inflammation of the mucosa. The presence, however, of mucopolysaccharides and mucoproteins in the normal secretions of more readily

available epithelial surfaces such as the gut and the urinary tract indicates that these substances may largely comprise normal healthy bronchial mucus. Under the stimulus of inflammation, it is probable that an increase in the permeability of the pulmonary vascular bed takes place with the likely result that albumin, being the smallest of the plasma proteins, is the first to make its way into the extra-cellular spaces of the mucosa. It is possibly at this site that the complex constituting the water-insoluble phase of the sputum gel, is formed between the extravasated albumin and the polysaccharides of the normal bronchial secretion. With the progression of inflammation, the initial " mucoid " sputum may become purulent, the deoxyribonucleo-protein complexes, described by Armstrong and White (1950), further contributing towards the viscidity.

SUMMARY

A method has been described by which pooled sputum can be homogenized by ultra-sonic vibration.

A water-soluble complex, containing 8·8 per cent of nitrogen, was obtained. There was evidence that nucleic acids were not present in this substance. Tiselius electrophoresis showed that the preparation was composed of at least five components.

One of the major components, accounting for approximately 34 per cent of the complex, was identified with human serum albumin. The other two major components, accounting for approximately 43 per cent of the complex, were shown to be a mucopolysaccharide fraction and a mucoprotein fraction.

Evidence was obtained that the albumin fraction was possibly bound in complex formation to form the insoluble phase of the sputum gel. It was suggested that the albumin fraction may have originated from the pulmonary vascular bed as a result of inflammatory change.

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