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Model Systems for the Evaluation of Mucolytic Drugs: Acetylcysteine and *S*-Carboxymethylcysteine

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Abstract—The therapeutic place of mucolytic drugs remains uncertain; clinical studies have seldom demonstrated significant benefit and the activity of such agents is poorly understood. In this study the effects of the mucolytic agents acetylcysteine (AC) and *S*-carboxymethylcysteine (SCMC) have been assessed in-vitro, using purified mucus gels and tracheal explant systems and in-vivo, in the mini-pig tracheal pouch model, in order to elucidate their mechanisms of action. A reduction in the elastic modulus (up to 70% over the frequency range 0·2–20Hz) was apparent after treatment of mucus gels in-vitro with AC ($P < 0·05$), but not with SCMC. Gel chromatography indicated that AC reduced the mucus glycoprotein to smaller subunits and a breakdown of gel structure was apparent when visualized using a cryofracture technique. SCMC treated gels were comparable with control samples. Mucus production was assessed in isolated rat trachea by monitoring the uptake and release of [^{3}H] glucosamine. AC (5–15 mM) did not affect secretion whereas SCMC (5 and 10 mM) reduced the production of radiolabelled material (24 and 37%, respectively) over 24 h ($P < 0·05$). Single oral doses of SCMC and AC (20 mg kg^{-1}) were administered to mini-pigs and mucus collected from tracheal pouches; no significant changes in the rheological or biochemical properties of the secretion could be determined. The in-vitro mucolytic activity of AC depends upon a direct action on the secretion, SCMC appears able to reduce production of the mucus glycoprotein. Wide inter-and intra-individual variation in the properties of the secretion would suggest that such effects are not readily demonstrated in-vivo.

Acetycysteine (AC) has been administered both orally and by inhalation in treatment of hypersecretory airway disease, but its therapeutic value remains uncertain. Recent clinical studies in chronic bronchitis have suggested that AC may reduce the incidence of bronchial infection (exacerbation) and the number of days when patients are incapacitated (Boman et al 1983; British Thoracic Society 1985; Parr & Huitson 1987). However, Millar et al (1985) found no significant difference in lung function, mucociliary clearance or sputum viscosity during AC therapy. Sheffner (1963) demonstrated that in-vitro AC decreases mucus viscosity by reducing disulphide bonds in the mucus glycoprotein. Subsequent studies have confirmed the mucolytic properties of AC in-vitro (Lightowler & Lightowler 1971) although some workers have reported that the elasticity of the secretion is reduced to a much greater extent than the viscosity (Takishima et al 1980; Marriott et al 1983a). This mucolytic activity has not been conclusively demonstrated in-vivo and other effects apparently unrelated to this direct action on mucus glycoprotein have been reported (Turner & Marriott 1983; Konradova et al 1985).

Another cysteine derivative, *S*-carboxymethylcysteine (SCMC) has also been widely used as a 'mucolytic' in the treatment of respiratory disease. This agent does not possess a free thiol grouping which apparently renders it unable to reduce the mucus glycoprotein to smaller subunits (Degand 1973). The activity of SCMC has not been clearly defined, but "mucoregulatory" properties have been proposed (Puchelle & Sadoul 1980). SCMC may reduce the secretory response to nervous mediators (Turner & Marriott 1983) or prevent pathological changes in the respiratory mucosa following exposure to inhaled irritants (Puchelle & Sadoul 1980). Clinical studies have shown changes in the rheological properties of sputum with both increases (Puchelle et al 1978) and decreases in viscosity (Edwards et al 1976; Muittari & Linnoila 1977). Increases in sputum volume with SCMC have been widely reported, but improvements in respiratory function are not consistent (Aylward 1974; Edwards et al 1976).

This study employs a combination of in-vitro and in-vivo experimental systems in order to clarify the effects of AC and SCMC on mucus secretions and indicate their therapeutic potential.

Materials and Methods

Materials

SCMC was obtained from Berk Pharmaceuticals Ltd, Eastbourne, East Sussex, UK. AC was purchased from BDH, Poole, Dorset, UK and gentamycin sulphate, amphotericin B-solubilised and medium 199 from Sigma Chemicals, Poole, Dorset, UK. [^{3}H]Glucosamine was obtained from Amersham International Ltd, Buckinghamshire, UK. Sepharose CL4B and CL2B were supplied by Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals were of general purpose or analytical grade as available.

Viscoelastic assessment of mucus gels

Mucus was isolated from the stomachs of freshly slaughtered pigs (*Suis scrofa domestica*), mixed with an equal volume of protease inhibitor solution (phenylmethylsulphonylfluoride 1 mM, ethylenediaminetetra-acetic acid 6·3 mM, sodium

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chloride 0·15 M, sodium azide 3 mM) and sheared in a blender for two periods of 30 s. The homogenized gel was then centrifuged at 16 000 *g* for 1 h at 4°C and the supernatant retained and filtered through washed glass wool. The supernatant was diluted 1 in 4 with protease inhibitor solution, applied to a column of gel filtration medium (Sepharose CL4, 9 cm diameter × 25 cm height) and eluted with further protease inhibitor solution. The absorbance of the eluate was continuously monitored at 280 nm and the excluded peak collected and concentrated by ultrafiltration. This concentrate, termed pig gastric mucin (PGM), was dialysed against 10 mM tris (hydroxymethyl)methylamine buffer (pH 7·4) and concentrated by ultrafiltration to a final concentration of 11·1% dry weight. Buffered solutions of AC and SCMC (0·06 M) were added (diluting the PGM to 10% dry weight) and the gels incubated at 37°C for 2 h before rheological assessment using an oscillating sphere magnetic microrheometer (James & Marriott 1982).

Mucus gel structure

PGM was exhaustively dialysed against distilled water at 4°C and concentrated to a gel by ultrafiltration. Aliquots of the gel mounted between two rivets were frozen in slushed nitrogen (−196°C), fractured by breaking the rivets apart and immediately transferred to the scanning electron microscope (SEM) stage (Phillips PSEM 501B). The sample was partially sublimed by raising the stage temperature to −80°C for 14 min. The surface was then sputter coated with gold (Emscope SP2000) and examined under the SEM. Test samples were treated with AC or SCMC (0·06 M) for 16 h at 25°C before SEM examination.

Gel exclusion chromatography

Molecular size changes in the mucus glycoprotein were estimated following 2 h incubation of a PGM solution (1·5–2 mg mL^{-1}) with AC or SCMC (0·06 M) at 37°C by gel filtration (Sepharose CL2B). Columns (1·7 cm diameter × 34 cm height) were eluted with 8 M urea to prevent aggregation of the mucin (Lethem et al 1984). Fractions were collected continuously and analysed for hexose (Winzler 1955), a general marker for the glycoprotein.

Tracheal explants

Male Wistar rats were killed and the trachea, including the larynx, removed immediately. Tracheas were washed in saline (0·9%), weighed, cut longitudinally and four sections placed in 5 mL of unlabelled tissue culture medium (gentamycin 0·5 mg mL^{-1}, amphotericin 1 μg mL^{-1}, tricine 25 mM and sodium bicarbonate 15 mM in Medium 199). The tracheas were incubated at 37°C in a 95% O_2:5% CO_2 atmosphere for 1 h then placed in fresh tissue culture medium containing [^{3}H] glucosamine (1 μCi mL^{-1}). The incubation was continued, the medium being replaced with fresh labelled medium after 3, 6, 9, 12, 24 and 27 h. AC (5–10 mM) or SCMC (5–15 mM) was present in the culture medium throughout the 27 h study. Proteins in 2 mL amounts of all the samples of medium were precipitated with trichloroacetic acid (TCA) (final concentration 5% w/v) centrifuged and the pellet washed twice by resuspending in 0·5 M NaOH before suspending in liquid scintillant (cocktail T) and counting (Beckman model LS 3133P) using an external standard to assess quenching.

In some cases the remaining portions of medium were combined, precipitated, washed and redissolved in potassium thiocyanate (2 M). This solution was applied to a column of gel filtration medium (Sepharose CL4B), eluted with thiocyanate and fractions mixed with liquid scintillant before counting.

Tracheal pouch in the mini-pig

Tracheal pouches were established in mini-pigs (Readman et al 1982). Mucus samples were flushed from the pouches with saline (0·9%), using gentle pressure from a syringe, and stored at −20°C before analysis. On study days AC or SCMC (20 mg kg^{-1}) was administered with the normal diet and samples collected immediately before, 4 h after the first dose, and after 24 h; no drug was administered in control experiments. Immediately after thawing the wet weight of each sample was determined and the viscoelastic properties assessed by creep compliance analysis (Petronics Viscoelastic Analyzer MR 8301). Creep curves were analysed in terms of

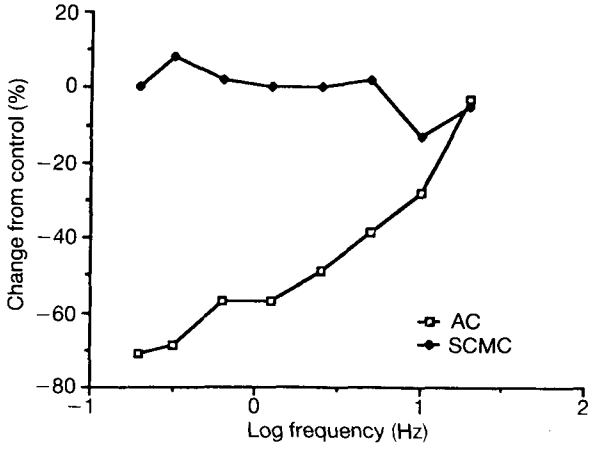

FIG. 1. The effects of SCMC and AC (0·06 M) on the storage (elastic) modulus of a purified mucus glycoprotein gel.

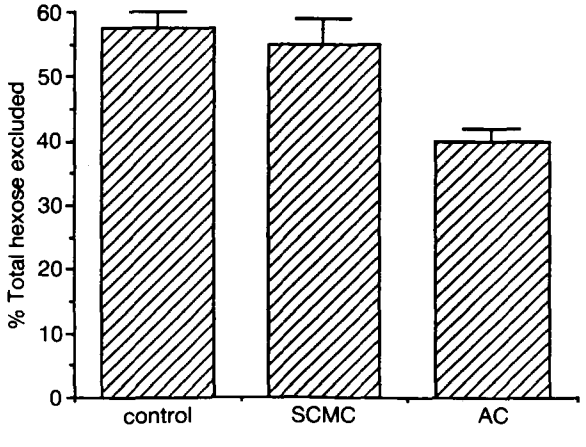

FIG. 2. The effects of SCMC and AC (0·06 M) on the gel exclusion chromatography (Sepharose CL4B) profile of a purified mucus glycoprotein preparation. The results are expressed as the percentage (±s.e.m.) total hexose recovered in the excluded peak.

Table 1. The effect of single oral doses of SCMC (20 mg kg^{-1}) and AC (20 mg kg^{-1}) on tracheal pouch mucus in the mini-pig.

Wet wt mg	% Dry wt	Viscosity Nsm^{-2} ($\times 10^2$)	Compliance m^2N^{-1} ($\times 10^{-2}$)	Hexose: Protein ratio	Fucose μg^*
Control (n=6) 1066±243	5·4±1·2	120±25	1·85±0·71	0·154±0·019	17·5±2·5
AC (n=4/5) 917±320	6·0±1·2	705±240	0·66±0·35	0·159±0·017	21·0±5·6
SCMC (n=4/5) 606±247	5·5±1·4	292±159	1·32±0·81	0·328±0·093	14·9±5·4

Mean values ±s.e.m. (*μg per 100 mg wet weight)

a line spectrum to produce a residual sheer viscosity and an instantaneous compliance. Samples were then analysed for hexose (Winzler 1955), protein (Bradford 1976) and fucose (Gibbons 1955).

Results

The results of the rheological assessment are presented as percentage changes from control values for G′ the storage (elastic) modulus over the frequency range 0·2–20Hz in Fig. 1. In order to overcome problems of variation between batches of mucus gel, each test sample was compared to control samples from the same batch. The statistical significance of changes from control values was calculated using the Mann-Whitney U-test, with P values of 0·05 or less taken to be significant ($n=12$). AC significantly reduced G′ at frequencies below 10 Hz but no change was apparent with SCMC. No consistent changes in G″ the loss (viscous) modulus were observed with either agent.

The hexose content of the excluded gel filtration fractions, expressed as a percentage of the total recovered from the column, are shown in Fig. 2. Control and SCMC treated material showed similar profiles with 55–58% of the total hexose excluded from the column. However, only 40% of the hexose containing material was present in the excluded peak following treatment with AC. The effects of acetylcysteine on gel structure visualized by SEM are shown in Fig. 3: the surface pore network is entirely disrupted in some areas and filaments show marked thickening from approximately 0·2–0·4 μm up to 1 μm. No differences between control gels and those treated with SCMC were observed.

Figs 4 and 5 show the cumulative TCA precipitable disintegrations min^{-1} (±s.e.m.) released by the tracheal explants over the total 27 h study period for AC and SCMC, respectively. The data were fitted to a regression equation

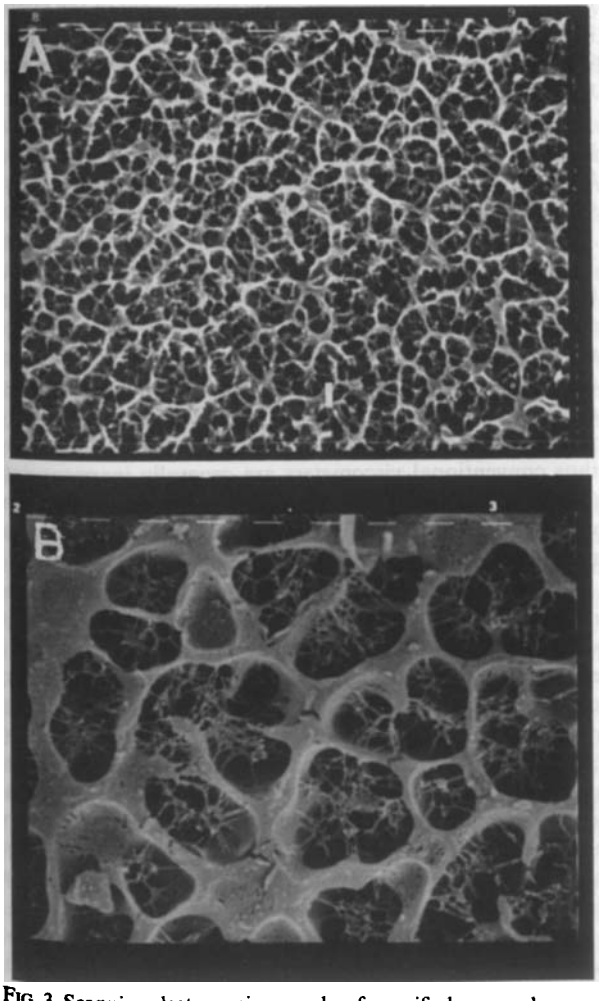

FIG. 3. Scanning electron micrographs of a purified mucus glycoprotein gel prepared by a cryofracture technique. A) Purified gel. B) Gel treated with AC (0·06 M). (Bar = 1 μm).

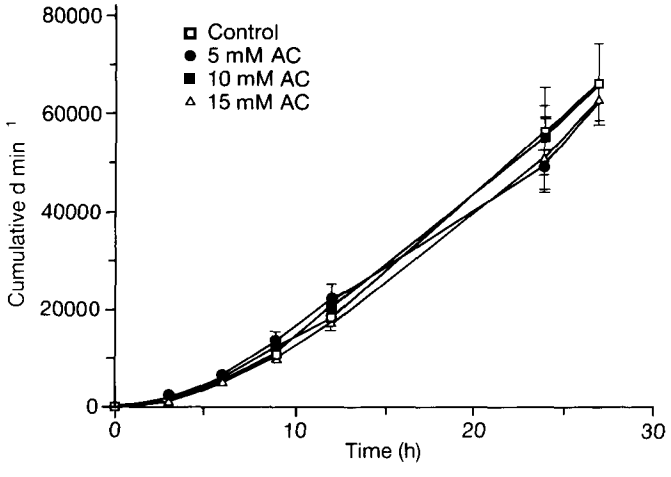

FIG. 4. The effect of AC (5–15 mM) on the release of radiolabelled mucin by rat tracheal explants incubated with [^{3}H]glucosamine over 27 h (±s.e.m.).

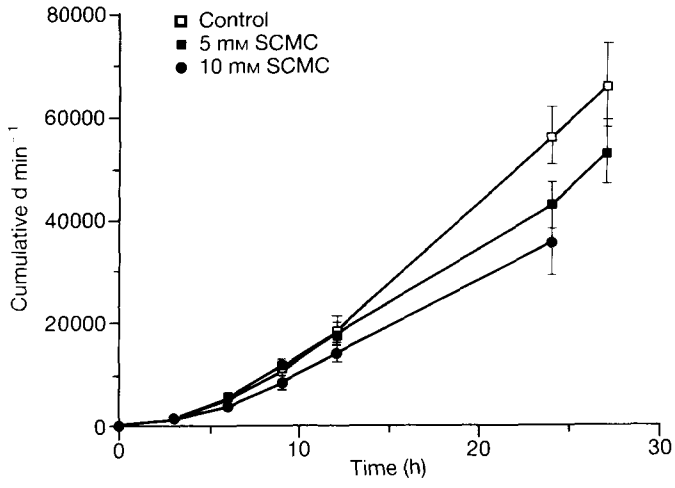

FIG. 5. The effect of SCMC (5–10 mM) on the release of radiolabelled mucin by rat tracheal explants incubated with [^{3}H]glucosamine over 27 h (±s.e.m.).

and compared using a modified *t*-test. AC (5–15 mM) did not affect the release of radiolabelled material. SCMC, 5 and 10 mM, reduced its release ($P < 0{\cdot}05$) by approximately 24 and 36%, respectively. Fig. 6 shows a typical gel filtration profile for the radiolabelled material precipitated with TCA. A large proportion of the total counts was located in the excluded peak, indicating that the labelled material is present in a high molecular weight species.

The effects of single doses of AC and SCMC on tracheal pouch mucus in the mini-pig are shown in Table 1. The results for control experiments were compared statistically with those for each of the drugs using the Mann-Whitney U-test. None of the parameters differed significantly ($P > 0{\cdot}05$) from control values. The individual values for both biochemical and rheological parameters reflect the inherent day to day variation in the physicochemical properties of the secretion.

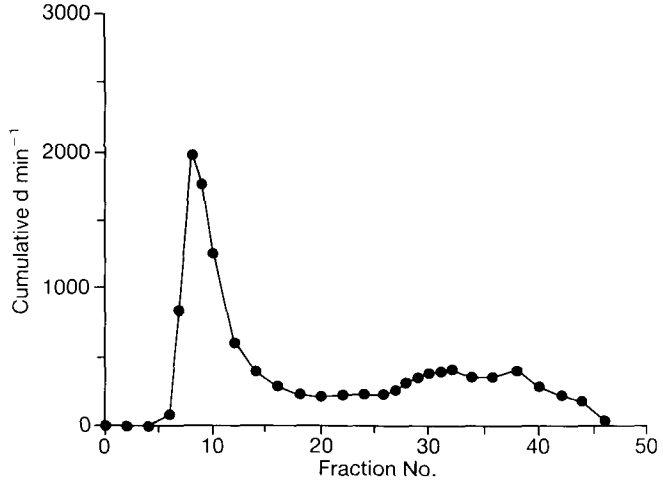

FIG. 6. A typical gel exclusion chromatography (Sepharose CL4B) profile for radiolabelled, trichloroacetic acid precipitated material released by rat tracheal explants incubated with [^{3}H] glucosamine.

Discussion

The physical properties of mucus secretions are essentially determined by the large molecular weight glycoproteins (mucins) which consist of a protein backbone with many oligosaccharide side chains. The peptide chain of human tracheobronchial and gastric mucin contains some non-glycosylated regions and these regions contain most of the cysteine residues. These mucus glycoproteins are thought to polymerize by formation of disulphide bonds in the non-glycosylated region of the protein core, probably involving interaction between cysteine residues (Allen 1978).

The purification of mucus glycoprotein by gel filtration has been widely documented (Snary & Allen 1971; Hatcher et al 1977) and such preparations are biochemically representative and retain their gel forming properties (Brown et al 1981; Marriott et al 1983a). Normal human tracheobronchial secretions are not readily obtainable and only in obstructive lung disease or infection is sputum produced. A purified glycoprotein preparation isolated from gastric mucus was identified as an appropriate system in which the effects of direct application of SCMC and AC on the viscoelastic properties of a mucus gel could be evaluated in-vitro (Marriott et al 1983a). (The activity of a mucolytic drug at all sites of mucus secretion requires consideration in the evaluation of therapeutic potential. Since SCMC and AC are both administered by the oral route, effects on gastrointestinal mucus may have additional implications.)

The elastic modulus G′ is the most important rheological parameter associated with successful mucociliary clearance (Gelman & Meyer 1979) and is proportional to the number of cross-linkages per unit volume of gel (Silberberg & Meyer 1982). AC reduced G′ by 28–38% in the frequency range 6-16Hz which parallels the ciliary beat frequency in the lung (Lopez-Vidriero & Clarke 1982), although changes in viscosity were minimal. Other workers have also demonstrated reduced elasticity with AC, and in one study this was accompanied by an increase in mucociliary transport rate on a ciliated epithelium (Martin et al 1980). Mucus is a viscoelastic material rather than a simple viscous fluid and thus conventional viscometers are generally inappropriate (Marriott & Davis 1978); major reductions in viscosity in-vitro may reflect the use of inappropriate rheological techniques.

SCMC did not affect the rheological properties of the mucus gel and no change in the molecular size of the mucin was observed by gel exclusion chromatography. However, a reduction in molecular size was observed with AC indicating the breakdown of the mucus glycoprotein to smaller fragments. The mucolytic activity of AC demonstrated by Sheffner (1963) is due to the reduction of the macromolecular glycoprotein to smaller subunits. This reduction in molecular size was apparent from gel exclusion chromatography where treatment of the glycoprotein preparation with AC considerably increased the amount of material recovered in the included volume of the gel.

A change in the structure of a mucus gel visualized by SEM was apparent after application of AC, but not after application of SCMC. Interpretation of the data obtained from SEM techniques has generally proved difficult because the "pore" structure depends upon both water content of the

sample and freezing rate (Parish et al 1982). However, the SEM cryofracture technique used in this study produced highly reproducible results; freezing conditions were rigidly controlled and gel samples showed uniform structure over the entire fractured surface. Similar changes in the gel structure following treatment with other reducing agents (Walters et al 1985) suggest that this is characteristic of a mucus gel which has undergone disulphide reduction.

The mucolytic activity of AC can be demonstrated by direct application of this agent to a mucus gel in-vitro. The combination of experimental techniques isolates both changes in the physical properties of the gel and in the molecular size of the mucus glycoprotein. In contrast SCMC, although another cysteine derivative, appears unable to influence the physical properties of mucus post-secretion suggesting that activity may be via an effect on mucus synthesis or secretion.

The production of mucins by the trachea in culture has been demonstrated by many workers; uptake of radiolabelled precursors, e.g. glucosamine, fucose and leucine (Adler et al 1981; Hartmann et al 1984), is generally observed in less than 10 h and release of radiolabelled glycoprotein after 1–120 h in culture. Thus, the synthesis of glycoprotein from simple sugars and peptides is rapid and the macromolecules produced are consistent with those isolated directly from the epithelia (Woodward et al 1982; Lloyd et al 1984). Gel filtration indicated that activity from the rat tracheal explants was predominantly included in a very high molecular weight species, similar to that isolated from rat trachea using a perfusion technique in-situ (Turner & Marriott 1983). SCMC reduced the release of labelled glycoprotein over the 27 h study suggesting a reduction in mucus synthesis or secretion. Initial experiments where explants were pre-incubated with [^{3}H]glucosamine and subsequent release rates determined in the presence of AC and SCMC showed no change in release with either agent. Thus the activity of SCMC appears to be due to an effect on either the uptake of the glucosamine precursor or on the synthesis of the glycoprotein rather than a direct effect on release rate. Such an effect may be of value in treatment of hypersecretory airway disease if evident in-vivo.

The tracheal pouch allows respiratory mucus samples to be easily collected without the use of anaesthetics, whilst normal nervous and systemic influences remain intact. The mini-pig tracheal pouch model has been identified as being eminently suitable for the evaluation in-vivo of drugs affecting mucus synthesis and secretion (Readman et al 1982). Extreme intra- and inter-individual variation in both rheological and biochemical properties of mucus samples from the mini-pig was observed during control and test periods of the study. Variation in the rheological parameters was particularly marked with 50–100% changes occurring during control periods in one individual; inter-individual variation was routinely $>100\%$. Similar wide variation in the properties of human sputum has been reported; sputum samples from one chronic bronchitic had a coefficient of variation of 40% (Lopez-Vidriero et al 1973). In the clinical situation this is complicated by the possibility of infection which further influences the properties of the secretion (Lopez-Vidreiro & Reid 1978a). In the present study, fucose determinations were undertaken because the sugar is reported to be a specific marker of mucus-type glycoprotein, being relatively uncommon in serum glycoproteins (Lopez-Vidriero et al 1973; Lopez-Vidriero & Reid 1978b). Hexose and protein indicate the general composition of the secretion and any effect on mucin secretion is likely to be reflected in the ratio of hexose to protein. No changes in either the biochemical markers or the rheological properties of the secretion could be demonstrated in the mini-pig model following oral administration of single doses of AC or SCMC. However, it appears likely that modest changes in the secretion would not be readily differentiated from the normal day to day variation.

One other study of SCMC in the mini-pig tracheal pouch model has been reported where a reduction in the hexose: protein ratio of the secretion was observed following a prolonged dosing period and it was suggested that this could be due to secretion of a less glycosylated glycoprotein molecule (Marriott et al 1983b). Activity may depend upon the higher more prolonged drug levels which are likely to be achieved following repeated dosage. The doses kg^{-1} body-weight of both AC and SCMC employed in this study are similar to those administered in man in treatment of hypersecretory airway disease.

The reduction in glycoprotein secretion observed in-vitro with SCMC and the rheological changes observed in-vitro with AC could not be demonstrated in this in-vivo model. However, the use of different animal species for the in-vivo and in-vitro studies could be relevant if activity is via mucus synthesis. Interspecies variation in the biochemical composition of mucins could reflect variation in synthetic enzymes, so an agent which affects synthesis in one species might have less activity in another species.

Pharmacokinetic studies have demonstrated that AC and SCMC are rapidly absorbed from the gastrointestinal tract and both have then been detected in respiratory secretions (Rodenstein et al 1978; Braga et al 1982). Thus it would appear that poor distribution to the respiratory tract is not a major limitation to activity in-vivo. However, the concentrations attained in plasma and bronchial tissue following oral dosage may be less than those employed in the in-vitro and ex-vivo investigations.

A reduction in secretion of mucus glycoproteins could be of therapeutic importance in the treatment of respiratory disease, possibly by preventing hypersecretion or facilitating mucociliary clearance. An agent which affects only the rheological properties of the secretion may improve mucociliary clearance, but would require dose titration to achieve the required viscoelastic properties. These studies suggest that the activity of AC depends upon a direct effect on the rheological properties of secreted mucus and that the production of mucins is essentially unchanged. However, the possibility of a protective effect against noxious agents such as that proposed by Boman et al (1983) has not been investigated. SCMC reduces the production of mucus glycoprotein, but does not act directly on the glycoprotein post-secretion. The continual variation in-vivo in the properties of respiratory mucus may be a major limiting factor when demonstrating efficacy of "mucolytic" drugs.

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