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Resolution of the Major Components of Human Lung Mucosal Gel and Their Capabilities for Reaggregation and Gel Formation†

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ABSTRACT: Lung mucosal gel is composed mainly of mucin glycoproteins and lower molecular weight (150 000–13 000) proteins. The gel can be solubilized at low concentrations (1 mg/mL) by dissociating agents in the absence of a reducing agent, but the components are aggregated and cannot be resolved by chromatography. Following dissociation of the aggregates by the addition of dithiothreitol, the proteins and glycoproteins can be resolved by chromatography on Sepharose 4B in a buffer containing both $NaDodSO_4$ and dithiothreitol. The mucins elute in the void volume and the proteins in the included volume near the salt peak. Further purification of the mucin glycoprotein fraction on Sepharose 2B and hydroxylapatite resulted in the resolution of major and minor mucin glycoproteins, neither of which contained any detectable serum albumin or lower molecular weight proteins characteristic of lung mucosal gel. The mucin glycoproteins were shown to be capable of forming a gel in the absence of both a dissociating solvent and a reducing agent. Thus, human lung mucosal gels behave differently than another human mucosal system, i.e., the viscid meconium gel—observed in certain cases of cystic fibrosis (meconium ileus)—which results from an interaction of albumin with mucin glycoproteins. Both thiol interactions, as indicated by the effect of dithiothreitol, and nonthiol interactions, as indicated by the effects of guanidine hydrochloride, urea, and sodium dodecyl sulfate, are involved in aggregation and gel formation of lung mucosal gel. Both types of interactions are also implicated in aggregation and gel formation of purified lung mucin glycoproteins. The properties of the carboxymethylated mucins support the conclusion from dithiothreitol experiments, but the results of carboxymethylation are more difficult to interpret because of the apparent effect on nonthiol as well as thiol interactions.

The mucosal layer which coats the surface epithelium of mammalian airways contains macromolecules which interact to form a gel. This lung mucosal gel (LM-gel)[1] is insoluble in physiological saline and provides a protective barrier for the underlying epithelium as well as the viscoelastic properties necessary for the functioning of the mucociliary escalator that

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[1] Abbreviations used: LM-gel, lung mucosal gel; MGP, mucin glycoprotein; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; Gdn·HCl, guanidine hydrochloride; NANA, *N*-acetylneuraminic acid; HA, hydroxylapatite.

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protects the alveoli from airborne particulate matter and bacteria (Litt et al., 1974).

LM-gel contains several components—mainly mucin glycoproteins (MGPs) and a series of lower molecular weight proteins (Havez et al., 1968; Roberts, 1974; Roussel et al., 1975; Boat et al., 1976; Feldhoff et al., 1976; Creeth et al., 1977; Rose et al., 1979). Whether these components are capable of forming a gel when separated from one another has not previously been clarified (Roberts, 1976). However, the viscid meconium gel obtained from patients with meconium ileus has been attributed to interactions of its major components—mucin glycoproteins and serum albumin (Schachter & Dixon, 1965; Young et al., 1958).

We report here our work on the resolution of the mucin glycoprotein and protein components of human LM-gel and show that in contrast to meconium the purified lung mucin glycoproteins are capable of aggregation and gel formation without the addition of albumin or the lower molecular weight proteins of LM-gel.

Experimental Section

Materials. $NaDodSO_4$ was obtained from BDH Chemicals Ltd.; hydroxylapatite, acrylamide, and bisacrylamide were from Bio-Rad; anti-A and anti-B serum was from Ortho Diagnostics, and Gdn·HCl was from Heico. Urea was deionized on a mixed bed resin AG 501-X8 prior to use. The human blood type A-specific lectin from *Phaseolus lunatus* was covalently attached to Sepharose 4B that had been activated with cyanogen bromide (Cuatrecasas, 1970).

Preparation of LM-Gel. The LM-gel, hypersecreted by an adult (blood type AB, secretor) with bronchial asthma, was used as the starting material. It was collected on ice and frozen at −20 °C until used. A number of samples collected during the same time period, during which there was no apparent change in the clinical status of the patient, were thawed and pooled. The LM-gel was diluted threefold with saline (v/v), homogenized, heated for 10 min in a boiling water bath, and dialyzed overnight vs. deionized water at 4 °C with two changes of water. The heating step produced no discernible change in the turbidity of the suspension. On centrifugation at 65000*g* for 1 h in a Beckman Model L-2 ultracentrifuge, the suspension was separated into an opaque gel and a clear supernatant. The gel phase was suspended in water and again sedimented by centrifugation. The extraction was repeated until the extract contained less than 10% (w/w) of the total solute. In some preparations the heating step was omitted to test the possible effects of heating on the resolution of the components.

Reduction of LM-Gel. Reduction, as well as reduction and carboxymethylation, was carried out at a concentration of 3–5 mg/mL of lyophilized sample according to the procedure of Hirs (1967), except that 1% $NaDodSO_4$ rather than 9 M urea was routinely used as the dissociating solvent and iodoacetamide rather than iodoacetate was used as the alkylating reagent. Both reduced and reduced carboxymethylated samples were dialyzed overnight in the dark vs. water at 4 °C, and the reduced carboxymethylated samples were dried by lyophilization.

Isolation Procedures. Chromatography on Sepharose 4B was performed on a 73 × 2 cm column equilibrated with eluant buffer (0.1% $NaDodSO_4$ and 0.15 M ammonium acetate, pH 7.0) or on a 83 × 2 cm column equilibrated with 6 M Gdn·HCl. A 100–125-mg amount of reduced carboxymethylated LM-gel in 7 mL of eluant buffer was applied per run. Chromatography on Sepharose 2B was performed on a 112 × 2 cm column equilibrated with the eluant buffer. For chromatography of the reduced samples, the eluant buffer was made 0.01 M in DTT, and 30–35 mg in 7 mL was applied per run. Hydroxylapatite chromatography was performed on a 32 × 0.8 cm column equilibrated with 0.1% $NaDodSO_4$–0.01 M sodium phosphate, pH 6.6, according to the procedure of Moss & Rosenblum (1972), except that a discontinuous gradient of 0.01, 0.15, 0.3, 0.5, and 1 M sodium phosphate, pH 6.6, was used. Recovery of neutral carbohydrate from Sepharose and hydroxylapatite columns was 90–100%. $NaDodSO_4$ was removed by dialysis vs. 10 volumes of 1 M urea–1 M NaCl for 2 days with one change of solution, followed by dialysis against 40 volumes of deionized water for 2 days with the water changed every 12 h. Analyses with $NaDod^{35}SO_4$ showed that the detergent was effectively removed by this procedure as less than 60 cpm/mL were detected in a sample that contained 41×10^6 cpm/mL prior to dialysis. After dialysis, the mucin samples were lyophilized. About 5–10% of the lyophilized reduced carboxymethylated MGP cannot be solubilized, even in 1% $NaDodSO_4$.

Analyses. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard (Cohn et al., 1947) and by computation from amino acid analyses. Amino acid analyses were performed on a Beckman Model 120-C analyzer using a single-column, single-temperature run on Durrum DC-1A resin. Samples were hydrolyzed for 20 h under vacuum in 6 N HCl, 100 °C (conditions selected from an hydrolysis curve). Cysteine was measured as the S-carboxymethylated derivative. Free thiol groups were measured by the 5,5′-dithiobis(2-nitrobenzoic acid) assay (Habeeb, 1972).

Hexosamines were determined from the amino acid analyses described above after hydrolysis for 4, 12, 20, and 28 h under vacuum in 6 N HCl at 100 °C. Hexosamines were also analyzed by the automatic amino acid procedure of Bella & Kim (1970), as were the hexosaminitols, after hydrolysis for 10 h in 6 N HCl at 100 °C. Neutral carbohydrate was measured by the phenol–sulfuric acid assay (Dubois et al., 1956) using galactose as a standard. Where necessary, a standard curve was determined by using an appropriate volume of the eluant buffer. In some instances the carbohydrate elution pattern could not be determined. For example, DTT had a marked and variable effect on color development and Gdn·HCl resulted in a strongly exothermic and sometimes explosive reaction. Sialic acid was measured by the thiobarbituric acid assay of Warren (1959) after hydrolysis at 80 °C for 1 h in 0.1 N H_2SO_4, sulfate by the barium chloranilate procedure of Spencer (1960) after hydrolysis for 12 h in 6 N HCl at 100 °C, fucose by the sulfuric acid–cysteine procedure of Dische & Shettles (1948), mannose by enzymatic assay after hydrolysis for 2 and 4 h in 2 N HCl at 100 °C, and uronic acid by the carbazole reaction (Davidson, 1966). In all assays involving acid hydrolysis, a hydrolysis curve was used to determine the optimal time and concentration conditions and where necessary to correct for destruction.

Light-scattering studies were carried out on a Farrand-Optical fluorometer F-4 using a primary filter of 327 nm and a broad-band secondary filter. The scattered light was measured at a 90° angle to the incident beam. The β-elimination reaction was performed according to the procedure of Iyer & Carlson (1971). Gel electrophoresis was carried out on 7% polyacrylamide gel according to the procedure of Weber & Osborn (1969) or on 1–5% polyacrylamide or 1% agarose gels (Holden et al., 1971). The gels were stained with Coomassie blue or periodic acid–Schiff reagents. Analytical density gradient centrifugation was carried out in a Beckman

Table I: Analyses of Gel and Soluble Fractions of Lung Mucus[a]

	preparations							
	I		II		III		IV	
	gel	soluble	gel	soluble	gel	soluble	gel	soluble
protein (mg)	196	243	160	88	126	215	582	161
hexose (μmol)	299	866	327	381	182	669	1049	584
NANA (μmol)	34	146	45	42	41	133	259	105
sulfate (μmol)	140	200	173	139	80	205	372	126
blood group A activity (millions of units)	2.0	25	ND	ND	5.5	10	5.1	3.2
(NANA + sulfate)/hexose	0.58	0.40	0.60	0.48	0.66	0.50	0.60	0.40
NANA/hexose	0.11	0.17	0.14	0.11	0.23	0.20	0.25	0.18
protein/hexose	0.66	0.28	0.49	0.23	0.69	0.32	0.55	0.28
sulfate/hexose	0.46	0.23	0.52	0.36	0.44	0.31	0.35	0.21

[a] Analyses were performed according to the techniques described under Experimental Section. ND = not determined.

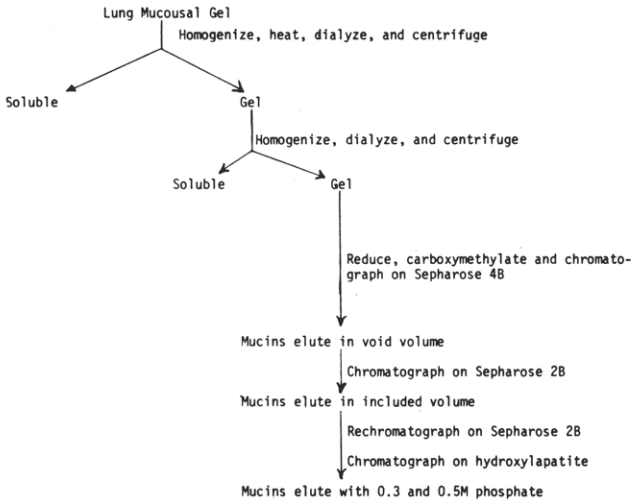

FIGURE 1: Flow diagram for the purification of human lung mucin glycoproteins.

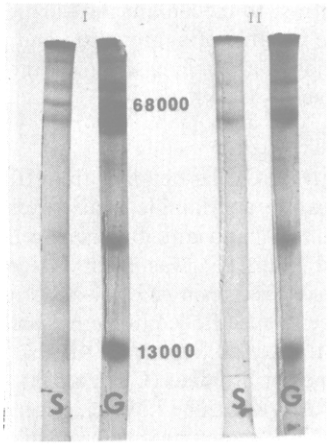

FIGURE 2: Electrophoresis profiles of the soluble (S) and gel (G) phases of preparations I and II. The soluble and gel phases were dissolved in a solution containing 1% $NaDodSO_4$, 0.01 M phosphate, pH 7.1, and 0.1% mercaptoethanol. The samples, 100 μg of protein, were layered on a 7% polyacrylamide gel (Weber & Osborn, 1969) electrophoresed at 3 MA/gel for 15 h, and the proteins were stained with Coomassie blue. Omission of mercaptoethanol resulted in a more intense staining at the top of the gel and the loss of the 13000, 25000, and 55000 molecular weight peptides. Albumin and cytochrome *c* were used as molecular weight markers.

Model E ultracentrifuge at 35000*g* for 12 h and then at 44000*g* for 12 h. The initial cesium chloride concentration was 1.400 g/mL, and at equilibrium the gradient ranged from 1.322 to 1.470 g/mL at 25 °C. Blood group titers were measured with 2 hemagglutinating units of anti-A serum and a 2% suspension of red blood cells (Kabat & Mayer, 1961).

Results

The general procedure used in the dissolution and chromatographic resolution of the components of LM-gel is illustrated in Figure 1. The steps involve extraction of the soluble components of lung mucus followed by dissolution of the gel phase in a solution containing a dissociating agent as well as an agent that disrupts disulfide linkages, chromatography on Sepharose 4B and 2B to separate the mucin glycoproteins from lower molecular weight protein constituents, and further resolution of the mucin glycoproteins by chromatography on hydroxylapatite.

Solubilization of LM-Gel. LM-gel through repeated extraction with saline and water (refer to Experimental Section for details) was resolved into an insoluble gel phase and a clear supernatant. Two classes of components were observed by polyacrylamide gel electrophoresis in the supernatant and gel phases (Figure 2): (a) high molecular weight glycoproteins that did not enter 7% polyacrylamide gel and (b) a number of lower molecular weight proteins (150000–13000). While both components were present in the soluble and gel phases, the concentration of the lower molecular weight proteins varied from preparation to preparation. In the soluble phases of preparations I and II, there was only a small amount of lower molecular weight proteins. However, in preparations III and IV the concentration of lower molecular weight proteins was similar in the supernatant and gel phases. The electrophoresis profiles of both the soluble and gel phases in both preparations were similar to those shown for the gel phases in Figure 2.

Analyses of four representative preparations, each of which contains material collected at different time intervals, are shown in the top section of Table I. The amount of hexose in the supernatant ranged from 36% (preparation IV) to 74–79% (preparations I and III), while the blood group activity varied from 39% (preparation IV) to 90% (preparation I). These analyses, normalized with respect to hexose, are shown in the bottom half of Table I. The NANA/hexose ratio is similar within a preparation for both phases, while the protein/hexose and sulfate/hexose ratios are usually higher for the gel components. In different preparations, however, the ratios of blood group activity, sulfate, NANA, and protein to hexose are variable, with the (NANA + sulfate)/hexose being the most consistent ratio. The same distribution pattern was observed whether the samples were or were not heated (90–100 °C for 10 min) prior to fractionation as shown by comparing the electrophoresis profiles of the supernatant and gel phases, the quantity of material in each phase (milligrams per fraction), and the ratio of carbohydrate to protein in each phase.

While the gel phase was insoluble in saline, it readily dissolved in solvents known to denature proteins (Gdn·HCl,

Table II: Solubility of Lung Mucus Gel[a]

solvent	% soluble (g/g) 5 mg/mL[b]	optical density 1 mg/mL	optical density 5 mg/mL
0.15 M NaCl	ND	0.700	
6 M Gdn·HCl	70	0.095	0.295
9 M urea	65	0.145	0.360
1% $NaDodSO_4$	84	0.295	0.250
0.15 M NaCl + 0.01 M DTT	ND	0.760	
6 M Gdn·HCl + 0.01 M DTT	85	0.040	
9 M urea + 0.01 M DTT	90	0.060	
1% $NaDodSO_4$ + 0.01 M DTT	95	0.070	

[a] The scattered light of the samples was measured at a 90° angle to the incident beam on a Farrand-Optical fluorometer F-4 using an excitation filter of 327 nm and a broad-band secondary filter. ND = not determined. [b] Mucin concentration.

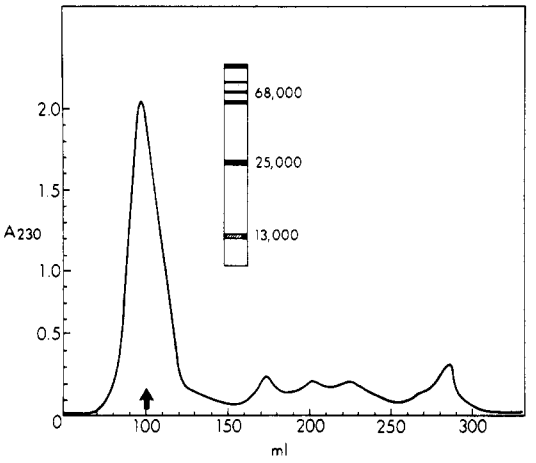

FIGURE 3: Chromatograph on Sepharose 4B (83 × 2 cm) of LM-gel (15 mg) eluted with 6 M Gdn·HCl. The void volume (↑) and the included peaks were pooled, dialyzed extensively vs. water, and lyophilized, and samples were prepared and electrophoresed as described in Figure 2. The electrophoresis profile of the void volume peak is shown. The small included peaks contained the 55 000, 25 000, and 13 000 molecular weight peptides.

urea, $NaDodSO_4$) as shown by light-scattering measurements in Table II. At lower concentrations (1 mg/mL) the solubility of LM-gel was increased in Gdn·HCl and urea. Further solubilization was achieved by adding the reducing agent, DTT, to the dissociating solvents. However, addition of DTT to LM-gel in physiological saline did not alter its solubility.

Separation of the Components of LM-Gel by Chromatography. Although the LM-gel components were solubilized by dissociating solvents, especially 6 M Gdn·HCl, they were not resolved by chromatography on either Sepharose 4B or 2B with 6 M Gdn·HCl as the eluant buffer. As shown in the chromatographic profile and electrophoretogram of Figure 3, most of the material eluted in the void volume fraction of a Sepharose 4B column. After dialysis this fraction contained all of the neutral carbohydrate and ~85% of the initial protein. Similar chromatographic behavior was observed after chromatography on Sepharose 2B using 6 M Gdn·HCl as the eluant buffer.

Separation of the glycoprotein and protein components was, however, achieved when elution was performed in the presence of a reducing agent and a dissociating solvent, and $NaDodSO_4$ and DTT were used throughout this study. Two peaks were obtained by chromatography on Sepharose 4B (Figure 4). The void volume peak (fraction I) contained 55% of the total neutral hexose, accounted for ~25% by weight of the starting material, and in some experiments contained traces of the lower molecular weight proteins that were removed by re-

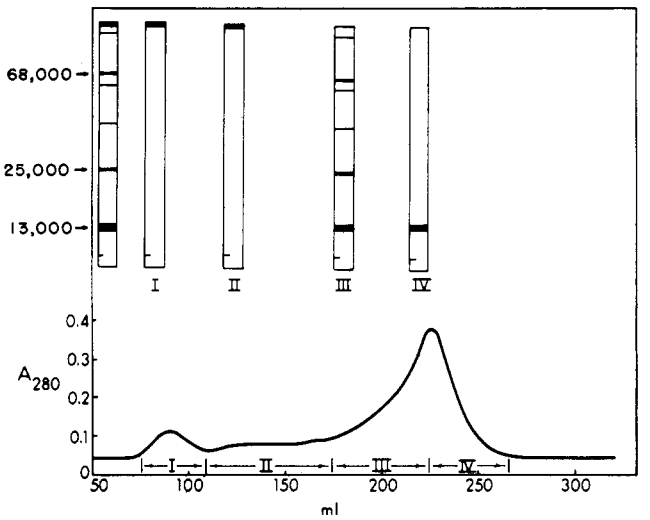

FIGURE 4: Chromatography of reduced LM-gel. LM-gel (30 mg) was dissolved in 7 mL of a solution containing 1% $NaDodSO_4$, 0.1% EDTA, 0.01 M DTT, and 0.1 M Tris, pH 8.0, and stirred for 4 h at room temperature. The solution was layered on a Sepharose 4B bed (73 × 2 cm), and the components were eluted with a buffer containing 0.1% $NaDodSO_4$, 0.01 M DTT, and 0.15 M ammonium acetate, pH 7.0. The electrophoresis profiles (100 μg of protein, stained with Coomassie blue) of LM-gel and of each fraction are shown in the upper half of the figure. The material at the top of the gel stained strongly with periodic acid–Schiffs reagent.

chromatography. Fraction I contained ~85% (by weight) carbohydrate (glucosamine, galactosamine, galactose, fucose, and sialic acid, but not mannose or uronic acid), exhibited blood group A and B specificity, was rich in serine, threonine, glycine, and proline, and on β-elimination reaction yielded galactosamminitol, α-aminobutyrate, an increase in alanine, and a decrease in serine and threonine. Fraction I, therefore, contains glycoproteins that have the same characteristics as mucin glycoproteins (Gottschalk, 1972). Fraction II comprised 9% by weight of the starting material and contained 19% of the total neutral hexose. The included peak (fractions III and IV) contained the lower molecular weight protein components as shown by gel electrophoresis profiles (Figure 4) and 26% of the neutral hexose as serum glycoproteins and as glycolipids. Similar results were obtained with LM-gel that had been heated prior to the initial centrifugation step and also with LM-gel that had been reduced and carboxymethylated in $NaDodSO_4$ and eluted with the eluant buffer.

Resolution of the Mucin Glycoproteins. Chromatography of fraction I on Sepharose 2B and hydroxylapatite as described below yielded major and minor MGP components. Similar chromatographic results were obtained with samples that were reduced and carboxymethylated in $NaDodSO_4$ and chromatographed in solvents containing $NaDodSO_4$ but no DTT.

The mucin glycoproteins from the void volume of several Sepharose 4B columns were pooled, lyophilized, and dialyzed vs. the eluant buffer. These dialyzed materials were chromatographed on Sepharose 2B (Figure 5). Three peaks were obtained: a minor glycoprotein fraction (IA) that eluted in the void volume, a major glycoprotein fraction (IB) that was included in the column, and a minor protein fraction. Rechromatography of the major included mucin fraction yielded a single peak (IB) with a constant ratio of hexose to absorbance at 280 nm. No lower molecular weight proteins were observed with the rechromatographed MGP IB fraction even when the electrophoretograms were overloaded (200 μg of protein). However, on rechromatography a peak was frequently observed at the salt peak (indicated by an arrow, Figure 5) which contained no detectable amino acids, amino sugars, or neutral

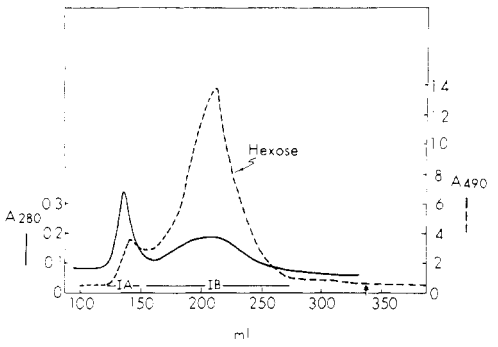

FIGURE 5: Chromatography of reduced carboxymethylated lung MGP on Sepharose 2B. The void volume eluates of the Sepharose 4B chromatographs (fraction I) were combined, lyophilized, and dissolved in 10 mL of eluant buffer (0.1% $NaDodSO_4$ and 0.15 M ammonium acetate, pH 7.0). The solution was layered on a Sepharose 2B bed (112 × 2 cm), and the components were eluted with the eluant buffer and assayed for protein at A_{280} (—) and for neutral hexose at A_{490} (---).

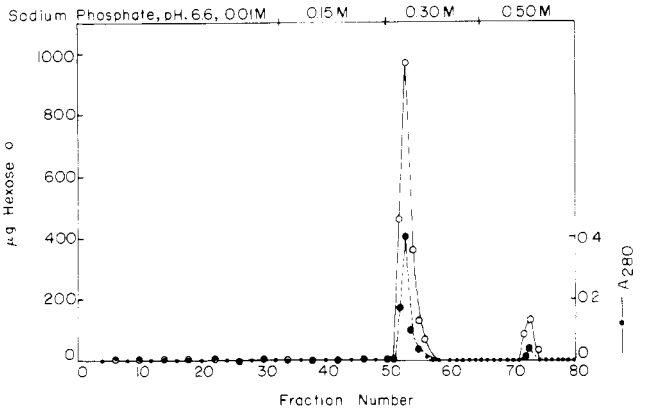

FIGURE 6: Chromatography of reduced carboxymethylated lung MGP on hydroxylapatite. The MGP fraction IB, after rechromatography on Sepharose 2B, was dialyzed vs. 0.1% $NaDodSO_4$ and 0.01 M phosphate, pH 6.6, layered on a hydroxylapatite column (25 × 0.6 cm), and eluted with 40–50 mL each of 0.01, 0.15, 0.30, and 0.50 M phosphate buffer in 0.1% $NaDodSO_4$. The mucins were assayed for protein at A_{280} (●) and for neutral hexose at A_{490} (○).

carbohydrate. Its weight was negligible as compared to the weight of the starting material and the mucin glycoprotein fractions IA and IB. The component appears to be a trace contaminant eluted from Sepharose 2B by the eluant buffer.

The included mucin fraction (IB) from Sepharose 2B, after dialysis vs. 0.1% $NaDodSO_4$ and 0.01 M phosphate, pH 6.6, was chromatographed on a column of hydroxylapatite. The hydroxylapatite was equilibrated with the same buffer and eluted with a stepwise phosphate gradient of 0.01, 0.15, 0.3, 0.5, and 1.0 M phosphate, pH 6.6. The eluted material was monitored by hexose assay and by absorbance at 280 nm. A major glycoprotein fraction eluted with 0.3 M phosphate, and a minor glycoprotein fraction eluted with 0.5 M phosphate (Figure 6). These two fractions contained ≥95% of the carbohydrate in the Sepharose 2B eluate, and both exhibited blood group activity. Rechromatography of the 0.3 and 0.5 M fractions on hydroxylapatite yielded single symmetrical peaks that eluted respectively with 0.3 and 0.5 M phosphate buffer. The major glycoprotein fraction accounted for ~20% by weight of the LM-gel.

Characteristics of the Purified Mucin Glycoproteins. The major reduced and carboxymethylated mucin glycoprotein (0.3 M HA) was analyzed by analytical density gradient centrifugation. At equilibrium it exhibited a single sharp symmetrical band with a buoyant density of 1.454 g/mL at 25 °C. No bands at the lower densities characteristic of non-

Table III: Amino Acid and Carbohydrate Composition of the Major Lung Mucin Glycoprotein

amino acid	residues/ 1000 residues	amino acid	residues/ 1000 residues	carbohydrates	nmol/mg
Asp	40	Met	6.0	GlcNAc	448
Thr	232	Ile	17	GalNAc	461
Ser	169	Leu	38	galactose	741
Glu	43	Tyr	16	fucose	319
Pro	128	Phe	22	NANA	348
Gly	71	Lys	32	sulfate	720
Ala	76	His	20		
Cys[a]	13	Arg	35		
Val	42				

[a] Cysteine was measured as *S*-(carboxymethyl)cysteine.

Table IV: Affinity Chromatography of Lung Mucin Glycoproteins on *P. lunatus*-Sepharose 4B[a]

	BGA[b] units	hexose (nmol)	sp act. (BGA units/ nmol of hexose)
lung mucin	2560	601	4.3
nonadsorbed mucin-1	1408	330	4.3
nonadsorbed mucin-2 (after readsorption of mucin-1)	915	231	4.0

[a] Sequential adsorption of the blood group activity was performed as follows. 500 μg of lung MGP (fraction IB) in 500 μL of saline was incubated with 1 mL of *P. lunatus*-Sepharose 4B for 30 min at 25 °C. The adsorbent was removed by centifugation, washed, and analyzed for hexose and blood group A activity. The experiment was repeated by using this nonadsorbed mucin-1 sample. [b] BGA = blood group A.

glycosylated proteins were observed. The weight-average molecular weight of the major reduced and carboxymethylated lung MGP, as estimated on a Sepharose 2B column calibrated with Blue dextran 2000 and T-500 dextran, is ~2×10^6. This value is within the range of molecular weight values [$(0.57–2.3) \times 10^6$] reported for reduced lung MGP by Creeth et al. (1977), although it is higher than the weight-average value of 0.5×10^6 reported by Meyer (1977) for reduced carboxymethylated lung MGP.

The major lung MGP contains 15% protein by weight and is rich in serine, threonine, and proline, which comprise 53% (residues/1000 residues) of the total amino acids (Table III). It is rich in carbohydrates and contains *N*-acetylgalactosamine, *N*-acetylglucosamine, galactose, fucose, and sialic acid (Table III), but no mannose, glucose, or uronic acid, and exhibits blood group AB specificity. No glycoproteins lacking blood group A reactive oligosaccharide chains were noted by affinity chromatography; i.e., a constant specific activity (blood group units per nanomoles of hexose) was noted throughout sequential adsorption on *P. lunatus*–Sepharose 4B (Table IV).

Studies on Aggregation and Gel-Forming Characteristics of the Components of LM-Gel. The gel-forming ability of the separated mucin glycoprotein and protein components was investigated. Fractions were pooled as indicated in Figure 4, dialyzed extensively vs. 1 M urea–1 M NaCl and H_2O, lyophilized, and suspended in water. Fractions I and II formed a gel after removal of $NaDodSO_4$ and DTT and as discussed above fraction I contained mucin glycoproteins. Fraction II also contained mucins; however, they constituted a minor fraction of the LM-gel and have not yet been further characterized. Fraction III contained protein-rich material that was essentially soluble, whereas the ~13000 molecular weight

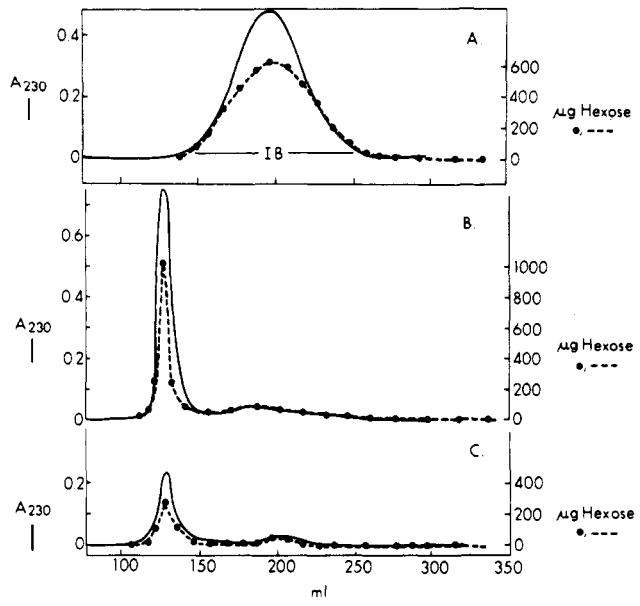

FIGURE 7: Chromatography of reduced carboxymethylated human lung MGP on Sepharose 2B with the following eluant buffers: (A) 0.1% $NaDodSO_4$ and 0.15 M ammonium acetate, pH 7.0; (B) 0.2 M NaCl; (C) 0.15 M ammonium acetate, pH 7.0. After dialysis and lyophilization, 10 mg of fraction IB from Figure 7A was solubilized in 10 mL of 0.2 M NaCl, loaded on a Sepharose 2B column equilibrated with 0.2 M NaCl, and eluted with 0.2 M NaCl. The void volume fraction in Figure 7B was pooled, dialyzed vs. 0.15 M ammonium acetate, and chromatographed on Sepharose 2B with ammonium acetate as the eluant. A_{230} (—); micrograms of hexose (-●-).

protein(s) of fraction IV formed a granular precipitate. The protein components in fractions III and IV, however, were capable of aggregation, and in a solvent containing 6 M Gdn·HCl, but not DTT, the proteins of each fraction eluted in the void volume of a Sepharose 4B column.

The major and minor lung mucin glycoproteins of fraction I, resolved by chromatography on Sepharose 2B and hydroxylapatite, formed a gel in the absence of $NaDodSO_4$ and DTT. Dissolution of this gel, in contrast to the original LM-gel, required the addition of both DTT and $NaDodSO_4$.

Substitution of 84% of the thiol groups by carboxymethylation with iodoacetamide resulted in a mucin that at a concentration of 10 mg/mL formed a translucent voluminous gel in water or saline. The gel dissolved upon the addition of $NaDodSO_4$; DTT was not necessary. At low mucin concentrations (1 mg/mL) this mucin sample was soluble and eluted in the included volume of a Sepharose 2B column when the solvent contained $NaDodSO_4$. In the absence of $NaDodSO_4$, however, the MGP eluted in the void volume (Figure 7). After carboxymethylation in $NaDodSO_4$ the solubility behavior of the 84% carboxymethylated mucin samples was not altered. However, after a second cycle of reductive carboxymethylation in $NaDodSO_4$, the mucins were fully carboxymethylated, soluble in water or saline at a mucin concentration of 10 mg/mL, and in the absence of $NaDodSO_4$ eluted in the included volume of a Sepharose 2B column. Since $NaDodSO_4$ and not DTT affects the gel properties of the partially substituted MGP and carboxymethylation of the remaining groups eliminates the need for $NaDodSO_4$, the carboxymethylation of at least the remaining thiol groups has affected interactions that apparently do not involve disulfide bridges.

Discussion

One of the major facets of current studies on LM-gels is concerned with the resolution of the gel constituents, their chemical and physical characterization, and the determination of the manner in which particular constituents and chemical groups affect aggregation and gelation reactions. In our studies concerning the chemical characterization of human LM-gel constituents, we encountered two major problems: (1) variation in the solubility of LM-gel in saline and, contrary to our expectation, the ineffectiveness of reducing agents for increasing solubility and (2) aggregation of solubilized LM-gel components, resulting in contamination of the mucin fractions on chromatography. While the variation in solubility is a troublesome feature of the system, the phenomenon is important since it indicates the presence of an unknown factor(s) that affects the tenacity of molecular interactions between the gel constituents.

All LM-gel samples could be partially dissolved by repeated extractions with 10–20 volumes of physiological saline or distilled water. In a few preparations as much as 40–60% of the dry weight of the gel was solubilized in this fashion, but in most preparations ~75% of the material remained in the gel phase. LM-gel was not solubilized by the addition of a reducing agent to a saline suspension, but it was solubilized in dissociating solvents (Gdn·HCl, urea, $NaDodSO_4$) (Table II). This behavior was in contrast to some studies in the literature where dissolution of LM-gel was achieved by addition of thiol reagents (Havez et al., 1968; Roberts, 1976). However, it was consistent with another study (Roberts, 1974) in which LM-gels from a number of sources were soluble in 6 M urea with some samples dissolving more rapidly than others. At present we do not know the reason for these differences in response to thiol reagents.

The components solubilized by dissociating solvents were still aggregated and in the presence of dissociating solvents eluted in the void volume of Sepharose 4B or 2B columns (Figure 3). However, on addition of dithiothreitol to these solvents, the MGP and protein components were separated by chromatography on Sepharose 4B. These studies suggest that for human LM-gel at least two processes are involved in gelation: (a) an aggregation reaction that is inhibited by reducing agents and (b) gel formation by soluble aggregates through a process that is inhibited by dissociating solvents (Figure 8).

The human lung MGPs were resolved into major and minor components by chromatography on Sepharose 2B and on hydroxylapatite. The same resolution could be obtained without the addition of DTT if the gel components were carboxymethylated prior to chromatography—the single carboxymethylation cycle resulting in 84% substitution of the thiol groups. However, $NaDodSO_4$ was still necessary since in its absence the carboxymethylated MGP and proteins were not resolved by chromatography on Sepharose 4B and the MGP eluted in the void volume when chromatographed on Sepharose 2B. When the carboxymethylated MGP was taken through a second reduction carboxymethylation cycle, the product exhibited neither aggregation behavior nor the capability of forming a gel in physiological saline. This behavior is described in reactions 1–3. Partial carboxymethylation

$$\text{partially (84\%) carboxymethylated MGP} \xrightarrow{\text{0.15 M saline}} \text{aggregates} \rightarrow \text{gel} \quad (1)$$

$$\text{partially (84\%) carboxymethylated MGP} \xrightarrow{NaDodSO_4} \text{no aggregates} \quad (2)$$

$$\text{carboxymethylated MGP} \xrightarrow{\text{0.15 M saline}} \text{no aggregates} \quad (3)$$

eliminated the requirement of DTT to dissociate the aggregates but not the need for $NaDodSO_4$ (reactions 1 and 2). The

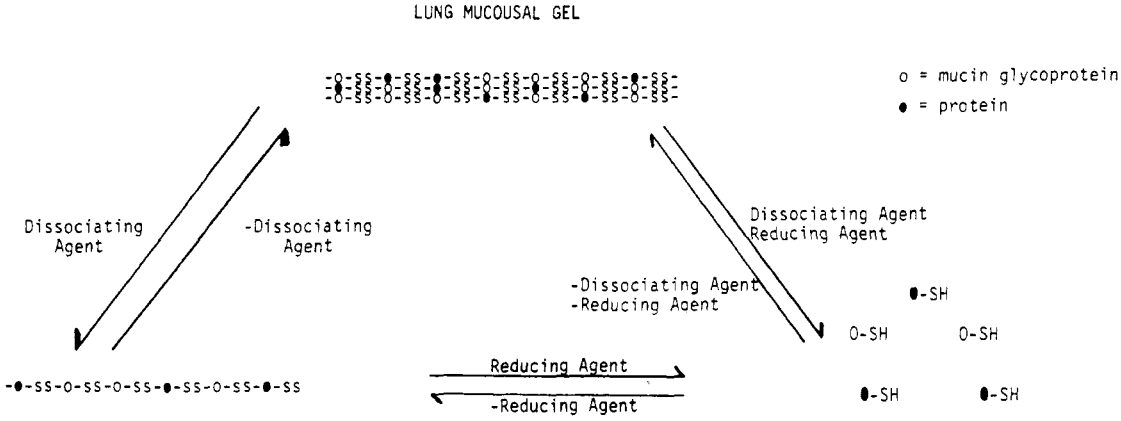

FIGURE 8: Schematic illustration of aggregation and gel formation by the components of human LM-gel.

results of complete carboxymethylation (reaction 3) are apparently caused by the presence of the bulky polar carboxymethyl groups rather than by a blockage in disulfide formation since aggregation of the partially carboxymethylated MGP was inhibited in a solvent containing $NaDodSO_4$ but not DTT.

The mucin described in the present report has properties in common with sample G of LM-gels studied by Roberts (1974, 1976)—i.e., the carboxymethylated MGP eluted in the void volume of Sepharose 4B and in the included volume following proteolytic digestion (Rose et al., 1979). More detailed comparisons cannot be made since the carbohydrate analysis of sample G mucin has not been reported and serum proteins constituted a significant portion of the purified gel G mucin. While it was suggested by Roberts (1976) that the nonmucin proteins of sample G were linked to the mucin through disulfide bridges, it is not possible to unequivocally draw the same conclusion from the study of the material described in the present report. As in the case of gel G, the gels described in the present report yield mucins contaminated with nonmucin proteins when the mucins are prepared by fractional ethanol precipitation of a 6 M urea solution. Subsequent chromatography on Sepharose 4B results in the elution of both the MGP and protein components in the void volume as shown in Figure 3. However, since the protein components of fraction III and of fraction IV aggregate in solvents containing a denaturant and lacking a thiol reagent and the aggregates elute in the same fraction as the mucins, it is not possible to conclude which components are interacting with each other.

We agree in spirit with some aspects of the model structure of mucin G suggested by Roberts (1976). However, we do not feel that the effects of carboxymethylation can be used in this instance for determining structural features dependent on disulfide bridges. Carboxymethylation of the mucin described in the present report has effects on interactions that are also affected by DTT and on some interactions that are affected by $NaDodSO_4$ in the absence of DTT.

In summary, we have studied the LM-gel of a single individual in order to eliminate genetic variation as a source of differences noted between different preparations. The MGPs have been resolved from the nonmucin proteins and have been further purified by chromatography on Sepharose 2B and on hydroxylapatite. These lung mucins have been analyzed and shown to aggregate and gel by interactions involving the polypeptide chain—interactions involving not only thiol but also nonthiol groups. They have been shown to be capable of gel formation in the absence of albumin or any of the proteins associated with LM-gel. Thus, human LM-gel clearly behaves differently than the viscid meconium gel obtained from patients with meconium ileus where gel formation results from an interaction of mucin glycoproteins and albumin (Schachter & Dixon, 1965; Young et al., 1958) and behaves similarly to porcine gastric (Snary et al., 1970; Robson et al., 1975) and bovine cervical mucus (Gibbons & Sellwood, 1973).

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Structural Changes in Glycogen Phosphorylase As Revealed by Cross-Linking with Bifunctional Diimidates: Phosphorylase *b*†

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ABSTRACT: Glycogen phosphorylase *b* was cross-linked with a homologous series of diimidates (maximal effective lengths, 3.7–14.5 Å) in the presence of various activators, inhibitors, and substrates under conditions where no polymers were formed. The cross-link products were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the gels were evaluated by densitometry. From the changes of cross-link patterns the following main conclusions were drawn. (1) In dimeric phosphorylase *b* there are at least two lysyl pairs at the subunit interface (contact m) whose ϵ-NH_2 groups are within 3.7 Å. (2) When dimeric phosphorylase *b* associates to form tetramers, the nearest two lysyl ϵ-NH_2 groups are ~8 Å apart at the interdimer interface (contact d). (3) The conformational change caused by adenosine 5′-monophosphate (AMP) promotes cross-linking at contact m and induces tetramerization of the enzyme with consequential cross-linkability at contact d. (4) In contrast to AMP, the other activator, inosine monophosphate, does not elicit any change in cross-linking. (5) Adenosine 5′-diphosphate (ADP) and adenosine 5′-triphosphate (ATP) diminish cross-link formation between NH_2 groups located within a distance of 4–9 Å at contact m. (6) Glucose 1-phosphate mimicks the effect of ADP, but when applied together with AMP it amplifies the effect of the latter (heterotropic interaction). (7) Glucose, caffeine, and glycogen decrease cross-link formation with the longer reagents owing to the dissociation of tetrameric phosphorylase *b*. The binding of glucose 6-phosphate does not seem to induce structural changes detectable in the cross-linkability of contact m. All four effectors diminish the influence of AMP.

It has been a long-lasting challenge to elucidate the structural basis of the catalytic and regulatory properties of glycogen phosphorylase (EC 2.4.1.1), the key enzyme of glycogen breakdown. Important advances have recently been made to this end: the structure of both phosphorylase *a* (Fletterick et al., 1976) and phosphorylase *b* (Weber et al., 1978) has been solved at 3-Å resolution, and the complete amino acid sequence of the phosphorylase subunit (841 residues) has been determined (Titani et al., 1977). The binding sites for the various substrates and effectors have been located in the three-dimensional structure, and the details of the transition from the inactive to the active conformation (Helmreich et al., 1967; Buc, 1967) are beginning to take shape (Weber et al., 1978; Kasvinsky et al., 1978a,b; Dreyfus et al., 1978).

Cross-linking with bifunctional reagents followed by sodium dodecyl sulfate gel electrophoresis, a fairly simple chemical technique, has proved to be a useful tool in the study of oligomeric enzymes as regards the number and arrangement of subunits (Davies & Stark, 1970; Hajdu et al., 1976). We deemed that this method might provide information, complementary to that available, about the structural events in phosphorylases on the following grounds.

(1) By using a homologous series of diimidates, we can measure interlysyl distances on the surface of a protein. Namely, the cross-linking of a given lysine pair by such a reagent will be influenced, among others, by the distance and reactivity of the ϵ-NH_2 groups. It is expected that a conformational change involving these lysines will affect the propensity for cross-linking in a different manner for reagents of various lengths by altering NH_2 group distances and/or reactivities.

(2) Allosteric transitions propagating from one subunit to the other are bound to include structural changes near the subunit contacts, and cross-linking reports mainly about this area. The allosteric nucleotide binding site in phosphorylase is also near the subunit contact region (Fletterick et al., 1976; Weber et al., 1978).

(3) Cross-linking data, in contrast to the X-ray picture, are related to the protein in solution; hence, they may reflect the dynamics of protein structure. Clearly, cross-linkability of a lysine pair will also be influenced by the movements of the two side chains relative to each other.

(4) In knowledge of the three-dimensional structure of phosphorylase, tentative assignments can be made to some cross-links which may later be checked by X-ray analysis. In

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