Gel Electrophoresis of Mucous Glycoproteins. II. Effect of Physical Deaggregation and Disulfide-Bond Cleavage*

Kenneth G. Holden,† Nelson C. F. Yim, Lee J. Griggs, and Jerry A. Weisbach

ABSTRACT: Gel electrophoresis was used to study the effect of physical deaggregation and disulfide-bond cleavage on mucous glycoproteins. Although deaggregation with urea or sodium dodecyl sulfate did not produce subunits from the mucins studied, electrophoresis in the presence of detergent did produce more compact bands. By contrast, the migration of serum proteins was strongly influenced by sodium dodecyl sulfate. In porous gels, all serum components migrated very rapidly and were clearly separated from mucins, thus providing a convenient technique for examining whole secretions or detecting the presence of impurities in mucin preparations. Reduction with dithioerythritol converted porcine and canine submaxillary mucins and canine tracheal mucin into lower molecular weight species. The reduced materials were still very large, but the decrease in molecular weight was sufficient to permit their facile characterization by electrophoresis in porous gels. Disulfide-bond reduction had no observable effect on bovine or ovine submaxillary mucins.

Mucous glycoproteins are difficult to characterize by the usual physical methods because of their great molecular size, polydispersity, and the viscous nature of their solutions. Recently, we have adapted gel electrophoresis to this purpose by taking advantage of the greatly increased porosity provided by Agarose gels (Holden *et al.*, 1971). Although most mucins were observed to migrate under these conditions, problems stemming from molecular interactions were not completely eliminated. Thus, the tendency for mucous glycoproteins to form gels at moderate concentration and to adhere tenaciously to supporting media are clear evidence of intermolecular forces which interfere with their separation and characterization by physical methods.

Detergents, such as SDS,[1] often have a profound effect on protein conformation and subunit structure at relatively low concentration (Tanford, 1968) and might be expected to break up mucin complexes dependent on physical forces such as hydrophobic or ionic interactions. Furthermore, SDS has been successfully employed for the estimation of protein molecular weights by gel electrophoresis (Shapiro *et al.*, 1967; Shapiro and Maizel, 1969; Weber and Osborn, 1969) and provided a technique which might be readily applied to mucins. This same method employs reducing agents to ensure cleavage of inter- and intramolecular disulfide bonds prior to electrophoresis. As the very high molecular weight of some mucins might be explained by the linking of subunits through intermolecular disulfide bonds (Dunstone and Morgan, 1965), we have now investigated the gel electrophoresis of mucins in the presence of SDS with and without disulfide-bond cleavage.

Materials and Methods

All materials and methods were as described previously (Holden *et al.*, 1971) with the following modifications and additions. Canine tracheal mucus produced by a trachael pouch (Wardell *et al.*, 1970) was the source of CTM_1. The mucus, kindly supplied by Dr. Chakrin, was solubilized in buffered 6 M urea, centrifuged at 100,000*g* to remove a small amount of cellular debris, and chromatographed on 1% or 2% Agarose columns equilibrated with the same solvent. Fractions containing the first peak, which occurred near the calculated void volume, were combined, dialyzed, and lyophilized to give CTM_1. Details of the preparation and characterization of this material, which represents approximately half of the macromolecules present in canine tracheal mucus, will be reported subsequently. CSM_1 was obtained from canine submaxillary saliva in an analogous manner. Sodium dodecyl sulfate was purchased from Eastman Kodak[2] and dithioerythritol from Pierce Chemical Co.

Sample and running gels as well as the electrode buffer solution all contained 0.1% SDS. When dithioerythritol was used, it was added to the sample solution (*ca.* 0.5%) which was maintained at 40° with gentle magnetic stirring for 30 min prior to addition of Agarose. In early experiments, 0.5% dithioerythritol was also added to the running gel but was later omitted when it was found to be unnecessary. Electrophoresis times were decreased to one-half those used without SDS (*viz.*, 7.5% acrylamide, 1 hr; 3% acrylamide–0.5% Agarose, 45 min; 1.5% acrylamide–0.5% Agarose, 37 min; 1% Agarose, 30 min). The slowest moving mucins (CTM_1, CSM_1 and PSM) were also examined in 1% Agarose gels under more vigorous electrophoresis conditions (5 mA/tube for 45 min).

Results

Figures 1 and 2 compare the electrophoretic patterns of canine serum, human fibrinogen, unfractionated canine submaxillary mucin, and purified bovine, ovine, and porcine submaxillary mucins with and without prior reduction of disulfide bonds. As previously noted (Holden *et al.*, 1971)

* From the Research and Development Division, Smith Kline and French Laboratories, Philadelphia, Pennsylvania 19101. *Received March 3, 1971.*

† To whom correspondence is to be addressed.

[1] Abbreviations used are: SDS, sodium dodecyl sulfate; DTE, dithioerythritol; CS, canine serum; HF, human fibrinogen; BSM, CSM, OSM, and PSM for bovine, canine, ovine, and porcine submaxillary mucins, respectively; CTM, canine tracheal mucir; CTM_1 and CSM_1, the high molecular weight fractions of CTM and CSM.

[2] One lot of SDS from this supplier gave completely unsatisfactory results and was found to be significantly impure as judged by its melting point, infrared spectrum, and solubility. We later found that SDS supplied by Pierce Chemical Co. was quite satisfactory and much less expensive.

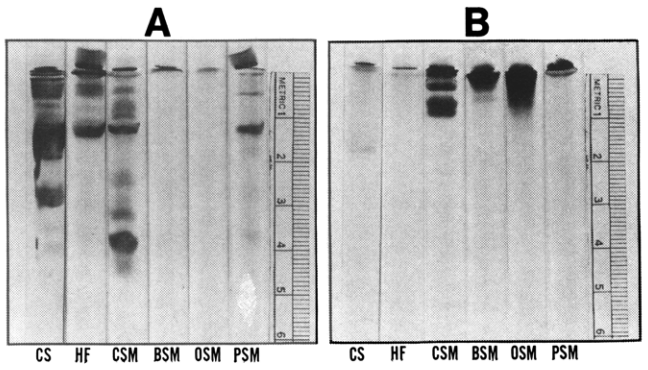

FIGURE 1: Electrophoresis of canine serum (CS), human fibrinogen (HF), unfractionated canine submaxillary mucin (CSM), and purified bovine, ovine, and porcine submaxillary mucins (BSM, OSM, and PSM) in 7.5% acrylamide gels containing SDS. In this and subsequent figures duplicate gels are stained for protein (A) with coomassie brilliant blue and for carbohydrate (B) with periodic acid–Shiff's reagent. The sample gel is at the top of the picture with the zero point of the scale placed at the sample–running gel interface; the anode is at the bottom. The smearing of the major protein positive band in CS is an artifact which was not reproduced in subsequent runs.

purified submaxillary mucins stained poorly for protein (A) and strongly for carbohydrate (B); the reverse was true for canine serum proteins and human fibrinogen. Rather pronounced changes in the patterns of CS and HF were evident after disulfide-bond reduction. Specifically, new bands of higher mobility were produced and much more of the applied sample entered the running gel. At least four new bands staining weakly for carbohydrate could be detected in CS after disulfide-bond reduction. On the other hand, significant changes in the electrophoretic patterns of the mucins were not evident. The general pattern of unfractionated CSM remained unchanged. The apparent small increase in mobility of the protein-positive bands was not the result of reduction but was due to minor deviations in the electrophoresis conditions. Repetition of the experiment showed no significant differences. The smearing of the carbohydrate-positive bands and corresponding weakly positive protein bands and their slightly increased mobility after reduction were reproducible. BSM, OSM, and PSM appeared to be essentially unchanged by reduction. Different samples of PSM were used in the two experiments which explains the apparent disappearance of the protein impurities seen in Figure 1 but not in Figure 2. When

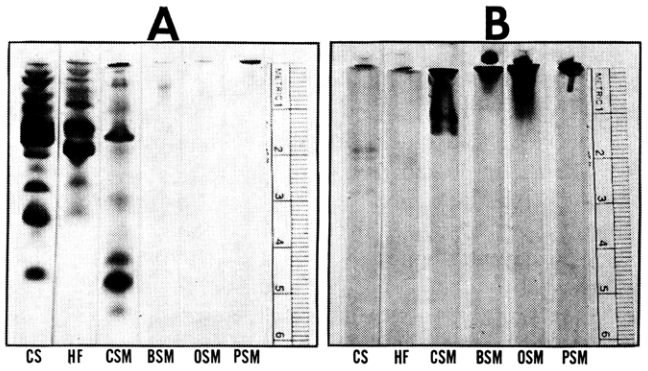

FIGURE 2: 7.5% Acrylamide–0.1% SDS–DTE. The vertical band near the top of the PSM gel stained for carbohydrate (B) was caused by fracturing of the gel during the staining process.

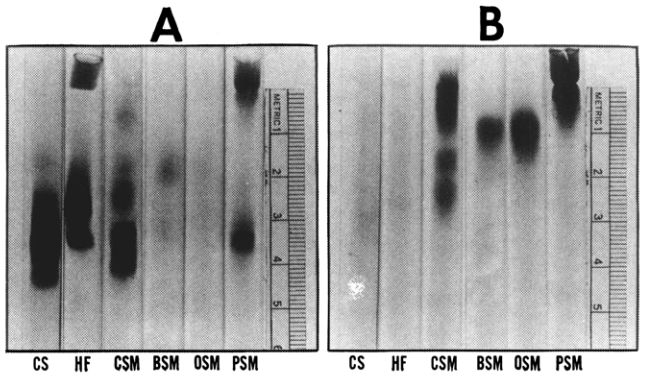

FIGURE 3: 1.5% Acrylamide–0.5% Agarose–SDS.

the same samples were used no significant difference was detectable between native and reduced PSM under these conditions.

Figures 3 and 4 compare the same samples in a much more porous gel medium composed of 1.5% acrylamide–1% Agarose. All CS components readily entered the running gel forming several overlapping bands of high mobility. After reduction, the bands were coalesced even more. A similar picture was presented by HF. While the strongly protein-positive components of CSM behaved in an analogous manner, those rich in carbohydrate did not. Before reduction, the major high molecular weight, carbohydrate-rich component of CSM just migrated out of the sample gel. After reduction, a significant increase in mobility resulted but this component was still clearly resolved from the protein-rich components. Two of the purified mucins, BSM and OSM, easily entered the more porous gel and appeared to be unaffected by disulfide-bond reduction. Although PSM entered even this more porous gel with great difficulty, disulfide-bond reduction did seem to increase its mobility to some degree. A protein impurity was again observed in PSM but had the same relative mobility after reduction.

Similar experiments were also carried out in a gel of intermediate porosity, 3% acrylamide–0.5% Agarose, and in a 1% Agarose gel which is comparable to 1.5% acrylamide–0.5% Agarose but slightly more porous. The results only served to confirm those shown in Figures 1–4 and are not presented.

To more clearly establish that disulfide-bond reduction has a significant effect on some mucins, we compared their behavior under more vigorous electrophoretic conditions. In-

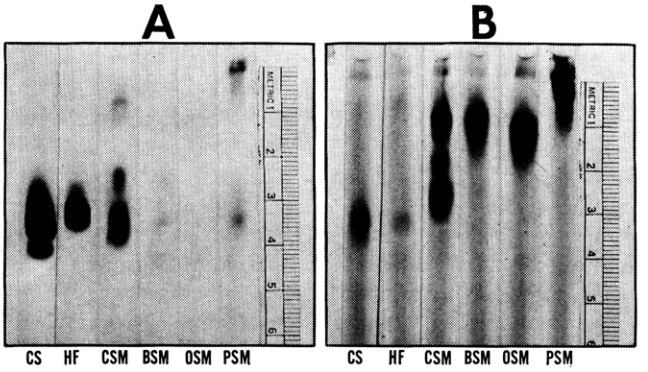

FIGURE 4: 1.5% Acrylamide–0.5% Agarose–SDS–DTE.

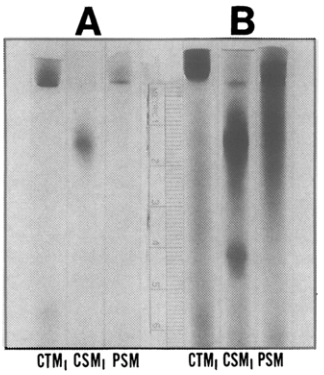

FIGURE 5: Extended electrophoresis of the high molecular weight fractions of canine tracheal mucin (CTM_1), caniine submaxillary mucin (CSM_1), and purified porcine submaxillary mucine (PSM) in 1% Agarose containing SDS.

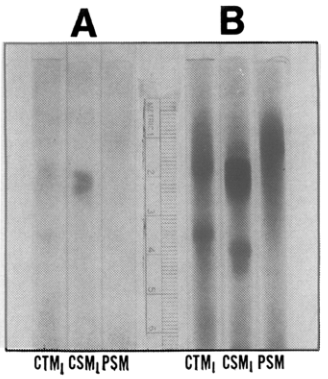

FIGURE 6: As in Figure 5 but following pretreatment with DTE.

cluded in this study was a sample of canine tracheal mucin which had been fractionated by gel filtration on Agarose to give a high molecular weight fraction, CTM_1. Also examined was a similarly derived fraction of canine submaxillary mucin, CSM_1. The same samples of BSM, OSM and PSM purified by conventional methods were also investigated. Figures 5 and 6 show how some of these materials behaved in 1% Agarose gels when the amperage and electrophoresis time were increased. Prior to disulfide-bond reduction (Figure 5) CTM_1 did not enter the running gel, most of it accumulating at the sample–running gel interface. On the other hand, CSM_1 readily moved into the running gel producing a doublet of rapidly moving bands staining moderately for carbohydrate but not detectably for protein and a slower moving major band which appeared to be composed of two or three barely resolved components. In the case of PSM much of the applied material was trapped at the sample–running gel interface but a significant amount entered to produce a very diffuse band of low mobility. Figure 6 shows the result of disulfide-bond reduction. In this case, the samples and gels were prepared at the same time and coelectrophoresed with those shown in Figure 5, the only difference being the addition of a small amount of dithioerythritol to half of each sample shortly before the run. The most obvious effect of disulfide-bond reduction was on CTM_1 which was converted to two major bands of relatively high mobility. CSM_1 showed a small but significant increase in mobility for the major group of bands located near the top of the running gel. Under the same conditions, the lower doublet was unaffected. PSM, which only partly entered the gel before reduction, entered smoothly to give a single diffuse band. Under the same conditions, no significant change could be detected with BSM or OSM.

Discussion

Since the molecular weights of mucous glycoproteins are approximately 10^6 or greater (Pigman and Gottschalk, 1966), it is likely that their biosynthesis is achieved through some sort of subunit assembly (Caspar, 1966). Downs and Pigman (1969, 1970) have suggested the occurrence, in BSM and OSM, of very small (*ca.* 28 amino acid) repeating units joined through peptide bonds. However, additional types of subunit assembly have been recognized. For example, the urinary glycoprotein of Tamm and Horsfall (1952) can be split into much smaller subunits by the action of physical deaggregating agents such as urea (Maxfield and Davis, 1963) or guanidinium chloride (Stevenson and Kent, 1970). High concentrations of urea are also useful for the solubilization of respiratory and gastrointestinal tract mucus (Waldron-Edward and Skoryna, 1966; Skoryna and Waldron-Edward, 1967). Certain covalent bonds, particularly disulfides, join peptide chains in a variety of macromolecules (Cohen, 1966). The presence of interchain disulfide bonds in mucins is not generally acknowledged since those most fully characterized (BSM and OSM) possess only trace amounts of sulfur-containing amino acids (Tettamanti and Pigman, 1968). However, the effect of disulfide-bond-cleaving reagents on ovarian cyst glycoproteins (Dunstone and Morgan, 1965), gastric mucus (Sheffner, 1963; Snary *et al.*, 1970; Kim and Horowitz, 1970), and respiratory tract mucus (Sheffner, 1963; Lieberman, 1968; Hirsch *et al.*, 1969) suggests that interchain disulfide bonds might be an important means of subunit attachment in such mucins.

These observations led us to examine the effect of physical deaggregation and disulfide-bond cleavage on mucous glycoproteins using the electrophoretic systems previously developed (Holden *et al.*, 1971). Initially, we tried 6 M urea as a physical deaggregating agent; however, no significant effect on mucin migration was observed in acrylamide gels, and high concentrations of urea prevented the solidification of Agarose. On the other hand, SDS was compatible with Agarose and had the expected pronounced effect on serum proteins, causing the components of CS to migrate as a single band of high mobility in 1% Agarose. Contrary to the behavior of serum proteins, mucins were not noticeably affected by the presence of SDS except for a tendency to form somewhat more compact bands than observed previously (Holden *et al.*, 1971). The practical value arising from this observation is that electrophoresis in large-pore gels containing SDS offers an effective way of separating serum proteins from mucins. This technique is particularly useful for examining whole secretions, as in the case of CSM where significant amounts of serum-type proteins may be present, or for detecting the presence of such impurities in isolated mucin preparations as demonstrated by the PSM used in this study.

Since neither urea nor SDS appeared to produce mucin subunits it was concluded that, if such subunits exist, they are joined by covalent bonds. A similar conclusion was reached with regard to BSM using gel chromatography (Bettelheim and Laurent, 1966). Attention was therefore focused on the possible role of disulfide bonds in mucous glycoprotein subunit attachment. Since complete cleavage is often prevented by conformational shielding of some sites in native proteins, SDS was used in conjunction with dithioerythritol (Cleland, 1964) to promote unfolding of peptide chains. Under these

conditions many components of CS were clearly converted to lower molecular weight species, and HF was similarly affected (McKee *et al.*, 1966). Although little change in the mucins could be detected in 7.5% acrylamide, a significant drop in molecular weight was suggested for the major mucin of CSM and perhaps PSM as judged from their behavior in 1.5% acrylamide–0.5% Agarose gels. Under more vigorous electrophoretic conditions in 1% Agarose, *a clear effect of disulfide-bond reduction was evident for some mucins.* The major mucin of canine tracheal mucus (CTM_1) appears to be very highly aggregated through disulfide bonds, since it failed to enter 1% Agarose prior to reduction. Nuclear RNAs up to 10^8 daltons were observed to migrate under similar conditions (Peacock and Dingman, 1968). Although the effect of reduction on CSM was less pronounced, an order of magnitude approximation suggests that the average molecular weight decreases by a factor of three.[3] In the case of PSM, a very polydisperse system with an upper molecular weight range sufficient to prevent much of it from entering the running gel is converted into a less disperse population of considerably lower molecular weight. Since it was conceivable that the higher mucin aggregates observed prior to disulfide-bond reduction were artifacts of the preparation procedure, we examined fresh secretions of CTM and CSM maintained under a nitrogen atmosphere to prevent oxidative coupling of sulfhydryl groups. However, they behaved exactly as the previously isolated samples, and it follows that disulfide bonds do play a significant role in determining the molecular weights of these mucous glycoproteins *in vivo*. Furthermore, the reduced subunits may be of particular value in terms of an approach to the study of mucous glycoproteins. Thus, superaggregates of the apparent size of CTM_1 and PSM are not amenable to study by most physical methods. But, the relatively smaller, more homogenous products produced by disulfide-bond reduction are readily characterized by the electrophoretic methods described here and, presumably, by other methods as well.

[3] This value was obtained by the method of Peacock and Dingman (1968) and, in this case, requires several additional assumptions which may not be entirely valid. As such, it represents only a rough estimate based on the available data.

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