Gel Electrophoresis of Mucous Glycoproteins. I. Effect of Gel Porosity*

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ABSTRACT: Great molecular size, the capacity to form viscoelastic gels or very viscous solutions at relatively low concentration, and a high degree of polydispersity are properties of mucous glycoproteins that make their characterization exceedingly difficult. With a view toward developing a suitable micro method of characterization, the electrophoresis of some representative mucins was studied in gels with a range of sieving properties. Canine serum served as a control protein mixture and human fibrinogen was used as a model protein of high molecular weight and extended shape. These substances along with unfractionated canine submaxillary mucin, and purified bovine, ovine, and porcine submaxillary mucins were investigated in gels composed of: 7.5% acrylamide, 3.0% acrylamide–0.5% Agarose, 1.5% acrylamide–0.5% Agarose, and 1% Agarose. The more porous gels containing Agarose or Agarose with a small amount of acrylamide allow migration of very large molecules and are suitable for the characterization of microgram quantities of mucous glycoproteins. Although excellent for detecting nonmucin impurities, the usual protein stains were found to be quite insensitive to mucins; however, staining for carbohydrate with periodic acid–Schiff's reagent proved effective. With the full range of gels described, mucin purity can be conveniently assessed. Some indication of relative molecular weight, polydispersity, and carbohydrate–protein composition can also be obtained for mucin mixtures or purified components.

The glycoproteins of epithelial mucous secretions represent a class of biological macromolecules whose degree of purity and characterization in general are not readily established by the usual physical methods (Gibbons, 1966). Besides having a high molecular weight (*ca.* 10^6), mucous glycoproteins are extended, thread-like molecules, and their effective molecular size is much greater than that of globular proteins of the same molecular weight. A further increase in molecular domain occurs at low ionic strength because of their high charge density (Bettelheim *et al.*, 1962). Even at low concentrations mucous glycoproteins form very viscous solutions; at moderate concentrations (*ca.* 1%) viscoelastic gels result. There is also a tendency for these molecules to adhere tenaciously to supporting media used in some fractionation procedures. Furthermore, all mucous glycoprotein preparations that have been carefully examined have been found to be polydisperse. This complicates the interpretation of separation and purification procedures since such materials can theoretically always be subfractionated.

As part of projected studies on the chemistry and biosynthesis of mucous glycoproteins, we required a sensitive method for characterizing and assessing the purity of relatively small mucin samples (<1 mg). Although gel electrophoresis has emerged as a particularly powerful technique for most proteins, its application to mucous glycoproteins has not been very successful (see, for example, Gibbons, 1966; Katzman and Eylar, 1966; Pigman and Tettamanti, 1968; Tettamanti and Pigman, 1968; De Salegui and Plonska, 1969). Purified mucins were observed not to migrate appreciably in acrylamide gels in spite of their high charge density. In retrospect, this is not surprising when one considers their great molecular size. Commonly used 7.5% acrylamide gels have an average pore size estimated to be about 50 Å and therefore exhibit extreme frictional resistance to relatively large extended molecules such as fibrinogen with molecular dimensions of 38 × 700 Å (Ornstein, 1964). Mucous glycoproteins are considerably larger; for example, bovine submaxillary mucin has a molecular length of 4360–8960 Å, depending on the solvent (Bettelheim *et al.*, 1962). It is therefore evident that a large increase in pore size is required before mucins could be expected to migrate appreciably in gels. Although some increase in pore size is achieved by lowering the acrylamide concentration, one rapidly reaches the point where the resulting gels become fluid and unmanageable.

Recently, Agarose and Agarose–acrylamide composite gels have been effectively used for the separation of substances with very large molecular dimensions, such as RNA (McIndoe and Munro, 1967; Peacock and Dingman, 1968; Ringborg *et al.*, 1968), DNA (Takahashi *et al.*, 1969), and ribosomes (Dahlbert *et al.*, 1969). It is evident from these studies that, although Agarose gels are very porous, they do possess sieving properties for very large molecules. This report describes the application of gels having a range of sieving properties to the electrophoretic characterization of mucous glycoproteins.

Materials and Methods

Purified submaxillary mucins (BSM, OSM, and PSM)[1] prepared by standard methods (Tettamanti and Pigman, 1968; De Salegui and Plonska, 1969) were the generous gifts of Drs. S. Roseman and W. Pigman. Additional samples of PSM were kindly supplied by Dr. D. Carlson. Unfractionated CSM was obtained through the courtesy of Dr. L. Chakrin who collected submaxillary saliva from anesthetized laboratory beagles stimulated with metacholine (0.3 mg/kg). The mandibular ducts were cannulated with polyethylene tubing;

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[1] Abbreviations used are: BSM, CSM, OSM, and PSM for bovine, canine, ovine, and porcine submaxillary mucins, respectively; CS, canine serum; HF, human fibrinogen.

TABLE I: Gel Compositions (Volumes in Milliliters).

Working Solutions	Sample Gel 1% Agarose	Running Gels 1% Agarose	0.5% Agarose–1.5% Acrylamide	0.5% Agarose–3.0% Acrylamide	7.5% Acrylamide
Water	0.35[a]	5.4	5.7	3.9	9.6
Sucrose		7.5	7.5	7.5	
Buffer	0.01	2.4	2.4	2.4	2.4
DMAPN			1.5	1.5	1.5
Acrylamide			1.8	3.6	9.0
Agarose	0.21	8.7	4.3	4.3	
Persulfate			0.8	0.8	1.5
	0.57	24.0	24.0	24.0	24.0

[a] For convenience and greater accuracy, a large quantity of appropriately diluted buffer was prepared and used for dissolving all samples.

and, after discarding the first few milliliters, collection was continued for approximately 2 hr. The saliva was maintained at ice temperature during collection and was then exhaustively dialyzed against deionized water at 4° and lyophilized. Canine serum was freshly prepared prior to electrophoresis. Human fibrinogen (Mann Research Laboratories) was used without purification. *N*-Acetylneuraminic acid was synthesized by the method of Kuhn and Baschang (1962). Commercial sources of other materials were as follows: Agarose (Marine Colloids' "Seakem" distributed by Bausch & Lomb); acrylamide, *N,N'*-methylenebisacrylamide, dimethylaminopropionitrile (Eastman Kodak); basic fuchsin (Fisher Scientific); coomassie brilliant blue (ICI America, Inc.).

The preparation of Agarose–acrylamide gels closely followed the method of Peacock and Dingman (1968) with the necessary modifications for their use with analytical columns rather than slabs. The 7.5% acrylamide gel and buffer were adapted from 5% gels described by Peacock *et al.* (1965). Working solutions are defined below, and the composition for each gel in terms of the working solutions is given in Table I.

Working solutions (diluted to the indicated volume with distilled water) are as follows: (1) sucrose, 15 g/100 ml; (2) buffer, 108 g of Tris–9.3 g of Na_2EDTA–55 g of boric acid/1000 ml (pH 8.2); (3) DMAPN, 6.4 g of dimethylaminopropionitrile/100 ml; (4) acrylamide, 19 g of acrylamide–1 g of *N,N'*-methalenebisacrylamide/100 ml; (5) Agarose, 0.344 g in 12.5 ml; and (6) persulfate, 1.6 g of ammonium persulfate/100 ml.

Agarose solution was prepared just before each run as described below. Persulfate and sucrose solutions were freshly prepared each week. All solutions except Agarose and buffer were stored at 4°.

Gel Preparation. Mucin samples (360–400 μg) were accurately weighed and dissolved in the indicated amount of buffer and water. Although satisfactory solutions could usually be obtained by magnetic stirring at room temperature for 1 hr, more reproducible results were obtained by gently stirring the mucin overnight at 4°. Canine serum (5–6 μl) was diluted in an analogous fashion. In a typical electrophoretic run twelve glass tubes (75 × 5 mm i.d.) were arranged vertically, their bottoms sealed with paraffin film. After dissolving the Agarose by refluxing for 15 min, the solution was cooled to 60° and introduced into the premixed gel components (except for persulfate) maintained at 40°. Persulfate was then added; and, after thoroughly mixing for a few seconds, the gels were cast without delay. Casting was best accomplished by two operators. One introduced the running gel (*ca.* 1.3 ml) using a warm syringe with a short length of capillary tubing attached to the needle. With the tip of the tubing maintained just below the rising liquid level, one tube was filled to a height of 66 mm. The second operator immediately layered on the sample gel (*ca.* 0.15 ml) with a warm disposable pipet to within 1 mm of the top of the tube. All the tubes were filled sequentially in this manner, casting two gels for each sample (one for protein and the other for carbohydrate staining). Finally a drop of buffer corresponding to the concentration of the sample gel was carefully layered on and geling was allowed to proceed at room temperature for 1 hr. The following modifications of the above general method are necessary. For 7.5% acrylamide gels all operations were carried out at room temperature. Composite gels containing 3% acrylamide solidified so rapidly that it was not possible to cast 12 gels. In this case the running gel solution should be divided into three parts to which persulfate is added as each is used.

Electrophoresis. The paraffin was removed from the bottom of the tubes and replaced by a piece of dialysis membrane secured with a small ring of plastic tubing. This prevents the gels from sliding out of the tube during electrophoresis and is necessary for gels other than 7.5% acrylamide. Care should be taken to avoid trapping air bubbles during this operation. The tubes were placed in a standard electrophoresis cell (*e.g.*, Canalco or Shandon), and the reservoirs were filled with buffer diluted 1:9 with distilled water. Power was applied at the rate of 2.5 mA/tube at approximately 100 V for the following times depending on gel composition: 1% Agarose, 1 hr; 1.5% acrylamide 0.5% Agarose, 1.25 hr; 3% acrylamide–0.5% Agarose, 1.5 hr; 7.5% acrylamide, 2 hr.

Staining. After electrophoresis the gels were removed from their tubes, and one gel from each pair was stained for protein and the other for carbohydrate. Protein bands were located by soaking the gels in a 0.25% solution of coomassie blue in 7.5% acetic acid for at least 1 hr followed by continuous washing with 7.5% acetic acid passed through a bed of charcoal to remove leached dye. Carbohydrate was revealed by first washing the gels well with 7.5% acetic acid to fix the glycoproteins and remove sucrose. At least seven washes (10 ml each) separated by a 30 min soaking interval are required. After oxidation for 1 hr with 0.2% periodic acid in the same solvent, the gels were washed as before and then stained with fuchsin solution. The latter was prepared by stirring 1 g of basic fuchsin with 1.9 g of sodium metabisulfite in 100 ml of 0.2 N hydrochloric acid for at least 3 hr at 4°. After adding a little charcoal and stirring for a few minutes, the mixture was filtered by suction through a glass fiber mat. The colorless solution was stored in a sealed bottle at 4°.

Sialic Acid Analysis. The thiobarbituric acid method (Warren, 1959) was used. Interference from gel components was determined by analyzing gels containing known amounts of pure *N*-acetylneuraminic acid. Analysis of sialic acid bound to macromolecules within the gel matrix was accomplished by first dialyzing the gels against distilled water to remove interfering sucrose followed by hydrolysis with 0.1 N H_2SO_4 at 80° for 1 hr to release sialic acid and solubilize the gel.

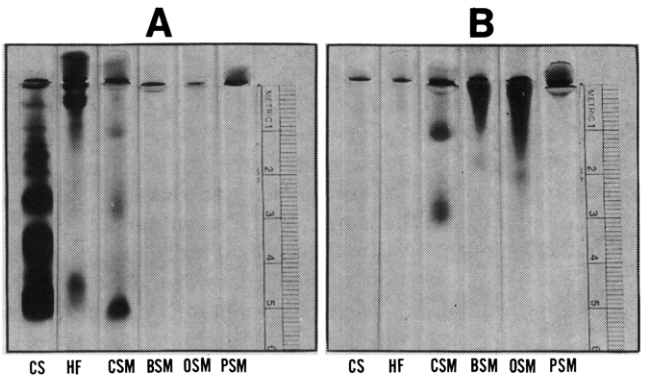

FIGURE 1: Electrophoresis of canine serum (CS), human fibrinogen (HF), unfractionated canine submaxillary mucin (CSM), and purified bovine, ovine, and porcine submaxillary mucins (BSM, OSM, and PSM) in 7.5% acrylamide gels. In this and subsequent figures, duplicate gels are stained for protein (A) with coomassie brilliant blue and for carbohydrate (B) with periodic acid–Schiff'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.

Results

Figures 1–4 show the electrophoretic patterns of canine serum, human fibrinogen, and submaxillary mucins in a series of gels of decreasing acrylamide content. In each case the set on the left (A) is stained for protein and that on the right (B) for carbohydrate. As expected, canine serum components (CS) and human fibrinogen (HF) stained strongly for protein but weakly or not at all for carbohydrate. The reverse was true for the submaxillary mucins. Lack of appreciable protein staining was most pronounced for BSM and OSM although positive staining was clearly visible when these substances were strongly concentrated at the sample–running gel interface (Figures 1 and 2). Even when the submaxillary mucins had moved well into the more porous gels (Figures 3 and 4), extremely weak protein-positive bands corresponding to the major carbohydrate-positive band could always be detected; however, they faded rapidly and are not easily visible here in the case of BSM or OSM.

Figure 1 shows that most canine serum (CS) components readily entered 7.5% acrylamide gels as expected, although some carbohydrate-rich components appeared to be trapped at the sample–running gel interface. The human fibrinogen (HF) sample is quite heterogeneous but appeared to enter the running gel with difficulty. Also from Figure 1, it can be seen

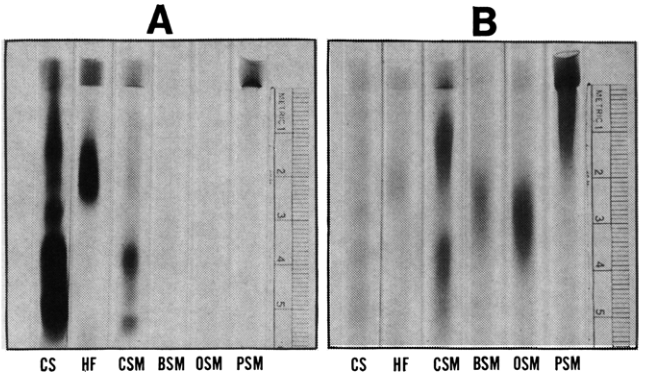

FIGURE 3: 1.5% Acrylamide–0.5% Agarose.

that unfractionated CSM contains a rapidly moving component which stained only for protein, and had the same mobility as albumin. Two other components staining for both protein and carbohydrate were also evident, but the bulk of the applied material was located at the sample–running gel interface. For BSM and OSM, appreciable amounts of material entered the running gel; but, except for a weak leading band, there was no resolution. The major band tailed back toward the junction of the sample and running gels where a significant amount of the applied sample remained as judged from the intensity of both protein and carbohydrate stains. Doubling the time of the electrophoretic run did not significantly increase the amount of mucin entering the running gel; only an expansion of same general pattern was observed. In the case of PSM, essentially none of the sample entered the 7.5% acrylamide running gel.

When the acrylamide content was reduced to 3% (Figure 2) a great increase in the amount of fibrinogen (HF) entering the running gel was observed, and the heterogeneity revealed by 7.5% acrylamide was still evident. The prominent components of unfractionated CSM which were seen in Figure 1 were less evident and appeared as weak bands near the bottom of the gel. Other features remained essentially unchanged.

Figure 3 shows the result of decreasing the acrylamide content to 1.5%. In this case resolution of serum components was decreased since sieving is greatly reduced. Fibrinogen now appeared essentially homogeneous. The most dramatic changes, however, were with the submaxillary mucin samples. It is clear that the major carbohydrate positive component of CSM which had failed to enter the less porous gels now migrated readily. BSM and OSM easily penetrated this gel, and

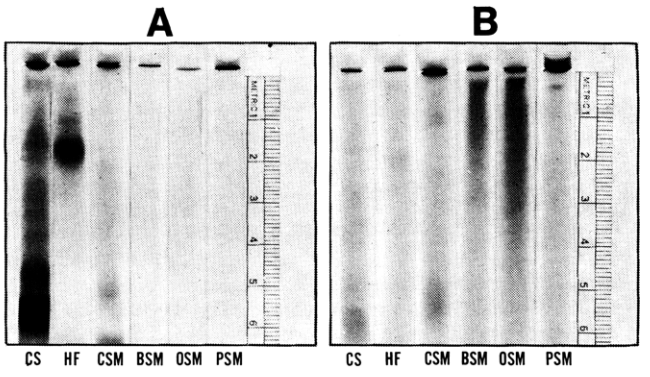

FIGURE 2: 3% Acrylamide–0.5% Agarose.

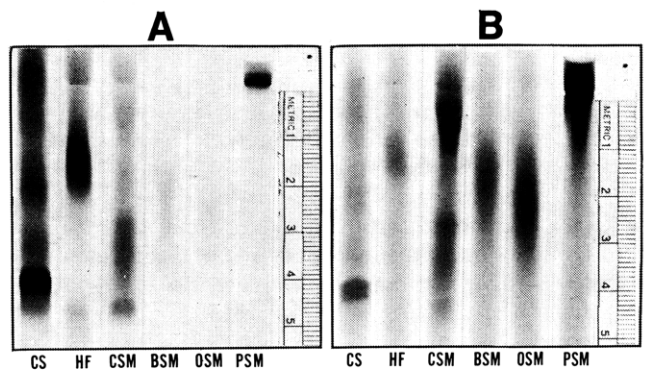

FIGURE 4: 1% Agarose.

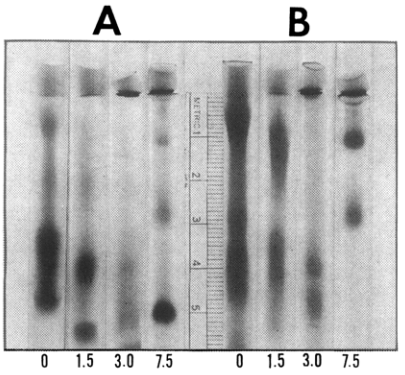

FIGURE 5: Electrophoresis of unfractionated canine submaxillary mucin (CSM) in a series of gels of increasing acrylamide content stained for protein (A) and carbohydrate (B). The duration of the electrophoretic run was adjusted according to gel composition so that the most rapidly migrating component approached the end of the gel in each case.

no material was retained at the sample–running gel interface. PSM partially entered the running gel, but much of it remained behind.

Results obtained with a 1% Agarose running gel (Figure 4) were similar to those obtained with the 1.5% acrylamide–0.5% Agarose composite gel; however, more intense staining was usually observed.

Figure 5 more clearly shows the effect of changes in gel porosity for a single sample, unfractionated CSM. The porosity decreases from left to right corresponding to an increased acrylamide content. Although the fastest moving component appears to migrate with equal facility in all the gels regardless of porosity, this is not the case since electrophoresis time was increased to compensate for the increased resistance to migration provided by higher acrylamide content (see Methods section).

Possible methods for sample application were investigated quite thoroughly and require some commentary. Initial experiments showed that the migration of mucous glycoproteins was quite sensitive to gel discontinuities. Thus, if the running gel was allowed to solidify before the sample gel was applied, an accumulation of sample often occurred at the interface even when the gels were of identical composition. This effect could be minimized by adding 5% by weight of sucrose to the running gel solution so that the sample gel could be easily layered over it before either had solidified.

The sample gel buffer was a 1:5 dilution of the same buffer used in the electrode compartments and running gel. By lowering the conductivity of the sample region in this manner, zone sharpening is readily achieved (Hjerten *et al.*, 1965) without the necessity of employing the more complicated discontinuous buffer and spacer gel system of Ornstein (1964). Although increased zone sharpening (and therefore resolution) may be obtained by decreasing the conductivity still further, we found that this again led to undesirable accumulation of mucous glycoproteins at the sample–running gel interface.

The failure of submaxillary mucins to exhibit appreciable protein staining suggested the possibility that they might be partially eluted from the more porous gels during the rather extensive washing with 7.5% aqueous acetic acid required for destaining. The difficulty of fixing proteins in Agarose gels has previously been noted (Davidsen, 1968). To test this possibility, we measured the sialic acid content of Agarose gels before and after washing; the wash solution was also assayed. Using unfractionated CSM[2] and purified OSM as examples, we found that, in each case, 85% of the sialic acid was still present in the gels after thorough washing (15 10-ml portions) comparable to that used in staining for either protein or carbohydrate. The remaining 15% was accounted for by the wash solution. These results prove that the poor protein staining exhibited by submaxillary mucins is not due to loss of a major part of the sample during gel washing.

Another point that was more clearly settled using the same assay was the proportion of applied sample that actually entered the running gel. After electrophoresis of unfractionated CSM in the usual way, the sample gel and adhering interface were cut away from the running gel and analyzed separately. Unfortunately acrylamide interfered with the assay, and results were only obtained for the more porous gels. On this basis 1% Agarose permitted migration of 95% of the sample; with 0.5% Agarose containing 1.5% acrylamide, 90% of the sample entered the running gel.

Discussion

The results of this study show that mucous glycoproteins penetrate conventional 7.5% acrylamide gels with great difficulty, but that they can be effectively studied in more porous gels composed of Agarose or Agarose–acrylamide combinations. Because they are relatively simple to prepare, 1% Agarose gels are recommended for preliminary studies. Agarose was also used for the sample gel because it has minimal sieving properties and has the added advantage of not exposing the sample to the possible degradative effects of persulfate or photolysis required for acrylamide polymerization. A sample gel is preferred to layering in a sucrose gradient because it allows one to estimate how much remains at the origin.

The very weak protein staining exhibited by submaxillary mucins is surprising since they are 40–60% protein by weight (Pigman and Gottschalk, 1966). However, most protein stains depend on physical binding through electrostatic and van der Waals forces, and it is likely that such binding is unfavorable when the protein backbone is partially masked by carbohydrate chains with a high negative charge density. Because of differences in protein and carbohydrate content and the marked differences in affinity for coomassie brilliant blue[3] demonstrated by mucous glycoproteins as compared to other proteins, it is very difficult to estimate the relative amounts of each present in a mixture unless duplicate gels are stained with periodic acid Schiff's reagent. This may explain a report (Dabbons and Draus, 1968) in which purified mucins were claimed to migrate readily in 15% acrylamide gels on the basis of protein staining. Other reports have indicated some degree of success with whole human submaxillary (Caldwell and Pigman, 1965; Gugler *et al.*, 1967) and parotid (Meyer and Lamberts, 1965) salivas in acrylamide gels under somewhat different conditions. However, the very large amounts of sample applied and the lack of appreciable carbohydrate staining found in the running gels indicate that the protein-positive bands observed by these investigators were most probably due to minor constituents of lower molecular weight.

Among the properties of mucous glycoproteins that create

[2] Unfractionated CSM was found to contain 15–18% sialic acid. Purified OSM is reported to have a sialic acid content of 22.5–25.0% (Pigman and Gottschalk, 1966).

[3] Essentially the same results were obtained with the more commonly used Amido Schwarz, but it was even less sensitive.

difficulties when conventional methods of separation are applied, high molecular weight is most effectively dealt with by the methods developed in the present study. The use of very porous Agarose and Agarose–acrylamide composite gels for large molecules is based on similar studies with nucleic acids (McIndoe and Monroe, 1967; Peacock and Dingman, 1968; Ringborg *et al.*, 1968; Takahashi *et al.*, 1969). Those results clearly showed that distance migrated is inversely related to molecular weight. However, this concept cannot be applied directly to molecular weight estimations for mucous glycoproteins since their charge to mass ratio may vary over a considerable range. On the other hand, the electrophoretic mobilities of bovine, ovine, and porcine submaxillary mucins in solution are quite similar near pH 7 (Pigman and Tettamanti, 1968), and it follows that the differences in mobility observed here are due largely to differences in molecular weight. On this basis BSM and OSM appear to be of comparable size while PSM is clearly much larger. This ranking by molecular weight does not coincide with the generally accepted values determined from sedimentation and light-scattering studies (Pigman and Gottschalk, 1966) which place BSM at the upper end of the scale (4×10^6) followed by OSM (10^6) and PSM (0.8×10^6). More recent values (Tettamanti and Pigman, 1968) for BSM (0.375×10^6) and OSM (0.394×10^6) place the mucins in the same relationship suggested by our work provided the earlier figure for PSM is retained. Some very recent studies (Payza *et al.*, 1970) with BSM and PSM using sedimentation and light-scattering techniques showed that the molecular weights obtained vary within large limits depending on the source of the sample, method used, and experimental conditions employed. While the results obtained in this study are only qualitative, electrophoresis in a series of gels of different porosities offers a means of judging the relative molecular weights of mucins. Samples of like mucins prepared in different laboratories behaved similarly under all conditions of electrophoresis investigated.

The unique physical properties of mucous glycoprotein solutions created several unusual problems. Normally, for maximum resolution, it is necessary to either apply the sample in a very small volume or cause the macromolecular components to concentrate into a small volume just prior to separation as with disc electrophoresis (Ornstein, 1964). However, neither of these techniques is directly applicable to mucous glycoproteins. Concentrated solutions of these molecules are themselves gels composed of a thickly intertwined molecular network, and movement through even the most porous medium is impossible. With the discontinuous buffer system of Davis (1964) most of the sample accumulated at the sample–running gel interface, and a hypersharp,[4] distorted band of high mobility was also produced. We therefore used a continuous buffer system and achieved additional zone sharpening by diluting the sample buffer as recommended by Hjerten *et al.* (1965). However, dilution below 20% again led to accumulation of material at the sample–running gel interface. Thus, it would appear that the great increase in resolving power provided by disc electrophoresis cannot be fully utilized for mucous glycoproteins without causing serious aggregation problems. Experiments designed to reduce such aggregation have been carried out and are described in the following paper.

Finally, the question of polydispersity *vs.* heterogeneity of mucin preparations can be settled with some confidence using the present method. Although trace impurities were detected in some purified mucin samples, the usual observation was a single, slowly migrating, diffuse band. The broadness of the band appears to accurately reflect the degree of polydispersity, since serum proteins which do not exhibit polydispersity gave much sharper bands under the same conditions. The ability of gel electrophoresis to detect heterogeneity when present is illustrated by resolution of unfractionated CSM into a number of discrete components and the differences in mobility noted for mucins from different species.

[4] The use of this term as it applies to mucin separations is discussed by Gibbons (1966).

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