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Studies of the Bulbo-Urethral (Cowper's)-Gland Mucin and Seminal Gel of the Boar

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1. Moving-boundary electrophoresis of the mucin from the Cowper's gland of the boar revealed a sharp single peak at pH values from 1.1 to 9.0 and an isoelectric point of 1.1. 2. Neuraminidase treatment of the mucin, which removed at least 96% of the sialic acid groups, decreased the electrophoretic mobility at pH 4 from -7.4×10^{-5} (for the mucin) to -0.64×10^{-5} cm^2 V^{-1} s^{-1}. 3. Ultracentrifugal sedimentation values of $s_{20,w}$ showed a marked dependence on concentration. A hyperfine peak, similar to that given by ovine submaxillary secretion, persisted throughout the run at higher concentrations. Ultracentrifugal studies further showed a very low value for the diffusion coefficient ($D_{20,w}$ -1.57×10^{-8} cm^2/s). 4. Calculation of the approximate molecular weight from comparable $s_{20,w}$ and $D_{20,w}$ values gave a provisional value of 6.5×10^6. 5. Two proteins present in the boar vesicular secretion known as protein A and protein H (the haemagglutinating protein) were shown to promote the swelling of the mucin to form the characteristic rigid elastic gel of boar semen. It is suggested that protein A molecules particularly (mol.wt. 2.8×10^4) cross-link with the long molecules of the mucin to form the seminal gel.

The CGM† of the boar is the source of the seminal gel that is a normal constituent of boar semen (McKenzie, Miller & Bauguess, 1938). At the time of ejaculation the white mucin is extruded into the urethra as a large number of more or less discrete 'beads'. If the semen is stored at room temperature the beads continue to absorb water for several hours. Eventually a coherent mass of a translucent tough elastic gel occupies all or almost all of the semen volume.

Hartree (1962) explored the composition of the CGM and found that it was a mucoprotein containing (on a dry-weight basis) up to 25% of sialic acid (*N*-acetylneuraminic acid). All the sialyl residues were liberated by bacterial neuraminidase and the predominant sugar residues were sialic acid and (after hydrolysis) galactosamine. It would thus appear that the CGM is analogous to OSM (Gottschalk, 1960; Graham & Gottschalk, 1960; Pigman & Gottschalk, 1966). This paper reports extensions of these analogies.

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† Abbreviations: CGM, Cowper's-gland mucin; OSM, ovine submaxillary-gland mucin; RDE, receptor-destroying enzyme (crude neuraminidase); RDE-M, CGM that had been treated with RDE; N-M, CGM that had been treated with purified neuraminidase.

The swelling of CGM can be simulated *in vitro* by adding small fragments of this mucin to the secretion from the boar seminal vesicles. The main protein components of boar vesicular secretion and of seminal plasma have now been characterized as proteins A, B and H. Of these, proteins A and H are basic, and H is a powerful haemagglutinin (Boursnell & Coombs, 1966). These proteins can be separated in relatively pure form by gel filtration (Boursnell & Briggs, 1969). It therefore seemed profitable to examine the interaction of these proteins with CGM in more detail.

MATERIALS AND METHODS

Collection and storage of CGM. Cowper's glands, obtained from fresh slaughterhouse material, were compressed in a vice and the extruded CGM was collected and stored at −20°C. It contained 20.3% of *N*-acetylneuraminyl residues on a dry-weight basis. Stock solutions (50mg wet wt./ml) were prepared by stirring the CGM vigorously for several hours in 0.15M-NaCl at room temperature (19°C). Traces of cellular debris were removed by centrifuging at 10000*g*. Solutions were stored at 5°C in the presence of 0.025% (w/v) merthiolate (Eli Lilly and Co., Basingstoke, Hants., U.K.). This had no discernible effect on the reactions that we have studied.

Seminal plasma and gel. The seminal plasma was prepared from whole semen collected by means of an artificial

vagina from boars housed at the Animal Research Station. The gel was removed soon after collection by straining through muslin and the seminal plasma was obtained as a clear supernatant fluid by centrifuging down the spermatozoa. The seminal plasma was stored at −20°C until required.

Vesicular secretion. This was obtained from seminal-vesicle glands freshly excised from slaughterhouse material. The glands were allowed to drain naturally in a large polythene funnel. The secretion was centrifuged and stored at −20°C.

Separation of proteins A, B and H from vesicular secretion or seminal plasma. These separations were carried out as described by Boursnell & Briggs (1969). Protein A was separated as the supernatant fluid (A Dial) remaining after the complete precipitation of the associated proteins B and H (as the composite B Dial). This precipitation was effected by prolonged dialysis against a buffer of pH 8 and I 0.015 (Boursnell & Nelson, 1965). After the B Dial had been redissolved in buffer at pH 4 and I 0.3, proteins B and H were separated by gel filtration through Sephadex G-200 [Pharmacia (G.B.) Ltd., London W.13, U.K.]. The fractions were concentrated as described below and the pH values and ionic strengths of the three protein solutions were adjusted as required by dialysis against the appropriate buffer or water.

Method of concentrating mucin and protein solutions. This was carried out as described by Boursnell, Cole & Briggs (1968) by tying the dilute solution in a Visking cellophan sac and compressing the sac between dry plaster of Paris blocks of suitable porosity under a 6 kg weight. The degree of concentration was followed by weighing the sac at intervals.

Determination of N-*acetylneuraminic acid.* Free and total *N*-acetylneuraminic acid were assayed by the methods of Warren (1959) and Svennerholm (1957) respectively. The Ehrlich reaction (Werner & Odin, 1952) was used as a semi-quantitative test for sialyl compounds.

Protein determinations. The Lowry, Rosebrough, Farr & Randall (1951) modification of the Folin–Ciocalteu method was used for the routine determination of protein in concentrated gel-filtration fractions. N content was used as a more rigorous measure of the concentrations of CGM and other proteins. A micro-Kjeldahl method was employed and the ammonia was distilled from a Markham (1942) apparatus into a modified Conway & O'Malley (1942) boric acid-screened indicator. A sharper end-point was obtained by using Methyl Red recrystallized twice from aq. 50% (v/v) ethanol, and a sensitivity of 1 μg of N could be obtained by back-titration with 71.4 mM-HCl from a microburette.

The total N content of CGM was determined after equilibration of the freeze-dried mucin against an atmosphere of 52% relative humidity (at 20°C), which was maintained in a desiccator containing the triple system $Na_2Cr_2O_7 2H_2O$ (solid and saturated solution) and air plus water vapour (Rottenberg & Boursnell, 1966). The water content of the equilibrated mucin was determined by drying to constant weight over P_2O_5.

Moving-boundary electrophoretic mobility. Electrophoretic mobility was determined with a much modified Perkin–Elmer Model 38A apparatus with a 2 ml cell. Conductivity was determined by means of a Cambridge Instrument Co. meter and a conductivity cell that was immersed in the constant-temperature tank of the electrophoretic apparatus.

Neuraminidase (from Vibrio cholerae). Two samples were used: (1) a crude enzyme supplied by Burroughs Wellcome Ltd., Beckenham, Kent, U.K., as Receptor Destroying Enzyme (RDE); (2) a purified enzyme from Hoechst Pharmaceuticals, Brentford, Middx., U.K., free from aldolase and proteolytic activity and containing 500 units/ml.

Release of sialic acid from CGM. (1) With RDE. Stock CGM solution (2 ml) was incubated with 3 ml of the enzyme and 3 ml of buffer (93 mM-tris, 64 mM-maleic acid, 15 mM-$CaCl_2$ and 0.01% merthiolate, pH 5.6) for 18 h at 20°C. The mixture was then dialysed for 24 h against sodium–potassium phosphate buffer, pH 7.8 and I 0.1. The dialysed solution of RDE-M contained 1.2 μmol of sialic acid. In a control experiment, 2 ml of CGM solution was treated in the same way, but the enzyme solution was replaced by an equal volume of water. In this case the product contained 13.6 μmol of sialic acid. Thus 91% of the sialyl residues had been liberated during incubation with the enzyme. On the basis of the composition of the mucin (Hartree, 1962) it was calculated that the solution of RDE-M contained 1.0–1.1 mg of protein/ml.

(2) With purified neuraminidase. Because the enzyme is more stable at lower temperatures, prolonged treatment of the mucin at 5°C resulted in a more complete removal of sialyl residues than did incubation at 20°C or 37°C with the same concentration of enzyme (23 units/ml).

Stock CGM solution (8 ml) was diluted with 13 ml of the tris–maleate–Ca^{2+}–merthiolate buffer used above and 1 ml of neuraminidase. The mixture was dialysed at 5°C for 16 days against eight changes of buffer diluted with water to the same extent (i.e. 13 vol. to 22 vol.). The amount of sialic acid in the dialysis sac was 56.1 μmol originally and not more than 2.5 μmol after 16 days. Thus about 96% of the sialyl residues had been released. The solution of N-M was concentrated until its weight had decreased by about 90%. Calculations based upon the sialic acid content of the stock CGM showed that the concentrated solution contained about 16 mg of protein/ml. A solution of neuraminidase in water was made up to contain a concentration equal to that in the N-M solution. Both solutions were dialysed for 24 h against sodium–potassium phosphate buffer, pH 7.8 and I 0.1.

N-*Acetylneuraminic acid.* This was prepared from boar seminal gel (Srivastava, Adams & Hartree, 1965) and the synthetic acid was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. The two samples behaved identically and as pure compounds on paper electrophoresis in butan-1-ol–pyridine–water (6:4:3, by vol.) (Blix & Lindberg, 1960) and in butan-1-ol–propan-1-ol–0.1 M-HCl (1:2:1, by vol.) (Warren, 1960). *N*-Glycollyl groups could not be detected in the sample from seminal gel, nor in the gel itself, by the sensitive method of Klenk & Uhlenbruck (1957). This means that less than 0.5% of *N*-glycollylneuraminic acid was present. *O* Acetyl groups could not be detected in CGM by the method of Bertolini & Pigman (1967). Thus no labile *O*-acetylated *N*-acetylneuraminic acid is present and the sialic acid of CGM must be virtually all *N*-acetylneuraminic acid.

*Poly-*L-*lysine.* Poly-L-lysine (mol.wt. 52000) was produced by Miles–Yeda, Rehovot, Israel, and salmine

sulphate was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Amino acid composition of N-M protein. A sample of N-M was dialysed at 5°C against three changes of water saturated with toluene and was then freeze-dried. Of this preparation 8.58mg was dissolved in 3.0ml of redistilled 6M-HCl. Three 0.9ml portions of this solution were hydrolysed at 105°C in sealed evacuated tubes for 27, 51 and 70h respectively. Insoluble black residues were removed by filtration through glass wool and the filtrates and washings from each hydrolysis were separately combined and each was evaporated to dryness over KOH pellets. The three residues were each dissolved in 1.5ml of water and duplicate 0.2ml portions were examined in a Beckman Amino Acid Analyzer.

The tryptophan/tyrosine ratio was determined by the method of Goodwin & Morton (1946).

Alkaline β-carbonyl elimination. This reaction, and subsequent amino acid analyses, were carried out on N-M as described by Tsiganos & Muir (1969).

RESULTS

Moving-boundary electrophoresis of CGM and N-M. Samples of the stock CGM solution were dialysed against buffers of various pH values and *I* 0.1 (Miller & Golder, 1950) and subjected to moving-boundary electrophoresis. Single fine peaks were always obtained. The results are summarized in Fig. 1. As might be expected from the high

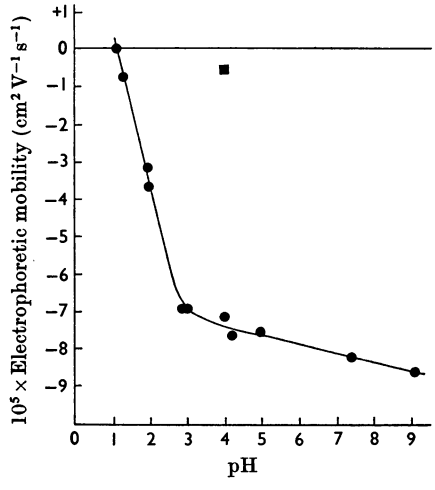

Fig. 1. pH–mobility curve of CGM in various buffers at *I* 0.1 (Miller & Golder, 1950). The potential gradient was about 7V/cm between pH2 and 9 but fell to 1.4V/cm at pH1.1. pH1.1 was obtained by dialysis of CGM against 0.1M-HCl. Other pH values were determined on the CGM solution after dialysis against the buffer. ●, Mobilities determined on 'ascending' boundaries, i.e. those migrating to the positive electrode and progressing into the buffer; almost identical values were obtained from the 'descending' boundaries. ■, Mobility at pH4 of N-M.

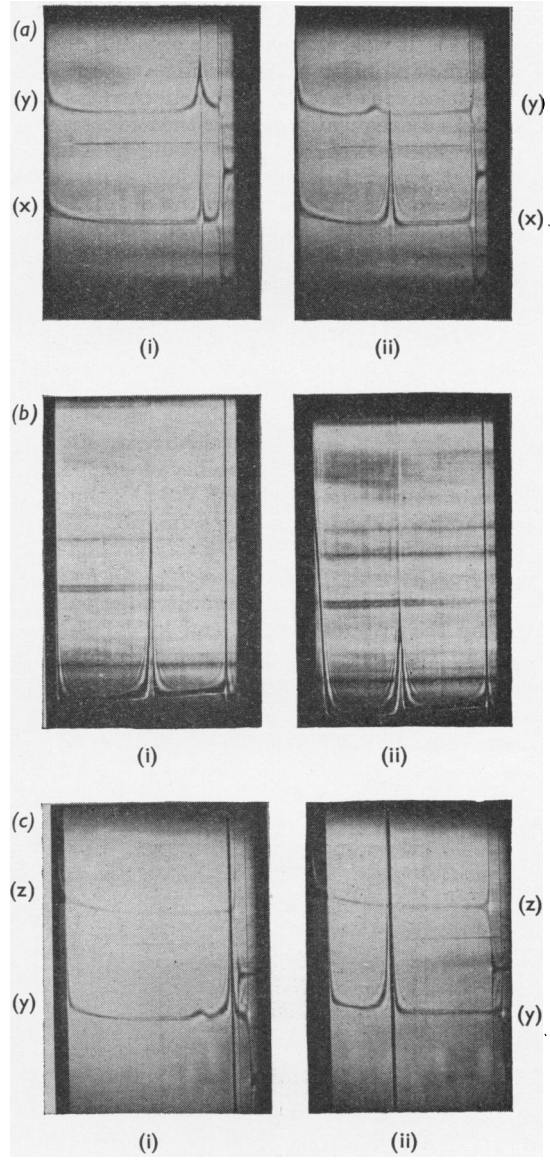

Fig. 2. Ultracentrifuge patterns of: (*a*) simultaneous sedimentation runs on (x) dialysed CGM (concn. 2mg dry wt./ml) and (y) RDE-M (concn. 1.5mg dry wt./ml) [the protein concentrations (Kjeldahl) of (x) and (y) were about 1.0mg/ml; times after full speed (259000*g*) were (i) 8min and (ii) 48min]; (*b*) diffusion run on dialysed CGM used in (*a*) (x), with a synthetic-boundary cell [times after full speed (7000*g*) were (i) 16min and (ii) 272min]; (*c*) simultaneous sedimentation runs on (y) N-M (concn. 10mg/ml) and (z) purified neuraminidase alone at the same concentration as in (*c*) (y) [times after full speed (259000*g*) were (i) 16min and (ii) 176min]. All solutions were equilibrated against phosphate buffer, pH7.8 and *I* 0.1, before centrifuging. A wedge cell (used in the twin rotor) was used for (*a*) (y) and (*c*) (z).

sialic acid content, the isoelectric point is very low (close to 1). It was not possible to obtain a reversal of the sign of mobility without using a hydrochloric acid solution considerably more concentrated than 0.1 M and a consequent increase of the ionic strength. At pH values less than 1 there would be a risk of significant removal of sialyl residues even at the low experimental temperatures. At all pH values there was little or no difference between the mobilities derived from the ascending and descending boundaries, nor was any self-sharpening observed.

The solution of N-M recovered from the ultracentrifuge run (see below) was further examined electrophoretically after dialysis at pH 4 and I 0.1 to determine any change in mobility caused by the removal of at least 96% of the sialic acid. Fig. 3(*b*) shows a single peak with a mobility of -0.60×10^{-5} instead of about -7.4×10^{-5} cm^2 V^{-1} s^{-1}, the value for the untreated mucin at the same pH.

Ultracentrifuge studies of CGM solution and of the mucin from which much of the sialic acid had been enzymically removed. The ultracentrifuge patterns given by the untreated CGM and by RDE-M are shown in Fig. 2(*a*) (x and y respectively). The untreated mucin exhibited a single hypersharp peak having a sedimentation coefficient ($s_{20,w}$) of 7.19S at a concentration of 2mg dry wt./ml. The behaviour of this peak, which persisted throughout the run, is analogous to that of OSM (Gottschalk & McKenzie, 1961) and suggests a very slow diffusion. An attempt was therefore made to measure the diffusion coefficient ($D_{20,w}$) by observations on the same sample during a prolonged ultracentrifuge run at slow speed, a synthetic-boundary cell being used (Fig. 2*b*). Measurements of the areas of the hyperfine peaks at successive stages of the run showed no change. A Gaussian form of the peak persisted throughout; this was established from measurements made with a measuring microscope (Cambridge Instrument Co.) and calculations as set out by Bennett & Boursnell (1962). The diffusion coefficient was 1.57×10^{-8} cm^2/s. The ultracentrifuge runs with RDE-M showed a peak which was quite sharp initially but which dispersed during the run. The $s_{20,w}$ value was 8.65S.

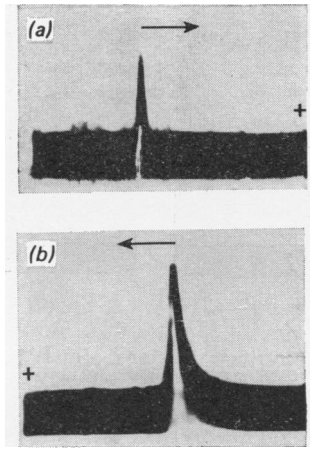

Fig. 3. Electrophoretic diagrams obtained with: (*a*) CGM, pH 4.1, at 186min after the start, mobility -7.40×10^{-5} cm^2 V^{-1} s^{-1} (ascending boundary progressing into buffer); (*b*) N-M, pH 4.0, at 58min after the start, mobility -0.64×10^{-5} cm^{2}V^{-1} s^{-1} (descending boundary progressing into protein; other peaks partly masked on photographic plate by subsequent exposures; descending and ascending boundaries almost identical in appearance and mobility). Buffers of I 0.1 were used. The potential gradients were about 7V/cm.

Fig. 2(*c*) shows the behaviour of N-M in the ultracentrifuge. Although N-M had been depleted of at least 96% of its sialyl residues, its ultracentrifuge peak ($s_{20,w}$ 2.72S) remained sharp throughout the run. However, the solution of N-M had a concentration about 16 times that of the corresponding RDE-M, where the fairly sharp initial peak broadened greatly during the run.

The control neuraminidase solution centrifuged at the same time (Fig. 2*c*, z) contained too little protein to show any peaks.

Determination of the apparent molecular weight of CGM and of protein A by sedimentation and diffusion measurements. By means of the Svedberg & Pedersen (1940) equation:

$$M = \frac{\boldsymbol{R}\,T\,s_{20,w}}{D_{20,w}(1-\bar{v}\rho_{20})}$$

it is possible to calculate the apparent molecular weight (M), where $\boldsymbol{R}$ is the gas constant (8.31×10^7), T the absolute temperature of the run (293°K), $s_{20,w}$ the sedimentation coefficient, $D_{20,w}$ the diffusion coefficient, ρ_{20} the density of the solution (g/ml) at 20°C and $\bar{v}$ the partial specific volume (0.66). The value for $\bar{v}$ of 0.66ml/g was assumed, this being the mean of the four values given by Gibbons (1966) for the similar ovine and bovine submaxillary secretions. Of Gibbons's values, two were calculated, one assumed (Gibbons, 1963) and only one was measured (with an error of ±1%) (Gottschalk & McKenzie, 1961).

Strictly, the above equation only holds for dilute solutions in which solute molecules move independently and obey the gas laws. Thus the value for the molecular weight (6.55×10^6) derived from the equation is only provisional. However, experiments have shown that on gel filtration through a column of Sepharose 2B (which is claimed by the suppliers [Pharmacia (G.B.) Ltd.] to exclude molecules greater than 7×10^6 mol.wt.) the CGM appears

almost exclusively in the first runnings after 1 void volume. This suggests that the exceptionally high value obtained from the above equation is not an overestimate. [Since this work was submitted our attention has been drawn to the paper by Dishon, Weiss & Yphantis (1967), which would suggest that the value of the diffusion coefficient may be too high and the above molecular weight too low, possibly by a factor of 2.] Thus the CGM appears to have a considerably higher molecular weight than the OSM. Gottschalk & McKenzie (1961) derived a value of about 1×10^6 for the latter, although by a different method.

A similar examination of a solution of protein A (0.93g/dl) gave an $s_{20,w}$ value of 2.38S and a $D_{20,w}$ value of 7.83×10^{-7}. From these values, assuming a likely value for $\bar{v}$ of 0.74, an apparent molecular weight of 2.8×10^4 was derived.

Concentration-dependence of $s_{20,w}$ values of CGM. There is a considerable discrepancy between the $s_{20,w}$ values for N-M and RDE-M, the peak of the former, more concentrated, solution moving much more slowly in the ultracentrifuge than the peak of the latter. To investigate this phenomenon further, samples of RDE-M were concentrated and re-run on the ultracentrifuge. The $s_{20,w}$ value was now considerably lower than before and much less diffusion was evident. The $s_{20,w}$ values obtained with successive dilutions of the concentrated solution showed progressive increases.

This concentration-dependence was established in more detail for the stock CGM itself. A sample was dialysed against buffer, pH 7.8 and I 0.1, concentrated slightly and re-dialysed. Dilutions of this material were ultracentrifuged, a wedge cell being used for comparison purposes. A persistent hypersharp peak was manifest at concentrations greater than about 0.3g dry wt./dl. A plot of the reciprocal of $s_{20,w}$ against concentration was linear, and extrapolation to zero concentration gave an $s_{20,w}$ value of 13.6S (Fig. 4). The equation for the straight line, derived by linear regression, is

$$1/s_{20,w} = (0.0769\pm0.0075)+(0.335\pm0.015)c$$

where c is the concentration of CGM in g/dl.

Ultracentrifuge pictures obtained with different concentrations of CGM show that, above a concentration of about 0.3g/dl, a hypersharp peak persists throughout the run of 96min at 279000g; with progressively lower concentrations the hypersharp property disappears more quickly.

Amino acid composition of N-M protein. All duplicate analyses for each hydrolysis time were in close agreement. The results, after linear extrapolation to zero time, are given in Table 1, calculated as g of amino acid residue/100g of freeze-dried N-M, and compared with the amino acid analysis of OSM by Gottschalk & Simmonds (1960).

Table 1. *Comparative amino acid analyses of CGM and OSM*

Amino acid	Amino acid composition* (g of amino acid residue/100g dry wt. of N-M or OSM)	
	N-M	OSM†
Lysine	1.15	1.32
Histidine	1.07	0.27
Arginine	3.16	2.97
Aspartic acid	3.18	2.63
Threonine	8.62	5.94
Serine	3.76	6.27
Glutamic acid	5.18	4.02
Proline	4.69	4.72
Glycine	2.42	4.54
Alanine	3.29	4.05
Cystine (half)	1.59	1.37
Valine	3.55	3.55
Methionine	0.31	0.0
Isoleucine	1.06	1.64
Leucine	2.51	2.52
Tyrosine	1.33	0.61
Phenylalanine	1.32	1.75
Tryptophan	0.87	0.0
Total‡	49.04	48.17

* Calculation allows for the loss of 1 molecule of H_2O per peptide link.

† Values from Gottschalk & Simmonds (1960).

‡ The values for the total amino acids in N-M and OSM are fortuitously similar and merely reflect the fact that N-M and OSM have similar protein/oligosaccharide ratios (w/w).

Determinations of total N on an equivalent quantity of the neuraminidase revealed that the enzyme used for the removal of the sialyl groups contributed considerably less than 1μg of hydrolysed protein to a portion taken for amino acid analysis. Consequently the amino acid contribution from this source was negligible.

After β-carbonyl elimination 66% of the threonine residues and 44% of the serine residues were destroyed. There were no significant changes in other amino acid residues.

Identification of the proteins in boar vesicular secretion and seminal plasma responsible for the swelling of CGM. To study the factor in vesicular secretion that induces swelling of CGM, a more convenient and reproducible assay was required than the one based on the increase in size of small portions of the mucin mentioned in the introduction. It was found that addition of very small quantities (<0.01 vol.) of vesicular secretion to the stock CGM solution caused the latter to set rapidly to a clear firm gel. On the basis of this observation an assay was developed involving the use of serial doubling dilutions.

Initial tests in which proteins A, B and H were added to CGM solutions immediately ruled out protein B as the causative agent. Both proteins A and H in sufficient concentration caused an apparently immediate gelling of the CGM.

Serial doubling dilution of proteins A and H against CGM. Dilutions of proteins A or H were carried out in 0.15M-sodium chloride in 8mm × 50mm tubes as in a normal serological titration by using one accurate 0.1ml blow-out pipette. Stock solution of CGM (0.1ml) was added to each tube and the contents were immediately vigorously mixed. The end point of the titration was taken as that dilution in which a firm gel was formed which would not flow down the tube when it was inverted. The end point was quite sharp, and with the accurate dilutions and determinations of total N employed we could determine the relative amounts and ultimately the molecular ratio of protein A to the CGM necessary to form a firm gel under these conditions.

In this determination the following values were used for protein A and CGM respectively: total N [in the tube at the end-point (1:32 dilution)] 2.5μg and 288μg; N (% of dry wt.) 10.0 and 12.7 (McIntosh & Boursnell, 1966); mol.wt. 2.8×10^4 and 6.55×10^6. From these values calculation shows that the combining ratio of protein A to CGM is 0.04 on a weight basis and that 9.4 molecules of protein A combine with one of CGM.

Protein A represents approx. 80% of the total proteins in vesicular secretion and seminal plasma (Boursnell, Nelson & Cole, 1966). The titre of undialysed vesicular secretion against CGM showed a (protein A in vesicular secretion)/(protein in CGM) ratio that was similar to the ratio obtained with pure protein A and CGM.

Tests with protein H demonstrated that its concentration (w/v) at the end point of the titration was only marginally more than that of protein A.

Investigation of the gelling action of sections of the protein A peak obtained by gel filtration (A Seph). To determine whether the gelling reaction of protein A obtained by dialysis of seminal plasma against buffer, pH 8 and I 0.015, (A Dial), was not due to a minor contaminant, a sample of boar seminal plasma was separated into its components by gel filtration as described by Boursnell *et al.* (1968). The components emerge in the order B, H and A. The last two are incompletely separated, H being represented by a shoulder on the leading edge of the large peak A. The combined H+A peak was collected in five fractions. These were separately concentrated and, after protein determinations, were titrated against CGM.

All five fractions caused gelling and in fractions 2–5 the titres were proportional to the weights of protein A in the last tube that showed gelling. In the case of fraction 1 (representing the H shoulder) there was a marginal increase in the minimum weight of the protein that was required for the gelling reaction. A similar increase was noted in the experiment with pure protein H (above).

These results suggest that the gelling reaction is promoted by proteins A and H rather than by a minor contaminant of very high activity.

Effects of different factors on the gelling reaction. Expts. I were carried out with pure protein A (see the Materials and Methods section) and Expts. II with a possibly less pure protein A, prepared by dialysing seminal plasma against water.

Expt. I. When stock CGM solution was diluted with an equal volume of 0.15M-sodium chloride it yielded very soft gels with protein A. The concentration of the stock solution was in fact the lowest that would give a firm gel on addition of protein A. The titre remained unchanged over the temperature range 19–50°C, although it was slightly lower at 4°C. Ionic strengths between 0 and 0.3 produced no differences in titre: above 0.5 the gels were softer and it was difficult to determine the end points.

Expt. II. The gelling reaction occurred as readily in 0.05M-sodium phosphate buffers as in sodium chloride solution. The titre was scarcely affected by pH values in the range 5.0–8.0 but was marginally highest at pH 6. The titre was unaffected by 1mM-EDTA or by 10mM-calcium chloride, although higher concentrations (>0.1M) of calcium chloride and magnesium chloride decreased the titre. Heating of protein A for 30min at 80°C abolished the gel reaction, as this causes almost total coagulation of the protein (Boursnell & Nelson, 1965). If stock CGM solution was heated in the same way the gel reaction persisted, although the gel was less firm. A sample of N-M that had been concentrated to be equivalent to the stock CGM solution did not gel on the addition of protein A. However, a sample of CGM from which 70% of sialyl residues had been removed enzymically gave rise to gels that were only slightly less firm than those given by the stock solution.

CGM does not yield a clot when mixed with serum albumin at pH 3.5 as do some submaxillary mucins (Hashimoto, Tsuiki, Quintarelli & Pigman, 1961).

Effect of other basic proteins on CGM. A poly-L-lysine solution was made up in buffer, pH 7 and I 0.3 (Miller & Golder, 1950), to a concentration of 5mg/ml (i.e. eight times the minimum concentration of protein A needed for gelling). Titration of this against stock CGM solution gave an amorphous white blob in the first three tubes.

A similar titration at the same concentration with salmine sulphate solution (neutralized to pH 5 with sodium hydroxide) merely produced a haze in

the first tube. Higher concentrations of salmine gave rise to a stringy precipitate. No typical gel was produced in any of these tests.

Self-gelling of CGM in very dilute hydrochloric acid. Samples of stock CGM solution were dialysed at 40°C against pre-chilled (4°C) hydrochloric acid in the concentration range 0.5–0.01 M. With decreasing acidity, increasing opacity of the gel was observed. There was a sharp difference in viscosity between those in 0.025 M- and 0.01 M-hydrochloric acid, and the gel dialysed against the lower concentration of acid set solid. Bubbles trapped within the gel did not rise when the sac was inverted or when the sac was palpated. There appeared to be only a slight partial reversal of the gelling when the CGM, dialysed at 0.01 M-hydrochloric acid, was transferred, still in the cellophan sac, to 0.5 M-hydrochloric acid.

DISCUSSION

The amino acid analyses of CGM are very similar to those of OSM (Gottschalk & Simmonds, 1960). Of the total amino acids, aspartic acid plus glutamic acid represent 17% (CGM) and 17.8% (OSM), and lysine plus arginine 8.8% (CGM) and 8.9% (OSM). Although threonine plus serine together total almost the same in the two mucins, 25.2% (CGM) and 25.4% (OSM), the ratio of threonine to serine is considerably higher in CGM (2.29) than in OSM (0.95). As with OSM the tryptophan and methionine contents are low but the histidine and tyrosine contents are both considerably higher than in OSM.

The high serine, threonine and sialic acid contents of CGM and the high content of galactosamine in hydrolysates of CGM (Hartree, 1962) strongly suggest close structural analogies between CGM and OSM. In the latter the carbohydrate consists mainly of 6-α-D-sialyl-*N*-acetylgalactosamine residues linked to hydroxyl groups of serine and threonine (Gottschalk, 1960; Pigman & Gottschalk, 1966). The additional neutral sugars in OSM may be constituents of sialo-oligosaccharides containing more than two residues as in porcine submaxillary-gland mucin (Carlson, 1966, 1968). As far as is known all oligosaccharide units of submaxillary mucins carry one sialic acid residue. Thus a tentative estimate of the number of units can be deduced from the sialic acid content of the mucin. If such a calculation is made for the sample of CGM used for the amino acid analyses and the β-carbonyl elimination reaction the number is 4830/molecule of mol.wt. 6.5×10^6. The decrease in serine and threonine residues by β-elimination was 4920 out of a total of 8300. This agreement suggests that the oligosaccharide units of CGM are, like those of submaxillary mucins, linked glycosidically to serine and threonine residues.

The electrophoretic mobilities of CGM confirm that the charge on the mucoprotein is largely due to the very high sialic acid content. When most (>96%) of the sialyl groups are removed enzymically, the mobility changes (at pH 4 and I 0.1) from -7.4×10^{-5} to -0.6×10^{-5} cm^2 V^{-1} s^{-1}. The still relatively high negative value for the residual material suggests that it retains some strongly charged groups. These could possibly be residual (<4%) sialic acid, but certainly the high proportion of dicarboxylic acids, as in OSM (Gottschalk & Simmonds, 1960), could contribute effectively to the negative charge on the CGM stripped of the greater part of its sialic acid.

The ultracentrifuge results also confirm other similarities of the properties of CGM and OSM. The occurrence of a hypersharp peak with a very small diffusion coefficient is characteristic of these highly charged sialyl glycoproteins. We find, however, that the hypersharp peak of CGM is not entirely eliminated when almost all the sialyl groups are removed enzymically. The still relatively negatively charged product retains some degree of association, as revealed by a fine peak in the initial stages of the ultracentrifuge run. This fine peak tends to disperse, especially at low concentrations.

The considerable dependence of $s_{20,w}$ values on concentration also links the properties of CGM with those of OSM, where a similar marked dependence was also shown by Gottschalk & McKenzie (1961). These authors obtained an $s^0_{20,w}$ value of about 8.5S by plotting the reciprocal of their $s_{20,w}$ values against concentration. A similar plot (Fig. 4) gave a value of 13.6S for the CGM. However, although the $s_{20,w}$ values derived for these two mucins by similar methods are different, the slopes of the lines relating $1/s_{20,w}$ to concentration are very similar, and indeed are statistically indistinguishable.

It would appear that both protein A (isoelectric point 8.8) and the haemagglutinating protein H

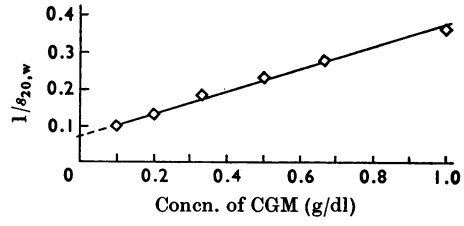

Fig. 4. Plot of $1/s_{20,w}$ values for different concentrations of CGM in phosphate buffer, pH 7.8 and I 0.1. Concentrations were calculated from determinations of total N on the freeze-dried material as described in the text.

(isoelectric point 9.4) are the agents in vesicular secretion that promote the formation from CGM of the rigid but elastic gel that is characteristic of ejaculated boar semen. The mechanism may well be an electrostatic cross-linking of a long high-molecular-weight protein possessing a strong negative charge by a smaller basic protein. The formation of a rigid gel may demand a basic protein of rather specific dimensions. Neither salmine (mol.wt. about 4000) nor poly-L-lysine (mol.wt. 52 000) produce the typical gel given by protein A (mol.wt. 28 000). We find that the protein A/CGM molar ratio necessary for the gelling reaction in the serial dilution tests is about 10.

It is tempting to postulate that, under natural conditions, both proteins H and A are concerned in the cross-linking. Even though protein H is more basic it is likely that some of the cross-linkages are still formed by protein A molecules, on the evidence of Boursnell & Briggs (1969) that the concentration (w/v) of protein A is about ten times that of protein H in vesicular secretion.

Evidence that protein H is at least partially involved comes from a comparative study of the haemagglutinin titres of protein H in vesicular secretion and seminal plasma. The dilution of vesicular secretion occurring during ejaculation to form seminal plasma, as shown by fructose, ergothioneine and citric acid determinations (Mann & Glover, 1954), is about 5. Many protein determinations of our own have confirmed that this fivefold dilution is also valid for the vesicular proteins. However, the usual haemagglutinin titres of seminal plasma (11–13 tubes) are considerably less than one-fifth of those of vesicular secretion (16–18 tubes). Combination of part of the protein H with CGM would help to explain this hitherto puzzling discrepancy.

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