Biochem. J. (1983) **209**, 893–896
Printed in Great Britain

Further evidence for a flexible and highly expanded spheroidal model for mucus glycoproteins in solution

Stephen E. HARDING,* Arthur J. ROWE† and J. Michael CREETH‡
**Department of Biochemistry, Medical School, University of Bristol, Bristol BS8 1TD, U.K., †Electron Microscopy Laboratory, School of Biological Sciences, Adrian Building, University of Leicester, Leicester LE1 7RH, U.K., and ‡Department of Medicine, University of Bristol, Bristol Royal Infirmary, Bristol BS2 8HW, U.K.*

(*Received 25 November 1982/Accepted 16 December 1982*)

The flexible and greatly expanded roughly spherical model for mucus glycoproteins proposed earlier, on the basis of hydrodynamic and n.m.r. data, is supported by new hydrodynamic results on a bronchial glycoprotein from a cystic-fibrosis patient. Furthermore, images from electron microscopy of this molecule and a lower-molecular-weight mucus glycoprotein (which closely resembles a glycopolypeptide) appear to be at least consistent with this model.

The dominant role of secretory glycoproteins in determining the characteristic protective features of mucus has now been well established (Allen *et al.*, 1976; Creeth, 1977; Avery-Jones, 1978), and many workers have attempted, sometimes contradictorily, to correlate their conformation with biological function. The general structure (Pigman, 1977*a*) for a monomeric macromolecule of a hydrophobic single polypeptide core, surrounded almost completely by serine- or threonine-linked oligosaccharides of variable chain length, is now widely accepted, but data relating to the gross conformation in solution have been conflicting. Although the hydrodynamic properties (Gibbons & Glover, 1959; Creeth & Knight, 1967, 1968; Snary *et al.*, 1971, 1974; Gibbons, 1972; Mantle *et al.* 1981) appear to indicate consistently a roughly spherical immensely expanded random-coil model, electron-microscope studies have suggested a quite different picture (Slayter *et al.*, 1974; Lamblin *et al.*, 1979), with the molecules appearing as highly elongated structures, of axial ratio about 100:1. We now present fresh evidence that the former model is applicable, and suggest a reason for the earlier discrepancies.

Mucus glycoproteins from a wide variety of sources have the general characteristic features of very high concentration-dependence of sedimentation coefficients (represented by the parameter k_s) and very high intrinsic viscosity $[\eta]$. Use of the ratio $k_s/[\eta]$ for a well-characterized blood-group-B-specific glycoprotein (Creeth & Knight, 1967), for example, indicates a spheroidal particle of axial ratio <2:1 (see Rowe, 1977) and a swelling ratio of approx. 60 (v/v). Further evidence for the very high excluded volume has come from measurement of the molecular excluded volume via the second virial coefficient (Creeth & Knight, 1968). Allen and co-workers have arrived at the same basic conclusions for pig gastric and pig colonic mucus (Allen *et al.*, 1976; Snary *et al.*, 1971, 1974; Mantle *et al.*, 1981). Further support that glycoproteins in solution are in general spheroidal (axial ratio <10:1) as opposed to extended rigid rods derives from the fact that they do not give rise to any observable birefringence when subjected to shear (Creeth & Knight, 1967). One notable exception is the relatively high molecular-weight bovine cervical mucin, for which birefringence has been observed (Gibbons & Glover, 1959). However, this observation was accounted for in terms of an elastic deformable spheroidal model with random-coil properties. Evidence for the flexibility derives from the fact that the Huggins constant for the concentration-dependence of the reduced viscosity generally lies in the range 0.3–0.5 (Creeth & Knight, 1967; Snary *et al.*, 1971) characteristic of random-coil behaviour (Flory, 1953), from the temperature-dependence of sedimentation coefficients (Creeth & Knight, 1967) and from n.m.r. studies (Barrett-Bee *et al.*, 1981, 1982).

Some electron-microscope studies have given, however, very persuasive evidence that these molecules exist as extended stiffened rods of axial ratio approx. 100:1, after rotary shadowing of molecules air-dried on to mica. This model has been proposed both for thiol-reduced glycopolypeptides and for native glycoprotein molecules; for example, a study (Slayter *et al.*, 1974) on blood-group-A, -B and -H substances has clearly shown extended rods. Al-

though these structures are again the predominant feature of a study on human bronchial-mucus glycoproteins (Lamblin *et al.*, 1979), other 'bizarre' features are also evident, and neutral glycoproteins appear as flat sheet-like structures. Although a general model of stiffened rods was implied from the appearance of the micrographs, good agreement between the lengths predicted by electron microscopy and sedimentation coefficients is actually obtained for a flexible-coil model (p. 40 of Lamblin *et al.*, 1979).

In an attempt to throw some light on these discrepancies, we have examined a human bronchial glycoprotein, CF PHI, prepared by a slight modification of the method of Creeth *et al.* (1977). In the dispersal of the sputum in CsBr solution, mixed proteinase inhibitors were present. After the usual two density-gradient centrifugations, bovine pancreatic ribonuclease and deoxyribonuclease were used to remove residual traces of nucleic acids. The enzymes were subsequently removed by a third density-gradient centrifugation in CsCl. The final product was examined by analytical density-gradient ultracentrifugation, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with staining for protein, and gel electrophoresis with staining for nucleic acid (Bridge, 1979). These techniques would have detected (*a*) lipid, as a thickening of the meniscus, (*b*) free protein and (*c*) degraded nucleic acids. The freedom from lipid is expected from the findings by Bhaskar & Reid (1981) that repeated preparative density-gradient centrifugations break up stubborn lipid–glycoprotein complexes, whereas the sensitivity of the electrophoretic tests is recognized (Creeth *et al.*, 1979) to be even greater than centrifugation (Creeth & Horton, 1977). The final product was a glycoprotein whose analytical composition was similar to those reported by Creeth *et al.* (1977), and with similar physical properties. For example, the reduced viscosity at 1 mg/ml was 104 ml/g, and its weight-average molecular weight M_w^0 was 2.25×10^6; both measurements were made on material in 1 M-NaCl, phosphate-buffered to pH 6.8. Thus by all normally accepted criteria (Pigman, 1977*b*) it was a typical sialic acid-rich mucus glycoprotein (*N*-acetylneuraminic acid content 13%).

A 4 mg/ml solution in 1 M-NaCl buffered to pH 6.8 was found to give no observable birefringence when subjected to shear, whereas that for a known extended rod, myosin, under similar conditions did so. The second virial coefficient, B, was then determined by using ultra-short-column sedimentation-equilibrium experiments and from a slope ($\equiv 2B$) of a plot of the reciprocal of the apparent weight-average molecular weight versus concentration (Fig. 1). The value calculated, $1.5 \times 10^{-4} \pm 0.3 \times 10^{-4}$ ml · mol · g^{-2}, represents a lower

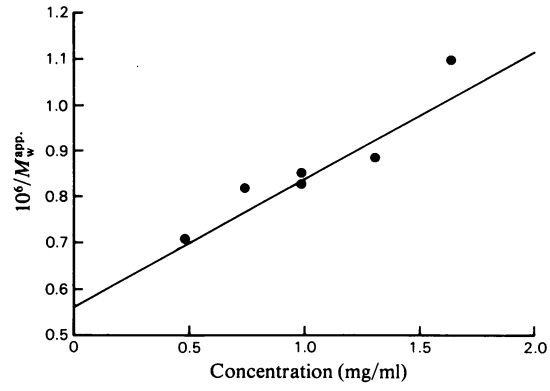
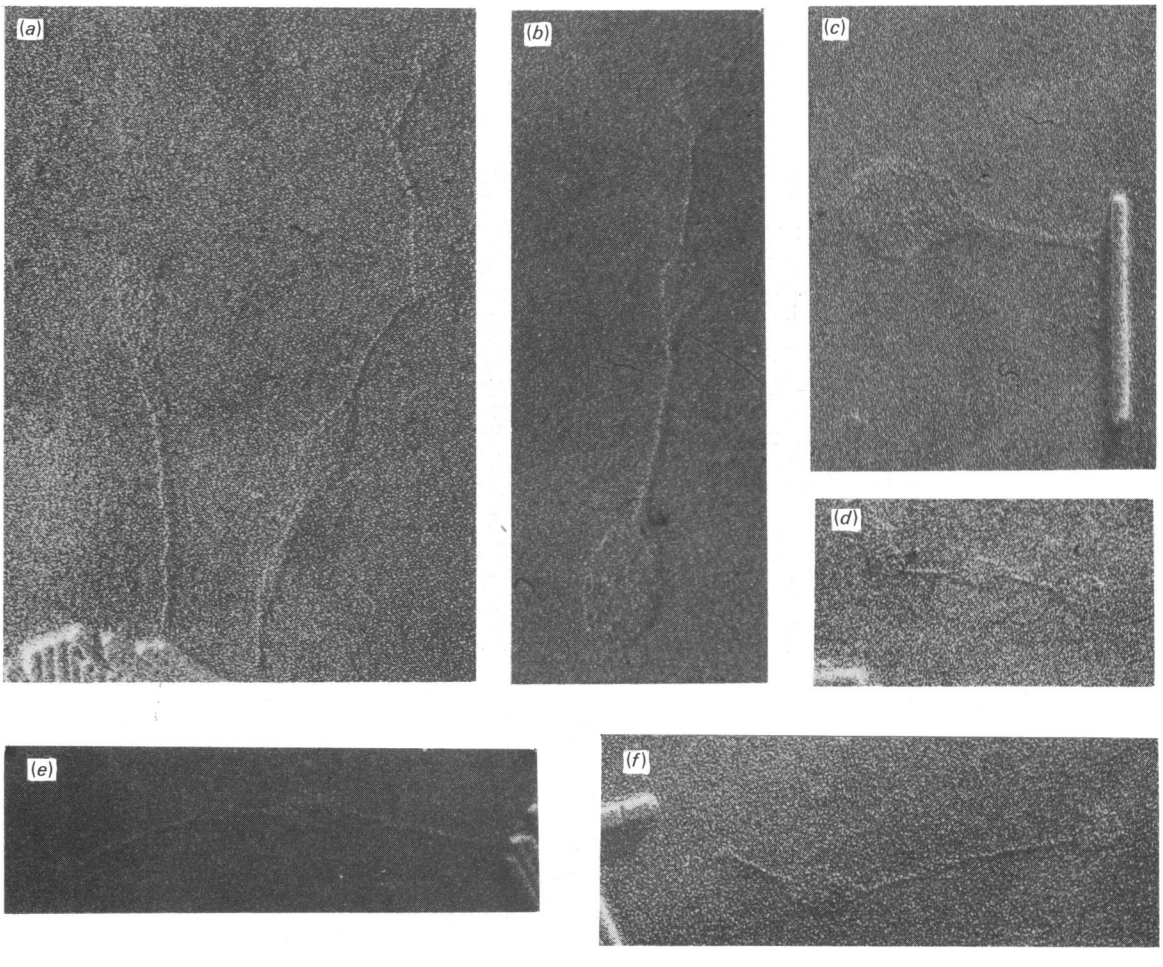


Fig. 1. *Extrapolation of apparent molecular weights for glycoprotein CF PHI*

Freeze-dried glycoprotein was dissolved in 1 M-NaCl and buffered to pH 6.8 with a phosphate buffer (I 0.10) of the following composition: 30 mM-NaCl, 16.7 mM-Na_2HPO_4, 16.7 mM-NaH_2PO_4, 2 mM-NaN_3 and 1 mM-EDTA. The apparent molecular weights were determined by using the technique of ultra-short solution columns (approx. 0.7 mm), recorded by using Rayleigh optics, and interpreted by following method I of Van Holde & Baldwin (1958).

limit [owing to the effects of association (Fujita, 1962; Teller, 1973; Harding & Creeth, 1982)] for the contribution from thermodynamic non-ideality. Assuming the suppression of charge effects, the molecular co-volume $U = 2BM^2$ (Tanford, 1961; Jeffrey *et al.*, 1977) $= 1.2 \times 10^9$ ml/mol, and hence the excluded volume (in ml/g) $u' \equiv U/M = 600$ ml/g. Assuming a spherical particle, the specific volume $v_s = u'/8 \sim 75$ ml/g. Since the partial specific volume, $\bar{v}$, is 0.633 ml/g (Bridge, 1979), the swelling ratio $v_s/\bar{v}$ is found to be approx. 120.

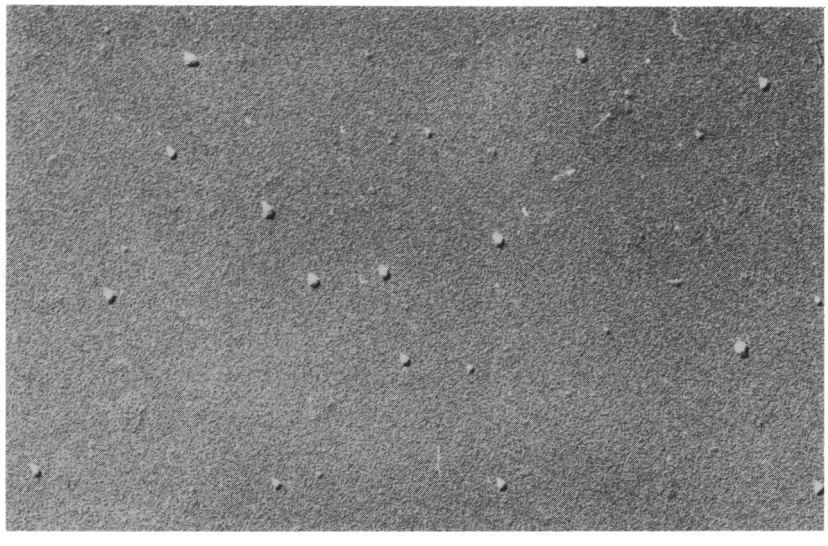
Although there is considerable uncertainty on conclusions from electron microscopy with regard to solution structures, it would be instructive to see whether images of metal-shadowed material were at least consistent with the solution data. The problems of flattening due to air-drying of droplets sprayed on to cleaved mica were considered to be less than those of subjecting normally highly solvated material to concentrated glycerol, and hence, following Slayter *et al.* (1974), the former technique was used. Electron microscopy of unidirectional platinum-shadowed glycoprotein CF PHI revealed a rather complex situation (Plate 1). Most of the material present appeared as very flattened 'low-profile' areas, barely resolvable from the background. From these low-profile areas radiated one or two narrow thread-like extensions, typically 100–200 nm in length. By using tobacco-mosaic-virus particles as calibration markers, a maximum height



EXPLANATION OF PLATE 1

Electron micrographs of platinum-shadowed glycoprotein CF PHI, air-dried after spraying on to mica

These molecules appear to consist of 'low-profile' areas that have one or two thread-like extensions (*a* left, *c* and *e*). These extensions frequently attach to a virus particle and also overlap and attach to each other (*a* right, *b*, *d* and *f*). Magnifications × 104 000 (magnifications were calibrated by using a diffraction-grating replica to ±2%).



EXPLANATION OF PLATE 2

Electron micrographs of 603/AmS mucus glycoprotein from an ovarian cyst, after platinum-shadowing air-dried material after spraying on to mica

Tobacco-mosaic-virus particles were used as a calibration standard. The shadowed width (Willison & Rowe, 1980*a*) for an 'end-on' tobacco-mosaic virus was measured to be 19.2nm: the correction for metal cap (Willison & Rowe, 1980*b*) applied to glycoprotein widths was therefore 4.2nm. Hence the corrected width of the particles was 15.0 ± 1.9nm. From their shadow lengths, by using 'edge-on' tobacco-mosaic virus as a calibration, a particle height of 9.1 ± 1.5nm was inferred. An estimate for the molecular weight can be found from the relation $M_r = N_A \cdot V_e/\bar{v}_s$, where N_A is Avogadro's number, V_e the particle volume and $\bar{v}_s$ the specific volume (of particle + associated solvent). By taking the average particle dimensions as 15.0nm × 15.0nm × 9.1nm and assuming that after air-drying there is still a nominal hydration ($\bar{v}_s$ approx. 1ml/g), $M = 575\,000 \pm 150\,000$, consistent with solution measurements (Creeth *et al.*, 1982). Magnification ×56000.

of 1.5 nm was estimated for these thread-like extensions, and from the sharpness of their definition it is inferred that their width in the substrate plan was of the same order (i.e. they are not flattened tapes). The height above substrate of the low-profile areas was very low indeed at their perimeter, barely resolvable and perhaps 0.8 nm or less. It is clearly possible that they could be thicker in the centre, however. All these micrographs were of material prepared from very dilute solutions (approx. 10 μg/ml): at higher concentrations similar regions were seen, but extensive interaction between the particles made it very difficult to resolve individual particles.

The molecular weights of the low-profile areas can be estimated very approximately, assuming them to be oblate ellipsoids of double axes 1–1.5 nm × 'length' × 'width', and are in the range 1×10^6–6×10^6. These will be upper estimates, no allowance for possible non-perfect packing having been made. The thread-like extensions, diameter 1.5 nm, will have a mass of 25000/100 nm length. It is thus very clear that the latter cannot be other than a small component of the whole molecule. On a semi-quantitative basis, the particles appear in numbers corresponding to that expected for particles of this mass, when sprayed over a range of solute concentrations. Moreover, as the solution was made up of specimen free of salts and other low-molecular-weight components, dissolved into glass-distilled water, no component other than the mucus glycoprotein could be present in the quantity observed. Preparation in distilled water was found to be desirable for the additional reason that the high double-layer repulsive forces (Verwey & Overbeek, 1948) would decrease the tendency of the macromolecules to aggregate and simultaneously (through high intramolecular repulsive forces from *N*-acetylneuraminic acid residues) allow maximum rod-forming tendency.

These observations may possibly be reconciled with the earlier observations of stiffened rods (Slayter *et al.*, 1974; Lamblin *et al.*, 1979) in the following way. The linear extensions that we have observed are of similar length to the rods described earlier, but our estimated thickness of 1.5 nm is much lower than the estimates, made with the use of rotary shadowing, of 3–5 nm. The latter technique relies largely on 'decoration' by migrating metal ad-atoms to provide contrast (Willison & Rowe, 1980*a*). Although excellent for revealing conformation of extended molecules, it is a technique that is specifically designed to exaggerate width, the latter effect being related to geometric abruptness of the specimen/substrate transition. Subsequent calculations of widths are accordingly of low precision. Unidirectional shadowing, on the other hand, is simpler in its operation, and gives results of adequate accuracy in this size range.

The low-profile areas that we see could clearly be derived from highly swollen particles of low asymmetry, which flattened on to the substrate on drying down. This is our provisional conclusion, although clearly the future use of methods that might maintain the supposed three-dimensional order is indicated. Rotary shadowing, particularly of specimens dried down from 100 mM-ammonium acetate (which can leave a slight residue), would not be expected to resolve such structures. We therefore consider it possible that the difference between our own and the earlier results is purely methodological.

To see how typical our results might be, we have also taken electron micrographs of a lower-molecular-weight ovarian-cyst glycoprotein (Creeth *et al.*, 1974) [fraction 603/AmS, M_w^0 approx. 585000 (Creeth *et al.*, 1982), *N*-acetylneuraminic acid content approx. 6%]. Although a native glycoprotein, it is one of a class (see Donald, 1973) that has so little naked protein segment that it virtually constitutes a glycopolypeptide. For fraction 603/AmS, only particles of low asymmetry were observed after unidirectional shadowing (Plate 2). The measured widths (15.0 ± 1.9 nm) were considerably larger than the estimated heights (9.1 ± 1.5 nm), consistent with the known molecular weight and with a degree of flattening, but clearly to a much smaller extent than in the case of glycoprotein CF PHI. These results obtained with fraction 603/AmS confirm the plausibility of a generally spherical model for the gross conformation of mucus glycoproteins. The possibility is that a small part (less than 10% of the mass) may be present as linear extensions, but the alternative that these latter, as seen by us with glycoprotein CF PHI and by earlier workers (Slayter *et al.*, 1974; Lamblin *et al.*, 1979), arise as artifacts during drying down cannot be excluded, particularly for such immensely swollen macromolecules, where hydration is likely to play an important role in stabilizing the native conformation in solution. Alternatively, the absence of linear extensions for fraction 603/AmS is consistent with the absence of naked protein segment, unlike the more typical 'mucin' CF PHI, which has the observed extensions.

Attempts to extend our model to cover larger glycoproteins, e.g. pig gastric mucin, unfortunately proved unsuccessful, owing to the high tendency of these molecules to aggregate, even when dissolved in distilled water alone. X-ray fibre diffraction studies on pig gastric mucin performed by Dr. E. D. T. Atkins on our material have revealed no discernible ordering, which may indicate an absence of rod forms. It is possible therefore that our model may be applicable to a wider range of mucus glycoproteins. This would be consistent with the principal biological function of mucus in immobilizing water and forming a space-filling layer.

Finally, our model is consistent with the high proline contents (10–20% of amino acid content) found in mucus glycoproteins: proline has the effect of introducing bends into a polypeptide chain, and hence may assist the coiling of these substances into spheroidal domains.

S. E. H. is in receipt of an award from the Lister Institute of Preventive Medicine.

References

Allen, A., Pain, R. H. & Robson, T. H. (1976) *Nature (London)* **264**, 88–89

Avery-Jones, F. (1978) *Br. Med. Bull.* **34**, 1–4

Barrett-Bee, M., Bedford, G. & Loftus, P. (1981) in *Mucus in Health and Disease* (Chantler, E. N., Elder, J. B. & Elstein, M., eds.), vol. 2, pp. 107–111, Plenum Press, New York

Barrett-Bee, M., Bedford, G. & Loftus, P. (1982) *BioSci. Rep.* **2**, 257–263

Bhaskar, K. R. & Reid, L. (1981) *J. Biol. Chem.* **256**, 7583–7589

Bridge, J. L. (1979) Ph.D. Thesis, University of Bristol

Creeth, J. M. (1977) *Mod. Probl. Paediatr.* **19**, 34–45

Creeth, J. M. & Horton, J. R. (1977) *Biochem. J.* **161**, 449–463

Creeth, J. M. & Knight, C. G. (1967) *Biochem. J.* **105**, 1135–1145

Creeth, J. M. & Knight, C. G. (1968) *Chem. Soc. Spec. Publ.* **23**, 303–313

Creeth, J. M., Bhaskar, K. R., Donald, A. S. R. & Morgan, W. T. J. (1974) *Biochem. J.* **143**, 159–170

Creeth, J. M., Bhaskar, K. R., Horton, J. R., Das, I., Lopez-Vidriero, M.-J. & Reid, L. (1977) *Biochem. J.* **167**, 557–569

Creeth, J. M., Bridge, J. L. & Horton, J. R. (1979) *Biochem. J.* **181**, 717–724

Creeth, J. M., Cooper, B., Donald, A. S. R. & Clamp, J. R. (1982) *IRCS Med. Sci. Libr. Compend.* **10**, 548–549

Donald, A. S. R. (1973) *Biochim. Biophys. Acta* **317**, 420–436

Flory, P. J. (1953) *Principles of Polymer Chemistry*, p. 310, Cornell University Press, Ithaca

Fujita, H. (1962) *Mathematical Theory of Sedimentation Analysis*, pp. 235–298, Academic Press, New York

Gibbons, R. A. (1972) in *Glycoproteins: Their Composition, Structure and Function* (Gottschalk, A., ed.), vol. 5, part A, pp. 31–157, Elsevier, Amsterdam

Gibbons, R. A. & Glover, F. A. (1959) *Biochem. J.* **73**, 217–225

Harding, S. E. & Creeth, J. M. (1982) *IRCS Med. Sci. Libr. Compend.* **10**, 474–475

Jeffrey, P. D., Nichol, L. W., Turner, D. R. & Winzor, D. J. (1977) *J. Phys. Chem.* **81**, 776–781

Lamblin, G., Lhermitte, M., Degand, P., Roussel, P. & Slayter, H. S. (1979) *Biochimie* **61**, 23–43

Mantle, M., Mantle, D. & Allen, A. (1981) *Biochem. J.* **195**, 277–285

Pigman, W. (1977*a*) in *Glycoconjugates* (Horowitz, M. I. & Pigman, W., eds.), vol. 1, p. 132, Academic Press, New York

Pigman, W. (1977*b*) in *Glycoconjugates* (Horowitz, M. I. & Pigman, W., eds.), vol. 1, pp. 181–188, Academic Press, New York

Rowe, A. J. (1977) *Biopolymers* **16**, 2595–2611

Slayter, H. S., Cooper, A. G. & Brown, M. C. (1974) *Biochemistry* **13**, 3365–3371

Snary, D., Allen, A. & Pain, R. H. (1971) *Eur. J. Biochem.* **24**, 183–189

Snary, D., Allen, A. & Pain, R. H. (1974) *Biochem. J.* **141**, 641–646

Tanford, C. (1961) *Physical Chemistry of Macromolecules*, pp. 195–196, John Wiley and Sons, New York

Teller, D. C. (1973) *Methods Enzymol.* **27**, 346–441

Van Holde, K. E. & Baldwin, R. L. (1958) *J. Phys. Chem.* **62**, 734–743

Verwey, E. J. W. & Overbeek, J. Th. G. (1948) *The Theory of the Stability of Lyophobic Colloids*, Elsevier, Amsterdam

Willison, J. H. M. & Rowe, A. J. (1980*a*) in *Practical Methods in Microscopy, Vol. 8: Replica Shadowing and Freeze Etching Techniques* (Glauert, R. M., ed.), pp. 83–86, 136, 254, North-Holland, Amsterdam

Willison, J. H. M. & Rowe, A. J. (1980*b*) in *Practical Methods in Microscopy, Vol. 8: Replica Shadowing and Freeze Etching Techniques* (Glauert, R. M., ed.), pp. 259–268, North-Holland, Amsterdam