Structural Studies on Lens Proteins

By CHI-CHING MOK AND S. G. WALEY

Nuffield Laboratory of Ophthalmology, University of Oxford

(Received 21 November 1966)

The sequence around the thiol group in lens proteins has been investigated. The proteins were converted into their carboxy[^{14}C]methyl derivatives and submitted to partial acid hydrolysis, or digested with proteolytic enzymes. Acid hydrolysis of bovine α -crystallin gives *N*-seryl-(*S*-carboxymethyl)cysteine, Ser-CMCys (Waley, 1965*a*), but this dipeptide is not obtained from β -crystallin or γ -crystallin. Trypsin and chymotrypsin also give different peptides from the three crystallins. The radioactive peptide from α -crystallin and chymotrypsin has the sequence Ser-CMCys-Ser-Leu; another peptide, Asp-Leu-Leu-Phe, was also identified. The radioactive peptides obtained from bovine α -crystallin are probably also obtained from human α -crystallin, and from bovine and human albuminoid (the insoluble lens protein). α -Crystallin has been fractionated by chromatography in urea on DEAE-cellulose. Comparison of the fractions by peptide 'mapping', and immunochemically, shows that they fall into two classes. The fraction eluted first differs from the later fractions, but the later fractions resemble each other. The first fraction may represent impurities, or it may be a structurally different sub-unit of α -crystallin.

The proteins of the lens have been studied for many years, but the main problems of structure and function are still unsolved. Structural methods are useful in the characterization of proteins lacking a characteristic biological property, and have the advantage that alterations during isolation are more likely to affect the conformation than the primary structure. α -Crystallin, which comprises about a third of the soluble lens proteins and is closely related to the insoluble protein albuminoid (Woods & Burky, 1927; Thomann, 1962; Ruttenberg, 1965; Waley, 1965*b*), contains 1 thiol group/sub-unit (Waley, 1965*a*). This feature has now been utilized in comparisons of α -crystallin with other lens proteins, and forms the basis for a chemical method of recognizing α -crystallin.

Further study of the nature of α -crystallin demands a knowledge of how many different kinds of peptide chain are present. α -Crystallin is a large molecule, made up of 30–50 sub-units (Bloemendal, Bont, Jongkind & Wisse, 1962; Björk, 1964; Bloemendal, Bont, Benedetti & Wisse, 1965; Spector & Katz, 1965; Wisse, Zweers, Jongkind, Bont & Bloemendal, 1966), and often obtained in several forms. Chromatography of bovine α -crystallin, for example, gives two (Björk, 1963) or four (Papaconstantinou, Resnik & Saito, 1962; Spector, 1965) peaks, and the nature of the differences between the fractions is not clear. Similarly, chromatography of the disaggregated protein in urea gives several peaks (Björk, 1964; Spector, 1965) that are quite similar in amino acid composi-

tion. We have now compared these fractions by immunodiffusion and by peptide 'maps' and conclude that the fractions fall into two groups, whose nature is discussed below.

MATERIALS AND METHODS

Preparation of bovine α -crystallin. Material was extracted from the cortex of ox lenses by stirring 160 g. of ox lenses for 60 min. with 500 ml. of water. The insoluble fraction (albuminoid) was removed by centrifuging for 15 min. at 10000*g* at 5°, and the supernatant was brought to pH 5 with *N*-HCl at 4° and made 13% (v/v) in ethanol. The precipitate was collected by centrifuging and dissolved in 150 ml. of 40 mM-NaHCO₃, and the solution dialysed against water for 16 hr. The precipitation at pH 5 in 13% (v/v) ethanol was then repeated twice more. Ammonium sulphate (65 g.) was then added to 325 ml. of solution containing about 11 g. of protein, at room temperature, at pH 6.8; the precipitate was rejected; the supernatant was treated with 18.5 g. of ammonium sulphate, the protein was collected and redissolved, and the solution was dialysed and freeze-dried to give 8 g. of α -crystallin.

Albuminoid and α -crystallin (from human lenses). Pooled cataractous human lenses (2.2 g.) were ground with 16 ml. of pH 8 buffer (50 mM-tris-1 mM-EDTA, 0.02% in sodium azide), and the albuminoid was separated by centrifuging and washed five times with this buffer, once with water and once with acetone; the yield was 0.22 g. Part (10 ml.) of the original extract was applied to a 100 cm. long $\times$ 2.5 cm. diam. column of Bio-Gel P-300 (Calbiochem. Ltd., Basingstoke, Hants.) and eluted with the pH 8 buffer. The fractions comprising the first peak were pooled and the α -crystallin was precipitated at pH 5 in 13% (v/v) ethanol. The

precipitation was repeated and the protein was recovered by dialysis and freeze-drying; the yield was 18 mg.

Bovine albuminoid. The capsules were removed from ox lenses, the lenses were minced by forcing through a Fischer mincer and ground in a mortar, and the viscous mass was centrifuged for 20 min. at 20000g to remove incompletely disrupted fibres. The supernatant (16.2 g.) was diluted with 100 ml. of the pH 8 buffer and stirred for 16 hr. at 4°. The precipitate was collected by centrifuging and washed four times with the buffer and three times with water; 100 mg. of albuminoid was obtained.

Separation of α -, β - and γ -crystallins from calf lens. This was carried out by stepwise elution from DEAE-cellulose as described by Spector (1964).

Carboxymethylation of proteins. This was carried out with sodium iodo[2-¹⁴C]acetate in 8M-urea (Waley, 1965a). The carboxymethylated protein was isolated by precipitation with acetone containing 0.4% (v/v) of 6N-HCl, and washed five times with this acid acetone and then five times with acetone.

Amino acid analysis. Amino acids were identified by thin-layer chromatography on cellulose powder. Propan-2-ol-formic acid-water (20:1:5, by vol.) followed by 2-methylpropan-2-ol-butan-2-one-aq. NH₃ (sp.gr. 0.88)-water (5:3:1:1, by vol.) were used as solvents (Heathcote & Jones, 1965); twofold development in the second solvent aided the separation of leucine and isoleucine. Identification of the amino acids in the hydrolysate of a small peptide was greatly aided by the addition of radioactive amino acids. A hydrolysate of the protein from *Chlorella vulgaris* (The Radiochemical Centre, Amersham, Bucks.) was used (specific activity about 640 μ Ci/mg.), 100 μ Ci being dissolved in 0.4 ml. of water, and 1 μ l. of this solution was added to 5 μ l. of hydrolysate. If the thin-layer plate was spotted with 4 $\times$ 0.5 μ l. of hydrolysate, there was sufficient radioactivity to detect the marker amino acids by radioautography overnight; on the other hand, the added radioactive amino acids were not present in large enough amounts to be detected with ninhydrin. The plates were sprayed with 0.05 g. of ninhydrin and 0.02 g. of hydrindantin in 10 ml. of ethanol containing 0.25 ml. of acetic acid, heated at 95° for 5 min., and then sprayed with ethanol containing 1% (v/v) of saturated aq. Cu(NO₃)₂ and 2% (v/v) of 10% (w/v) HNO₃.

Quantitative amino acid analysis was carried out on the EEL amino acid analyser by Dr D. B. Hope, to whom we are much indebted.

Paper electrophoresis and paper chromatography. These procedures were carried out as described by Waley (1965a).

Enzymes. Chymotrypsin (three-times crystallized), carboxypeptidase A treated with di-isopropyl phosphorofluoridate, and crystallin peptin were from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.); TPCK-trypsin (trypsin treated with chloromethyl L-1-toluene-p-sulphonamido-2-phenylethyl ketone) was from Seravac Laboratories, Maidenhead, Berks.

RESULTS

Comparisons of α -crystallin with β -crystallin and γ -crystallin

Partial acid hydrolysis. Suspensions of the carboxy[¹⁴C]methyl-proteins (about 1 mg.) in acetic

Fig. 1. Radioautograph after electrophoresis at pH 4 for 4 hr. at 10 v/cm. of products from partial acid hydrolysis at 100° of carboxy[¹⁴C]methyl-proteins. The symbols α , β and γ stand for bovine α -crystallin, β -crystallin and γ -crystallin.

acid (0.1 ml.) were treated with concentrated hydrochloric acid (0.1 ml.); the clear solutions were heated at 100° for 11 min. and then freed from acid by evaporation, followed by adding water and evaporating three times. Paper electrophoresis at pH 4 for 4 hr. at 10 v/cm. followed by radioautography gave the results shown in Fig. 1. α -Crystallin gave Ser-CMCys* (Waley, 1965a), but β -crystallin and γ -crystallin did not give this dipeptide.

Enzymic hydrolysis. Portions (about 5 mg.) of the carboxymethyl-proteins suspended in 0.2 ml. of 2% (w/v) ammonium hydrogen carbonate were treated with 0.2 mg. of chymotrypsin in 20 μ l. of water, or with 0.2 mg. of TPCK-trypsin in 20 μ l. of 1 mM-calcium chloride in 1 mM-hydrochloric acid. The mixtures were kept for 4.5 hr. at 37°; after 2 hr., further 5 μ l. portions of the enzymes were added. The chymotryptic digests were evaporated and the residues taken up in 80 μ l. of water. The tryptic digests were acidified to pH 4 and the soluble fraction was evaporated; the residue was dissolved in 80 μ l. of acetic acid-water-pyridine (3:12:10, by vol.). The results after electrophoresis and radioautography are shown in Fig. 2(a).

α -Crystallin gives rise to few radioactive peptides, but β -crystallin and γ -crystallin, which are relatively rich in thiol groups, give many radioactive S-carboxy[¹⁴C]methylpeptides. The patterns of the ninhydrin-positive bands in Fig. 2(b) show structural differences among α -, β - and γ -crystallins.

* Abbreviation: CM, carboxymethyl.

radioactive peptides with the same mobility from bovine α -crystallin and human α -crystallin (Fig. 3).

*Sequence round the cysteine residue
in α -crystallin*

Carboxy[^{14}C]methyl- α -crystallin was digested with chymotrypsin (enzyme/substrate ratio 1:40), and the digest, containing about 2000 counts/sec. and about 400 mg. of peptides, was fractionated on a 140 cm. long $\times$ 2 cm. diam. column of Sephadex G-25 equilibrated with 0.03% ammonia containing 0.2% of thiodiglycol (Fig. 4). The fractions (41–45) containing the radioactive peptide were concentrated and submitted to successive steps of electrophoresis at pH 4 on Whatman no. 3 paper for 4.5 hr. at 10 v/cm., electrophoresis at pH 7 at 80 v/cm. for 1.5 hr. and chromatography in butan-1-ol-acetic acid-water-pyridine. After each step the radioactive peptide was detected by radioautography overnight. After electrophoresis at pH 7 the radioactive peptide (IA1) was incompletely separated from a faster (non-radioactive) peptide (IA2); after a further fractionation by paper chromatography both peptides were obtained pure and their amino acid compositions determined (Table 1). Peptide IA1 is (Ser₂,CMCys,Leu) and peptide IA2 is (Asp,Leu₂,Phe). The anionic mobility of peptide IA2 at pH 7 shows that aspartic acid rather than asparagine is present.

The C-terminal amino acids were determined with carboxypeptidase; the reaction mixtures were fractionated by electrophoresis at pH 7 (80 v/cm. for 40 min.), and the neutral spots were identified as leucine from peptide IA1 and phenylalanine from peptide IA2. The hydrolysates of the acidic spots lacked these amino acids.

The N-terminal amino acids were identified by

treatment with 1-dimethylaminonaphthalene-5-sulphonyl chloride and hydrolysis (Gray & Hartley, 1963a); the fluorescent derivatives were identified by thin-layer chromatography (Waley, 1965a) and by electrophoresis at pH 7 as those of serine from peptide IA1 and of aspartic acid from peptide IA2.

These results show that the structure of peptide IA1 is either Ser-CMCys-Ser-Leu or Ser-Ser-CMCys-Leu, and that the structure of peptide IA2 is Asp-Leu-Leu-Phe. The structure of peptide IA1 was further studied by partial acid hydrolysis of about 0.1 μ mole in acetic acid-concentrated hydrochloric acid (1:1, v/v) at 100° for 5.5 min. The products were fractionated by paper electrophoresis at pH 4 followed by paper chromatography in butan-1-ol-acetic acid-water-pyridine, detected by radioautography and spraying with 0.025% ninhydrin in acetone, and hydrolysed. There were two main products: one was radioactive and gave CM-cysteine and serine on hydrolysis; the other was not radioactive and gave serine and leucine on hydrolysis. The structure of peptide IA1 may now be deduced to be: Ser-CMCys-Ser-Leu.

The structures of these peptides were also studied by the 'dansyl-Edman' procedure (Gray & Hartley, 1963b) on about 0.2 μ mole of each peptide. The results were consistent with the structures given above.

*Comparison of fractions obtained after
chromatography of α -crystallin in urea*

Separation of fractions. Chromatography was carried out in buffers containing 6.2 M-urea (Spector, 1965); the buffers also contained dithiothreitol (1 mg./l.). α -Crystallin (200 mg.) was dissolved in 2 ml. of the first buffer (1 mM-tris, pH 7.9), and after 30 min. at room temperature the solution was

Fig. 4. Exclusion chromatography of chymotryptic digest of carboxymethyl- α -crystallin. The column (145 cm. long $\times$ 2 cm. diam.) of Sephadex G-25 was eluted with 0.03% ammonia containing 0.2% of thiodiglycol at about 45 ml./hr., and 7.2 ml. fractions were collected; 20 μ l. portions were taken for counting. —, Transmission at 254 m μ ; ○—○, radioactivity.

Table 1. Amino acid compositions of chymotryptic peptides from α -crystallin

The values are given in μ moles found, in one-fifteenth of the sample, and molar ratios were calculated so that the sum was integral.

Amino acid	Peptide IA1		Peptide IA2	
	(μ mole found)	(molar ratio)	(μ mole found)	(molar ratio)
Asp	—	—	0.047	1.08
Ser	0.130	2.04*	—	—
Leu	0.068	0.97	0.080	1.84
Phe	—	—	0.047	1.08
CMCys	0.070†	1.00	—	—

* From radioactivity.

† Corrected, assuming 10% loss on hydrolysis.

Fig. 5. Chromatography of α -crystallin on DEAE-cellulose in 6.2M-urea. Stepwise elution was carried out with 1M-tris buffer, pH 7.9 (A), 40mM-tris buffer, pH 7.9 (B), 50mM-tris buffer, pH 7.9 (C), 100mM-tris buffer, pH 7.4 (D), and 0.1M-sodium borate buffer, pH 11, containing 0.4M-KCl (E).

applied to a 26cm. long $\times$ 3cm. diam. column of DEAE-cellulose equilibrated with this buffer. Stepwise elution with buffers filtered through a small column of DEAE-cellulose gave four main peaks (Fig. 5); the small amount of material in fraction 2 was not studied further. The fractions comprising each peak were pooled and freed from urea by chromatography on Sephadex G-25 in 5mM-ammonium hydrogen carbonate, and the protein was recovered by freeze-drying. The thiol group was no longer present in the proteins from the fractions, and carboxymethylation and digestion gave little radioactivity in any of the peptides.

A number of other methods of fractionation under disaggregating conditions were investigated. Guanidine hydrochloride is the most powerful dissociating agent for proteins, but countercurrent distribution on α -crystallin in a 3M-guanidine hydrochloride-phenol system gave only one peak. Chromatography on Amberlite IRC-50 with urea and acid (compare Wilson & Smith, 1959) was also unsuccessful. Chromatography of carboxy[^{14}C] methyl- α -crystallin on CM-cellulose with acetate buffers containing 7M-urea gave several peaks, but the results were not reproducible (Dr P. M. Honovar, personal communication); partial acid hydrolysis of the proteins from the peaks gave Ser-CMCys.

Immunochemical comparison of fractions. Antiserum to lens proteins was prepared and concentrated by the method of Manski, Auerbach & Halbert (1960). Immunodiffusion was carried out in 3% (w/v) agar gel in 0.05M-sodium phosphate buffer, pH 7.4; the samples were tested at a concentration of 1%. Fractions 3-5 showed reactions of identity with each other, and with α -crystallin, as found by

Fig. 6. Comparison of peptides from tryptic digestion of fractions obtained by chromatography of α -crystallin in 6.2M-urea (Fig. 5). The digests of the fractions were first fractionated by electrophoresis at pH 4 for 4 hr. at 10 v/cm. The paper was then divided into six sections (from the cathode to the origin) and each section eluted with 0.03% ammonia. The eluates were dried, and the peptides dissolved in 20 μ l. of water and submitted to paper chromatography on Whatman no. 1 paper in butan-1-ol-acetic acid-water-pyridine. The six sections shown correspond to the eluates from the cathode (left) to the origin (right). The numbers denote the fractions in Fig. 5.

Björk (1964). Fraction 1 showed no precipitation lines; Björk (1964) also noted that the earliest chromatographic fraction gave only very weak precipitation lines, or none at all with some antisera. Absorption of the antiserum with fraction 3 prevented the precipitation line with fraction 4, and the converse was also true.

Structural comparison of fractions. Peptide 'maps' of tryptic digests of the fractions were compared. The samples were carboxymethylated, or oxidized with performic acid. The oxidation of 4mg. of protein in 0.26 ml. of performic acid was carried out for 2.5 hr. at -9° ; the protein was recovered either by dilution and freeze-drying, or by precipitation with 0.8 ml. of 25% (w/v) trichloroacetic acid, followed by washing with 10% (w/v) trichloroacetic acid, ethanol and ether (Kimmel, Kato, Paiva & Smith, 1962). The digestions with trypsin were carried out in 2% (w/v) ammonium hydrogen carbonate as described above. Comparison by paper electrophoresis at pH 4 indicated that there were some differences between fraction 1 and the other fractions. Thus the band nearest to the cathode gave a positive reaction for arginine (but not for histidine) with fractions 3-5, and a positive reaction for histidine (but not arginine) for fraction 1. Further comparisons were made by a modified two-dimensional method ('dissected fingerprints'; Ingram, 1963). After electrophoresis the paper was cut into six sections, and the peptides were eluted with 15mM-ammonia; the concentrated eluates

were then compared by paper chromatography in butan-1-ol-acetic acid-water-pyridine (Fig. 6). No consistent differences were noted among fractions 3-5, but there were distinct differences between fraction 1 and the other fractions.

DISCUSSION

Sequence around the cysteine residues in lens proteins. Bovine α -crystallin contains 1 cysteine residue/20 000 g. of protein (Thomann, 1959; Wisse *et al.* 1966) and partial acid hydrolysis of the carboxymethylated protein gives Ser-CMCys (Waley, 1965a). This is a characteristic of bovine α -crystallin shared by human α -crystallin and by bovine and human albuminoid. Immunological comparisons have led to the idea that in general the structural proteins of the lens are organ-specific rather than species-specific (Halbert & Manski, 1963), and so the resemblance between two mammalian α -crystallins is not unexpected. Nevertheless, this is the first structural comparison between lens proteins from different species. The insoluble albuminoid seems to be structurally very closely related to (or even identical with) α -crystallin (Ruttenberg, 1965; Waley, 1965b), and the present findings are in accord with this idea. The other structural lens proteins, comprising the β -crystallin and γ -crystallin fractions, have different sequences around their cysteine residues; the large number of radioactive spots seen in chromatograms of enzymic digests of these proteins suggested that structural work on these peptides would be difficult.

The sequence around the cysteine residue in α -crystallin is Ser-Cys-Ser-Leu, and this structure accounts for the ready formation of Ser-CMCys by brief acid hydrolysis of the carboxymethylated protein as peptide bonds from serine are readily split by acid to give peptides with *N*-terminal serine (Sanger, 1952). Analogously, the sequence Ser-CMCys-Thr-Thr in glyceraldehyde phosphate dehydrogenase readily gives Ser-CMCys (Perham & Harris, 1963). The peptide B previously obtained from carboxymethyl- α -crystallin (Waley, 1965a) was probably Ser-CMCys-Ser-Leu.

Heterogeneity of α -crystallin. α -Crystallin, both in the native and in the disaggregated form, is heterogeneous with respect to size (Spector & Katz, 1965), but this and many other physicochemical studies carried out have not resolved the question of the complexity of α -crystallin. Electrophoresis in the presence of urea or detergent may give as many as 12 or as few as two or three bands (Wisse *et al.* 1966; Bloemendal *et al.* 1965). Chromatography in the presence of urea gives four to seven peaks (Björk, 1964; Spector, 1965). The proteins comprising these peaks fall into two groups, here called 'early-peak proteins' and 'late-peak proteins'. The early-

peak proteins under Spector's (1965) conditions are those in the first peak, and the late-peak proteins are those in the other peaks (Fig. 5); under Björk's (1964) conditions, the first two peaks contain the early-peak proteins. Now the results that we have obtained show that the early-peak proteins are immunochemically and structurally distinct from the late-peak proteins, and the late-peak proteins form, immunochemically and structurally, one group. Thus the division into early-peak proteins and late-peak proteins is consistent both with the only specific biological property of α -crystallin and with our results from peptide 'maps' (Fig. 6).

Previous investigators have assumed that the early-peak protein, which forms 10-30% of the whole, should be regarded as α -crystallin. Björk (1964) found that the early-peak protein aggregated (after the urea had been removed by dialysis) and he obtained $S_{20,w}$ 13.2s, a value comparable with those obtained from the late-peak proteins. Moreover, the early-peak proteins represent the slowest bands seen on electrophoresis of α -crystallin in polyacrylamide gel in 7M-urea (Björk, 1964; Wisse *et al.* 1966); these bands are present when extensively purified α -crystallin is used, and Wisse *et al.* (1966) concluded that the complexity of the electrophoretic pattern was not due to impurities in α -crystallin. The amino acid composition of the early-peak protein (Björk, 1964; Spector, 1965) is consistent with this conclusion: proline and phenylalanine are relatively abundant, glycine and tyrosine are relatively scarce; these are features that distinguish α -crystallin from β -crystallin and γ -crystallin. Nevertheless, it is impossible to exclude the possibility that the early-peak proteins represent tenacious impurities, and further structural studies are required to establish the number of chemically distinct sub-units in α -crystallin.

The support of the Medical Research Council is gratefully acknowledged. This investigation was supported in part by a Fight for Sight Grant-in-Aid of the National Council to Combat Blindness Inc., New York, N.Y., U.S.A. We also thank the Staffs of the Oxford Co-operative Society and Eastwyck Farm Slaughterhouses for their co-operation.

REFERENCES

- Björk, I. (1963). *Exp. Eye Res.* **2**, 339.
- Björk, I. (1964). *Exp. Eye Res.* **3**, 1.
- Bloemendal, H., Bont, W. S., Benedetti, E. L. & Wisse, J. H. (1965). *Exp. Eye Res.* **4**, 319.
- Bloemendal, H., Bont, W. S., Jongkind, W. F. & Wisse, J. H. (1962). *Exp. Eye Res.* **1**, 300.
- Gray, W. R. & Hartley, B. S. (1963a). *Biochem. J.* **89**, 60p.
- Gray, W. R. & Hartley, B. S. (1963b). *Biochem. J.* **89**, 379.
- Halbert, S. P. & Manski, W. (1963). *Progr. Allergy*, **7**, 107.
- Heathcote, J. G. & Jones, K. (1965). *Biochem. J.* **97**, 15p.

- Ingram, V. M. (1963). In *Methods in Enzymology*, vol. 6, p. 847. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Kimmel, J. R., Kato, G. K., Paiva, A. C. M. & Smith, E. L. (1962). *J. biol. Chem.* **237**, 2525.
- Manski, W., Auerbach, T. P. & Halbert, S. P. (1960). *Amer. J. Ophthalm.* **50**, 315.
- Papaconstantinou, J., Resnik, R. A. & Saito, E. (1962). *Biochim. biophys. Acta*, **60**, 205.
- Perham, R. N. & Harris, J. I. (1963). *J. molec. Biol.* **7**, 316.
- Ruttenberg, G. (1965). *Exp. Eye Res.* **4**, 18.
- Sanger, F. (1952). *Advanc. Protein Chem.* **7**, 1.
- Spector, A. (1964). *Invest. Ophthalm.* **3**, 182.
- Spector, A. (1965). *Invest. Ophthalm.* **4**, 579.
- Spector, A. & Katz, E. (1965). *J. biol. Chem.* **240**, 1979.
- Thomann, H. (1959). *v. Graefes Arch. Ophthalm.* **160**, 219.
- Thomann, H. (1962). *v. Graefes Arch. Ophthalm.* **165**, 219.
- Waley, S. G. (1965a). *Biochem. J.* **96**, 722.
- Waley, S. G. (1965b). *Exp. Eye Res.* **4**, 293.
- Wilson, S. & Smith, D. B. (1959). *Canad. J. Biochem. Physiol.* **37**, 405.
- Wisse, J. H., Zweers, A., Jongkind, J. F., Bont, W. S. & Bloemendal, H. (1966). *Biochem. J.* **99**, 179.
- Woods, A. C. & Burky, E. L. (1927). *J. Amer. med. Ass.* **89**, 102.