The Mechanism of Folding of Globular Proteins

SUITABILITY OF A PENICILLINASE FROM *STAPHYLOCOCCUS AUREUS* AS A MODEL FOR REFOLDING STUDIES

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1. A homogeneous preparation of penicillinase (penicillin amido- β -lactamhydrolase, EC 3.5.2.6) was isolated and purified from cultures of *Staphylococcus aureus* by a simple two-stage procedure. 2. The native protein contains 20–30% helix as determined by optical-rotatory-dispersion and circular-dichroism measurements. Some 54($\pm$ 5)% of the 13 tyrosine residues are exposed to solvent molecules of diameter 0.44 and 0.94 nm. 3. Conditions that allow full recovery of enzymic activity and native conformation from the fully unfolded state in 4M-guanidinium chloride were defined. 4. Refolding of the protein was shown to be inhibited by intermolecular interaction, by small changes in ionization and by low concentrations (0.025M) of phenol.

Many monomeric globular proteins have been shown to regain their biological activity and three-dimensional conformation from the random-coil state in which virtually all groups along the polypeptide chain are exposed to the solvent (Anfinsen, 1967; Tanford, 1968). The final conformation appears to be identical with that of the native protein as produced by the cell, although the process of folding may well be different *in vivo*, since it occurs, most probably, during as well as after biosynthesis of the polypeptide chain. When a protein is allowed to fold *in vitro* there are no possible external templates or constraints to direct the process and, if the polypeptide is not to explore every possible conformation before settling in that one corresponding to the stable, native state, there must be the possibility of internal templates that can mark out a relatively short kinetic folding pathway. Intuitively, the latter mode of folding seems more reasonable, in view of the length and complexity of the former type of pathway (Levinthal, 1968).

Analyses of the relation of sequence to conformation have shown that secondary structures, i.e. α -helix, β -pleated sheet and turns, are determined to a very large extent by local interaction between amino acid side chains and the peptide backbone chain (for reviews, see Robson, 1974a,b; Robson & Pain, 1974a,b,c). Calculations of a helix-forming tendency for individual amino acid residues support this conclusion (Kotelchuck & Scheraga, 1969; Pain & Robson, 1970, 1971; Ralston & De Coen, 1974).

These results lead to the hypothesis that regions of secondary structure can be formed specifically and rapidly from the unfolded polypeptide, thus fulfilling the criteria required of nucleating structures in protein folding. Attainment of the native structure would then be achieved through a pathway of tertiary interactions directed by these secondary structures (for reviews, see Robson, 1974a,b). Experimental support for this concept of nucleation is given by the studies of Anfinsen and his co-workers on staphylococcal nuclease (Anfinsen, 1973) and of Tsong *et al.* (1972) on ribonuclease.

We have initiated an experimental study of protein folding to parallel the theoretical studies already reported (Robson & Pain, 1974a,b,c). To limit the internal constraints to those depending on non-covalent forces, we chose to study a penicillinase (penicillin amido- β -lactamhydrolase, EC 3.5.2.6; β -lactamase) from *Staphylococcus aureus*. This enzyme possesses no disulphide cross-links or thiol groups and contains 13 tyrosine and 7 phenylalanine residues, but no tryptophan. Its sequence (Ambler & Meadow, 1969; Ambler, 1975) shows close similarities to those of the penicillinases from *Bacillus licheniformis* and *Bacillus cereus*. It is rather larger than most proteins hitherto used for conformational kinetic studies, having a molecular weight of 28800. Evidence suggests that penicillinase enzymes from a variety of sources have conformations that are readily perturbed by, for example, penicillin analogues (Citri & Garber, 1961; Dyke, 1967; R. Virden & R. H. Pain, unpublished work) antibodies (Pollock, 1964), alkali (Mile *et al.*, 1970) and urea (Citri, 1958).

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Guanidinium chloride was chosen as the denaturing agent because of its effectiveness in unfolding globular proteins. Most proteins are fully denatured in concentrated guanidinium chloride solution (see, e.g., Tanford *et al.*, 1967) and are found to be random coils without detectable inter-residue interactions. This is important, because on folding the polypeptide is obliged to arrive at the native conformation starting with the minimum of structural cues and the results obtained will then be applicable to the problem of how a protein seeks out its most stable state. In this respect, denaturation by guanidinium chloride is preferable to thermal or pH denaturation alone because the products of the latter are usually ill-defined but still structured (see section IVA of Tanford, 1970; Kugiyama & Bigelow, 1973). The objection against the introduction of an additional chemical species can, in principle, be eliminated by extrapolation of the data to zero concentration of denaturant.

This paper deals with the isolation and characterization of the penicillinase enzyme and, in particular, the conditions governing reversibility of its conformational transitions.

Materials and Methods

Isolation and purification of penicillinase

Penicillinase was isolated from the supernatant of a growth of *Staph. aureus* constitutive mutant PC1 (Richmond, 1963), by using a modified growth medium (see below) and a simplified version of his method. Slope cultures were grown from single cells selected for penicillinase production and used to inoculate starter cultures grown in the absence of glucose, by shaking on an orbital incubator at 37°C overnight to a final $E_{620} = 1.8$; 1 vol. of this culture was used to inoculate 20 vol. of the main growth medium. Growth of the organisms was carried out in batches of 4 × 8 litres in 10-litre aspirators at 37°C, with strong aeration. Mechanical stirring did not increase the yield of enzyme and was therefore discontinued. Minimum quantities of silicone MS antifoam A were used. Penicillinase activity reaches a maximum after 5–6 h, with $E_{620} = 2.1$, and then falls rapidly. By harvesting at $E_{620} = 1.9$ –2.0, slightly lower yields were obtained, but the final purification was facilitated owing to the decreased proportion of non-penicillinase protein. The cells were separated from the supernatant as quickly as possible, by using an Alfa-Laval continuous centrifuge. Approx. 500 g of carefully de-fined phosphorylated cellulose (Whatman P-11) was added to 30 litres of culture supernatant. The aqueous slurry of cellulose P-11 was added until the penicillinase activity in the supernatant was decreased to 5% of its original value. The solution was left to settle for 60 min, then the supernatant was removed by decantation and the cellulose

packed into a 6 cm-diam. column and washed with 0.05 M-ammonium acetate until the eluate showed $E_{275} \leq 0.3$. The penicillinase was then eluted with 80% satd. $(\text{NH}_4)_2\text{SO}_4$ containing 0.01 M-EDTA, pH 5.5–6.0. The crude enzyme was freeze-dried after a single dialysis against 20 vol. of water and subsequent vacuum dialysis. The enzyme at this stage was approx. 70% pure on a specific-activity basis. It was then purified further in small batches as required.

Batches (250 mg) of this crude product were dissolved in water at 10 mg/ml, dialysed against 2 litres of 8% satd. $(\text{NH}_4)_2\text{SO}_4$ /0.05 M-sodium acetate, pH 5.0, and, after filtration, applied to a column (30 cm × 2.5 cm) of P-11 cellulose equilibrated with the same buffer. The column was washed at 4°C with the same buffer and then eluted with 11% satd. $(\text{NH}_4)_2\text{SO}_4$ /0.05 M-sodium acetate, pH 5.0. The largest peak is symmetrical and contains the penicillinase activity. The product was vacuum dialysed and used without freeze-drying. Overall yield was in the range 300–350 mg of purified penicillinase/30-litre batch. The enzyme is stable at 4°C in 8% satd. $(\text{NH}_4)_2\text{SO}_4$ or in 0.2 M-sodium phosphate at pH 7 over a period of several months.

The conditions under which penicillinase is eluted and separated from contaminating protein have to be varied with different batches of P-11 cellulose. Concentrations of up to 11% satd. $(\text{NH}_4)_2\text{SO}_4$ have been used. The column may usually be regenerated with 80% satd. $(\text{NH}_4)_2\text{SO}_4$ and re-used five or six times before separations become inadequate.

Growth medium. Oxoid Casamino acids, 10 g; Oxoid yeast extract, 10 g; Na_2HPO_4 (anhydrous), 13.6 g; KH_2PO_4 (anhydrous), 3.3 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g; trace-metal solution, 0.02 ml; demineralized water, 1000 ml. Glucose (5 g) was autoclaved separately and added to the growth medium. Trace-metal solution (Novick, 1963): $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.5% (w/v); $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5% (w/v); $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5% (w/v); $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.2% (w/v); conc. HCl, 10% (w/v). Slopes contained growth medium as above.

Assay of penicillinase

Enzyme activity was measured by using a modification of the spectrophotometric assay of Jansson (1965), by using potassium benzylpenicillin recrystallized three times from methanol/diethyl ether as substrate. The change of light-absorption, ΔE_{235} , reflects the degree of hydrolysis of the β -lactam ring and is followed by using a Unicam SP.800 spectrophotometer, with scale expansion to 0–0.4 extinction unit full scale and fitted with a thermostatically controlled cell holder. The assay solution consists of 0.04% penicillin G (K^+ salt), 0.1 M-sodium phosphate buffer, pH 6.5, and 1 mM-EDTA. The concentration of penicillinase in the cuvette in $\mu\text{g/ml}$ is given by $2.5 \times (\Delta E/\text{min})$.

If the background extinction is greater than 1.7, as

for example when growth media are being assayed, ΔE_{240} may be followed and related to the units at 235 nm by the relation $\Delta E_{235} = 1.60 \times \Delta E_{240}$. A similar assay procedure has been reported by Waley (1974).

Polyacrylamide-gel electrophoresis

This was performed by the procedure of Davis (1964). For electrophoresis in sodium dodecyl sulphate, the protein was first incubated for 1 h in 1% (w/v) sodium dodecyl sulphate at 37°C, followed by electrophoresis with 1% sodium dodecyl sulphate added to the gel.

Specific extinction coefficient

The specific extinction coefficient was redetermined for staphylococcal penicillinase by the procedure of Crumpton & Wilkinson (1963). Measurements in triplicate gave a value of $E_{1\text{cm}}^{1\%} = 6.87 \pm 0.02$ at 276.5 nm. This contrasts with the previous value of 12.1 (Richmond, 1963), but is close to that of Ambler (1975).

Ultracentrifugal analysis

Sedimentation-velocity experiments were carried out by using a Beckman model E analytical ultracentrifuge.

Difference spectroscopy

Measurements of difference spectra were made by using a Beckman DB-GT double-beam spectrophotometer and also a Hilger-Gilford recording spectrophotometer with expanded scale output. Both were equipped with thermostatically controlled cell holders.

Dilutions were made from a protein stock solution of 6 or 12 mg/ml to give $E_{276.5} = 1.2$ in a 10 mm-light-path cuvette. Buffers and protein stock solution were filtered through 0.22 μm Millipore filters immediately before use. Solvent-perturbation difference-spectroscopy measurements were made by using techniques similar to those described by Herskovits & Laskowski (1962).

The absorption maximum for penicillinase is at 276.5 nm and the main difference spectrum maximum is at 286.5 nm. The absence of turbidity was confirmed at 320 nm.

Optical rotation and circular dichroism

O.r.d. (optical rotatory dispersion) was measured by using a Bendix-Ericson Polarmatic 62 recording spectropolarimeter equipped with jacketed 1 mm- and 10 mm-light-path cells. Results are expressed in terms of reduced mean residue rotation (Urnes & Doty, 1961):

$$[\bar{m}]_{\lambda} = \frac{3}{n^2 + 2} \cdot \frac{M_0}{100} \cdot [\alpha]_{\lambda}$$

when $[\alpha]$ is the specific rotation, n is the refractive index of the solvent at the wavelength of measurement and M_0 the mean residue molecular weight, calculated to be 112 for penicillinase from the data of Ambler & Meadway (1969).

C.d. (circular-dichroism) spectra were obtained by using a Cary 61 circular-dichroism instrument by kind permission of Dr. W. B. Gratzer, Department of Biophysics, King's College, London; 1 mm-light-path cells were used at ambient temperature. Mean residue ellipticities were calculated from the measured ellipticity θ by the expression:

$$[\theta]_{\lambda} = \frac{\theta}{10} \cdot \frac{M_0}{lc}$$

where θ is in degrees, l is the optical path length in cm, and c the concentration in g/ml.

Results and Discussion

Native protein

Penicillinase can be isolated from the culture supernatant of *Staph. aureus* strain PC1 grown to optimum supernatant activity of the enzyme by a simple, two-stage batch and column-chromatography procedure. In early runs, a gel-filtration step on Sephadex G-100 was included between the two phosphorylated-cellulose steps, by using a column 90 cm $\times$ 9 cm. This was not found in any way to affect the purity of the product and was discontinued. An important modification to the growth medium previously used (Richmond, 1963) was to replace the costly sodium glycerophosphate with P_i . Trial growths with concentrations of P_i up to 0.5 M showed that the yield of penicillinase activity is not markedly increased above 0.1 M, and a final concentration of 0.12 M- P_i was used.

A major advance in obtaining a purified product in high yield was the recognition that the ratio of non-penicillinase protein to penicillinase in the culture supernatant rises steeply as the turbidity owing to bacterial growth reaches a maximum. Although the penicillinase activity also rises, the enzyme is much less easy to separate from its contaminants and the overall yield of purified enzyme is decreased if growth is not stopped, in this case by rapid centrifugation, before the growth plateau is reached.

The product has a specific activity of 50 P.T.U. (Pollock & Torriani units)/ μg (Pollock & Torriani, 1953), which compares with 40 P.T.U./ μg obtained by Richmond (1963). Polyacrylamide-gel electrophoresis at pH 7.1 shows the presence of one band only. Calibrated gel electrophoresis in sodium dodecyl sulphate again reveals only a single band, which migrates with a velocity corresponding to mol.wt. 29000. Sedimentation-velocity analysis indicates a single, symmetrical boundary with a sedimentation coefficient $s_{20,w} = 2.63\text{S}$ at a protein concentration of 6.0 mg/ml.

O.r.d. and c.d. spectra are characteristic of a protein containing an appreciable proportion of residues in an α -helical conformation. From the value of $E_{1\text{cm}}^{1\%} = 6.87$ at 276.5 nm, the value of $[m']_{234} = -3750$ was calculated, which indicates a helix content of 21% (Simmons *et al.*, 1961). The ellipticity at 208 nm, $[\theta] = -12100$, corresponds to a helix content of 28% (Greenfield & Fasman, 1969). Such estimates must be regarded as approximate only (Bayley, 1973).

Further information about the conformation of the molecule can be obtained from an assessment of the environment of the tyrosine chromophores. A study of solvent perturbation by difference spectroscopy shows the occurrence of a strong difference-spectrum maximum at 286.5 nm, associated with a red shift of the tyrosine spectrum. For ethylene glycol and for sucrose the change in extinction owing to the spectral shift was shown to be directly proportional to the concentrations of perturbant over the range 0–30% (w/v). Thus, it may be assumed that no conformational transition is being effected by these additives.

The values of $\Delta E_{286.5}/E_{276.5}$ for the perturbation by the two solutes of penicillinase and of the model compound *N*-acetyl-L-tyrosine ethyl ester are given in Table 1. From the ratio of the results for penicillinase and *N*-acetyl-L-tyrosine ethyl ester the proportion of exposed tyrosine residues in the enzyme is $53(\pm 10)\%$ and $55(\pm 5)\%$ for ethylene glycol and sucrose respectively. These residues are thus equally exposed to perturbants of 0.44 and 0.94 nm diameter. This is in keeping with observations on proteins whose structures have been determined by X-ray crystallography, where many tyrosine residues appear to be located in or near the protein surface.

Reversibility of unfolding

Before attempting to analyse thermodynamic and kinetic parameters for conformational transitions in a protein, it is necessary to ensure that the transitions are fully reversible. Reversibility is frequently hindered, as was found for penicillinase from *B. cereus* (Citri *et al.*, 1960), for arginine kinase (R. H.

Table 1. Spectral perturbation of penicillinase

Perturbant	Stokes radius*	$\Delta E_{\text{max.}}/E_{\text{max.}}$		Proportion of tyrosine residues exposed in penicillinase
		<i>N</i> -Acetyl-L-tyrosine ethyl ester	Penicillinase	
Sucrose (20%, w/v)	0.47 nm	0.040 (± 0.002)	0.022 (± 0.002)	55 (± 10)%
Ethylene glycol (20%, w/v)	0.22 nm	0.072 (± 0.002)	0.038 (± 0.002)	53 (± 5)%

* Herskovits & Laskowski (1962).

Fig. 1. Equilibrium curves for the unfolding and refolding of penicillinase in guanidinium chloride as followed by optical rotation at 234 nm

(a) Temperature 20°C. (b) Temperature 30°C. O, Samples diluted from 0.1 M-KCl/0.01 M-sodium phosphate, pH 7.0, into guanidinium chloride dissolved in the same buffer. Δ, Samples diluted with the same buffer from 2 M-guanidinium chloride/0.1 M-KCl/0.01 M-sodium phosphate.

Pain & R. Ryder, unpublished work) and for lysozyme (Pain, 1974), by aggregation of the partly or fully unfolded species. By using difference-spectroscopy techniques, measurements at 320nm indicate the appearance of light-scattering owing to aggregation of the unfolded protein in solutions of guanidinium chloride stronger than 3M at 20°C and 2M at 30°C. The kinetics of this phenomenon, which are higher than second order, become faster with increasing guanidinium chloride concentration and with higher temperature. The effects of protein concentration and pH on reversibility were investigated by dialysis of solutions of penicillinase in 4M-guanidinium chloride to a final solvent concentration of 0.02M-guanidinium chloride, recovery of enzymic activity being taken as the criterion of recovery of the native conformation. Recovery was estimated as a function of pH over the range pH3–10. At concentrations of less than 1.5mg of protein/ml and at pH values between 5.5 and 7.2 recovery of activity was consistently above 90% and, on average, close to 100%. The lower pH limit reflects the presence of an acid-induced conformational transition which has a pK of 3.6 (Al-Shamaony, 1972). No equivalent transition has been found on the alkaline side, but changes in ionization can inhibit correct refolding without necessarily disturbing the native conformation. Spectroscopic and optical-rotation experiments confirm that, within these limits, the unfolding of penicillinase is fully reversible and that, within experimental error, this reversibility is not accompanied by hysteresis (Robson & Pain, 1976). Thus conditions for thermodynamic reversibility of the unfolding of the enzyme (Tanford, 1968) can be defined. These experiments underline the stability of intermolecular interactions between regions of the protein molecules usually concealed from the solvent.

The effect of increasing concentrations of guanidinium chloride on the optical rotation at the Cotton-effect trough at 234nm is shown in Fig. 1. A single transition occurs to a plateau with a value of mean residue rotation corresponding to that of a fully unfolded polypeptide chain (Simmons *et al.*, 1961).

Although the value of $[m']_{234}$ continues to become less negative with increasing guanidinium chloride concentration, there is no evidence of a further conformational transition. The increased scatter of optical-rotation values at higher guanidinium chloride concentration, together with the above evidence for aggregation, probably accounts for the apparently sloping plateau on the high guanidinium chloride side of the transition. Dilution back to low guanidinium chloride concentration of samples of penicillinase allowed just to come to a constant value of $[m']_{234}$ in 2M-guanidinium chloride shows that the transition is thermodynamically reversible (see Fig. 1). Between 20° and 30°C the position of the equilibrium is unaltered, indicating a value of ΔH near to zero for this transition.

Anfinsen (1967) reported that tyrosine analogues hindered the folding of ribonuclease from the unfolded state. For example, phenol at 0.1M decreased the recovery of activity to less than 1%. Similar experiments have been performed with penicillinase by using all the naturally occurring amino acids except cysteine and tyrosine (*N*-acetyltyrosine amide was tested at 6mM). Penicillinase (1.5mg/ml) was refolded from 4M-guanidinium chloride solution into 0.1M-Tris/HCl, pH6.5, in the presence of the amino acid at 0.05M (asparagine and tryptophan at 0.025M; aspartic acid and glutamic acid at 0.013M). Recovery of activity was compared with that obtained under similar conditions but in the absence of amino acid (102%), and was within the range 85–100% for all the amino acids tested, indicating no significant effect on the refolding of the protein. The tyrosine analogue, phenol, at a comparable concentration of 0.025M, resulted consistently in a recovery of only 50% of the activity. It is reasonable to suggest, therefore, that this inhibitory phenomenon may be a more general one. The effectiveness of such low concentrations suggests a specificity of action for phenol and possibly, therefore, a specific role for tyrosine in folded protein structures.

References

- Al-Shamaony, L. A. (1972) M.Sc. Thesis, University of Newcastle upon Tyne
- Ambler, R. P. (1975) *Biochem. J.* **151**, 197–218
- Ambler, R. P. & Meadway, R. J. (1969) *Nature (London)* **222**, 24–26
- Anfinsen, C. B. (1967) *Harvey Lect.* **61**, 95–116
- Anfinsen, C. B. (1973) *Science* **181**, 223–230
- Bayley, P. M. (1973) *Prog. Biophys.* **27**, 1–76
- Citri, N. (1958) *Biochim. Biophys. Acta* **27**, 277–281
- Citri, N. & Garber, N. (1961) *Biochem. Biophys. Res. Commun.* **4**, 143–146
- Citri, N., Garber, N. & Sela, M. (1960) *J. Biol. Chem.* **235**, 3454–3459
- Crumpton, M. J. & Wilkinson, J. M. (1963) *Biochem. J.* **88**, 228–234
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
- Dyke, K. G. H. (1967) *Biochem. J.* **103**, 641–646
- Greenfield, N. & Fasman, G. D. (1969) *Biochemistry*, **8**, 4108–4115
- Herskovits, T. T. & Laskowski, M. (1962) *J. Biol. Chem.* **237**, 2481–2492
- Jansson, J. A. (1965) *Biochim. Biophys. Acta* **99**, 171–172
- Kotelchuck, D. & Scheraga, H. A. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **62**, 14–21
- Kugiyama, M. & Bigelow, C. C. (1973) *Can. J. Biochem.* **51**, 581–585
- Levinthal, C. (1968) *J. Chim. Phys.* **65**, 44–45
- Mile, I., Csanyi, V. & Ferencz, I. (1970) *Acta Biochim. Biophys. Acad. Sci. Hung.* **5**, 33–39
- Novick, R. P. (1963) *J. Gen. Microbiol.* **33**, 121–136
- Pain, R. H. (1974) *Faraday Discuss. Chem. Soc.* **57**, 203–204

- Pain, R. H. & Robson, B. (1970) *Nature (London)* **227**, 62–63
- Pain, R. H. & Robson, B. (1971) *Proc. Eur. Biophys. Congr.* **1**, 33–37
- Pollock, M. R. (1964) *Immunology* **7**, 707–723
- Pollock, M. R. & Torriani, A. M. (1953) *C. R. Hebd. Séances Acad. Sci. Ser. D* **237**, 276–278
- Ralston, E. & De Coen, J. L. (1974) *J. Mol. Biol.* **83**, 393–420
- Richmond, M. H. (1963) *Biochem. J.* **88**, 452–459
- Robson, B. (1974a) in *Amino-acids, Peptides and Proteins* (Sheppard, R. C., ed.), vol. 4, pp. 224–229, The Chemical Society, London
- Robson, B. (1974b) in *Amino-acids, Peptides and Proteins* (Sheppard, R. C., ed.), vol. 5, pp. 180–187, The Chemical Society, London
- Robson, B. & Pain, R. H. (1974a) *Biochem. J.* **141**, 869–882
- Robson, B. & Pain, R. H. (1974b) *Biochem. J.* **141**, 883–897
- Robson, B. & Pain, R. H. (1974c) *Biochem. J.* **141**, 899–904
- Robson, B. & Pain, R. H. (1976) *Biochem. J.* **155**, 331–344
- Simmons, N. S., Cohen, C., Szent-Gyorgyi, A. G., Wetlaufer, D. B. & Blout, E. R. (1961) *J. Am. Chem. Soc.* **83**, 4766–4767
- Tanford, C. (1968) *Adv. Protein Chem.* **23**, 122–283
- Tanford, C. (1970) *Adv. Protein Chem.* **24**, 1–95
- Tanford, C., Kawahara, K. & Lapanje, S. (1967) *J. Am. Chem. Soc.* **89**, 729–749
- Tsong, T. Y., Baldwin, R. L., McPhie, P. & Elson, E. L. (1972) *J. Mol. Biol.* **63**, 453–475
- Urnes, P. & Doty, P. (1961) *Adv. Protein Chem.* **16**, 401–544
- Waley, S. (1974) *Biochem. J.* **139**, 789–790