The Effect of Zinc on the Activity and Fluorescence of Carbonic Anhydrase Holoenzymes

By T. ROBIN HESKETH* and MICHAEL T. FLANAGAN

National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.

(Received 23 September 1974)

The addition of Zn^{2+} to human carbonic anhydrase B holoenzyme was shown to enhance the protein fluorescence, and this enhancement was correlated with the inhibition of the *p*-nitrophenyl acetate esterase activity. The affinity for the inhibitory Zn^{2+} was increased when the ionic inhibitors, acetate or chloride, were added, suggesting that the inhibitory Zn^{2+} -binding site is within the region of the protein that undergoes an anion-induced conformational change. A similar fluorescence enhancement was observed when Zn^{2+} was added to human carbonic anhydrase C and to bovine carbonic anhydrase, demonstrating that the binding site is not a thiol group. Circular-dichroism studies showed that the C isoenzyme but not the B isoenzyme underwent a major conformational change in the presence of Zn^{2+} . A mechanism for the Zn^{2+} -induced fluorescence enhancement was suggested on the basis of studies with simple compounds.

Mammalian erythrocyte carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) is a single-polypeptide-chain protein of molecular weight 30000 and contains one Zn^{2+} atom, essential for activity, in a co-ordination complex at the active site. In human erythrocytes the enzyme is present as two isoenzymes designated CA B and CA C. In the first paper characterizing this enzyme Meldrum & Roughton (1933) reported that the addition of excess of Zn^{2+} to the holoenzyme is inhibitory. Very few cation-inhibition studies have subsequently been performed on the holoenzyme, but those that have suggest that cations that might be expected to compete with Zn^{2+} for a binding site are also inhibitory. Lindskog & Nyman (1964) state that several transition elements are inhibitors of bovine and human carbonic anhydrase at concentrations exceeding $10\mu\text{M}$. Batolska (1963) has reported that lead inhibits the human enzyme, and Magid (1967) has shown that Cu^{2+} is a strong inhibitor of human isoenzyme C and a weak inhibitor of human isoenzyme B. Hodgen *et al.* (1969) and Johnson & Walker (1970) have demonstrated the inhibition of the testicular enzyme of rats and domestic fowl by Cd^{2+} .

In all these studies the enzymic activity of carbonic anhydrase was measured by following the dehydration of bicarbonate or the hydration of CO_2 . As all cations in the above studies form insoluble salts with bicarbonate it is impossible to ascertain whether the inhibition is a direct effect of the metal on the enzyme or is simply due to the removal of the substrate.

* Present address: Department of Pharmacology, University Medical School, Hills Road, Cambridge CB2 2QD, U.K.

In this paper we report the inhibition by Zn^{2+} of the hydrolysis of *p*-nitrophenyl acetate by carbonic anhydrase. Neither *p*-nitrophenyl acetate nor the products of this reaction form insoluble zinc salts. We also observed that the addition of Zn^{2+} to solutions of carbonic anhydrase causes an enhancement of the protein fluorescence and we have correlated this enhancement with the Zn^{2+} inhibition of the enzyme. Further, we have shown that the anion-induced conformational change in the enzyme, demonstrated by King & Burgen (1970), increases its affinity for the inhibitory Zn^{2+} ion.

Materials and Methods

The B and C isoenzymes of carbonic anhydrase were prepared from human erythrocytes by the method of Armstrong *et al.* (1966). The storage and preparation of stock solutions of the proteins have been described (Flanagan & Hesketh, 1974), as have the methods of enzymic activity and protein concentration measurement. $E_{280}^{1\%}$ was taken as 16.3 for human isoenzyme B and as 18.7 for human isoenzyme C at pH 7.0 (Nyman & Lindskog, 1964). Bovine carbonic anhydrase was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. $E_{280}^{1\%}$ was taken as 19.0 (Nyman & Lindskog, 1964).

Absorption spectra were measured with a Unicam SP. 8000 spectrophotometer, enzymic assays were performed in a Unicam SP.700 and fluorescence spectra were measured with an Aminco-Bowman spectrofluorimeter. The enzyme fluorescence was excited at 280 nm and the emission observed at 325 nm. Circular-dichroism spectra were measured with a

Roussel-Jouan CD 185 model II dichrograph. Values of 259, 261 and 263 residues per molecule were taken for human isoenzymes B, C and bovine carbonic anhydrase respectively in calculating the mean residue molecular ellipticities (Lindskog *et al.*, 1971). Complete details of these techniques have also been described (Flanagan & Hesketh, 1974).

AnalaR-grade sodium acetate, zinc acetate, ZnSO_4 , KCl, K_2SO_4 , acetic acid, H_2SO_4 and HCl were obtained from BDH Chemicals Ltd. (Poole, Dorset, U.K.), guanidinium chloride was a BDH Aristar reagent and *p*-nitrophenyl acetate and 1,10-phenanthroline were BDH laboratory reagents. Human serum albumin, bovine serum albumin, L-tryptophan, L-tyrosine and their derivatives were obtained from Sigma (London) Chemical Co. Ltd. (Kingston-upon-Thames, Surrey, U.K.).

Tris, despite its low buffering capacity at pH 7.0, was used, as strong buffers in this pH region either form insoluble Zn^{2+} salts or chelate Zn^{2+} very strongly. All pH values were measured in the final experimental solutions. When studying the effect of acetate on the Zn^{2+} interaction, solutions at both pH 3.3 and 7.0 were required. As these pH values are at the extremes of the acetate buffering capacity the pH values of the final experimental solutions were obtained by appropriately adjusting the relative proportions of 250 mM-sodium acetate and 250 mM-acetic acid.

Fig. 1. Inhibition and fluorescence enhancement of human isoenzyme B by Zn^{2+}

(a) Mean residue molecular ellipticity at 270 nm, $[\theta]_{\text{res.}, 270}$; (b) percentage inhibition of esterase activity; (c) percentage fluorescence enhancement at 325 nm. The concentrations of human isoenzyme B were (a) $17.9 \mu\text{M}$, (b) $1.1 \mu\text{M}$ and (c) $1.6 \mu\text{M}$. The buffer was 250 mM-Tris- H_2SO_4 , pH 7.0, and the temperature was 25°C .

Fig. 2. Effect of Cl^- on the fluorescence enhancement of human isoenzyme B

■, 100 mM-Tris-HCl-240 mM-KCl, pH 7.0; ▲, 100 mM-Tris- H_2SO_4 -240 mM-KCl-80 mM- K_2SO_4 , pH 7.0; ●, 100 mM-Tris- H_2SO_4 -80 mM- K_2SO_4 , pH 7.0. The concentration of human isoenzyme B was $1.4 \mu\text{M}$ and the temperature was 25°C .

Results

Fluorescence enhancement and Zn^{2+} inhibition of human isoenzyme B

The increase in fluorescence intensity of human isoenzyme B caused by the addition of Zn^{2+} to the protein solution is shown in Fig. 1. The buffer solution was 250 mM-Tris- H_2SO_4 , pH 7.0, and ZnSO_4 was added to a maximum concentration of 33 mM. The addition of Zn^{2+} did not alter the wavelength of maximum emission, the uncorrected value of which was 325 nm. The percentage inhibition of the enzyme by the Zn^{2+} under identical conditions was calculated from the rates of hydrolysis of *p*-nitrophenyl acetate and is also shown in Fig. 1.

A 50% loss of enzymic activity occurs at a concentration of 6.5 mM- Zn^{2+} . The degrees of fluorescence enhancement and of enzymic inhibition are identical for a given Zn^{2+} concentration.

The reversibility of the Zn^{2+} inhibition was demonstrated by diluting a maximally inhibited sample from 24 mM- to 0.4 mM- Zn^{2+} , a non-inhibitory concentration. Full activity was restored to the enzyme.

The mean residue molecular ellipticity at 270 nm is included in Fig. 1, showing that under the above conditions the aromatic Cotton effects are undiminished. These features of the circular-dichroism spectrum are a sensitive measure of the conformation of this enzyme; therefore this result shows that the concentrations of Zn^{2+} used do not cause human isoenzyme B to unfold (Flanagan & Hesketh, 1974; Beychok *et al.*, 1966).

Effect of anionic inhibitors

To determine whether the behaviour of the enzyme with respect to zinc differed in the presence of a

univalent anionic inhibitor we have repeated the zinc titration of Fig. 1 in the presence of Cl^- . The results in 100mM-Tris- H_2SO_4 -80mM- K_2SO_4 , 100mM-Tris-HCl-240mM-KCl and 100mM-Tris- H_2SO_4 -240mM-KCl-80mM- K_2SO_4 , pH 7.0, are shown in Fig. 2. When SO_4^{2-} is replaced by Cl^- the concentration of Zn^{2+} required to give 50% of the maximum fluorescence enhancement is decreased from 5.0 to 0.23mM. In this case the ionic strength remains constant but the replacement of SO_4^{2-} by Cl^- may well alter the concentration of free Zn^{2+} , as Cl^- and SO_4^{2-} do not chelate Zn^{2+} equally strongly (Sillén & Martell, 1964a,b; Izatt *et al.*, 1969). To show that the change in affinity of the enzyme for the Zn^{2+} was not an artifact caused by such an effect the experiment was performed in which both Cl^- and SO_4^{2-} were added. If the apparent affinity change is due to the stronger chelating power of SO_4^{2-} the enhancement should occur at the same or higher Zn^{2+} concentration than when SO_4^{2-} alone is present. However, if it is due to a genuine change in the affinity of the protein the enhancement will occur at Zn^{2+} concentrations nearer those obtained with

Fig. 3. Fluorescence enhancement in the presence of acetate

(a) Mean residue molecular ellipticity at 270nm and an enzyme concentration of 20.0 μM ; (b) percentage fluorescence enhancement at an enzyme concentration of 1.0 μM . ■, Human isoenzyme C; ●, human isoenzyme B; ▲, bovine carbonic anhydrase; each solution was adjusted to pH 7.0 with 250mM-sodium acetate-acetic acid in the presence of zinc acetate in the concentrations shown. ○, Human isoenzyme B in 250mM-sodium acetate-acetic acid, pH 3.3, after 20h. The temperature in all experiments was 25°C.

Fig. 4. Pseudo-first-order plot of fluorescence enhancement of human isoenzyme B

The concentrations of Zn^{2+} in 100mM-Tris-HCl-240mM-KCl, pH 7.0, were 1.67mM (●), 2.50mM (□), 3.33mM (▲), 6.67mM (○), 10.0mM (■), 13.33mM (△) and 1.67mM (▽) in 100mM-Tris- H_2SO_4 -240mM-KCl-80mM- K_2SO_4 , pH 7.0. The concentration of human isoenzyme B was 2.0 μM and the temperature was 25°C. F_t , F_0 and F_∞ are the fluorescence intensities at time t , at zero time and on completion of the reaction.

Cl^- alone, although slight displacement of the characteristic may occur owing to the higher ionic strength. Experimentally the latter situation occurs (Fig. 2). The maximum fluorescence enhancement of approx. 29% is independent of the nature of the anion.

The presence of the anionic inhibitor acetate also shifts the fluorescence enhancement characteristic to lower Zn^{2+} concentrations (Fig. 3). If the pH of a solution of human isoenzyme B is lowered below pH 4.0 a major conformational change occurs in which there is extensive unfolding of the protein (Flanagan & Hesketh, 1974). The Zn^{2+} -induced fluorescence enhancement in 250mM-sodium acetate-acetic acid at pH 3.3 is compared with that at pH 7.0 in Fig. 3. The pH 3.3 solution was incubated for 20h at 4°C to ensure that denaturation was complete (Flanagan & Hesketh, 1974). The enhancement is still found with the acid-denatured enzyme, although the concentration of Zn^{2+} required for half-maximum enhancement has been increased from 1.3 to 24mM.

Kinetics of fluorescence enhancement

The rate of increase of fluorescence of human isoenzyme B which takes place on the addition of Zn^{2+} has been measured in the presence of Cl^- . The data are presented as pseudo-first-order plots in Fig. 4. As the Zn^{2+} concentration greatly exceeds that of the protein these plots should also be linear for a second-order reaction (Laidler, 1965).

The reaction is clearly neither first- nor second-order but is biphasic, the first step being the most rapid. This rapid step is dependent on the Zn^{2+} concentration, but the subsequent reaction does not appear to be so. Replacement of Cl^- by SO_4^{2-} accelerates the rate of enhancement to such an extent that we could not measure it. Addition of SO_4^{2-} to the Cl^- buffer does not produce this acceleration (Fig. 4), demonstrating that the removal of the Cl^- ion is necessary.

Comparison of human isoenzymes B and C and bovine carbonic anhydrase

The Zn^{2+} -induced fluorescence enhancement curve for human isoenzyme C in 250mM-acetate, pH7.0, is shown in Fig. 3. The plateau value of enhancement is similar to that found for the B isoenzyme (30%), but the onset of enhancement occurred at a lower Zn^{2+} concentration. The half-maximum enhancement concentrations were 0.2 and 1.3mM for human isoenzymes C and B respectively. The curve for bovine carbonic anhydrase is also shown in Fig. 3 and is very similar to that of human isoenzyme C. In all three cases no gross protein-conformational changes occur, as indicated by the circular-dichroism spectra. The mean residue molecular rotation values at 270nm are also included in Fig. 3.

When Zn^{2+} is added to human isoenzyme C in solutions containing Cl^- or SO_4^{2-} the initial increase in fluorescence is followed by a slow decrease, and this is mirrored by large changes in the circular-dichroism spectra. The rate of this reaction is

Fig. 5. Kinetics of the Zn^{2+} -induced conformation change of human isoenzyme C

The Zn^{2+} concentrations were 0.25mM (●), 2.5mM (■) and 25mM (▲). The concentration of human isoenzyme C was 16.0 μM in 100mM-Tris-HCl-240mM-KCl, pH7.0, and the temperature was 25°C. The change in mean residue molecular ellipticity was followed at 270nm and the overall spectral change is shown in Fig. 6.

Fig. 6. Circular-dichroism spectra of human isoenzyme C

····, Spectrum of the folded enzyme in 250mM-sodium acetate-acetic acid, pH7.0; —, spectrum of the enzyme in 25mM- Zn^{2+} in 100mM-Tris-HCl-240mM-KCl, pH7.0, on completion of the Zn^{2+} -induced conformational change, i.e. after 115h. ---, Spectrum of the acid-unfolded enzyme in 10mM-HCl, pH2.0, after 20h. - · - · - , Spectrum of the guanidine-unfolded enzyme in 5.4M-guanidinium chloride-250mM-sodium acetate-acetic acid, pH7.0, after 20h. The concentration of the human isoenzyme C was 14.0 μM in each case and the temperature was 25°C.

dependent on the concentration of Zn^{2+} , as shown in Fig. 5, where the change in mean residue molecular ellipticity is plotted against time. The final circular-dichroism spectrum is shown in Fig. 6 together with the spectra of the native, the acid-denatured and the guanidinium chloride-denatured enzyme. In the two denatured-enzyme spectra the aromatic fine structure characteristic of the native enzyme has disappeared, but with enzyme incubated with Zn^{2+} a new, weaker, aromatic fine structure appears.

Model compounds

In an attempt to determine the cause of the fluorescence enhancement the effect of Zn^{2+} on the fluorescence of several simple aromatic compounds in 250mM-acetate, pH7.0, was examined. The positive results are shown in Fig. 7. L-Tryptophan shows an enhancement in the same Zn^{2+} -concentration range as the enzyme, but the extent of enhancement is greater. L-Tyrosine shows a much weaker effect. No enhancement was observed with L-tryptophylglycine, L-tryptophan amide, L-tryptophan methyl ester, N-acetyl-L-tryptophan, N-acetyl-L-tryptophan amide or L-tyrosylglycine. A very marked enhancement was found with 1,10-phenan-

Fig. 7. Zn^{2+} -induced fluorescence enhancement of simple compounds

▲, 1,10-Phenanthroline; ●, L-tryptophan; ■, L-tyrosine. Excitation wavelengths: ▲, 268 nm; ●, 280 nm; ■, 270 nm. Emission wavelengths: ▲, 362 nm; ●, 340 nm; ■, 300 nm. The concentrations were $2.4 \mu\text{M}$ (▲), $6.7 \mu\text{M}$ (●) and $17.0 \mu\text{M}$ (■), each in 250 mM-sodium acetate-acetic acid, pH 7.0 and the temperature was 25°C .

throle (phen), a powerful zinc-chelating agent. This enhancement curve was stepped, probably indicating transitions between the different chelated species $\text{Zn}^{2+}(\text{phen})$, $\text{Zn}^{2+}(\text{phen})_2$ and $\text{Zn}^{2+}(\text{phen})_3$ (Banks & Bystroff, 1959). The wavelength of maximum emission, 349 nm (uncorrected), was unaltered on enhancement, as was that of L-tryptophan, 340 nm (uncorrected). The fluorescence enhancement corresponds to an increase in quantum yield, as the absorption spectra of L-tryptophan and L-tyrosine were unaltered on the addition of Zn^{2+} ; there was a small change in the absorption spectrum of 1,10-phenanthroline, but the emission was excited at an isosbestic point.

No enhancement was found when Zn^{2+} was added to human and bovine serum albumin under conditions closely similar to those in which Rao & Lal (1958) have shown Zn^{2+} binding to occur.

Discussion

In this paper we have shown that the inhibition of carbonic anhydrase by Zn^{2+} is due to a direct effect of the metal on the enzyme. The problem confronting previous workers, i.e. the removal of substrate as an insoluble zinc salt, has been overcome by utilizing the esterase activity of the enzyme. Nonetheless a detailed study of the inhibition kinetics could not be undertaken for two reasons: (1) ZnCl_2 solutions are acidic, requiring a strong buffer solution to maintain neutral pH and, as most buffers, including Tris and acetate, are good metal-ion-chelating agents, the concentration of free Zn^{2+} in solution cannot readily be calculated; (2) the high K_m value for *p*-nitrophenyl acetate (Verpoorte *et al.*, 1967) and the

very low solubility of this substrate (Armstrong *et al.*, 1966) preclude any accurate analysis of the ester hydrolysis kinetics. This is not too great a disadvantage, as we have shown that the addition of Zn^{2+} to the enzyme enhances the fluorescence and that this enhancement is proportional to the inhibition.

The Zn^{2+} -induced fluorescence enhancement is facilitated by univalent anionic inhibitors of human isoenzyme B. King & Burgen (1970) have shown that a conformational change takes place in human isoenzyme B on the binding of univalent anions, resulting in the exposure of tryptophan residues to the solvent. From our results it may therefore be inferred that the site for the inhibitory Zn^{2+} ion is within part of the molecule undergoing this conformational change.

Normally the juxtaposition of a heavy atom and a fluorophore, either temporarily by collision or permanently by substitution, quenches the fluorescence by facilitating inter-system crossing (Murrell, 1963). Consequently the enhancement of aromatic fluorescence by Zn^{2+} may seem unusual. However, Bhatnagar & Forster (1965) (see also Parker, 1968), in a study of the fluorescence of 8-hydroxyquinoline and its metal chelates, observed that the heavy-atom effect did not appear to operate in the case of metals with a closed shell and of atomic number less than 49. Zn^{2+} , with an atomic number of 30, forms chelates with its 3d orbitals filled. Additionally, if one examines the data of Bhatnagar & Forster (1965), it is clear that the lighter metals, including Zn^{2+} , in fact enhance the ligand fluorescence. The Zn^{2+} is chelated in these complexes to a quinoline nitrogen and phenolate oxygen atom. We have also observed the enhancement of fluorescence of 1,10-phenanthroline when this ligand forms a complex with Zn^{2+} . In this complex the metal is chelated to the two heterocyclic nitrogen atoms, and it is tempting to extrapolate this mechanism to the amino acid L-tryptophan. Unfortunately several studies of the chelation of Zn^{2+} by L-tryptophan (Albert, 1950; Perkins, 1953; Weber & Simeon, 1971) and L-tyrosine (Albert, 1952; Perkins, 1953; Weber & Simeon, 1971) have concluded that these are bidentate ligands and the molecular strain generated by a third link via the indole nitrogen or phenol oxygen atom would be too great for stability. A more likely explanation for the enhancement observed with these compounds is that on chelation the α -amino group, normally protonated at pH 7.0, is deprotonated, as the free amino group is the effective ligand for a metal cation (Albert, 1950, 1952). As the protonated amino group is a quencher of the fluorescence of L-tryptophan and L-tyrosine (Weinryb & Steiner, 1971; White, 1959) the neutralization of this group will lead to an increase in quantum yield. Zn^{2+} -induced fluorescence en-

hancement does not occur after modification of the amino or carboxyl group, which may be expected if, as seems likely, the modification prevents Zn^{2+} chelation.

Both mechanisms discussed for the model systems are feasible in the case of the proteins: either direct chelation to a tryptophan residue or chelation near a tryptophan residue neutralizing an adjacent protonated amino group. If the latter mechanism is operative the amino group must be close to the tryptophan residue, as Weinryb & Steiner (1968, 1971) have shown that the quenching by an α -amino group declines sharply with distance. In the protein the quenching would probably arise from a side-chain group, for example, a lysine ϵ -amino group or an imidazole imino group. Other possible ligands for Zn^{2+} are thiol (Klotz *et al.*, 1952) and carboxyl residues (Rao & Lal, 1958; Tanford, 1952). These, together with imidazole, have been suggested in the case of albumin, which we found to show no fluorescence enhancement on binding Zn^{2+} . The participation of a free thiol group is possible in human isoenzymes B and C, both of which possess such a group, but seems unlikely, as bovine carbonic anhydrase, which possesses no cysteine (Lindskog *et al.*, 1971), shows a fluorescence enhancement characteristic similar to that of human isoenzyme C.

Several ligands may be involved in the metal-binding site, as Zn^{2+} generally forms complexes with high co-ordination numbers, often six, more commonly four (Cotton & Wilkinson, 1966). The change in affinity for the inhibitory Zn^{2+} on the addition of a univalent anion suggests that the spatial arrangement of the ligands binding this Zn^{2+} ion is disturbed when the protein undergoes the conformational change induced by the anion. Our results indicate that one or more tryptophan residues are either within or close to the zinc-binding site. As King & Burgen (1970) have shown that at least one tryptophan residue is exposed to the solvent as a result of the anion-induced conformational change, it is reasonable to conclude that the residues observed to be perturbed in the two experiments are the same. King & Roberts (1971) have also shown that a histidine residue, normally buried, is exposed to the solvent on the addition of a univalent anion. Considering the ubiquity of histidine as a zinc ligand (Liljas *et al.*, 1972; Lipscomb *et al.*, 1969; Rao & Lal, 1958; Tanford, 1952) the participation of this residue also seems possible.

The kinetics of enhancement suggested that although the greater part of the increase in fluorescence is due to the direct interaction with the metal, a minor proportion is not. The smaller component follows first-order kinetics and would therefore appear to be a purely protein-dependent phenomenon. The most probable interpretation of

these results is that Zn^{2+} itself induces a conformational change, which in turn perturbs the environment of a tryptophan residue. The dependence of the kinetics on the anion present provides additional evidence that the Zn^{2+} -binding site is perturbed by the anion-induced conformational change.

In acid solution carbonic anhydrase exists in a partially unfolded state (Beychok *et al.*, 1966; Lindskog *et al.*, 1971; Flanagan & Hesketh, 1974). The observation that the enhancement of fluorescence by Zn^{2+} also occurs in the acid-unfolded enzyme suggests the retention of sufficient structure to maintain the integrity of the Zn^{2+} -binding site, although perturbation of the protein in the region of the site is clearly reflected in the decrease in Zn^{2+} affinity.

We are grateful to Dr. R. W. King for critical comments on the manuscript.

References

- Albert, A. (1950) *Biochem. J.* **47**, 531–538
- Albert, A. (1952) *Biochem. J.* **50**, 690–697
- Armstrong, J. McD., Myers, D. V., Verpoorte, J. A. & Edsall, J. T. (1966) *J. Biol. Chem.* **241**, 5137–5149
- Banks, C. V. & Bystroff, R. I. (1959) *J. Amer. Chem. Soc.* **81**, 6153–6158
- Batolska, A. (1963) *Nauch. Tr. Nauchnoizsled. Inst. Ohrana Truda Prof. Zaboljavanija* **10**, 55–59
- Beychok, S., Armstrong, J. McD., Lindblow, C. & Edsall, J. T. (1966) *J. Biol. Chem.* **241**, 5150–5160
- Bhatnagar, D. C. & Forster, L. S. (1965) *Spectrochim. Acta* **21**, 1803–1807
- Cotton, F. A. & Wilkinson, G. (1966) *Advanced Inorganic Chemistry*, p. 610, John Wiley and Sons, New York
- Flanagan, M. T. & Hesketh, T. R. (1974) *Eur. J. Biochem.* **44**, 251–259
- Hodgen, G. D., Butler, W. R. & Gomes, W. R. (1969) *J. Reprod. Fert.* **18**, 156–157
- Izatt, R. M., Eatough, D., Christensen, J. J. & Bartholomew, C. H. (1969) *J. Chem. Soc. A* **47**, 53
- Johnson, A. D. & Walker, G. P. (1970) *J. Reprod. Fert.* **23**, 463–468
- King, R. W. & Burgen, A. S. V. (1970) *Biochim. Biophys. Acta* **207**, 278–285
- King, R. W. & Roberts, G. C. K. (1971) *Biochemistry* **10**, 558–565
- Klotz, I. M., Urquhart, J. M. & Fiess, H. A. (1952) *J. Amer. Chem. Soc.* **74**, 5537–5538
- Laidler, K. J. (1965) *Chemical Kinetics*, 2nd edn., chapter 1, McGraw-Hill, New York
- Liljas, A., Kannan, K. K., Bergstén, P. C., Warra, I., Fridborg, K., Strandberg, B., Carlbon, U., Järup, L., Lövgren, S. & Petef, M. (1972) *Nature (London) New Biol.* **235**, 131–137
- Lindskog, S. & Nyman, P. O. (1964) *Biochim. Biophys. Acta* **85**, 462–474
- Lindskog, S., Henderson, L. E., Kannan, K. K., Liljas, A., Nyman, P. O. & Strandberg, B. (1971) *Enzymes*, 3rd edn., **5**, 587–665

- Lipscomb, W. N., Hartsuck, J. A., Quijcho, F. A. & Reeke, G. N. (1969) *Proc. Nat. Acad. Sci. U.S.* **64**, 28–35
- Magid, E. (1967) *Scand. J. Haematol.* **4**, 257–270
- Meldrum, N. U. & Roughton, F. J. W. (1933) *J. Physiol. (London)* **80**, 113–142
- Murrell, J. N. (1963) *The Theory of the Electronic Spectra of Organic Molecules*, pp. 296–297, Methuen and Co., London
- Nyman, P. O. & Lindskog, S. (1964) *Biochim. Biophys. Acta* **85**, 141–151
- Parker, C. A. (1968) *Photoluminescence of Solutions*, pp. 473–478, Elsevier, Amsterdam
- Perkins, D. J. (1953) *Biochem. J.* **55**, 649–652
- Rao, M. S. N. & Lal, H. (1958) *J. Amer. Chem. Soc.* **80**, 3226–3235
- Sillén, L. G. & Martell, A. E. (1964a) *Chem. Soc. Spec. Publ.* **17**, 243–244
- Sillén, L. G. & Martell, A. E. (1964b) *Chem. Soc. Spec. Publ.* **17**, 289–290
- Tanford, C. (1952) *J. Amer. Chem. Soc.* **74**, 211–215
- Verpoorte, J. A., Mehta, S. & Edsall, J. T. (1967) *J. Biol. Chem.* **242**, 4221–4229
- Weber, O. A. & Simeon, V. (1971) *Biochim. Biophys. Acta* **244**, 94–102
- Weinryb, I. & Steiner, R. F. (1968) *Biochemistry* **7**, 2488–2495
- Weinryb, I. & Steiner, R. F. (1971) in *Excited States of Proteins and Nucleic Acids* (Steiner, R. F. & Weinryb, I., eds.), pp. 293–295, Plenum Press, New York, and MacMillan Press, London
- White, A. (1959) *Biochem. J.* **71**, 217–220