The Interactions of Haem with Ligandin and Aminoazo-Dye-Binding Protein A

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1. The interactions of ferriprotoporphyrin IX with ligandin and aminoazo-dye-binding protein A result in absorption spectra in the Soret region characteristic of the ligand in its monomeric state. 2. Both proteins are able to bind ferrous as well as ferric haem. 3. Ferriprotoporphyrin IX is bound at a single site on both proteins. At pH 7.0, I 0.16M, difference-spectrophotometric measurements gave association constants of 10^7 and 4×10^6 litre/mol for ligandin and protein A respectively. Under the same conditions fluorescence-quenching experiments gave an association constant of 2×10^7 litre/mol for ligandin. 4. Bilirubin, bromosulphophthalein and oestrone sulphate each compete with haem for binding by the two proteins. 5. Ferriprotoporphyrin IX bound to both ligandin and protein A is able to form co-ordination complexes with CN^- , but not, to any measurable extent, with either N_3^- or F^- . From these results it is suggested that binding by the two proteins may not involve the haem iron atom. 6. Both haem-protein complexes give rise to measurable extrinsic Cotton effects in the Soret region. 7. The formation and properties of the ligandin- and protein A-haem complexes are compared with those of haem-albumin, haemoglobin, myoglobin and other haemoproteins.

Evidence has been presented (Ketterer *et al.*, 1976a,b) that the intracellular transport and catabolism of haem in the liver may involve its binding by ligandin and aminoazo-dye-binding protein A, two proteins present in substantial amounts in the cytosol of this tissue (Ketterer *et al.*, 1967; Litwack *et al.*, 1971; Fleischner *et al.*, 1972; Ketterer *et al.*, 1976d). Studies of the interactions of haem with the two proteins should contribute to the understanding of their roles in these processes and are the subject of the present paper.

The binding of ferriprotoporphyrin IX by ligandin was first observed by Meuwissen *et al.* (1972). Using a method based on the partition of the ligand between Sephadex G-10 and the protein in solution, these authors reported one strong and several weaker sites for haem binding to ligandin. In a subsequent study (Meuwissen *et al.*, 1974) an association constant of approx. 10^8 litre/mol (pH 7.4, 4°C) was found. Much weaker binding was reported by Kamisaka *et al.* (1975), who exploited the large induced ellipticities associated with bilirubin bound to ligandin to monitor competition effects between this ligand and ferriprotoporphyrin IX. The results of these experiments, in combination with a previously determined association constant of 5×10^7 litre/mol for bilirubin, yielded an association constant of approx. 10^5 litre/mol (pH 7.4, 25°C) for ferriprotoporphyrin IX, on the assumption that both ligands share the same binding site. The quenching of the intrinsic fluorescence of

ligandin, which accompanies the binding of ferriprotoporphyrin IX, was used by Ketley *et al.* (1975) to measure the extent of interaction; these authors found an association constant of 10^7 litre/mol (pH 7.5, 25°C), but their approach did not allow the stoichiometry of binding to be determined.

Aminoazo-dye-binding protein A, which on the basis of its physical and non-covalent binding properties appears to be identical with Z-protein (Levi *et al.*, 1969; Mishkin *et al.*, 1972), has not yet been studied with regard to its binding of haem.

In view of the disagreement about the affinity of ligandin for ferriprotoporphyrin IX, the uncertainty as to the number of binding sites for the ligand on this protein and the complete lack of binding data for protein A, we decided to investigate in more detail the interactions of ferric haem with the two proteins. Because of the instability of aqueous solutions of the ligand (see, e.g., Falk, 1964), long equilibration times are undesirable in such studies, and we therefore chose the relatively rapid techniques of absorption spectrophotometry and fluorescence spectroscopy to measure binding. The studies by Brown *et al.* (1970) provided us with a means of determining the extent of dimerization of ferric haem in the protein-ligand solutions under investigation, an aspect of the problem not taken into account by previous authors. In addition, the interactions of the two proteins with ferriprotoporphyrin IX, some aspects of the co-ordination chemistry of the haem-protein

complexes and the extrinsic Cotton effects resulting from binding were examined.

Experimental

Materials

Unless otherwise stated all chemicals were of reagent grade, from BDH Chemicals, Poole, Dorset, U.K. Ferriprotoporphyrin IX, bilirubin, oestrone sulphate, glutathione and bovine serum albumin were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Disodium bromosulphophthalein [disodium 3,4-(1',2',5',6'-tetrabromobenz)-5,5-bis-(4-hydroxyphenyl-3-sulphonate)furan-2-one] was from G. T. Gurr, High Wycombe, Bucks., U.K. Sephadex G-100 and CM-Sephadex C-50 were from Pharmacia (G.B.) London, W5 5SS, U.K. Whatman DEAE-cellulose was from Hospital and Laboratory Supplies, London EC1M 6BB, U.K. Ampholines were from LKB Instruments, Croydon, Surrey, U.K.

Methods

Preparation of proteins. The proteins were prepared as described previously (Tipping *et al.*, 1976).

Preparation of solutions. Protein solutions were prepared as described previously (Tipping *et al.*, 1976).

Stock solutions of ferriprotoporphyrin IX (0.2–0.5 mM) and bilirubin (1 mM) were prepared in 5–10 mM-NaOH and their concentrations determined spectrophotometrically by using $\epsilon_{385} = 58400 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for ferriprotoporphyrin IX (Dawson *et al.*, 1969) and $\epsilon_{440} = 52000 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for bilirubin (Blauer & King, 1970). These solutions were used within 1 h of preparation. Solutions of 1 mM-oestrone sulphate, 1 mM-bromosulphophthalein, 1 M-KF, 1 M- NaN_3 , 1 M-KCN and 1 M- $\text{Na}_2\text{S}_2\text{O}_4$ were prepared by dissolution in buffer.

Spectrophotometry. Measurements were made with Pye Unicam SP.1800 double-beam spectrophotometer by using cells of 0.1, 0.5, 1.0 and 4.0 cm light-path. The temperature of the cell compartment was 24–28°C. Titrations were carried out by adding equal volumes of stock solutions of ferriprotoporphyrin IX from a micro-syringe to both sample and reference cell, the former containing a measured volume of protein solution, the latter an equal volume of buffer. Measurements were made immediately after mixing and no significant time-effects were observed up to 10 min in any of the solutions investigated.

Fluorescence spectroscopy. Measurements were made with a Perkin-Elmer Hitachi MPF 3L spectrofluorimeter. The cell compartment was maintained at 27°C. Protein solutions (2.0 ml, 0.024–1.2 μM)

were titrated with small volumes of stock solutions of ferriprotoporphyrin IX by using a micro-burette. Absorbances were kept below 0.1. At protein concentrations of 0.6 and 1.2 μM , small time-effects were observed, the fluorescence decreasing slightly for 2–3 min after the addition of the ligand before reaching a constant value.

Circular dichroism. Measurements were made with a Cary 61 spectropolarimeter at 24°C.

Analysis of fluorescence-quenching results. If the intrinsic fluorescence of a protein is quenched by the binding of ligand molecules, a binding isotherm may be derived with no assumptions about the stoichiometry or the response of different binding sites by performing experiments at more than one protein concentration. It is only necessary to assume that the relative fluorescence is a unique function of the degree of saturation of the binding sites (Halfman & Nishida, 1972). If $[L]_t$, $[L]_b$ and $[L]_f$ are the concentrations of total, bound and unbound ligand respectively then mass-balance requires that

$$[L]_t = [L]_b + [L]_f$$

which can be rewritten as

$$\frac{[L]_f}{[P]} = R - \bar{\nu}$$

where $[P]$ is the total protein concentration, $\bar{\nu} = [L]_b/[P]$ and $R = [L]_f/[P]$. For a series of plots of relative fluorescence intensity, F/F_0 (where F is the intensity in the presence of a given amount of ligand and F_0 the intensity in the absence of ligand), against R , a line of constant ordinate will intersect the curves at points of equal $\bar{\nu}$. A plot of R values for these points against $1/[P]$ will then give $[L]_f$ and $\bar{\nu}$ from the slope and intercept respectively. A series of such plots enables a complete binding isotherm to be constructed (Beaven *et al.*, 1974).

Results and Discussion

Absorption spectra

In the concentration range accessible to spectrophotometric measurements (approx 0.1–400 μM), neutral aqueous solutions of ferriprotoporphyrin IX are composed largely of dimers (Brown *et al.*, 1970) and, as shown in Fig. 1 (curves *a–c*), their spectra in the Soret region consist of broad bands of low intensity ($\epsilon \approx 50 \text{ litre} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$). Because of the variation of the extent of dimerization with concentration, these spectra do not obey Beer's Law. In the presence of ligandin and aminoazo-dye-binding protein A sharper bands of higher intensity ($\epsilon \approx 90 \text{ litre} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$), with maxima at longer wavelengths (400–410 nm) are observed (Fig. 1, curves *d* and *e*). These resemble the spectra of methaemalbumin (Beaven *et al.*, 1974) and ferric horseradish peroxidase

Fig. 1. Effects of ligandin and protein A on the absorption spectra in the Soret region of ferriprotoporphylin IX

Curves (a), (b) and (c) refer to ferriprotoporphylin alone at concentrations of 1, 10 and 100 μM respectively. Curves (d) and (e) are the spectra of 1 μM -ferriprotoporphylin IX in the presence of ligandin (74 μM) and protein A (70 μM) respectively.

Fig. 2. Difference spectra resulting from the binding of ferrous and ferric haem by ligandin and protein A

----, Difference spectra between ferriprotoporphylin IX (5.3 μM)-protein complexes and ferriprotoporphylin IX alone (5.3 μM); —, effect of adding dithionite (final concn. 4 mM) to both cuvettes. (a) Ligandin (34 μM); (b) protein A (22 μM).

(Willick *et al.*, 1969) and almost certainly result from the binding of monomeric haem by the proteins with the concomitant dissociation of dimers. The spectra of the haem-protein complexes shown in Fig. 1 (curves *d* and *e*) refer to protein:haem molar ratios of approx. 70:1, but identical results for both proteins were obtained at higher haem concentrations, corresponding to molar ratios as low as approx. 30:1. This demonstrates that curves (*d*) and (*e*) are the spectra of ferriprotoporphylin IX completely bound to ligandin and protein A respectively. At lower molar ratios (approx. 1–2:1) the intensities of the Soret bands of both haem-protein systems decreased and the maxima moved to shorter wavelengths, indicating that under these conditions a significant fraction of the total haem is unbound.

Fig. 2 shows the effect of the reducing agent dithionite on difference spectra arising from the binding of ferriprotoporphylin IX by ligandin and protein A. In both cases new difference spectra result; it is evident therefore that both proteins are able to bind ferrous as well as ferric haem.

After the addition to ligandin- and protein A-ferriprotoporphylin IX solutions of excesses of bilirubin, bromosulphophthalein or oestrone sulphate, each of which binds to both proteins (Levi *et al.*, 1969; Meuwissen *et al.*, 1972; Kamisaka *et al.*, 1975; Ketterer *et al.*, 1975, 1976*d*), spectra similar to curves (*a*)–(*c*) of Fig. 1 (i.e. spectra of unbound haem) are observed, indicating that both haem-protein complexes are formed reversibly and that haem shares the binding sites of the two proteins with the other ligands.

Previous equilibrium-dialysis measurements (Ketterer *et al.*, 1976*c*) of the displacement by ferriprotoporphylin IX of [^3H]oestrone sulphate bound to ligandin suggested that the nature of the interaction between haem and this protein was different from that of other ligands. The present spectral studies do not support such a conclusion; it seems most likely that the anomalous competition effects of

the previous study were only apparent and resulted from the monomer-dimer equilibrium of unbound ferriprotoporphyrin IX, which was not taken into account.

Determination of binding parameters

Although it appears that ferriprotoporphyrin IX bound to ligandin and protein A is monomeric, it is clear from Fig. 1 that neither bound species gives rise to exactly the same spectrum as that of the unbound monomeric ligand, since curves (a)–(c) are isosbestic at 411 nm with $\epsilon = 32 \text{ litre} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$, whereas curves (d) and (e) are near their maxima at this wavelength ($\epsilon = 84$ and $74.5 \text{ litre} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$ respectively). These differences allow the binding of ferriprotoporphyrin IX by the two proteins to be measured by difference spectrophotometry. It is most satisfactory to carry out titrations at the isosbestic point (411 nm) of spectra of haem alone, since at this wavelength the difference in absorbance (ΔE) between a given protein-haem solution and its protein-free counterpart is linearly related to the concentration of bound ligand, irrespective of the extent of dimerization of the unbound ligand, as long as it is assumed that all bound ligand is characterized by the same spectrum. Concentrations of unbound ligand can be calculated by subtracting bound from total ligand concentration. The approach is summarized by the equations

$$[L]_b = \Delta E / \Delta \epsilon$$

$$[L]_f = [L]_t - \Delta E / \Delta \epsilon$$

where $[L]_t$, $[L]_b$ and $[L]_f$ are as defined in the Experimental section and $\Delta \epsilon$ is the difference in extinction coefficient at 411 nm between bound and free ligand ($\Delta \epsilon = 52 \text{ litre} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$ for ligandin and $42.5 \text{ litre} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$ for protein A). Spectrophotometric titrations of ligandin and protein A with ferriprotoporphyrin IX are shown in Fig. 3. These were carried out at protein concentrations ($5\text{--}8 \mu\text{M}$) sufficiently low to ensure that a significant amount of the total haem present was unbound.

In order to determine the parameters characterizing the binding of monomeric ferriprotoporphyrin IX by the two proteins it is necessary to take into account the dimerization equilibrium which, as Brown *et al.* (1970) have shown, can be written

$$K_D = \frac{[L_2][H^+]}{[L]^2}$$

or at fixed pH

$$K_D^{\text{obs.}} = \frac{[L_2]}{[L]^2}$$

where L and L_2 are monomeric and dimeric haem respectively. The mass-balance for haem is therefore

$$\begin{aligned} [L]_t &= [L]_b + [L]_f \\ &= [L]_b + [L] + 2[L_2] \\ &= [L]_b + [L] + 2K_D^{\text{obs.}} [L]^2 \end{aligned}$$

If $K_D^{\text{obs.}}$ is known then the individual contributions of monomer and dimer to $[L]_f$ can be calculated. By measuring the spectra of a series of solutions of ferriprotoporphyrin IX ($0.1\text{--}200 \mu\text{M}$) over a range of pH, Brown *et al.* (1970) obtained a value of 4.5 for K_D at an ionic strength of 0.10M. Following their method we found $K_D^{\text{obs.}} = 10^7 \text{ litre/mol}$ at pH 7.0, I 0.16M, and this value was used to calculate $[L]$ and $[L_2]$ at each of the titration points of Fig. 3. Scatchard plots referring to monomeric haem were then constructed (Fig. 5).

We also used fluorescence spectroscopy to measure binding. On the addition of haem to solutions of ligandin the native fluorescence of the protein is quenched considerably and the emission maximum changes from 320 to 315 nm. From the dependence on protein concentration of the quenching (Fig. 4), the Scatchard plot shown in Fig. 5 was constructed. This plot again refers to monomeric ligand only. Protein A contains no tryptophan and only two tyrosine residues per molecule (Ketterer *et al.*, 1976d) and consequently displays little native

Fig. 3. Difference-spectrophotometric titrations of ligandin and protein A with ferriprotoporphyrin IX

○, Ligandin (initial concn. $7.2 \mu\text{M}$, final concn. $6.8 \mu\text{M}$);
□, protein A (initial concn. $5.1 \mu\text{M}$, final concn. $4.7 \mu\text{M}$).

Fig. 4. Quenching of the intrinsic fluorescence of ligandin by ferriprotoporphyrin IX

Protein concentrations (μM): 0.024 ($\blacktriangle$); 0.048 ($\square$); 0.12 ($\blacksquare$); 0.60 ($\circ$); 1.2 ($\bullet$). Excitation was at 280nm, emission at 320nm.

fluorescence. For this reason we did not undertake fluorescence-quenching experiments with this protein.

Fig. 5 shows that ligandin and protein A both bind monomeric ferriprotoporphyrin IX at a single site; these results justify the assumption made above that all bound haem is characterized by a single spectrum for both proteins. From the slopes of the Scatchard plots referring to spectrophotometric experiments, we find association constants of 10^7 and 4×10^6 litre/mol for ligandin and protein A respectively. The fluorescence-quenching data give an association constant of 2×10^7 litre/mol for ligandin, which is in reasonable agreement with the spectrophotometrically determined value, bearing in mind the complexity of the system under study.

Thus the affinities of the two intracellular proteins ligandin and protein A for ferriprotoporphyrin IX are slightly lower than that of human serum albumin ($K = 5 \times 10^7$ litre/mol for the primary binding site at pH 7.5; Beaven *et al.*, 1974), one to two orders of magnitude lower than that of rabbit hemopexin (Morgan & Muller-Eberhard, 1972) and approx. 10^{-5} that of human apomyoglobin ($K \approx 10^{12}$ litre/mol

Fig. 5. Scatchard plots for the binding of ferriprotoporphyrin IX by ligandin and protein A

Ligandin: $\circ$, difference-spectrophotometric results; $\bullet$, fluorescence-quenching results. Protein A: $\square$, difference-spectrophotometric results. The slopes and intercepts were calculated by the method of least-square analysis.

Fig. 6. Absorption spectra of ferriprotoporphyrin IX (----) and its ligandin complex (—) in the presence of 4mM-NaCN

The concentration of ferriprotoporphyrin IX was $1 \mu\text{M}$ and the concentration of ligandin was $50 \mu\text{M}$.

Fig. 7. Circular-dichroism spectra of the ferrous and ferric haem complexes of ligandin and protein A

—, Ligandin-ferriprotoporphyrin IX; ····, ligandin-ferroprotoporphyrin IX; ----, protein A-ferriprotoporphyrin IX; - · - ·, protein A-ferroprotoporphyrin IX. The concentrations of haem and protein were 10 and 50 μM respectively in each case. $[\theta]$ refers to haem.

at pH 9.1; Gibson & Antonini, 1963). These comparisons, together with the ease of displacement of bound haem by other ligands (see above), show that, like albumin, neither ligandin nor protein A is a specific haem-binding protein, i.e. ferriprotoporphyrin IX is one of a number of compounds containing hydrophobic moieties that are bound by these two proteins (cf. Litwack *et al.*, 1971; Kamisaka *et al.*, 1975; Ketterer *et al.*, 1975, 1976d).

Co-ordination chemistry

After the addition of CN^- , the Soret band of free ferriprotoporphyrin IX becomes more intense and undergoes a red shift to 422 nm (Fig. 6). In the presence of ligandin (Fig. 6) and protein A, shifts to still longer wavelengths (430 and 426 nm respectively)

are observed. The simplest interpretation of these spectral differences is that CN^- is able to co-ordinate to the iron atom of ferric haem whether the latter is bound or free, i.e. that the formation of co-ordinate complexes in the presence of ligandin and protein A does not necessitate the dissociation of the haem-protein complexes.

In common with free ferriprotoporphyrin IX (Keilin, 1955) and methaemalbumin (Beaven *et al.*, 1974), ferric haem bound to ligandin and protein A does not form spectroscopically distinguishable complexes with N_3^- or F^- under the conditions of our experiments. On the other hand ferric haemoproteins such as methaemoglobin, metmyoglobin, catalase and ferric peroxidase (see, e.g., Smith & Williams, 1970), in which the fifth co-ordination position of the iron atom is co-ordinately linked to an

amino acid residue of the protein, do form complexes with N_3^- and F^- . It may be that neither ligandin nor protein A are able to form co-ordinate bonds with the iron atom of haem; binding to these two proteins would then arise from interactions with the porphyrin moiety only. Methaemalbumin is believed to be formed similarly (Beaven *et al.*, 1974). Further, if the iron atom is not involved in binding it is unlikely that there is much difference between the association constants for ferri- and ferro-protoporphyrin IX.

Circular dichroism

The complexes of ferrous and ferric haem with ligandin and protein A give rise to measurable Cotton effects in the Soret region (Fig. 7). Because of its symmetry, free haem is not optically active and the ellipticities must therefore arise from haem-protein interactions. Induced Cotton effects of similar magnitude have been reported for methaemalbumin (Beaven *et al.*, 1974) and of somewhat greater magnitude (3–10-fold) for haemoglobin (Nagai *et al.*, 1969), myoglobin (Nagai *et al.*, 1969; Willick *et al.*, 1969), horseradish peroxidase (Willick *et al.*, 1969) and leghaemoglobin (Nicola *et al.*, 1975). On theoretical grounds Hsu & Woody (1971) have proposed that the Soret Cotton effects of haemoglobin and myoglobin arise from the interaction of the haem group with nearby aromatic residues of the protein, rather than from, for instance, non-planarity of the bound haem. It is possible that the circular-dichroism spectra shown in Fig. 7 are generated by similar interactions.

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References

- Beaven, G. H., Chen, S. H., D'Albis, A. & Gratzer, W. B. (1974) *Eur. J. Biochem.* **41**, 539–546
- Blauer, G. & King, T. E. (1970) *J. Biol. Chem.* **245**, 372–381
- Brown, S. B., Dean, T. C. & Jones, P. (1970) *Biochem. J.* **117**, 733–739
- Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (eds.) (1969) *Data for Biochemical Research*, 2nd edn., p. 316, Oxford University Press, Oxford
- Falk, J. E. (1964) *Porphyrins and Metalloporphyrins*, p. 46, Elsevier, Amsterdam
- Fleischner, G., Robbins, J. & Arias, I. M. (1972) *J. Clin. Invest.* **51**, 677–684
- Gibson, Q. H. & Antonini, E. (1963) *J. Biol. Chem.* **238**, 1384–1388
- Halfman, C. J. & Nishida, T. (1972) *Biochemistry* **11**, 3493–3498
- Hsu, M. C. & Woody, R. W. (1971) *J. Am. Chem. Soc.* **93**, 3515–3525
- Kamisaka, K., Listowsky, I., Gatmaitan, Z. & Arias, I. M. (1975) *Biochemistry* **14**, 2175–2180
- Keilin, J. (1955) *Biochem. J.* **59**, 571–579
- Ketley, J. N., Habig, W. H. & Jakoby, W. B. (1975) *J. Biol. Chem.* **250**, 8670–8673
- Ketterer, B., Ross-Mansell, P. & Whitehead, J. K. (1967) *Biochem. J.* **103**, 316–324
- Ketterer, B., Tipping, E., Meuwissen, J. A. T. P. & Beale, D. (1975) *Biochem. Soc. Trans.* **3**, 626–630
- Ketterer, B., Srai, K. S. & Christodoulides, L. (1976a) *Biochim. Biophys. Acta* **428**, 638–689
- Ketterer, B., Srai, K. S. & Tipping, E. (1976b) *Biochem. Soc. Trans.* **4**, 202–204
- Ketterer, B., Tipping, E., Beale, D. & Meuwissen, J. A. T. P. (1976c) in *Glutathione: Metabolism and Function* (Arias, I. M. & Jakoby, W. B., eds.), pp. 243–257, Raven Press, New York
- Ketterer, B., Tipping, E., Hackney, J. F. & Beale, D. (1976d) *Biochem. J.* **155**, 511–521
- Levi, A. J., Gatmaitan, Z. & Arias, I. M. (1969) *J. Clin. Invest.* **48**, 2156–2167
- Litwack, G., Ketterer, B. & Arias, I. M. (1971) *Nature (London)* **234**, 466–467
- Meuwissen, J. A. T. P., Ketterer, B. & Mertens, B. B. E. (1972) *Digestion* **6**, 293
- Meuwissen, J. A. T. P., Ketterer, B., Heirwegh, K. P. M. & De Groote, J. (1974) *Biol. Gastroenterol.* **4**, 322
- Mishkin, S., Stein, L., Gatmaitan, Z. & Arias, I. M. (1972) *Biochem. Biophys. Res. Commun.* **47**, 997–1003
- Morgan, W. T. & Muller-Eberhard, U. (1972) *J. Biol. Chem.* **247**, 7181–7187
- Nagai, M., Sugita, Y. & Yoneyama, Y. (1969) *J. Biol. Chem.* **244**, 1651–1653
- Nicola, N. A., Minasian, E., Appleby, C. A. & Leach, S. J. (1975) *Biochemistry* **14**, 5141–5149
- Smith, D. W. & Williams, R. J. P. (1970) *Struct. Bonding (Berlin)* **7**, 1–45
- Tipping, E., Ketterer, B., Christodoulides, L. & Enderby, G. (1976) *Biochem. J.* **157**, 211–216
- Willick, G. E., Schonbaum, G. R. & Kay, C. M. (1969) *Biochemistry* **8**, 3729–3734