Ferritin Iron Uptake and Release

STRUCTURE–FUNCTION RELATIONSHIPS

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Iron uptake and release by ferritin molecules of different iron contents show similar profiles. These are discussed in relation to the structure of the ferritin molecule. Two models of iron uptake and release are considered. One involves iron oxidation–reduction sites on the protein. The other allows direct interaction of reagents with the iron-core crystallites. It is concluded that the second model accounts better for the experimental results presented now and in previous publications.

Ferritin molecules consist of a protein shell (apoferritin) surrounding an inorganic core of microcrystalline hydrous ferric oxide phosphate (Harrison & Hoy, 1973). Ferritin preparations contain mixtures of molecules of various iron content from iron-free apoferritin to full ferritin containing a maximum of 4500 iron atoms (Rothen, 1944; Fischbach & Anderegg, 1965). Ferritin can be formed from apoferritin Fe(II) and an oxidant, and its iron is most readily released by reduction to Fe(II). Chelating agents with a high affinity for Fe(III) may also slowly mobilize ferritin iron (Wöhler, 1963; Pape *et al.*, 1968).

The construction of apoferritin from sub-units with intersubunit channels gives the molecule the properties of a sieve (Harrison, 1964). Fe(II) ions entering the molecule are trapped inside by conversion into polynuclear hydrous ferric oxide. Such polynuclear species forming outside the protein shell would also be unable to enter. Thus redox mechanisms may be required for ferritin iron uptake and release *in vivo*. This hypothesis has been restated by Niederer (1970) as the ‘penetration hypothesis’. In addition, Niederer showed that apoferritin has a catalytic effect on the oxidation of its iron.

In a recent series of papers (Harrison *et al.*, 1967; Macara *et al.*, 1972, 1973*a,b,c*; Hoy *et al.*, 1974*a,b*) we have presented the results of experiments on the uptake and release of iron from horse spleen ferritin. From these we have concluded that (a) ferritin catalyses iron oxidation in such a way that the ferric oxide hydrate product remains inside the molecule, in agreement with Niederer (1970) and Harrison (1964), (b) the main role of apoferritin in this process is to initiate the formation of stable crystal nuclei on to which further hydrous iron oxide can be deposited and (c) the rates at which iron-containing ferritin molecules both

accumulate and release iron depend on the number of iron atoms already present in the molecule and, more specifically, on the amount of surface area on the iron-core crystallites available for uptake or release.

Our ‘crystal growth’ hypothesis has been criticized by Bryce & Crichton (1973) and Crichton (1973), who favour the view that both uptake and release of ferritin iron are controlled solely by oxidation–reduction sites on the protein shell. In the present paper we discuss the two alternative models of ferritin iron incorporation and mobilization and provide further evidence, which, we believe, supports our view that the iron-core crystallite surface is directly involved in these processes. Since ferritin function must be seen in relation to the structure of the molecule, a brief outline is given of the current status of the X-ray analysis of apoferritin, which is proceeding in this laboratory.

Materials and Methods

Horse spleen ferritin (twice-crystallized, cadmium-free) was purchased from Miles Laboratories Inc., Kankakee, Ill., U.S.A. Ferritin fractions of different iron content were obtained by density gradient centrifugation as described by Hoy *et al.* (1974*a*), two density ranges being used to improve resolution. Apoferritin was prepared from ferritin by dialysis against 0.1 M-thioglycollic acid–0.1 M-sodium acetate solutions, pH 4.25, followed by exhaustive dialysis against glass-distilled water and precipitation with 30% (w/v) $(\text{NH}_4)_2\text{SO}_4$.

Iron uptake by ferritin fractions

Equilibrium density gradient centrifugation gives ferritin fractions continuously varying in iron content. Fractions were removed carefully from the gradient tubes from the top with a syringe and densities

obtained by weighing accurately measured volumes. Iron concentrations were obtained from the absorbance at 420nm ($E_{1\text{cm}}^{1\% \text{Fe}} = 100$) and a reasonably accurate estimate of the protein concentration in each fraction was obtained by using previous calibrations of densities as a function of Fe/protein ratios (Fischbach & Anderegg, 1965). The fractions were dialysed against glass-distilled water to remove CsCl and then against 100mM-imidazole buffer, pH 7.4. Volumes were taken such that an equal amount of protein and therefore of ferritin molecules were present in each fraction and the fractions were again mixed over two overlapping density ranges 1.30–1.62 and 1.62–1.77 g/ml. Fractions with buoyant density greater than 1.77 g/ml were not used. The fraction with lowest density included any apoferritin present. Each set of combined fractions was made up to 3.0ml with 100mM-imidazole-HCl buffer and then divided into two equal parts; 0.5ml of a solution containing a 10-fold excess of oxidant (20mg of KIO_3 and 100mg of $\text{Na}_2\text{S}_2\text{O}_3$ in 2ml of water) was added to one set and 0.5ml of water to the other (the oxidant for this set being dissolved molecular O_2). To each set a small volume of a freshly prepared aqueous solution of $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ labelled with ^{59}Fe was added. The total amount of iron added was calculated to give approximately an average of 100 Fe atoms per ferritin molecule. After a period to allow for iron incorporation into the mixed ferritin fractions, solid CsCl was added and the mixture fractionated as before. The new fractions were then analysed for total iron and density and for ^{59}Fe as described by Hoy *et al.* (1974b).

Uptake of iron added stepwise

Ferrous iron was added in successive steps to a solution containing apoferritin and an excess of oxidant ($\text{KIO}_3\text{--Na}_2\text{S}_2\text{O}_3$) in imidazole buffer, pH 7.40, as described by Macara *et al.* (1972). The reaction was followed by measuring absorbance at 520nm in a Beckman DB recording spectrophotometer. The iron was added as increments of 470 Fe atoms per protein molecule and the incorporation was allowed to proceed to completion before more iron was added.

Iron release

Iron was released from ferritin fractions as described by Hoy *et al.* (1974a) with 6.3mM-1,10-phenanthroline at pH 5.0 [wrongly given in Hoy *et al.* (1974a) as 63mM].

Results

Iron uptake by ferritin fractions

The distribution of labelled iron incorporated per molecule is plotted in Fig. 1 as a function of the total iron atoms per molecule present in each fraction.

The results obtained with excess of KIO_3 and those with molecular O_2 as oxidant follow the same trend. The graph shows clearly that molecules do not take up an equal amount of iron (this would give a horizontal straight line). More iron is incorporated into those molecules which contain about 1200 Fe atoms (between one-quarter and one-third of the maximum of 4500) than into those containing more or less iron than this. If the same results are expressed as Fe atoms incorporated per Fe atoms present a different trend is obtained showing that iron atom for iron atom most is deposited into those molecules which contain least iron (Fig. 2).

Fig. 1. Effect of iron content on uptake and release of ferritin iron plotted as iron atoms incorporated or released per molecule of ferritin fraction

●, ○, Uptake of ^{59}Fe by mixtures containing equal numbers of molecules of different iron content. Iron was added as $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$. Oxidant: ●, 10-fold excess of $\text{KIO}_3\text{--Na}_2\text{S}_2\text{O}_3$; ○, molecular O_2 . ■, Initial rate of release of iron from ferritin fractions of different iron content with 1,10-phenanthroline. Bars represent ± 1 s.d. from average values.

Fig. 2. Effect of iron content on uptake and release of ferritin iron plotted as iron atoms incorporated or released per iron atoms present in the fraction

Symbols are as for Fig. 1.

Uptake of iron added stepwise

The initial rates of iron uptake obtained with each increment of iron added are plotted in Fig. 3 as a function of average iron content (i.e. cumulative total of Fe atoms per protein molecule). As in Fig. 1 iron is incorporated preferentially into molecules containing between one-quarter and one-third their full complement of iron.

Iron release

When 1,10-phenanthroline was added to the ferritin fractions a pink colour was obtained immediately. This may be attributed to a small amount of 'loosely bound' iron. The bulk of the iron is released much more slowly. The initial rates of release of this slowly mobilized iron, obtained at constant protein concentration are plotted in Fig. 1 as a function of iron atoms per molecule. It can be seen that the points lie on the same curve as that obtained for iron uptake, with a maximum release for molecules containing about 1200 iron atoms. Attempts were made to determine the amount of 'loosely bound iron' by direct measurement of the initial release and by extrapolation of the slow release to zero time. These results showed clearly that the loosely bound iron was not simply related to protein concentration when different fractions were compared, but was also a function of molecular iron content. At constant protein the amount of rapidly released iron increased, as iron content increased, up to about 1500 iron atoms per molecule. Unfortunately there was too much scatter in the results to establish a clear trend for molecules of higher iron content. In one set of experiments the loosely bound iron followed a pattern

Fig. 3. Initial rates of iron uptake by ferritin molecules of different average iron contents

Iron was added in successive amounts each corresponding to 470 Fe atoms per molecule. Initial rates for each amount added are plotted against cumulative total of iron atoms previously incorporated per molecule.

similar to that found for the more slowly released species.

Discussion

Structure-function relationships in the uptake and release of ferritin iron

Importance of micelle surface. The accumulation of iron by ferritin molecules has been shown in the two different types of experiment described above to depend in a complex way on molecular iron content. Those molecules which contain 1000–1500 Fe atoms, or about between one-quarter and one-third of their total complement, apparently have the greatest avidity for iron. This result is not a quirk due to peculiarities of the non-physiological oxidant, $\text{KIO}_3\text{--Na}_2\text{S}_2\text{O}_8$, since it is also obtained with molecular O_2 as oxidant. Moreover in a completely different experiment Miller & Perkins (1969) found a similar dependence, when studying the transfer of iron from transferrin to ferritin in Tris-HCl buffer at pH 7.4. Ferritins of different average iron contents were produced by partial reductions of native ferritin and the transfer of ferritin iron was mediated by ascorbic acid and AMP. The results of Miller & Perkins (1969), replotted in the manner of Figs. 1 and 3, are shown in Fig. 4. The release experiments show that those molecules which have the highest avidity

Fig. 4. Effect of iron content of ferritin on the transfer of iron from $^{59}\text{Fe}_2$ -transferrin to part-saturated ferritin, obtained by partial reduction

These are the data of Miller & Perkins (1969) but with iron content expressed as iron atoms per molecule.

Fig. 5. Diagrammatic two-dimensional representation of ferritin molecules of different iron content

The protein shell is composed of subunits surrounding a central cavity which contains one or more microcrystals of hydrous ferric oxide, shown as a hexagonal network. The size and number of these microcrystals present per molecule is variable. The 'free surface' of the crystallite is a function of the crystallite size and the extent to which it is covered by protein. In the series (a)–(d) the 'free surface' per molecule (shown as a thick line) first increases and then decreases as indicated in Fig. 6 (curve A). The 'free surface'/crystallite volume ratio (i.e. free surface per iron atom) continuously decreases, however (see Fig. 6, curve B). To account for the observations of Figs. 1 and 2 it is proposed that addition of iron to or removal of iron from ferritin takes place at the iron-core crystallite surface, as summarized in Fig. 8. (e) and (f) represent the possibility that some molecules contain more than one crystallite, so that there may be intranuclear competition for crystal growth and resolution. If the crystallites block entry through the intersubunit channels, growth may cease before the molecule contains its maximum possible complement of iron, as in (f). The hydrous ferric oxide crystallites actually consist of close-packed layers of oxygen with interstitial iron. Note that the iron atoms are structurally and functionally heterogeneous in the sense that those near the surface are more readily released than those in internal sites. Few iron atoms are directly attached to protein.

for iron, also release iron most rapidly to 1,10-phenanthroline. When the results are expressed not as iron uptake or release per molecule but per iron atom then the largest rates are obtained with fractions containing least iron (see Fig. 2).

How can these results be explained? In previous papers we have suggested that uptake and release occurred at the surface of the micelle crystallite and for purposes of computation the latter was treated as a spherical segment inside a spherical protein shell. Such a model would give maximum iron uptake and release with molecules that were half full of iron oxide, if uptake and release occurred only at the plane surface of the segment. A more realistic version of this model is indicated in Figs. 5(a)–5(f). This shows a spectrum of ferritin molecules of different iron con-

tents such as may be found in a ferritin preparation. The drawings, although purely schematic, are based on proposed structures for the iron core consisting of hexagonally close-packed layers with interstitial iron atoms (Towe & Bradley, 1967; Harrison *et al.*, 1967). The relative proportion of surface to total iron atoms is a function of the size and number of crystallites and decreases as the crystallite increases in size [compare molecules of Figs. 5(a)–5(d) and see curve B of Fig. 6]. Because the crystallites are confined inside the protein shell, the amount of free surface decreases as the molecules accumulate more iron [compare Figs. 5(c) and 5(d)]. In some cases complete filling of the cavity may not be possible because the channels are blocked (Fig. 5f). If crystallite surface area is rate-determining in both

Fig. 6. 'Free surface' per molecule (A) and 'free surface per iron atom' (B) plotted as a function of percentage full (triangles in crystallite/total possible triangles per molecule %).

'Free surface' per molecule is the length of the thick edge in Figs. 5(a)–5(d) and the 'free surface per iron atom' is the length of the thick edge/number of triangles in crystallite from Figs. 5(a)–5(d). Compare with Figs. 1 and 2.

iron deposition and mobilization, a complex, but similar, dependence of both processes on iron content would be predicted. Calculations of surface variation for three dimensional models similar to those depicted in Fig. 5 are formidable, but, taking some simple cases, we have shown that maximum free surface area occurs with molecules containing less than 50% of their maximum iron content as has been found experimentally.

Plots representing 'free surface' per molecule and per iron atom as a function of iron atoms per molecule based on the simple two-dimensional models drawn in Figs. 5(a)–5(d) are shown in Fig. 6 (curves A and B respectively). These show trends similar to those in Figs. 1 and 2.

Support for our view that uptake and release occurs at micelle surfaces is provided by other experiments in which ^{59}Fe was added to a ferritin fraction containing ^{56}Fe and then released with thioglycollate (Hoy *et al.*, 1974a). When release of ^{59}Fe was compared with release of total Fe a 'first-in-last-out' principle was observed, provided iron was mobilized soon after addition of the label. After being left for 72h, however, the ^{59}Fe had mixed completely with the ^{56}Fe . This seems to confirm that iron atoms had indeed been added to the crystallite surface but had then migrated through the oxide lattice until complete exchange with Fe(III) already

present had occurred. Massover & Cowley (1973) have suggested that, in addition to surface effects of crystallite growth, entry of iron into lattice defects should be considered. They propose that the oxygen lattice structure is complete before all the iron is added to the micelle and that some iron may be taken up by diffusion into interstitial spaces. Conversely iron may be lost from some interstitial layers more readily than others. Their hypothesis, based on a structure proposed by Towe & Bradley (1967) for ferric hydrolysate (which has some interstitial planes with 'optional' occupancy) leads to the prediction that very full ferritin fractions (>3000 Fe atoms/molecule) should lose iron most rapidly and iron uptake should follow a similar pattern. The dependence we have observed, although not embracing fractions with more than 3750 Fe atoms/molecule is quite different from this.

Alternative models of iron uptake and release. Evidence has accumulated for the involvement of Fe(II)–apoferritin binding sites in the catalytic conversion of Fe(II) into ferric oxide hydrate (Niederer, 1970; Macara *et al.*, 1972, 1973b,c; Bryce & Crichton, 1973). The question to be reconsidered here is what is the nature of this involvement and are these sites also utilized in the reductive release of ferritin iron? Could the rapidly released iron observed by us and by several other workers (Mazur *et al.*, 1955; Jones & Johnston, 1967; Dognin *et al.*, 1973; Sirivech & Osaki, 1973) represent iron attached at these catalytic sites?

It has generally been assumed that the 'loosely bound iron' is attached at sites on the surface of the protein. Mazur *et al.* (1955) proposed that it was attached to thiol groups as Fe(II), since it gave a pink colour with $\alpha\alpha'$ -bipyridyl. This could, however, have resulted from the rapid photoreduction of an Fe(III) complex as occurs with the similar 1,10-phenanthroline complex (Jones & Johnston, 1967). Surface-bound iron was considered to be in equilibrium with the Fe(III) of the inner micelle by Jones & Johnston (1967) and by Dognin *et al.* (1973) following the proposal of Mazur *et al.* (1955). Jones & Johnston (1967) found that the more slowly releasable iron was removed by 1,10-phenanthroline at a rate which was independent of the concentration of the latter and proposed Scheme 1. The rate-determining step is the slow release of iron from the micelles in the interior of the ferritin molecule and its transport to the surface.

A model representing the view that all iron atoms entering or leaving the molecule do so by way of

Scheme 1. Removal of slowly releasable iron by 1,10-phenanthroline

Fig. 7. Model of ferritin iron uptake and release based on the assumption that all iron atoms entering or leaving the molecule do so by way of oxido-reduction sites

The sites $\odot$ are placed in narrow intersubunit channels, which restrict entry and exit to Fe^{3+} ions. Iron atoms at these sites might account for the observed 'loosely bound' iron.

oxido-reduction sites on the protein is drawn in Fig. 7, with the sites placed in the intersubunit channels towards the surface of the molecule. In this model no assumption need be made about the structure of the iron micelle except insofar as this may affect the rate at which iron is released from it or added to it. Logically there seem to be two tacit assumptions underlying this type of mechanism. (1) The channels can be penetrated by Fe(III) ions but not by reducing, oxidizing or chelating agents, otherwise these could react directly with the micelle. (2) There must be some mechanism ensuring that Fe(II) , entering the molecule, once oxidized to Fe(III) , moves inwards rather than outwards since there is no evidence that apoferritin exhibits ferroxidase activity except in relation to ferritin formation.

For this model to be reconciled with the dependence on iron content of both uptake and release processes described in this paper the rate-determining steps in the two processes would have to be respectively addition by iron to or removal of iron from the micelle. If this were the case it would be difficult to explain how release could proceed at such different rates with different reagents and under different conditions (see for example Bielg & Bayer, 1955; Jones & Johnston, 1967; Pape *et al.*, 1968; Sirivech & Osaki, 1973; Hoy *et al.*, 1974a). Phosphate is found attached to the micelles and there is evidence that sucrose and raffinose (F. A. Fischbach, personal communication) and phosphotungstate ions (Van Bruggen *et al.*, 1960) can penetrate into the interior of the molecule. If these can get in, then why should reducing agents like dithionite (Granick & Michaelis, 1943), thioglycollate and perhaps even FMNH_2 (Sirivech & Osaki, 1973) and chelating agents like nitrilotriacetate (Pape *et al.*, 1968) and perhaps deferrioxamine A (Wöhler, 1963), all known to be

Fig. 8. Model of ferritin iron uptake and release based on the assumption that iron atoms and small reagents enter the molecule relatively freely through wide [1–1.5 nm (10–15 Å) diameter] channels

The protein shell contains sites, U, at which crystal growth is initiated, but, once this has started, iron is added directly to the crystallite surface. There are no reduction sites on the protein. Iron release is from the surface of the iron-core microcrystals. Incomplete surface layers might account for the 'loosely bound' iron (LB) which is rapidly released.

capable of removing iron from ferritin, not also enter the molecule and interact directly with the micelle? (If they did, a dependence on iron content similar for iron release with 1,10-phenanthroline would be predicted.) It has been suggested by Niederer (1970) that the inhibition by bivalent cations such as Zn(II) of iron release with dithionite is due to Zn(II) competing for reducing sites. An alternative explanation may well be that these cations are adsorbed on the micelle surface (Macara *et al.*, 1973a). Circumstantial evidence against a special reductive mechanism involving an apoferritin site which has different affinities for different reducing agents (Frieden & Osaki, 1974) is provided by our unpublished observations that reducing agents like dithionite, ascorbate and reduced riboflavins, which release iron from ferritin, also remove iron from ferritin iron cores stripped of protein, from haemosiderin and from $\gamma\text{-FeOOH}$, and at similar relative rates.

It seems unlikely from the above arguments that the 'loosely bound' iron has any special function in iron uptake-release mechanisms. Possibly it represents iron in poorly crystallized regions or incomplete micelle surface layers. An alternative or additional source of rapidly mobilizable iron may indeed be bound to the protein. Apoferritin has been found to bind about five metal ions per subunit, including

the trivalent terbium ion (Macara *et al.*, 1973c). The loosely bound iron observed by us varied from about $\frac{1}{2}$ Fe to 5 Fe released per subunit, although this was not independent of iron content, the lower values being obtained with low iron fractions.

The model of ferritin ion uptake and release which is simplest, and, we think, best fits the observations we have discussed here, as well as kinetic data we have described previously, is depicted in Fig. 8. This model is derived from the model for ferritin formation shown in Fig. 11(a) of Macara *et al.* (1972). It is supposed that the apoferritin shell contains channels that permit the relatively free passage of reducing and chelating agents and provide continuity between the inside and outside of the molecule. If reduced flavin mononucleotide is to enter and ferrioxamine is to leave the molecule, then the channels must be at least 1.3–1.4 nm (13–14 Å) across. Iron might be removed by 1,10-phenanthroline as Fe(II)(phen)₃ [about 1.5 nm (15 Å) in diameter] or as a smaller Fe(II)(phen)aq. complex. Iron-binding sites on the inner surface of the apoferritin shell are involved only in the initial formation of stable hydrous ferric oxide nuclei on to which further iron is added directly [see below and Macara *et al.* (1972)]. Redissolution is by direct interaction of reagent and micelle.

The model of Fig. 8 seems to be supported by a preliminary 0.6 nm (6 Å) resolution electron-density map of horse spleen apoferritin based on a single isomorphous derivative (R. J. Hoare, P. M. Harrison & T. G. Hoy, unpublished work). This shows the molecule as a hollow, nearly spherical shell with an inner cavity of about 6 nm (60 Å) in diameter penetrated by six channels each 1–1.5 nm (10–15 Å) wide lying along the molecular fourfold axes. These channels are probably large enough to allow the passage of riboflavins and ferrioxamine and certainly of S₂O₃²⁻, IO₃⁻, O₂, cysteine, thioglycollate and ascorbate. Two mercury atoms per subunit have been located in the map. They are covalently bound (as *p*-mercuribenzoate) to the protein, presumably to two cysteine thiol groups. One of these lies in a cavern off the fourfold channels. The other lies at the inner end of what appears to be a narrow channel running in from the outer surface but apparently not penetrating through to the inner cavity. Neither of these can really be said to be 'at the surface' as suggested by Mazur *et al.* (1955). The mercury derivative was fully active in iron accumulation so that these cysteine residues do not seem to be part of the 'active site' as suggested by Bryce & Crichton (1973). Moreover a ferritin which contains no cysteine has been isolated from amphibian erythrocytes (Theil, 1973) so this residue may have no function in iron uptake or release.

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References

- Bielig, H.-J. & Bayer, E. (1955) *Naturwissenschaften* **42**, 466–467
- Bryce, C. F. A. & Crichton, R. R. (1973) *Biochem. J.* **133**, 301–309
- Crichton, R. R. (1973) *Struct. Bonding* **17**, 67–134
- Dognin, J., Girardet, J. L. & Chapron, Y. (1973) *Biochim. Biophys. Acta* **297**, 276–284
- Fischbach, F. A. & Anderegg, J. W. (1965) *J. Mol. Biol.* **14**, 458–473
- Frieden, E. & Osaki, S. (1974) in *Metalloproteins* (Friedman, M., ed.), Plenum Press, New York, in the press
- Granick, S. & Michaelis, L. (1943) *J. Biol. Chem.* **147**, 91–97
- Harrison, P. M. (1964) in *Iron Metabolism: An International Symposium* (Gross, F., ed.), pp. 40–56, Springer-Verlag, Berlin
- Harrison, P. M. & Hoy, T. G. (1973) in *Inorganic Biochemistry* (Eichhorn, G. L., ed.), chapter 8, pp. 253–279, Elsevier, New York and London
- Harrison, P. M., Fischbach, F. A., Hoy, T. G. & Harris, G. H. (1967) *Nature (London)* **216**, 1188–1190
- Hoy, T. G., Harrison, P. M., Shabbir, M. & Macara, I. G. (1974a) *Biochem. J.* **137**, 67–70
- Hoy, T. G., Harrison, P. M. & Shabbir, M. (1974b) *Biochem. J.* **139**, 603–607
- Jones, M. M. & Johnston, D. O. (1967) *Nature (London)* **216**, 509–510
- Macara, I. G., Hoy, T. G. & Harrison, P. M. (1972) *Biochem. J.* **126**, 151–162
- Macara, I. G., Hoy, T. G. & Harrison, P. M. (1973a) *Biochem. Soc. Trans.* **1**, 102–104
- Macara, I. G., Hoy, T. G. & Harrison, P. M. (1973b) *Biochem. J.* **135**, 343–348
- Macara, I. G., Hoy, T. G. & Harrison, P. M. (1973c) *Biochem. J.* **135**, 785–789
- Massover, W. H. & Cowley, J. M. (1973) *Proc. Nat. Acad. Sci. U.S.* **70**, 3847–3851
- Mazur, A., Baez, S. & Shorr, E. (1955) *J. Biol. Chem.* **213**, 147–160
- Miller, J. P. G. & Perkins, D. J. (1969) *Eur. J. Biochem.* **10**, 146–151
- Niederer, W. (1970) *Experientia* **26**, 218–220
- Pape, L., Multani, J. S., Stitt, C. & Saltman, P. (1968) *Biochemistry* **7**, 613–616
- Rothen, A. (1944) *J. Biol. Chem.* **152**, 679–693
- Sirivech, S. & Osaki, S. (1973) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **32**, 629
- Theil, E. C. (1973) *J. Biol. Chem.* **248**, 622–628
- Towe, K. M. & Bradley, W. F. (1967) *J. Colloid Interface Sci.* **24**, 384–392
- Van Bruggen, E. F. J., Wiebenga, E. H. & Gruber, M. (1960) *J. Mol. Biol.* **2**, 81–82
- Wöhler, F. (1963) *Acta Haematol.* **30**, 65–87