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The effect of the iron saturation of transferrin on its binding and uptake by rabbit reticulocytes

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Polyacrylamide-gel electrophoresis in urea was used to prepare the four molecular species of transferrin: diferric transferrin, apotransferrin and the two monoferric transferrins with either the *C*-terminal or the *N*-terminal metal-binding site occupied. The interaction of these ^{125}I-labelled proteins with rabbit reticulocytes was investigated. At 4°C the average value for the association constant for the binding of transferrin to reticulocytes was found to increase with increasing iron content of the protein. The association constant for apotransferrin binding was $4.6 \times 10^6 M^{-1}$, for monoferric (*C*-terminal iron) $2.5 \times 10^7 M^{-1}$, for monoferric (*N*-terminal iron) $2.8 \times 10^7 M^{-1}$ and for diferric transferrin, $1.1 \times 10^8 M^{-1}$. These differences in the association constants did not affect the processing of the transferrin species by the cells at 37°C. Accessibility of the proteins to extracellular proteinase indicated that the transferrin was internalized by the cells regardless of the iron content of the protein, since in each case 70% was inaccessible. Cycling of the cellular receptors may also occur in the absence of bound transferrin.

The plasma transferrin molecule, which has two iron-binding sites, exists not only as the iron-saturated and iron-free forms, but also as two monoferric species with either the *N*- or *C*-terminal binding site occupied. At normal plasma iron concentrations random distribution of the metal on the available transferrin binding sites would give rise to a population of molecules of which 50% were apoprotein, 40% monoferric and 10% diferric (Lane, 1975). Although deviations from this random distribution have been reported (Leibman & Aisen, 1979), the predominent species in plasma is apotransferrin, and the major iron species are the monoferric transferrins.

Ferrokinetic studies have usually assumed that plasma transferrin iron is a homogeneous pool, with all atoms equally available to cells (Hosain & Finch, 1964). This implies that iron on monoferric and diferric transferrin would be equally assimilated by cells. However, more recent work has suggested that iron on diferric transferrin is more rapidly taken up by cells than is that on monoferric transferrin, both when the species were compared separately and when the species were in competition for iron donation (Huebers *et al.*, 1981, 1983).

The initial step in the uptake of transferrin iron by cells involves the binding of transferrin to a specific cell-surface receptor (Jandl & Katz, 1963). It is known that the receptor interacts more strongly with diferric transferrin than with apotransferrin, with a 30-fold difference in the apparent association constants (Young & Aisen, 1981). Some data also suggest that partially saturated transferrin preparations, which would be rich in the monoferric transferrin species, bind with an avidity intermediate between those for apotransferrin and diferric transferrin (Kornfeld, 1969). If this is so, it would help to explain the differences in the efficiency of iron delivery from monoferric transferrin and the diferric species.

In the present study we have used polyacrylamide-gel electrophoresis in 6M-urea to separate pure preparations of apotransferrin, diferric transferrin and the two monoferric transferrins. We have examined the interaction of these proteins with the transferrin receptor, by incubating them with reticulocytes at 4°C, and have followed the subsequent uptake of the transferrins during incubation with cells at 37°C.

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; MEM, minimal essential medium.

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Materials and methods

Transferrin purification

Transferrin was prepared from pooled and iron-saturated rabbit plasma by an established method (Baker *et al.*, 1968) modified to use DEAE-Sepharose (Pharmacia Fine Chemicals, Milton Keynes, Bucks., U.K.). This yielded diferric transferrin with an A_{470}/A_{410} ratio >1.4.

Radiolabelling of transferrin

Purified diferric transferrin was labelled with ^{125}I (Amersham International, Amersham, Bucks., U.K.) by using lactoperoxidase (Sigma Chemical Co., Poole, Dorset, U.K.) coupled to CNBr–Sepharose (Pharmacia) by the method of David & Reisfeld (1974); 0.5 mCi of ^{125}I/mg of protein was used, with carrier NaI added to give an I:transferrin molar ratio of 0.5:1. Unbound ^{125}I was removed by passage of the protein through Dowex 1-X8 (BDH, Romford, Essex, U.K.) equilibrated with 25 mM-Hepes, pH 7.5. Final specific radioactivity was in the range 200–300 c.p.m./ng of protein.

Preparation of the transferrin species

^{125}I-labelled diferric transferrin was dialysed for 3 days against three changes of 0.1 M-sodium citrate/acetic acid, pH 4.5. Citrate was then removed by exhaustive dialysis against deionized double-distilled water. The apotransferrin thus prepared was removed to an acid-washed tube, and a $Fe(III)Cl_3$–citrate solution (Fe:citrate molar ratio 1:20, pH 6.0) was added, together with $NaHCO_3$ and a Chelex 100 (Bio-Rad Laboratories, Watford, Herts., U.K.)-treated Hepes solution, pH 7.5, to give 10 mM-$NaHCO_3$ and 25 mM-Hepes. The final saturation of the transferrin with iron was approx. 50%.

Transferrin prepared as above was subjected to polyacrylamide-gel electrophoresis in 6 M-urea, as described previously (Makey & Seal, 1976; Young, 1982), but in preparative gel rods (10 mm diam.) with approx. 2 mg of protein applied to each rod. All apparatus used was acid-washed, and all chemicals were treated with Chelex 100 to decrease the contamination with iron. After electrophoresis for 1800 V-h the gels were examined visually and the separated molecular species of transferrin were excised from the gel. The gel between the origin and the slower migrating monoferric transferrin was used as the source of apotransferrin. The fractionated gels were homogenized in Chelex 100-treated 25 mM-Hepes in an acid-washed Dounce homogenizer. The samples were left for 2 h on ice, centrifuged at $2000g_{av.}$ for 10 min, and the supernatants were subjected to vacuum dialysis against 25 mM-Hepes, pH 7.5, to concentrate them and to remove the urea. Final protein concentrations were calculated from the known specific radioactivity of the original diferric transferrin preparation. The effectiveness of the preparative-scale separation of the transferrin species was checked by analytical-scale polyacrylamide-gel electrophoresis in urea. The four fractions containing apotransferrin, monoferric transferrin (*C*-terminal iron), monoferric transferrin (*N*-terminal iron) and diferric transferrin were subjected to this analytical procedure both before and after their incubation with reticulocytes as described previously (Young, 1982). The gels were sliced into 1 mm slices with a lateral gel slicer, and each slice was counted for radioactivity in the gamma-counter.

Reticulocytes

Reticulocytosis was induced in New Zealand White rabbits by the subcutaneous administration of 6 mg of neutralized phenylhydrazine hydrochloride (BDH)/kg body wt. daily for 5 days. After a further 4 days the animals were bled from the peripheral ear vein into heparinized tubes. Cells were washed three times in cold phosphate-buffered saline (Dulbecco & Vogt, 1954). They were then subjected to three 15 min incubations in MEM/Hepes (Gibco Europe, Uxbridge, U.K.) containing 1% bovine serum albumin (Sigma, Fraction V) at 37°C to remove endogenous transferrin (Hemmaplardh & Morgan, 1974). Reticulocyte numbers were estimated on a dry smear after staining with New Methylene Blue, and a total cell count was obtained with a Coulter counter.

Incubations

Titrations with ^{125}I-labelled transferrin. Methods for the measurement of the binding of transferrin by cells have been described previously (Young & Aisen, 1980, 1981; Bomford *et al.*, 1983). Briefly, duplicate samples of reticulocytes were incubated at 4°C in MEM/Hepes with 1% bovine serum albumin, 50 μM-desferrioxamine mesylate (CIBA Laboratories, Horsham, Sussex, U.K.) and a range of concentrations of ^{125}I-labelled diferric transferrin (0.05–10 μg/ml), apotransferrin (1–200 μg/ml) and each of the two monoferric transferrins (0.2–50 μg/ml). The presence of the chelating agent was necessary to retain the transferrin species at their original saturations with iron during the incubation (Young & Aisen, 1981). A parallel incubation in the presence of 1 mg of unlabelled diferric transferrin/ml was performed in each case to estimate non-specific binding, which was always less than 5% of the total binding. The final reticulocyte count in the incubations was 2.5×10^8/ml.

After incubation under air/CO_2 (19:1) at 4°C for 2 h the suspensions were layered on to 0.5 ml of cold

silicone oil (sp. gr. 1.04) (Aldrich Chemicals, Gillingham, Dorset, U.K.) and centrifuged for 30s at $9000g_{av.}$ in an Eppendorf model 7412 micro-centrifuge. The cell supernatants above the oil were aspirated, and subjected to analytical polyacrylamide-gel electrophoresis in urea. The surface of the oil was washed twice with 0.1M-NaOH to remove residual protein. This method of separating the cells and medium was used to avoid potential errors owing to different rates of loss of the transferrin species from the cell surface when using aqueous washing procedures. Cell pellets were then taken for radioactivity counting in an LKB Compugamma counter.

Titrations with diferric transferrin were also performed with reticulocytes that had not been subjected to the preincubations to remove endogenous transferrin, but rather were preincubated at 4°C in MEM/Hepes.

Cellular uptake of transferrin. Reticulocytes (2.5×10^8/ml) were incubated in triplicate in MEM containing 50 μM-desferrioxamine mesylate with 10 μg of purified ^{125}I-labelled apotransferrin, monoferric transferrin (*C*-terminal iron) and diferric transferrin/ml at 37°C and at 4°C with and without 1mg/ml of unlabelled diferric transferrin in order to correct for non-specific binding. Samples were then taken after 35min, and centrifuged through silicone oil as described above. The oil was aspirated and the cells were suspended in MEM containing 0.25% (w/v) Pronase (Calbiochem, Bishops Stortford, Herts., U.K.). After incubation for 30min at 4°C, silicone oil (0.5ml) was inserted under the cell suspension and the cells were separated by centrifugation at $9000g_{av.}$ for 30s. Both cell pellets and supernatants were retained for gamma-counting.

Results

The adaptation of the technique of polyacrylamide-gel electrophoresis in urea to a preparative scale described here gave a clear separation of the four transferrin species. The homogeneity of each preparation was demonstrated by analysis of the separated species, both before and after incubation with reticulocytes at 4°C. As the preparations were not altered by incubation with the cells, only the analyses after incubation are shown. After incubation with the cells for 2h at 4°C, 93% of the protein in the apotransferrin preparation shown, remained iron-free (Fig. 1*a*), with only small traces of the monoferric species detectable. The diferric transferrin in this experiment was similarly homo-

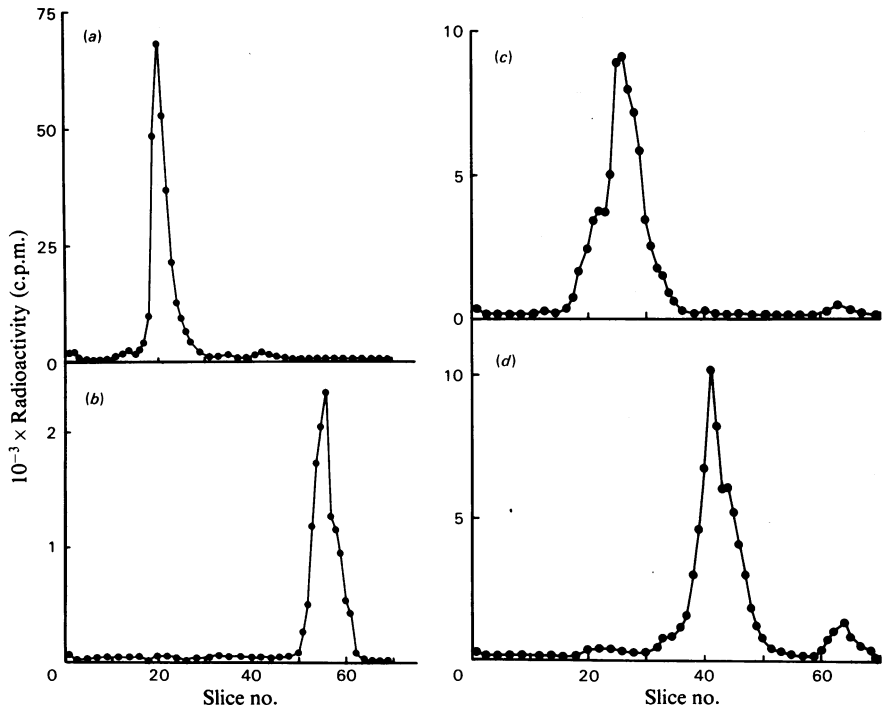

Fig. 1. *Analytical urea/polyacrylamide-gel electrophoresis of purified ^{125}I-labelled rabbit transferrin species*
Gels were sliced into 1mm slices and the radioactivity in each slice was measured. (*a*) Apotransferrin; (*b*) diferric transferrin; (*c*) monoferric transferrin (*C*-terminal iron); (*d*) monoferric transferrin (*N*-terminal iron).

geneous, with 91% of the radioactivity in the diferric-transferrin peak (Fig. 1*b*). The monoferric-transferrin preparations had at least 80% of the transferrin present as the required monoferric species (Figs. 1*c* and 1*d*). The *N*-terminal monoferric preparations contained a small amount (11%) of diferric transferrin, whereas the *C*-terminal preparations contained some apotransferrin.

When protein preparations such as those described were titrated against reticulocytes at 4°C, different values of the association constants for the binding of each species were obtained. Scatchard (1949) analysis of the data revealed that the number of sites to which each species bound at saturation was similar (Fig. 2). The slopes of the Scatchard plots gave values for the association constants of $2.9 \times 10^6 M^{-1}$ (apotransferrin) (Fig. 2*a*), $2 \times 10^7 M^{-1}$ (*C*-terminal monoferric), $2.7 \times 10^7 M^{-1}$ (*N*-terminal monoferric) (Fig. 2*b*) and $2.1 \times 10^8 M^{-1}$ (diferric) (Fig. 2*c*). Results from a series of experiments using different preparations of transferrins and cells gave values of $1.08 (\pm 0.35) \times 10^8 M^{-1}$ for diferric transferrin (mean ± S.D.; $n = 4$), $4.6 (\pm 1.8) \times 10^6 M^{-1}$ for apotransferrin ($n = 4$), $2.8 \times 10^7 M^{-1}$ for *N*-terminal monoferric transferrin (average; $n = 2$) and $2.5 \times 10^7 M^{-1}$ for *C*-terminal monoferric transferrin (average; $n = 2$). The average association constants for the different species were in the proportions 1:5.3:6.1:23.3 (apotransferrin:*C*-terminal monoferric:*N*-terminal monoferric:diferric). It was apparent that occupation of the iron-binding sites of transferrin influenced the binding of the molecule to cells. An investigation was therefore made of the effect of iron on the uptake of transferrin by cells at 37°C.

After incubation at 37°C for 35 min a different amount of each transferrin species was associated with the cells (Table 1). The concentration of the ligands used (10 μg/ml) was insufficient to achieve saturation of the available binding sites, and so the amount bound probably reflects the differences in the association constants for binding to the cells. When these cells were treated with 0.25% (w/v) Pronase at 4°C, a similar proportion (about 70%) of each ligand was inaccessible to the enzyme, indicating that all three transferrin species were internalized by the cells to a similar extent (Table 1). In contrast, when the cells were initially incubated at 4°C, over 70% of the cell-associated proteins remained accessible to the Pronase, indicating that they had remained on the cell surface. These results indicate that, although under the non-saturating conditions used different amounts of each transferrin species were bound by the cells, the degree of internalization was the same regardless of the iron content of the transferrin.

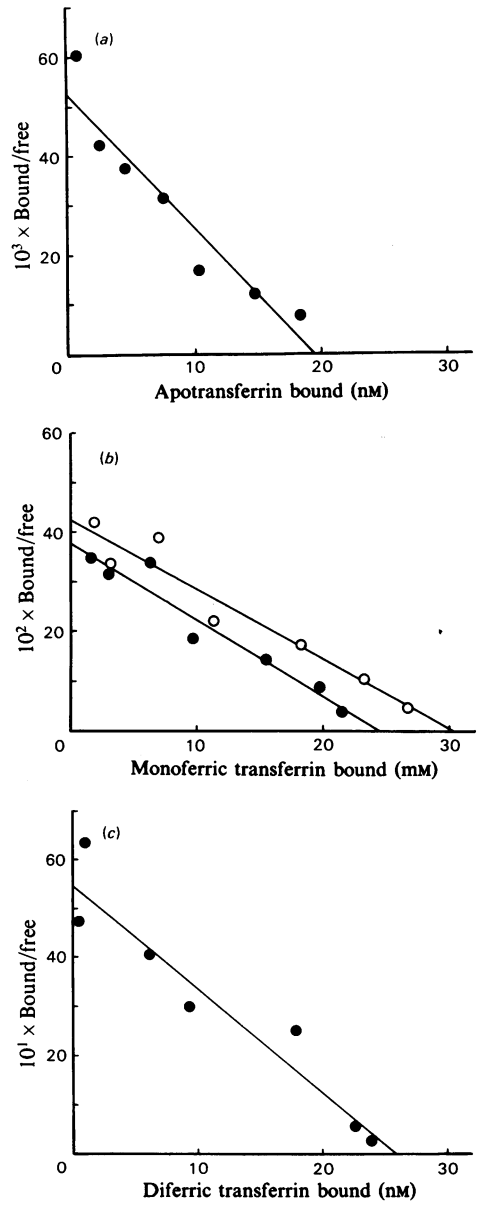

Fig. 2. *Scatchard analysis of the binding of ^{125}I-labelled transferrin by rabbit reticulocytes at 4°C*

The four molecular species of transferrin were prepared by polyacrylamide-gel electrophoresis in urea, and then their binding with increasing concentration to reticulocytes was measured. Binding data was corrected for non-specific binding and plotted by the method of Scatchard (1949): linear-regression analysis was used to find the line of best fit. The values for the association constants derived from the experiment shown are as follows: (*a*) apotransferrin, $2.9 \times 10^6 M^{-1}$; (*b*) monoferric transferrin (*C*-terminal iron; ○), $2 \times 10^7 M^{-1}$ and monoferric transferrin (*N*-terminal iron; ●), $2.7 \times 10^7 M^{-1}$; (*c*) diferric transferrin, $2.1 \times 10^8 M^{-1}$.

Table 1. *Pronase accessibility of transferrin bound by reticulocytes at 4°C and 37°C*

Reticulocytes (2.5×10^8/ml) were incubated at 4°C and 37°C for 35min in triplicate with 10μg of the purified ^{125}I-labelled transferrin species/ml. After centrifuging through silicone oil, the cells were resuspended in MEM containing 0.25% (w/v) Pronase and incubated for 30min at 4°C. The cells were again separated by centrifugation through oil, and both the cells and Pronase supernatants were counted for radioactivity to estimate Pronase-accessibility of the cell-associated ligands.

Ligand	Transferrin associated with cells at 37°C (ng/10^6 cells)	Percentage Pronase-accessible	
		4°C	37°C
Apotransferrin	7.5	73.9	35.7
Monoferric (*C*-terminal) transferrin	11.4	75.2	25.4
Diferric transferrin	16.7	78.7	32.7

Since the iron status of the transferrin did not appear to influence internalization, we decided to investigate whether unoccupied receptors were turned over by this endocytotic cycle. Cells were preincubated and washed at 37°C in transferrin-free medium to deplete the whole receptor population of transferrin (Hemmaplardh & Morgan, 1974). A second sample of cells was preincubated and washed at 4°C to deplete only the cell-surface receptor population and to prevent the exocytosis of intracellular receptor/transferrin complexes. Both sets of cells were titrated at 4°C with increasing amounts of ^{125}I-labelled diferric transferrin. The amount bound by the cells washed at 37°C was 100 ± 2ng/10^7 cells (mean + S.D.; $n = 3$) and cells washed at 4°C bound 94 ± 4ng/10^7 cells, indicating that both sets of cells had the same number of binding sites present on the surface.

Discussion

Much information on the function of transferrin has come from experiments on iron exchange *in vivo*. Interpretation of this type of data assumes that there is an homogeneous pool of transferrin iron which exchanges with the erythron and other tissues (Hosain & Finch, 1964). This assumption, however, may be incorrect. The early experiments of Fletcher & Huehns (1967, 1968) suggested that the two iron-binding sites on each transferrin molecule were functionally distinct and delivered iron to different tissues.

More recent studies have shown that the main dissimilarity may be between transferrin molecules containing one and two iron atoms. Iron delivery to reticulocytes from the diferric species was shown to be approx. 7 times that from the monoferric species when the two types of molecule were in competition at the same concentration (Huebers *et al.*, 1983). The results described here help to explain why this is the case, for we have shown that at 4°C diferric transferrin binds to its receptor with an affinity which is about 4 times that of the monoferric species. Although these values for the association constants will not hold at 37°C, the ratio of the binding constants is likely to be the same at the higher temperature. This argument is supported by the finding that at 37°C, with rat hepatocytes and rat transferrin, diferric transferrin binds with an association constant some 30 times that of apotransferrin (Young & Aisen, 1981). In the present experiments at 4°C, albeit with a different cell type, we found that apotransferrin bound with an association constant one-twenty-third of that for the binding of diferric transferrin. Thus, although the absolute values of the constants may vary with temperature, the relative avidity for binding remains similar. At 37°C, therefore, it can be expected that when monoferric and diferric transferrins are present at the same concentration, about four times as much diferric transferrin will be bound. As each diferric molecule will carry twice as much iron to the cell, the relative rates of iron delivery will be 8:1, a value in good agreement with the value of 7:1 found experimentally by Huebers *et al.* (1983).

The similar affinities of the two monoferric transferrins for the cell receptor suggest that these two species will deliver iron to cells at similar rates, and indeed this has been found to be the case when purified preparations of the two monoferric transferrins have been compared (van der Heul *et al.*, 1981). The low affinity of the apoprotein for the transferrin receptor suggests that, in spite of the preponderance of this species in normally saturated plasma, it probably does not interfere greatly in the iron-delivery process.

In spite of the influence of iron on the association constant of the different species for the transferrin receptor at 4°C, little effect of the iron on endocytosis of the protein was found. The same proportion (approx. 70%) of each protein species was inaccessible to Pronase after incubation at 37°C. This suggests that the strength of binding of the protein to the receptor does not trigger endocytosis, but that the receptor is internalized with whatever ligand is in occupation. It is unclear

whether the unoccupied receptor is also cycled into and out of the cell, but this is likely to be the case, since, if it were not, the incubation of the cells at 37°C, to deplete endogenous transferrin, would have driven all the receptors to the cell surface, but this was not found to be the case. Preincubation did not appear to influence the number of cell-surface receptors on the reticulocyte. Other work using rabbit reticulocytes has suggested that 90% of cell-associated transferrin is intracellular (Iacopetta & Morgan, 1983), and experiments using cultured human cells provided evidence that internalized transferrin remains associated with its receptor (Lamb *et al.*, 1983). The distribution of the receptor between the surface and the interior of these cells remained constant under different conditions of preincubation. These findings, taken with those reported here, suggest that incubation of reticulocytes in the absence of transferrin does not affect the internalization of the receptor and that, even under these conditions, only a minority of transferrin receptors are present on the cell surface.

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References

Baker, E., Shaw, D. C. & Morgan, E. H. (1968) *Biochemistry* **7**, 1371–1378

Bomford, A. B., Young, S. P., Nouri-Aria, K. & Williams, R. (1983) *Br. J. Haematol.* **54**, 93–101

David, G. S. & Reisfeld, R. A. (1974) *Biochemistry* **13**, 1014–1021

Dulbecco, R. & Vogt, M. (1954) *J. Exp. Med.* **99**, 167–199

Fletcher, J. & Huehns, E. R. (1967) *Nature (London)* **215**, 584–586

Fletcher, J. & Huehns, E. R. (1968) *Nature (London)* **218**, 1211–1214

Hemmaplardh, D. & Morgan, E. H. (1974) *Biochim. Biophys. Acta* **373**, 84–89

Hosain, F. & Finch, C. A. (1964) *J. Lab. Clin. Med.* **64**, 905–912

Huebers, H., Csiba, E., Josephson, B., Huebers, E. & Finch, C. A. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 621–625

Huebers, H. A., Csiba, E., Huebers, E. & Finch, C. A. (1983) *Proc. Nat. Acad Sci. U.S.A.* **80**, 300–304

Iacopetta, B. J. & Morgan, E. H. (1983) *J. Biol. Chem.* **258**, 9108–9115

Jandl, J. H. & Katz, J. H. (1963) *J. Clin. Invest.* **42**, 314–326

Kornfeld, S. (1969) *Biochim. Biophys. Acta* **194**, 25–33

Lamb, J. E., Ray, F., Ward, J. H., Kushner, J. P. & Kaplan, J. (1983) *J. Biol. Chem.* **258**, 8751–8758

Lane, R. S. (1975) *Br. J. Haematol.* **29**, 511–520

Leibman, A. & Aisen, P. (1979) *Blood* **53**, 1058–1065

Makey, D. G. & Seal, U. S. (1976) *Biochim. Biophys. Acta* **453**, 250–256

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672

van der Heul, C., Kroos, M. J., van Noort, W. L. & van Eijk, H. G. (1981) *Clin. Sci.* **60**, 185–190

Young, S. P. (1982) *Biochim. Biophys. Acta* **718**, 35–41

Young, S. P. & Aisen, P. (1980) *Biochim. Biophys. Acta* **633**, 145–153

Young, S. P. & Aisen, P. (1981) *Hepatology* **1**, 114–119