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Differential Sedimentation-Velocity and Gel-Filtration Measurements on Human Apotransferrin and Iron–Transferrin

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Differential measurements of sedimentation velocity showed that binding of 2 atoms of iron per molecule of human apotransferrin caused an increase in $s^0_{20,w}$ of about 1.8%. Gel-filtration experiments to compare the elution volumes of apotransferrin and transferrin radioactively labelled with iron showed that binding of the first atom to a molecule produced a decrease in Stokes radius of about 0.7%, and the binding of a second atom an equal decrement. These results confirmed that saturation of human transferrin with iron alters the conformation sufficiently to produce detectable changes in the hydrodynamic properties. They also indicate that the local changes brought about by successive addition of 2 atoms of iron are very similar, if not identical.

The biological importance of the transferrins, which convey iron in a stable chemical form to the sites of biosynthesis of proteins such as haemoglobin, has been recognized in the numerous studies that have been made on them. Besides biological investigations there have been many chemical and physicochemical investigations, especially ones employing spectroscopic methods. Although the binding of many metals other than iron has received attention, the goal of most of the studies has been to throw light on the structure and functioning of the iron complexes.

Apart from the visible development of colour that occurs when ferric iron attaches to apotransferrin, various other changes have been demonstrated. In particular, Bezkorovainy (1966*b*) claimed that there were changes of 1–2% in the sedimentation coefficients, translational diffusion coefficients and intrinsic viscosity when human apotransferrin became saturated with iron. No assessment of the errors involved in measuring these small changes was given, so that it was difficult to be sure of the reality of the differences. Aisen *et al.* (1970) found differences at finite concentrations between the sedimentation coefficients of transferrins free from, and half-saturated with, iron, but could not exclude the possibility that values extrapolated to zero concentration would be identical.

The present work was undertaken because the technique of making measurements of differential sedimentation velocity (Schumaker & Adams, 1968) afforded an opportunity to resolve these uncertainties, and the very strong binding of iron (available in radioisotopic forms) suggested that gel filtration, which is governed by Stokes radius and hence related to translational diffusion, could give valuable supplementary information.

MATERIALS AND METHODS

Preparation of human transferrin. Three batches of protein, isolated in different ways, were made as follows.

(1) A commercial sample (Behringwerke, Marburg-Lahn, Germany; lot no. 1168), which seemed analytically to be satisfactory except that it contained some heavier, presumably polymeric, material, was obtained in monomeric form by passage through Sephadex G-150 (Pharmacia Fine Chemicals, Uppsala, Sweden). About 300mg, dissolved in and dialysed against 0.3M-NaCl–50mM-tris–HCl buffer, pH7.2, was applied to a column (2.2cm×70cm) which had been prepared in the same solvent. A flow rate of 14ml/h was maintained by a Perpex peristaltic pump (LKB-Produkter AB) and 4.0ml fractions were collected after monitoring the effluent by an LKB Uvicord at 253.7nm. The later fractions from the main peak, representing about 50% of the original protein, when pooled gave a solution that was concentrated enough for many experiments, but a portion was dialysed under pressure in the same buffer to provide a stock for some of the sedimentation work.

(2) Pooled human serum was fractionated with $(NH_4)_2SO_4$ as described by Charlwood (1969). The volume of the filtrate was reduced to about one-quarter by dialysis under pressure against the buffer used for Sephadex G-150 and 50ml portions containing about 5g of protein were applied to a column of Sephadex G-150 (6.0cm×90cm) in the same solvent. The effluent, which flowed at 40–45ml/h, was monitored as before and collected in 9.0ml fractions. Two main peaks emerged, the second one consisting largely of albumin and transferrin as judged by disc electrophoresis (see below for

experimental conditions). Approximately 3.7g of this protein was obtained in 54ml by dialysis, initially under pressure, against 50mM-glycine–17mM-tris, pH8.7. Part (9ml) was used for preparative electrophoresis in polyacrylamide gel in the apparatus of Brownstone (1969). The gel (8%; 9.4cm diam.×4.6cm long) was made from Cyanogum 41 (British Drug Houses Ltd.) and treated in the usual way (Charlwood, 1969). It was possible to begin buffer circulation after half an hour because the protein had by then entered the gel. The albumin, which emerged first, was discarded and the material from the second peak was divided into a main portion and a leading shoulder. Corresponding fractions from the rest of the Sephadex G-150 product were obtained and pooled with these. The work involved was much reduced by the use of larger gels having treble the capacity (A. D. Brownstone, unpublished work). As disc electrophoresis showed that the leading shoulder was less pure transferrin than the remainder, the latter only was used for the final stage. It was dialysed, at first under pressure, against 0.12M-tris–HCl, pH8.0, and applied to a column (1.6cm×70cm) of DEAE-Sephadex A-50 (Pharmacia) equilibrated with this buffer. Elution at 14ml/h was carried out first by a gradient from a mixing device (Hegenauer, Tartof & Nace, 1965), which originally had 85ml of the 0.12M buffer in its inner compartment and 160ml of 0.16M-tris–HCl, pH8.0, in the outer. Elution was then completed with the 0.16M buffer. Monitoring of the effluent as before showed one main peak, the central (3.0ml) fractions of which were pooled and concentrated in the same way as part of batch 1.

(3) Serum was fractionated by salt precipitation in the same manner as for batch 2. The filtrate was dialysed against water to remove precipitant and then against 25mM-succinic acid–NaOH, pH5.45, before multimembrane electro-decantation (Fleetwood & Milne, 1967). The solution was pumped through the cell at 50ml/h with 350mA current passing and the cooling jackets were maintained at 3–4°C. For the first 3h the liquid flowing out of the top of the cell was fed back into the reservoir to improve the separation, but subsequently it was collected, dialysed against distilled water and freeze-dried. This product was stored at 4°C until required for the final stage of purification, which was the same as for batch 2 (chromatography on DEAE-Sephadex).

Quality of transferrins. The final products, as well as some intermediate samples, were checked by disc electrophoresis under conditions which were the same as previously used (Charlwood, 1969) except that the gel was of uniform strength (5%). Each of the three batches gave only a single band.

To test whether contamination by haem compounds had been avoided the E_{465}/E_{405} ratios were measured for the iron-saturated proteins. Satisfactory values, close to 1.4, were obtained.

Subsequently the sedimentation experiments showed that no significant amounts of stable polymer were present. This was confirmed by gel filtration, which also provided evidence for freedom from extraneous proteins.

Removal of iron. This was achieved by dialysis of the transferrins against changes of 10mM-EDTA–50mM-sodium acetate–acetic acid, pH4.8. This buffer was then replaced by the buffer used for all the sedimentation and Sephadex G-100 experiments (0.3M-NaCl–50mM-tris–HCl, pH8.0). Several successive small volumes were used to decrease the chances of fresh iron being acquired by the protein. When it was necessary to remove the transferrin from the dialysis bag to add a trace of radioactive iron, further dialysis was always done in tubing that had been through the EDTA treatment.

Attachment of iron. Ferric iron was added as the citrate or nitrilotriacetate. A stock solution of iron was prepared by dissolving a known weight of ferric nitrate crystals in water, adding the requisite volumes of sodium nitrilotriacetate and $NaHCO_3$ solutions, and diluting with water to give a final solution containing 50μg of iron/ml and about 1.5 molecules each of nitrilotriacetate and bicarbonate per atom of iron. The final pH was about 7. Suitable portions of this stock were dried in a desiccator to provide small amounts of solid, which could be dissolved in solutions of apoprotein to saturate it with iron. Although it was obvious from the development of the characteristic pink colour that uptake was rapid, to ensure that saturation was reached rather more than the amount of iron theoretically necessary was used and at least 2h was allowed before ultracentrifugation was begun.

^{59}Fe (as the citrate) and ^{55}Fe (as the chloride in 0.1M-HCl) were both obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Trace-labelling was carried out by adding a small amount of the ^{59}Fe solution to apoprotein contained in some of the buffer used for Sephadex G-100 work, to which had been added a small amount of $NaHCO_3$. Iron-saturated labelled transferrin was prepared similarly by adding to the apoprotein a mixture of ^{55}Fe and stock iron solution containing more than enough iron for saturation. For many column experiments it was necessary to dialyse the labelled material because the presence of residual chelating agent might have promoted exchanges (Bates, Billups & Saltman, 1967*a*,*b*; Aisen & Leibman, 1968) that would have vitiated the conclusions.

Differential sedimentation. Velocity measurements were all done at 59780rev./min in a Spinco model E ultracentrifuge with temperature control operating at 25°C. Records were made with schlieren optics, which included a Wratten 77A filter to isolate light of λ 546nm, and a phase plate. The first photograph in each experiment was taken when the peaks had separated from the menisci and ten photographs were recorded at intervals, generally of 8min.

Concentrations of protein were measured refractometrically, a specific refractive increment of 0.0018 being assumed. Below 1% concentration of protein differential measurements of sedimentation velocity were made in two 12mm cells, one of which had a wedge window. Apoprotein above 1% concentration was put in a 3mm cell and compared with a solution of approx. 0.5% albumin or transferrin in a 12mm cell with a wedge window. The contents of the 3mm cell were then replaced by an equivalent solution of iron-saturated transferrin, and the contents of the 12mm cell were thoroughly remixed before the second comparison was made.

Each photograph was measured twice by two observers, and the mean of the four estimates of the position of the maximum of the peak used in the calculations.

Diffusion measurements. These were done in the ultracentrifuge, at 4059rev./min, as previously described (Charlwood, 1969).

Gel filtration. All analytical experiments were done on a column (1.5cm×65cm) of Sephadex G-100 in the buffer specified above. Samples, which generally contained 2–3mg but in some instances up to 6mg of protein in 0.5–0.8ml, were introduced into the column under gravity and eluted by the pump operating at 7.2ml/h. Monitoring was generally done as before, and fractions were collected at intervals of 10min by means of an automatic timer. Tests were made to check the reproducibility of fraction size.

The protein concentrations of the fractions in the transferrin peak were obtained by measuring extinction at 280nm in micro-cells in a Unicam SP.500 spectrophotometer, appropriate solvent blanks being taken into account. When the main peak was due to apoprotein the composition of the mixture was such that the contribution to the extinction made by other transferrin species was always less than 5% of the total, generally much less. The volume at which a peak emerged from the column was calculated in the way described in the Calculations and Results section.

To calibrate the column a mixture was made of pig thyroglobulin (Brownstone, 1969), ovalbumin (Worthington Biochemical Corp.; lot no. 0A568) labelled with ^{131}I (see Charlwood, 1963*a*), bovine pancreatic ribonuclease (Koch–Light 41083) similarly labelled with ^{125}I, apotransferrin and free [^{125}I]iodide. The positions of the peaks of thyroglobulin and transferrin were determined by measurement of extinction, the other substances being located by counting of radioactivity (see below). The concentration of ovalbumin was sufficiently low not significantly to affect any of the measurements of extinction where slight overlap occurred with the transferrin peak. Separate tests were made to confirm that the location of iodine radioactivity was a reliable measure of the positions of both ovalbumin and ribonuclease. Another separate experiment was done to compare the measure of the internal volume of the column given by the free iodide with that given by an uncharged substance of small molecular weight, iodoacetamide, which was determined by extinction at 280nm.

Counting of radioactivity. Samples containing ^{59}Fe, ^{131}I or ^{125}I alone were measured, with automatic background subtraction, in a Packard Autogamma counter. For ^{55}Fe, either alone or in a mixture with ^{59}Fe, 0.5ml of the fraction was mixed with 4.0ml of emulsifier, consisting of 1 part (by vol.) of Beckman Bio-Solv (BBS-3) and 3 parts of scintillator, and then 4.0ml of scintillator added. This contained 0.5% 2,5-diphenyloxazole and 0.005% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in AnalaR toluene. The mixture was counted in a Beckman scintillation counter (LS 200B) by using the '^{3}H with ^{14}C' channel for ^{55}Fe and the '^{32}P with ^{3}H' channel for ^{59}Fe. Background subtraction was arranged, and a correction made for the cross-over (about 14%) of ^{59}Fe into the ^{55}Fe channel. The importance of this correction was decreased by ensuring that the initial ratio of $^{55}Fe/^{59}Fe$ (c.p.m.) was at least 2:1. Unmodified, this procedure for scintillation counting gave values that fell with time, possibly owing to adsorption or precipitation of the protein to which the iron was bound. This problem was overcome by decreasing the pH of the sample to about 4 by adding about 0.09ml of 0.1M-HCl before emulsifying, thereby releasing the iron from the protein. It was thus possible to avoid the more elaborate methods sometimes found necessary for dual counting of iron isotopes in the presence of protein (e.g. Katz, Zoukis, Hart & Dern, 1964).

CALCULATIONS AND RESULTS

Differential sedimentation. Calculations were based on the first method of Schumaker & Adams (1968). The computations gave the regression of

$$(1-kc_0)\times\ln[\{(r/r_0)^2-kc_0\}/(1-kc_0)]$$

on t, which equals $2\omega^2 s^{c0}$. [r = radial position of peak at time t; r_0 = initial (i.e. meniscus) position; ω = velocity of rotation (rad/s); c_0 = initial concentration of protein; k = coefficient of linear dependence of sedimentation coefficient, defined by $s^{c0}=s^0(1-kc_0)$, where s^c = sedimentation coefficient at concentration c (expressed in g/dl).]

Separate calculations were carried out for each cell and all values converted into $s_{20,w}$ by using density and viscosity data compiled by Svedberg & Pedersen (1940) for sodium chloride and data obtained by conventional methods for tris buffer. The difference plot proposed by Schumaker & Adams (1968) was not used as it gives δs^0, which is more sensitive to errors in the values of k than δs^{c0} is, particularly when each sample is compared with a standard in a separate experiment. The values of k were obtained from differential experiments, the results of which were calculated by using a provisional value of k based on earlier data (Charlwood, 1963*b*) and refined by a second approximation. This gave $k = 0.085$ for both apotransferrin and iron-saturated transferrin.

Even the results of the measurements of s^{c0} shown in Fig. 1 demonstrate that there is a significant difference in this characteristic between the apoprotein and iron-saturated protein. The distinction is so readily apparent because of the differential manner of measurement. The regression lines shown in Fig. 1 gave $k = 0.094$ and 0.089 for apotransferrin and iron–transferrin respectively, but statistically these values did not differ significantly, although the values of $s^0_{20,w}$ did. The difference, $0.07_3\pm0.02_7$S (S.D.), altered only very slightly to $0.07_4\pm0.01_9$S (S.D.) when all the results were recalculated with k values of 0.094 and 0.089 in place of 0.085. A more accurate assessment of the difference was obtained from Fig. 2, which makes fuller use of the pairing procedure. The difference in s^{c0} for the two forms of transferrin, δs^{c0}, is not significantly dependent on c_0, so that the best estimate is the mean, which is $0.09_1\pm0.01_6$S (S.D.). Thus not only do the sedimentation coefficients differ at finite concentrations but so do the extrapolated values.

A differential sedimentation experiment to ascertain the direct effects on density and viscosity

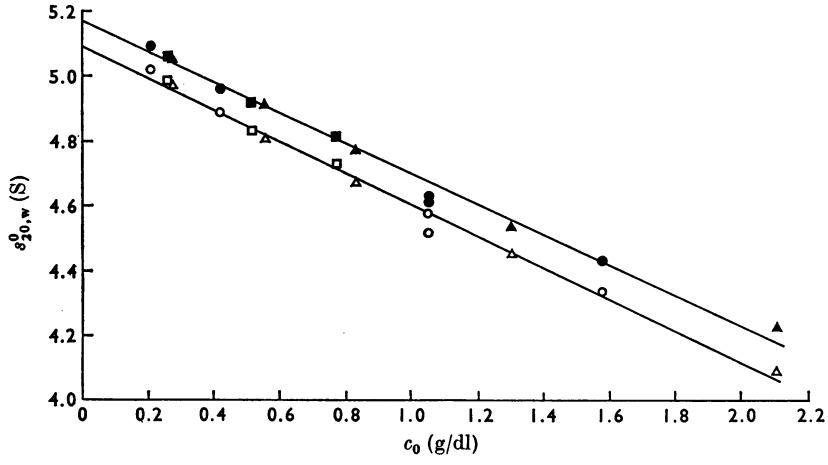

Fig. 1. $s_{20,w}$ values (Svedberg units) as a function of concentration for apotransferrin and iron-saturated transferrin are indicated by open and filled symbols respectively: ▲ and △, batch 1; ● and ○, batch 2; ■ and □, batch 3.

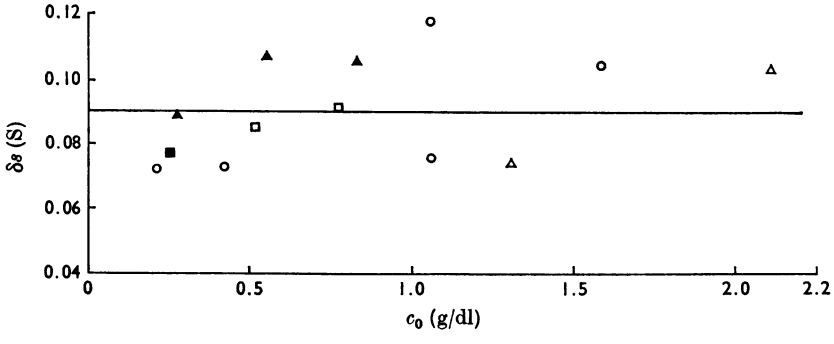

Fig. 2. Differences of $s_{20,w}$ (Svedberg units) for apotransferrin and iron-saturated transferrin as a function of concentration. Filled symbols distinguish experiments in which the apoprotein was contained in the cell with a wedge window: ▲ and △, batch 1; ○, batch 2; ■ and □, batch 3.

of the protein solutions occasioned by dissolving solid derived from the stock iron solution gave a ratio of 1.002_2 for the sedimentation coefficients of albumin (5mg/ml) in the absence and presence respectively of the extra solute. Since the latter was at double the highest concentration used in any transferrin experiment, any error in δs^{c_0} accruing from this source can be disregarded.

Gel filtration. The results of typical experiments are shown in Fig. 3. Although smooth curves have been drawn through the points, because this gives a clearer visual impression of the differences between the protein species, it would be more correct to compare the histograms. The elution volume of a peak was, indeed, found to be most reliably and consistently estimated by a simple procedure based on the histogram. A value for the total material of interest in the peak was obtained by summing the measurements of extinction or counting rate. The point by which half of this had emerged was assessed by summing the contributions of leading fractions until a total slightly less than 50% was reached, and expressing the deficiency as a proportion of what the next fraction contained. This is illustrated in Table 1. It has been ascertained by calculation that, if the concentration in the effluent is a Gaussian function of volume, the bias hereby introduced is quite small, despite the limited number of fractions which cover the peak. Moreover, random errors in the measurements of concentration would have little influence. Where two peaks are close in position, as for different transferrin species,

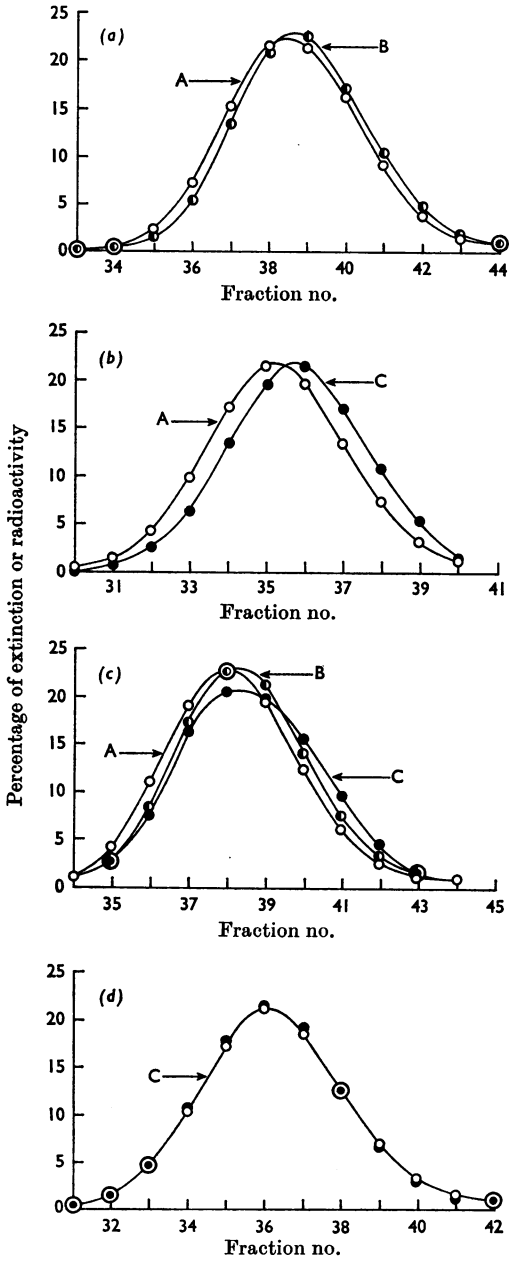

Fig. 3. Gel-filtration results on mixtures of apotransferrin (A), 1-iron-atom transferrin (B) and iron-saturated transferrin (C). Total protein (mg): (*a*) 3.6, (*b*) 1.9, (*c*) 4.1, (*d*) 2.2. In (*a*), (*b*) and (*c*) ○ corresponds to A, ◐ to B, ● to C. In (*d*), ○ corresponds to extinction, ● to radioactivity counts.

the difference between the elution volumes is affected little by any slight variations there may be in fraction size.

Elution volumes were related to Stokes radii by means of the equation derived by Ackers (1967), involving the error function complement (erfc), which may be written as:

$$\sigma = (V_e - V_0)/V_i = \text{erfc}[(a - a_0)/b_0] \qquad (1)$$

where V_0 = void volume of column, V_i = internal volume of column, V_e = elution volume of substance having Stokes radius a, and a_0 and b_0 are constants for a particular column. Thyroglobulin gave V_0, and V_i was obtained from the difference between the elution volumes of small molecules and thyroglobulin. By substitution of the V_e and a values for ovalbumin and ribonuclease in eqn. (1) a_0 and b_0 were obtained. For ovalbumin a was taken to be 29.0 Å (Creeth & Winzor, 1962) and for ribonuclease 19.2 Å was used (Laurent & Killander, 1964). The radius of apotransferrin then calculated from the elution volume was 36.3 Å and the mean difference between the elution volumes of apotransferrin and iron-saturated transferrin (see Table 2) led to a radius of 35.8 Å for the latter.

The effects of errors in the various parameters on the final estimate of Stokes radius were obtained by writing eqn. (1) for the standard proteins (ovalbumin and ribonuclease) as well as for transferrin, eliminating a_0 and b_0, and forming the partial differentials of the resulting relation. Substitution of typical values gave the results shown in Table 3, which were confirmed by numerical differentiation. In particular, although the elution volume of iodoacetamide was 0.3 ml greater than that of free iodide, it would have affected the final results by only 0.01% had the lower value been used in the calculations.

In comparative measurements the calculated Stokes radii of apotransferrin and iron–transferrin are affected almost equally by errors in parameters other than the elution volumes of the transferrins. The estimate of the magnitude of the change brought about by iron binding is thus determined mainly by the differences of these elution volumes. When the 'dead space' at the bottom of the column was minimized and the Uvicord omitted from the system there was no alteration in the differences. Thus mixing in the effluent must be quite small.

All three batches of protein gave results similar to those shown in Fig. 3 and which are summarized in Table 2. Where only a trace of iron was added to a large amount of transferrin it was assumed that the radioactivity corresponded with the species having 1 atom of iron (see the Discussion section). As total load of protein, within the limits given in the Materials and Methods section, did not affect the results (cf. Winzor & Nichol, 1965) all relevant experiments were included in the average. The elution volumes for the iron-saturated protein based

Table 1. *Calculation of peak position in gel-filtration experiments*

Fraction no.	31	32	33	34	35
Percentage of counts	0.62	2.88	9.27	18.93	24.42
Running total	0.62	3.50	12.77	31.70	
Fraction no.	36	37	38	39	40
Percentage of counts	21.48	13.39	6.18	2.21	0.62

Elution position = $34+(50-31.7)/24.42 = 34.75$

Table 2. *Gel-filtration differences between apotransferrin and iron–transferrin*

	Difference between apotransferrin and 1-Fe-atom form	Difference between apotransferrin and Fe-saturated form
Elution volume (ml)	$0.24\pm0.04_9$ (S.D.)	$0.50\pm0.05_7$ (S.D.)
Change in Stokes' radius (%)	0.6_4	1.3_8

Table 3. *Effect of errors in various parameters on the calculated value of Stokes radius*

Symbols have the significance attributed to them in the text. Subscripts 1, 2 refer to ovalbumin and ribonuclease respectively and absence of subscript implies transferrin.

$\partial a/\partial a_1 = 1.7$, $\partial a/\partial a_2 = -0.71$,
$\partial a/\partial V_1 = 1.0$, $\partial a/\partial V_2 = -0.27$, $\partial a/\partial V = -0.97$
$\partial a/\partial V_0 = -0.20$, $\partial a/\partial V_i = 0.013$

on extinction values and radioactivity counts coincided within the limits of error, differing by less than 0.04ml, thus incidentally proving that any extraneous protein present was too small in amount to influence the results.

DISCUSSION

The nature of the iron–transferrin species investigated requires consideration in the light of the characteristics of the binding sites. When iron attached to a chelate is added to a large excess of the protein, whether or not the binding sites have identical constants, the metal will transfer to the protein so that only a very small proportion of molecules will contain more than 1 atom of iron (Oncley, 1957; Wenn & Williams, 1968). At alkaline pH practically no iron will remain on the chelating agent, which can be removed by dialysis. It is important in many instances to remove chelating agent for reasons already stated.

The scatter of data in the literature for $s^0_{20,w}$ of transferrins has been discussed before (Charlwood, 1963*b*). The values in the current work, 5.09S and 5.16S for apotransferrin and iron-saturated transferrin respectively, are in excellent agreement with the previous values from these laboratories and with those of Roberts, Makey & Seal (1966). The difference (0.07S) between the two sedimentation coefficients coincides with the difference reported by Bezkorovainy (1966*b*), although the individual values were about 4% higher, possibly on account of the different solvent used. However, at present the best estimate for the difference is 0.09_1S, obtained by the differential method. An even more precise technique is now available for measuring differential sedimentation (Kirschner & Schachman, 1971), but its application would require very careful assessment of some of the factors that are rather less critical for the degree of accuracy reached here.

An increase in s could arise from the direct increase in molecular weight (M) due to the binding of iron, an increase in average M resulting from enhanced association, a relatively large decrease in partial specific volume ($\bar{v}$), or a change in conformation. Calculations of the type made by Schumaker (1968) ruled out the first possibility. Aisen *et al.* (1970) presented evidence that transferrin does not ordinarily form polymers near neutrality, although Bezkorovainy (1966*a*) showed that the situation is different at lower pH. The change in Stokes' radius demonstrated by gel filtration showed that a change in conformation was the correct explanation. This is supported by recent measurements of dielectric dispersion (Rosseneu-Motreff, Soetewey, Lamote & Peeters, 1971), which were, however, made under different conditions.

The validity of interpretation of the gel results depends on the correctness of the assumptions made about which molecular species was being located by the particular type of measurement. The precautions regarding relative amounts in mixtures can be assumed to have been adequate in this respect. The decay of ^{59}Fe produces, fortunately, a form of cobalt that is stable. Since cobalt is bound at

the same sites as iron, although it is not yet known whether it produces a similar change in conformation, a radioisotope would have complicated the situation.

If $\bar{v}$ is essentially unchanged when iron binds to transferrin the product $s \cdot a$ (because a is inversely proportional to the diffusion coefficient) should be only slightly higher, in the ratio 1.0034, owing to the increase in M occasioned by the binding of ferric and bicarbonate ions. The ratio found (1.004_2) agrees well within experimental error.

The absolute value of M was discussed before (Charlwood, 1963*b*), but no satisfactory reason has emerged for the large scatter in reported figures. Possibly the most reliable value is about 77000 (Greene & Feeney, 1968; Mann, Fish, Cox & Tanford, 1970), which is very close to 76600, the value obtained for iron-saturated transferrin by combining the sedimentation coefficient and Stokes radius measured in the present work and assuming $\bar{v} = 0.725$. This close agreement would not obtain if the Stokes radius had been calculated on the basis of 27.3 Å for ovalbumin (Laurent & Killander, 1964). The reason for preferring 29.0 Å is that it is based on later, probably more reliable, diffusion data for ovalbumin. Moreover, the Stokes radius to which this leads for apotransferrin (36.3 Å) corresponds to $D_{20,w} = 5.86 \times 10^{-7} cm^2 \cdot s^{-1}$, which may be compared with the values measured for batch 1. These were 6.14 ± 0.05 (S.D.) $\times 10^{-7} cm^2 \cdot s^{-1}$ and 5.97 ± 0.05 (S.D.) $\times 10^{-7} cm^2 \cdot s^{-1}$ at 0.4 and 0.8% concentration respectively.

The derivation of eqn. (1) has been criticized by Rodbard & Chrambach (1970), but so far as the calculation of small changes in Stokes radius is concerned it is unlikely to be misleading to any great extent.

No attempt was made to ensure that the human transferrins used here consisted of a single genetic variant, because Parker & Bearn (1962) showed that form C is predominant. It is assumed that the measurements made here relate to that form. Preliminary experiments indicate that both rabbit transferrin and the pink form of rat transferrin (Gordon & Louis, 1963) behave in a fashion similar to the human protein. The results obtained are valid only for the particular solvent used, and could conceivably be changed in another environment. The single chain of transferrin (Greene & Feeney, 1968; Mann *et al.* 1970) possibly has two rather similar parts with an iron-binding site on each. If it did consist of two linked subunits in this way, according to hydrodynamic theory (Bloomfield, 1966) the changes observed in the whole molecule could be accounted for by a change in the distance between the centres of the subunits, which, dependent to some extent on their shape, would be about 4%.

The results of the gel-filtration experiments show that addition of trace amounts of iron to transferrin produces a species of intermediate properties that corresponds almost certainly to the form having 1 atom of iron per molecule, whose existence has been established by free-boundary electrophoresis (Aisen, Leibman & Reich, 1966) and by isoelectric focussing (Wenn & Williams, 1968). It would be interesting to isolate sufficient to carry out ultracentrifuge and other studies, but unfortunately mobility differences are too small to permit ordinary electrophoresis to be used for this purpose, and there is a tendency for iron to be lost near the isoelectric point. However, it should be possible to take advantage of the chromatographic method recently worked out by Lane (1971). The gel-filtration characteristics of this form do not indicate that the binding of the first atom of iron has a more pronounced effect on the transferrin molecule than the binding of the second atom, as may be the case with some other properties of the molecule that may depend on more subtle features (Fletcher & Huehns, 1967).

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