The measurement of iron-binding capacity in serum and purified transferrin with the aid of chemical affinity chromatography

W. N. M. RAMSAY

From the Veterinary Unit, Department of Biochemistry, University of Edinburgh, Edinburgh

SYNOPSIS In a new method for the estimation of transferrin by iron-binding capacity iron is added as the tartrate in NaCl with about 10 mM bicarbonate. The excess iron is removed by passage through DEAE-Sephadex A-50 previously treated with the iron chelator disodium catechol-3,5-disulphonate. The iron remaining bound to transferrin is measured without protein precipitation by the use of ferrozine. The method is applicable to fresh, frozen, or lyophilized serum, purified transferrin, and some quality control preparations. Validation experiments confirm that transferrin in serum and in pure solution is saturated with iron and give some evidence of specificity. The possible use of commercially available transferrin preparations as analytical reference standards is discussed.

Current methods for the measurement of serum iron-binding capacity lack reproducibility (Dixon, 1973; Leggate and Crooks, 1972; Betts and Stuart, 1973; Fraser, 1973), especially when lyophilized serum is analysed (Bothwell, Conrad, Cook, Crosby, Fielding, Hallberg, Izak, Layrisse, and Ramsay, 1972). The widely used magnesium carbonate method (Ramsay, 1957) is not fully specific for transferrin (van der Heul, van Eijk, Wiltink, and Leijnse, 1972; Ramsay, 1973) and requires 2 ml serum.

This paper describes a method of improved sensitivity which is not affected by freezing or lyophilization and storage of serum for two months. Some quality control sera and purified transferrin can be analysed. Non-transferrin iron detected by the methods of Ramsay (1973) is virtually absent, and evidence is given that the transferrin present is saturated with iron.

The method depends on the familiar principle of adding excess iron and removing that thought not to be bound to transferrin. Measurement of the iron retained gives the total iron-binding capacity (TIBC). The saturating iron solution contains tartrate, which complexes with iron and permits the addition of NaHCO_3 to pH 7-8. Inclusion of ^{59}Fe allows the estimation of unsaturated iron-binding capacity (UIBC). In serum iron estimation the initial treatment with iron is omitted.

Non-transferrin iron is removed in a small column

of DEAE-Sephadex A-50 (Ramsay, 1973), previously treated with disodium catechol-3,5-disulphonate (Tiron, CDS). This reagent adheres to DEAE-Sephadex through the sulphonate ions while retaining its property of chelating with iron. Early workers (Johnson, 1964) used CDS to estimate Fe^{3+} , but it was found in preliminary experiments that in alkaline solution Fe^{2+} reacts equally well, even in an atmosphere of argon. Iron is determined spectrophotometrically in the eluate after the addition of 3 (2-pyridyl)-5,6-bis (sodium 4-phenyl sulphonate)-1,2,4-triazine (Ferrozine; Stookey, 1970) and ascorbic acid (Carter, 1971).

Measurements of TIBC on purified transferrin are related to E_{280} rather than to weight because most protein preparations contain small amounts of water, salts, and other contaminants. The experimental values of absorbance and TIBC are used to calculate the absorptivity of a solution of TIBC = 2 mol/l. This function, termed ϵ' for the purposes of this paper, should be equal to the molar absorptivity ϵ calculated from the molecular weight of transferrin and the specific absorbance $E^{1\%}_{1\text{cm}}$. Recent determinations of the molecular weight (Roberts, Makey, and Seal, 1966; Greene and Feeney, 1968; Mann, Fish, Cox, and Tanford, 1968; Palmour and Sutton, 1971; Charlwood, 1971) indicate a value close to 77 000 daltons. Published values for $E^{1\%}_{1\text{cm}}$ are few, and range from 10.9 (Behringwerke literature issued with

immunological standard transferrin) to 11.9 (Roberts *et al* 1966). It is therefore not possible to give an exact value for ϵ , but limits of approximately 84 000 to 92 000 can be set. Some of the reported variations in $E_{1\text{cm}}^{1\%}$ may result from overlooking the fact that E_{280} for Fe_2 -transferrin is about 25% higher than the value for apotransferrin. Preliminary experiments showed that in the range of conditions used in this work neither buffer composition nor pH affects E_{280} . Bates and Schlabach (1973) used a similar constant which recalculates to $\epsilon = 85\,000$ and is apparently equivalent to $E_{1\text{cm}}^{1\%} = 11.04$.

Iron-binding Capacity Method

Where available, reagents of Analar or similar quality should be used. Disodium catechol-3,5-disulphonate (Hopkin and Williams) varies in quality and only pure white finely crystalline batches should be used. Ferrozine is purchased from Ralph Emanuel Ltd. Three transferrin preparations have been studied: human transferrin, substantially iron free (Sigma), iron-free human transferrin (Behringwerke, Hoechst Pharmaceuticals Ltd), and standard human transferrin for immunological analysis (Behringwerke; preweighed 1 mg ampoules). Quality control sera were reconstituted according to the manufacturer's instructions.

Radioactivity is assayed in a Gamma guard 150 counter (Tracerlab (GB) Ltd). Electrophoresis is conducted in 7.5% polyacrylamide gel in tris-glycine buffer, pH 8.5. Gels stained with naphthalene black are scanned at 600 nm in a Gilford scanner. The spectrophotometer used (Pye-Unicam SP1800B) is calibrated with $\text{K}_2\text{Cr}_2\text{O}_7$ (Rand, 1969), since absolute absorbance values are required for calculating ϵ .

REAGENTS

The following reagents are required:

NaCl-NaHCO₃

Aqueous solution containing NaCl (0.14 mol/l) and NaHCO_3 (0.02 mol/l).

Saturating iron solution, 0.75 mmol/l

On the day of use, mix 0.5 ml ferric chloride (3 mmol/l in HCl, 20 mmol/l) with 0.1 ml ammonium (+)-tartrate (1 mol/l) and add 1.4 ml NaCl-NaHCO₃. The stock solutions are stable indefinitely. For UIBC measurements add $^{59}\text{FeCl}_3$ in HCl, 20 mmol/l (< 0.1 ml) before the NaCl-NaHCO₃.

Disodium catechol-3,5-disulphonate

A stock solution of disodium catechol disulphonate

(20 mmol/l in water, stable at 4 for one week) is diluted with 9 volumes NaCl-NaHCO₃ immediately before use.

Standard iron solution, 20 $\mu\text{mol/l}$

Prepare by dilution of a stock solution one hundred times more concentrated, containing $\text{FeSO}_4 \cdot (\text{NH}_4)_2 \cdot \text{SO}_4 \cdot 6\text{H}_2\text{O}$, 0.784 g in 1 l HCl, 2 mol/l. Oxidation during storage is harmless and both solutions are stable.

Ferrozine, 8 mmol/l in acetate buffer with ascorbic acid, 0.1 mol/l

Dissolve 23 mg ferrozine and 88 mg ascorbic acid in 5 ml acetate buffer (acetic acid 2.4 mol/l, sodium acetate 0.8 mol/l). The solution is stable for 10 days at 4.

DEAE-Sephadex A-50

The ion exchanger (4 g) is thoroughly mixed with NaCl-NaHCO₃ in a measuring cylinder (250 ml) and the gel is allowed to settle. The supernatant is discarded, replaced with fresh NaCl-NaHCO₃, and the process repeated six times to ensure equilibration of the DEAE-Sephadex with the salt solution. The suspension is finally made up to about 1.8 times the volume of the settled ion exchanger.

DEAE-Sephadex columns are supported on acid-washed glass paper discs in 1 ml disposable syringe barrels (Gillette Scimitar). Solutions are introduced without the formation of air locks by the use of extensions to disposable tips for a spring-loaded pipetter adjustable between 0.2 ml and 1 ml (Finnpipette). Eight cm extensions of polyvinyl tubing (Wright Scientific Ltd; 1.5 mm id) are needed for filling columns, 2.5 cm for adding serum and reagents. The appropriate settings for the pipette were found by gravimetric calibration to be close to those for unmodified tips, and delivery slowly executed was highly reproducible with the type of plastic tubing selected.

Immediately after thorough mixing, DEAE-Sephadex suspension (1.0 ml) is pipetted into a prepared syringe barrel by placing the tip of the extended pipette close to the base and withdrawing it smoothly as delivery proceeds. After the excess liquid has drained from the column, CDS in NaCl-NaHCO₃ (0.25 ml) is added, followed by sufficient DEAE-Sephadex (usually 0.6–0.7 ml) to give a total settled volume of 0.8 to 0.9 ml. The column is finally washed with NaCl-NaHCO₃ (0.5 ml). In preparing the column and analysis all solutions must be added slowly (10–15 s per 0.5 ml) with minimal disturbance of the column. Columns are conveniently prepared after the addition of saturating iron solution to serum samples, and

should be used within one and a half hours of preparation.

PROCEDURE

Saturating iron solution (0.1 ml) is added to serum (0.5 ml) and the mixture left to stand for 30 minutes. The treated serum (0.5 ml) is transferred to a DEAE-Sephadex column and all the eluate is collected in a clean dry tube. When the last trace of serum disappears into the column five successive portions of NaCl-NaHCO₃ (each 0.5 ml) are added in the same way. It is convenient to run 10–12 columns together, but each portion of eluant should be added as soon as the surface of the column is exposed. The recommended columns flow at a suitable rate, which should not be increased because 'tailing' is likely to follow.

Serum iron is measured by applying serum (0.5 ml) directly to a column.

Standard tubes contain standard iron solution (1 ml; 0.02 μ mol) and water (2 ml). The 'blank' solution contains water (3 ml). There is no advantage in running a 'blank' column with 6 $\times$ 0.5 ml NaCl-NaHCO₃ because the column removes any iron present in the salt solution. The UIBC counting standards contain radioactive saturating iron solution (0.05 ml) and water (2.95 ml). All counts are made to a standard error less than 1%.

Ferrozine solution (0.1 ml) is added to all tubes at least 15 min before the measurement of absorbance at 562 to 565 nm. The complex is stable for several days. If the DEAE-Sephadex eluate appears significantly coloured (it is usually tinted very pale pink by Fe₂-transferrin, but this should not be confused with the stronger colour given by haemoglobin) it is transferred to the cuvette and the spectrophotometer is set to zero before the addition of ferrozine.

CALCULATIONS

If the net absorbances of standard and unknown are

$$E_s \text{ and } E_u, \text{ then serum Fe } (\mu\text{mol/l}) = \frac{E_u}{E_s} \times 40$$

$$\text{and TIBC } (\mu\text{mol/l}) = \frac{E_u}{E_s} \times 48. \text{ If standard and}$$

unknown give C_c and C_u counts/min and the absorbance of the counting standard is E_c , then

$$\text{UIBC } (\mu\text{mol/l}) = \frac{C_u}{C_c} \times \frac{E_c}{E_s} \times 48.$$

Validation Studies

Ramsay (1973) used ion exchange and gel filtration to show that the iron measured in the magnesium

carbonate method (Ramsay, 1957) and that of Nielsen (1968) included 1.11% of non-transferrin iron. Using ⁵⁹Fe, similar tests have been applied to the new method. Preliminary work showed that the results are not affected by the substitution of NaCl-NaHCO₃ for the NaCl-tris-HCl buffer previously used or by the change in the ion exchange technique. Electrophoretic analysis has confirmed that the new technique removes albumin completely from serum. Rechromatography of the DEAE-Sephadex eluate on a fresh column indicates the completeness of recovery of the iron fraction sought. Gel filtration of the DEAE-Sephadex eluate on Sephadex G-150 is conducted by elution with NaCl-NaHCO₃ in acrylic resin columns 30 cm $\times$ 4 cm² (Wright Scientific Ltd), run by upward flow at 1.5 ml.hr⁻¹.cm⁻² (Watson-Marlowe pump, type MHRE22). At the end of each run residual iron is eluted by passage of CDS (5 μ mol in 1 ml NaCl-NaHCO₃), a procedure shown to be as effective as the citric acid formerly used. This technique is used to show how much of the iron is present in the molecular weight range corresponding approximately to transferrin.

The saturation of transferrin in the DEAE-Sephadex eluate is determined by using the observation of Bates and Schlabach (1973) that the nitrilotriacetate (NTA) complex of Fe³⁺ reacts rapidly with transferrin. A non-radioactive DEAE-Sephadex eluate is treated with 0.05 ml ⁵⁹Fe-NTA (containing 0.02 μ mol Fe³⁺ and 0.05 μ mol NTA) and analysed on Sephadex G-150. The proportion of the total radioactivity found in the fractions containing transferrin allows the degree of unsaturation of the transferrin in the original DEAE-Sephadex eluate to be calculated. Control experiments showed that the results were not falsified by the removal of iron from transferrin by unchelated nitrilotriacetate.

Results

PRELIMINARY EXPERIMENTS

The complete elution of native transferrin-bound iron from DEAE-Sephadex-CDS was demonstrated by the chromatography of serum from a rat three and a half hr after the subcutaneous injection of 4 μ Ci ⁵⁹Fe-citrate. In one experiment the eluate (3 ml) gave 4130 counts/min, while 0.5 ml serum diluted to 3 ml gave 4160 counts/min. In four experiments the difference between diluted and chromatographed serum was between -2% and +1%. Gel filtration of the same sera on Sephadex G-150 gave recoveries > 98.5% in fractions which peaked at 0.54–0.58 of the column volume. These recoveries were not altered by prior passage through DEAE-Sephadex.

The apparent TIBC found after treating a normal human serum with increasing quantities of iron is shown in the figure. The chromatographic technique clearly copes with a large excess of iron. Gel filtration of the first of the apparently saturated samples after treatment with ^{59}Fe -NTA showed it to be 99.8% saturated, whereas similar treatment of one of the earlier samples gave the expected level of about 80% saturation.

Fig Apparent total iron-binding capacity found after the addition of increasing amounts of iron to serum. Tested with ^{59}Fe -NTA for saturation of transferrin, two additional samples gave the following results: *80%, **99.8%

Exposure of serum and transferrin to the recommended excess of saturating iron for varying periods indicated that more than 95% of the final uptake was achieved within five minutes, but that complete saturation took place more slowly with purified transferrin. In one set of experiments two sera after 20 min gave apparent TIBC values 99.8% and 100% of those found after 60 minutes. For two transferrin solutions the corresponding figures were 98.8 and 98.3%. The small difference, which was repeatedly confirmed, is of practical significance in relation to the precise evaluation of ϵ' .

Table I shows that the results are not affected by moderate concentrations of ferritin or Imferon. Imferon eluted from DEAE-Sephadex decomposes slowly in the ferrozine reagent, and the iron estimations should therefore be completed within 30 minutes. The effect of varying concentrations of haemoglobin is also shown, together with the spectrophotometric compensation for the error which affects the results when the concentration exceeds 30 mg/100 ml.

A comparison of serum iron determinations made on seven horse and five human sera by the new method and that recommended by the International

Experiment	Fe added	Serum Fe	TIBC
Haemoglobin	0	35.2	52.8
	18	36.6	53.2
	36	41.2	57.5
	54	45.4	62.2
	54	34.8*	53.4*
Ferritin	0	22.2	46.3
	17	22.4	46.5
Imferon	0	13.0	40.0
	17	14.2	40.2

Table I Effect of haemoglobin, ferritin, and imferon on serum Fe and TIBC¹

¹All figures in $\mu\text{mol/l}$ Fe

*Spectrophotometer set to zero with sample in cuvette before addition of ferrozine

Committee for Standardization in Haematology (1971) gave a correlation coefficient $r = 0.984$. The mean by the new method was $0.54 \mu\text{mol/l}$ higher. This difference probably represents error introduced by ignoring non-specific absorbance in the DEAE-Sephadex eluate, because it was not seen in samples selected for freedom from haemolysis (see table III)

IRON-BINDING CAPACITY AND VALIDATION

Table II shows results obtained by applying the procedure and the validation tests to normal human and horse sera. Neither rechromatography on DEAE-Sephadex-CDS nor gel filtration on Sephadex G-150 removed significant amounts of iron from the radioactive eluate, while failure of the non-radioactive eluate to take up iron from ^{59}Fe -NTA indicated complete saturation of transferrin.

	Horse Serum	Human Serum
Serum Fe ($\mu\text{mol/l}$)	29.7	13.2
TIBC ($\mu\text{mol/l}$)	57.9	52.5
Transferrin saturation by ^{59}Fe -NTA (%)	99.8	100
UIBC ($\mu\text{mol/l}$)	28.0	37.9
^{59}Fe recovered in DEAE chromatography (%)	100	98
^{59}Fe -eluted from G-150 in transferrin molecular weight range (%)	98	99

Table II Validation of serum iron-binding capacity assays by the procedures described in the text

Table III summarizes comparisons between the new method and the MgCO_3 method, with the addition of ion exchange and gel filtration tests on the MgCO_3 supernatant (Ramsay, 1973). The new method gives lower results than MgCO_3 , but agreement is better after a correction is made for non-transferrin iron in the MgCO_3 supernatants. The quality control serum (Wellcome Two Assayed)

Specimen	Method	Serum Fe	TIBC	TIBC after DEAE	UIBC	UIBC	
						After DEAE	After G-150
Horse, fresh	1	39.7	69.1	60.4	30.4	23.4	—
	2	40.5		61.2		22.2	
Horse, frozen	1	25.3	74.0	71.2	46.0	45.3	45.0
	2	25.1		67.7		42.3	
Horse, ¹ lyophilized	1	23.6	60.8	50.0	36.5	26.3	26.0
	2	21.8		48.0		26.1	
Human, fresh	1	24.1	63.7	61.5	40.6	39.2	38.2
	2	24.4		59.8		36.6	
Human, fresh	1	23.2	57.9	54.5	34.2	32.5	32.2
	2	22.8		53.6		31.6	

Table III Analyses ($\mu\text{mol/l}$) of human and horse serum by (1) MgCO_3 method with screening of results by DEAE-Sephadex and Sephadex G-150 (Ramsay, 1973) and (2) the method now described

¹Wellcontrol Two Assayed; manufacturer's values: serum Fe 24.0, TIBC (MgCO_3) 57.3 $\mu\text{mol/l}$

shows this difference clearly. This material gave highly reproducible results when analysed on four separate occasions (table IV). Validation experiments on the first bottle analysed gave satisfactory results. In particular, the ^{59}Fe -NTA test indicated 99.6% saturation, giving further evidence that the original MgCO_3 results are too high.

Bottle	Serum Fe	TIBC	UIBC
1	21.8	48.0	26.1
2	22.3	48.7	26.6
3	23.0	46.9	24.6
4	22.6	47.6	25.1

Table IV Serum iron, total and unsaturated iron-binding capacity ($\mu\text{mol/l}$) on four bottles from the same batch of Wellcontrol Two assayed control system (see Table III)¹

¹Bottles were opened singly at intervals over ten weeks. Validation tests on samples from bottle 1 gave satisfactory results including 99.6% saturation of transferrin by the iron tartrate reagent.

STORAGE AND PRESERVATION

Storage of fresh serum up to two weeks at 4 does not affect the results.

Type of Specimen	Weeks of Storage	Temperature (C°)	Serum Fe	TIBC	UIBC
Fresh	—	—	29.7	57.9	28.0
Frozen	1	—50	30.2	57.1	28.1
	4	—50	28.8	56.6	28.6
	8	—50	28.9	56.0	27.2
	10	—50	29.4	56.5	27.3
Lyophilized	1	—50	30.2	56.9	27.9
	4	22	29.8	56.7	28.2
	8	22	30.2	56.2	26.0
	10	4	30.2	57.4	26.9
Mean			29.7	57.0	27.6
SD			± 0.6	± 0.5	± 0.8

Table V Effects of freezing, lyophilization, and storage of one lot of horse serum on serum Fe, total and unsaturated iron-binding capacity ($\mu\text{mol/l}$)¹

¹The results of validation tests on the analyses on this fresh serum are reported in table II.

Aliquots of the horse serum used for the experiments quoted in table II were measured into vials which were weighed, shell frozen, lyophilized, and capped. After storage for varying times at different temperatures the samples were reconstituted to the original weight with distilled water. Other serum aliquots were stored frozen at -50 . Analysis (table V) showed that neither frozen nor lyophilized specimens were affected by storage for up to two months. All the results afford a good example of the high reproducibility of the method.

PURIFIED TRANSFERRIN

Preliminary work on iron-free human transferrin (Behringwerke) confirmed its low iron content. Electrophoresis showed only a trace of β_2 -globulin, and although the conditions used would not have disclosed the presence of haemopexin, Bates and Schlabach (1973) found very little of this impurity in the same material. For the evaluation of ϵ' , solutions (1.5–3 mg/ml) in NaCl-NaHCO_3 were used for the measurement of TIBC, allowing 60 min for saturation with iron. E_{280} was measured on separate aliquots passed without iron treatment through DEAE-Sephadex without CDS, because

	Transferrin Preparation		
	Sigma	Behringwerke	Behringwerke (Standard)
TIBC ($\mu\text{mol/l}$)			
A, serum + transferrin	92.1	79.1	91.3
B, serum	55.2	52.5	48.7
C, transferrin	37.0	26.8	44.4
Transferrin recovered (A - B)	36.9	26.6	42.6
Transferrin expected ¹	36.4	26.9	44.0
ϵ' ²	88 300	89 200	86 400

Table VI Use of purified transferrin as reference material in iron-binding capacity assays

¹Calculated from observed absorbance of solution C and mean ϵ' found for each transferrin type (see text)

²Calculated from observed absorbance and TIBC of solution C in these experiments

this compound absorbs strongly at 280 nm and any breakthrough would vitiate the results. In general, analyses of pure transferrin require more care than serum, perhaps because the total amount of protein passing through the column is very small. In 20 experiments the mean value of ϵ' was 88 800 ($\text{SD} \pm 900$, range 87 200-90 600). Six measurements on human transferrin (Sigma), which appeared to be of comparable purity, gave $\epsilon' = 89 800$ ($\text{SD} \pm 1000$, range 88 300-91 000). Neither preparation gave different results after preliminary passage through either a large column (60 cm $\times$ 4 cm²) of Sephadex G-150 in NaCl-NaHCO₃ or a smaller column of Sephadex G-25 in NaClO₄ (Price and Gibson, 1972), Chelating agents which interfere with the method are thus absent, but complete freedom from chelating agents is not proved, because the analytical results were not affected by EDTA equivalent to approximately 25% of total iron-binding capacity. In two experiments on immunological standard transferrin (Behringwerke) ϵ' was 88 200 and 86 400. In several experiments tests with ⁵⁹Fe-NTA confirmed the full saturation of transferrin.

Although uncertainty in the value of ϵ makes it impossible to determine the precise accuracy of these highly reproducible values of ϵ' , the transferrin preparations studied should be suitable as reference materials. Appropriate control analyses might include a check evaluation of ϵ' and the assay of transferrin added to serum, and should ideally be accompanied by validation tests on the serum analysis. A possible procedure is outlined below:

1 Prepare a stock transferrin solution from a suitably pure sample by dissolving 16-20 mg in 1 ml NaCl-NaHCO₃.

2 Use this solution to prepare the following: A, serum (2.5 ml) + transferrin (0.25 ml); B, serum (2.5 ml) + NaCl-NaHCO₃ (0.25 ml); C, NaCl-NaHCO₃ (4.0 ml) + transferrin (0.40 ml).

3 (a) Measure TIBC on solutions A, B, C, by the method described. (b) Measure ϵ_{280} after passage

of solution C (0.5 ml) through DEAE-Sephadex (without CDS) with NaCl-NaHCO₃ (5 $\times$ 0.5 ml).

4 (a) Confirm that solution C gives a satisfactory ϵ' value. (b) Confirm that $\text{TIBC}_A - \text{TIBC}_B = \text{TIBC}_C$.

When immunological standard transferrin (Behringwerke) is used, at least 3 $\times$ 1 mg ampoules are required. Solution A and two lots of solution C are prepared by direct addition of serum or NaCl-NaHCO₃ to the opened ampoules. It must be assumed that ampoules contain identical quantities of transferrin.

Table VI gives one example of this procedure applied to each of the three preparations studied. The ϵ' values and the recoveries of transferrin judged by TIBC seem highly satisfactory.

Discussion

The complete removal of albumin and associated pigments by the chromatographic step is a major advantage, because the analysis is improved in speed and precision by the omission of protein precipitation. Columns can be prepared and used more quickly than might be expected, and a dozen specimens can be processed in about 45 minutes less time than is required for the MgCO₃ method. Although DEAE-Sephadex has some capacity for removing excess iron added to serum (Ramsay, 1973), the columns now used are very small. Preliminary studies showed that the iron-scavenging properties of CDS ensure a clean and complete separation of transferrin-bound iron from that less stably complexed. The column unfortunately has no clearing effect on lipaemic serum or on the turbidities found in some quality control sera, and these can only be handled by protein precipitation. Jaundiced serum, however, can be dealt with by spectrophotometric compensation for non-specific absorbance as recommended for haemolysed samples.

The experimental ϵ' values around 89 000 are within 1% of a figure calculated from the data of

Roberts *et al* (1966), who used very different methods. On the other hand, they are 5% higher than the lowest figure for the molar absorptivity of transferrin, obtained by assuming a molecular weight of 77 000 and $E_{1\%}^{1\text{cm}} = 10.9$. Several factors may contribute to the discrepancy, including traces of residual impurity in transferrin preparations, occasional low values for TIBC, error in either the molecular weight or $E_{1\%}^{1\text{cm}}$, or error in other absorbance measurements. The small possible margin of error in ϵ' should not prove an obstacle to the practical use of available transferrin preparations as reference materials. There seems no reason why immunological standards should not be calibrated in the same way, and it would be a logical advance to put all kinds of transferrin measurement on a common molar reference base.

While this work is personal, the author is indebted to his colleagues on the Iron Panel, ICSH, for much stimulating discussion. Thanks are also due to R. W. Brown for skilled technical assistance, to the Agricultural Research Council, which gave the radioactive counting equipment to the Faculty of Veterinary Medicine, University of Edinburgh, and to the Earl of Moray Endowment, University of Edinburgh, for a grant with which supplies of purified transferrin were purchased.

References

- Bates, G. W., and Schlabach, M. R. (1973). The reaction of ferric salts with transferrin. *J. biol. Chem.*, **248**, 3228-3232.
- Betts, C. A., and Stuart, B. (1973). Determination of serum total iron binding capacity. *J. clin. Path.*, **26**, 457.
- Bothwell, T. H., Conrad, M. E., Cook, J. D., Crosby, W. H., Fielding, J., Hallberg, L., Izak, G., Layrisse, M., and Ramsay, W. N. M. (1972). Studies on the standardization of serum iron and iron binding capacity assays. In *Modern Concepts in Hematology* edited by G. Izak and S. M. Lewis, pp. 67-160. Academic Press New York and London.
- Carter, P. (1971). Spectrophotometric determination of serum iron at the submicrogram level with a new reagent (ferrozine). 3(2-pyridyl)-5,6-bis(4-phenyl sulphonic acid)-1,2,4-triazine, disodium salt trihydrate. *Analyt. Biochem.*, **40**, 450-458.
- Charlwood, P. A. (1971). Differential sedimentation-velocity and gel-filtration measurements on human apotransferrin and iron-transferrin. *Biochem. J.*, **125**, 1019-1026.
- Dixon, P. (1973). Routine clinical measurements of transferrin in human serum. *Ann. clin. Biochem.*, **10**, 127-131.
- Fraser, C. G. (1973). Problems in the determination of serum total iron binding capacity. *J. clin. Path.*, **26**, 457-458.
- Greene, F. C., and Feeney, R. E. (1968). Physical evidence for transferrins as single polypeptide chains. *Biochemistry*, **7**, 1366-1371.
- van der Heul, C., van Eijk, H. G., Wiltink, W. F., and Leijnse, B. (1972). The binding of iron to transferrin and to other serum components at different degrees of saturation with iron. *Clin. chim. Acta*, **38**, 347-353.
- International Committee for Standardization in Hematology (1971). Proposed recommendations for measurement of serum iron in human blood. *Brit. J. Haemat.*, **20**, 451-453.
- Johnson, W. C. (1964). *Organic Reagents for Metals*, vol. 2, pp. 189-195. Hopkin and Williams, London.
- Leggate, J., and Crooks, A. E. (1972). Problems in quality control in determinations of serum total iron-binding capacity by the magnesium carbonate method. *J. clin. Path.*, **25**, 905-906.
- Mann, K. G., Fish, W. W., Cox, A. C., and Tanford, C. (1970). Single-chain nature of human serum transferrin. *Biochemistry*, **9**, 1348-1354.
- Nielsen, I. (1968). A gel filtration method for determining total iron binding capacity in serum. *Z. klin. Chem.*, **6**, 103-105.
- Palmour, R. M., and Sutton, H. E. (1971). Vertebrate transferrins, molecular weights, chemical compositions, and iron binding studies. *Biochemistry*, **10**, 4026-4032.
- Price, E. M., and Gibson, J. F. (1972). A reinterpretation of bicarbonate-free ferric transferrin EPR spectra. *Biochem. Biophys. Res. Commun.*, **46**, 646-651.
- Ramsay, W. N. M. (1957). The determination of total iron binding capacity of serum. *Clin. chim. Acta*, **2**, 221-226.
- Ramsay, W. N. M. (1973). The measurement of serum transferrin by iron-binding capacity. *J. clin. Path.*, **26**, 691-696.
- Rand, R. N. (1969). Practical spectrophotometric standards. *Clin. Chem.*, **15**, 839-863.
- Roberts, R. C., Makey, D. G., and Seal, U. S. (1966). Human transferrin: molecular weight and sedimentation properties. *J. biol. Chem.*, **241**, 4907-4913.
- Stookey, L. L. (1970). Ferrozine, a new spectrophotometric reagent for iron. *Analyt. Chem.*, **42**, 779-782.