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DETERMINATION OF THE UNSATURATED IRON-BINDING CAPACITY OF SERUM

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This paper deals with a modification of the methods suggested by Rath and Finch (1949) and Cartwright and Wintrobe (1949) for the determination of the unsaturated iron-binding capacity of the serum.

Transport of iron by the blood plasma may be effected through union with a β_1-globulin contained in the IV-7 fraction of Cohn (1945) ; this fraction has been called siderophyllin (Schade and Caroline, 1946 ; Laurell, 1947 ; Surgenor, Koechlin, and Strong, 1949). Various methods of determination indicate that the amount of siderophyllin present in 100 ml. of normal plasma is sufficient to bind about 300 μg. of iron. Only about one-third of this protein is normally saturated with iron in the plasma or serum, the metal constituting the serum iron. The sum of the iron and the further amount of iron required to saturate all siderophyllin (unsaturated iron-binding capacity or U.I.B.C.) gives a measure of the total iron-binding capacity of the serum.

Monasterio and Lattanzi (1943) and Waldenström (1944) measured the U.I.B.C. by the difference between serum iron levels before and after intravenous injection of an ionizable form of iron. Schade and Caroline (1946) employed a microbiological method based on the fact that *Shigella dysenteriae* grows only in the presence of ionized iron, and these authors noted the change in colour of the iron-binding protein when linked with the metal. Rath and Finch (1949) and Cartwright and Wintrobe (1949) estimated siderophyllin on the basis that the intensity of the red produced by union with iron is directly proportional to the amounts of the two components.

Premises for a Simplified Estimation of Unsaturated Iron-binding Capacity

The addition of sufficient ionic iron to a sample of serum to saturate all the siderophyllin present results in the appearance of a red colour, the intensity of which is directly proportional to the amount of the iron-binding protein ; the quantity of iron thus bound gives the most convenient measure of the amount of the protein.

The red compound formed in serum by adding ionized iron has E max 460 mμ, and is dissociated at *p*H values on the acid side of neutrality (Figs. 1 and 2). This behaviour parallels the known properties of crystalline siderophyllin combined with iron *in vitro*. The $E\frac{1\%}{1\text{ cm.}}$ for the pure substance was determined by Surgenor *et al.* (1949), but cannot be applied to serum, as interference may occur from the presence of such substances as bile pigments and haemoglobin (Rath and Finch, 1949 ; Cartwright and Wintrobe, 1949).

In the present work the change in optical density of normal and pathological sera has been studied after the addition of fixed increments of ionized iron. The procedure is carried out in the cell of

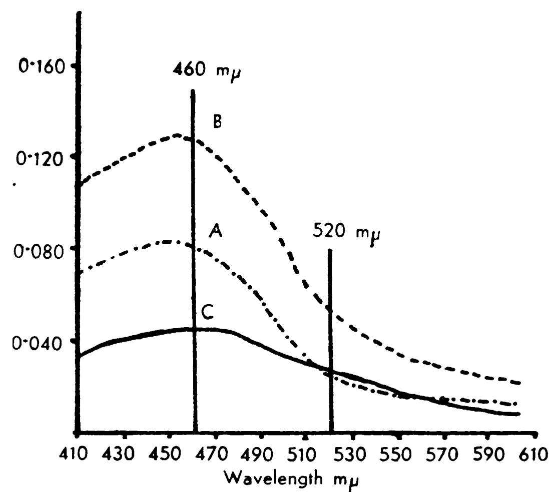

FIG. 1.—Absorption curve of serum before and after the addition of iron to saturate the siderophyllin fraction. A = serum plus saline. B = serum plus added iron. C = difference between A and B, representing absorption of iron-bound to protein.

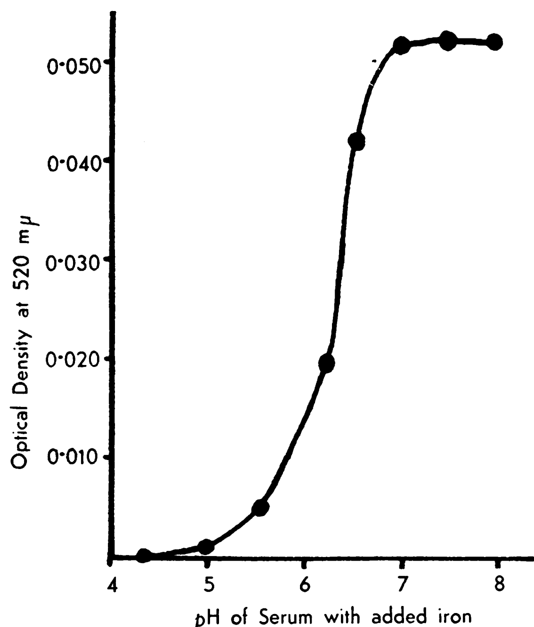

FIG. 2.—Optical density of serum at 520 mμ after the addition of iron at various hydrogen ion concentrations.

the Beckman spectrophotometer, and optimal conditions have been defined for the dilution of serum and the wavelength at which an increase in density is measured.

Dilution of the serum (1 ml.) is best made with 1.5 volumes of 0.85% sodium chloride solution. Veronal buffer is avoided, since the veronal itself combines with iron.

Fe^{++} is bound more rapidly and the reaction is more reproducible than with Fe^{+++}, as judged by the increase in optical density with time (Fig. 3).

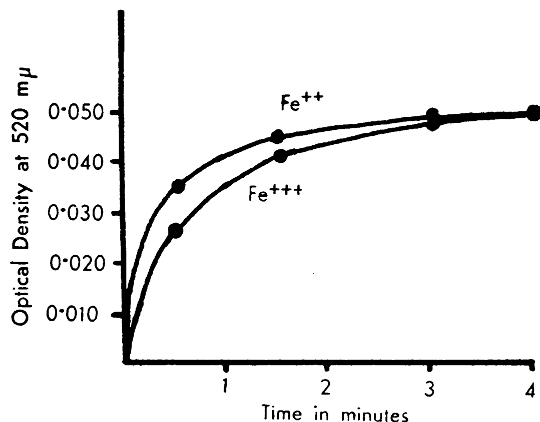

FIG. 3.—The rate of reaction of added iron in the ferrous and ferric states with the iron-binding protein of sera.

The optimum wavelength for measuring the increase in density has been found to be 520 mμ, as measurements at this wavelength are free from the influence of interfering substances observed at the maximum 460 mμ. The readings are made against a 0.85% sodium chloride solution blank.

Serum is separated at 37° C. from clotted venous blood samples. Estimations are carried out within 12 hours of taking the blood, or low values may be observed. No advantage has been found from Laurell's (1947) suggestion of first freezing the serum at −20° C. for 24 hours.

Construction of a Standard Calibration Curve

One hundred and fifty samples of sera from normal and anaemic individuals, and from normal and anaemic pregnant women, have been used. From 1 to 5 μg. of Fe^{++}, in increments of 1 μg., were added to samples of 1 ml., the increase in optical density at 520 mμ being recorded. Normal sera showed no further increase in density with the addition of Fe^{++} above 3 μg., but, with 15 sera from anaemic patients, saturation had not occurred after the addition of 5 μg. (1 μg. of Fe^{++} added to 1 ml. of serum is equivalent to the saturation of an unsaturated iron-binding capacity of 100 μg./100 ml.) For any observed increment in density $\triangle$D, the U.I.B.C. is the product of $\triangle$D and a factor K, derived from the average of the quotient of added iron in μg. over the determined average densities in the above experimental series (Table I).

TABLE I

DATA FOR THE CALIBRATION CURVE FOR DETERMINATION OF THE UNSATURATED IRON-BINDING CAPACITY OF SERUM

Quantity of Fe++ added to 1 ml. Serum	Average Increase of Optical Density at 520 = D	Coefficient of Variation (%)	$K = \frac{\mu g. \text{ of added } Fe^{++}}{D}$	No. of Sera Examined	Percentage of Total
1 μg.	0·0106	6·26	94·4	150	100
2 μg.	0·0221	3·73	90·7	141	94
3 μg.	0·0329	2·91	91·3	105	70
4 μg.	0·0437	2·50	91·8	30	20
5 μg.	0·0549	1·17	91·3	15	10
			Mean value of K = 91·9		

The average values for densities after each increment of iron are shown in Table I. In Fig. 4 the average increments in optical density of the

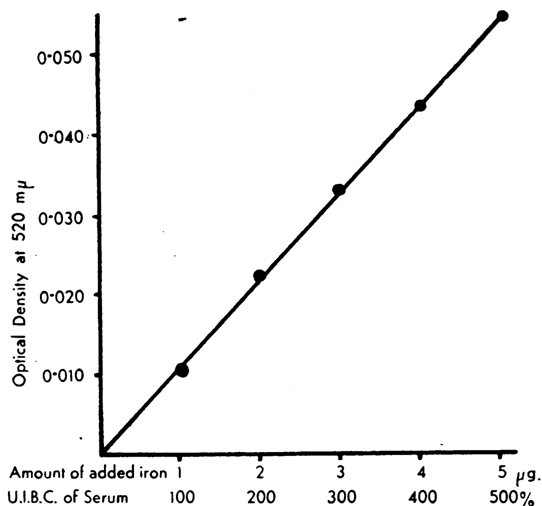

FIG. 4.—Standard calibration curve for determination of the unsaturated iron-binding capacity of serum, based on the average increases in optical density after the addition of fixed increments of ferrous iron to 150 samples of serum.

serum at 520 mμ are plotted against the corresponding amount of added iron. The points lie in a straight line. The $E^{1\%}_{1\,cm.}$ of serum siderophyllin may be derived, knowing that 0.4 μg. of iron saturate 0.321 mg. of the protein (Surgenor *et al.*, 1949). The average increase in density of 1 ml. of serum diluted to 2.5 ml. on the addition of 1 μg. of iron is approximately 0.011 at 520 mμ and 0.019 at 460 mμ ; this increase in density corresponds to the addition of 0.4 μg. of iron to 1 ml. of diluted serum or to its binding by 0.321 mg. of siderophyllin. Hence 1 g./100 ml. of serum siderophyllin saturated with iron will have a density of $\frac{0.019 \times 1000}{0.321 \times 100} = 0.59$ at 460 mμ. This is in good agreement with the value of 0.57 obtained by Surgenor *et al.* (1949) for the crystalline protein saturated with iron.

Technique

Standard Iron Solution.—This contains 70 μg. of Fe^{++} per ml. To make the solution, 122.5 mg. of Analar ferrous ammonium sulphate are dissolved in 5 ml. of N-acetic acid, and the volume made up to 250 ml. with double-distilled water (0.1 ml. of the solution added to 1 ml. of serum does not change the *p*H of the latter).

Procedure.—First 1 ml. of serum is measured directly into the Beckman spectrophotometer cell and diluted with 1.5 ml. of 0.85% sodium chloride solution in double-distilled water. The blank cell is filled with 2.5 ml. of the saline solution alone. The density of the test cell is then read against the blank at 520 mμ, and 0.1 ml. of the standard iron solution added to the test, mixed with a glass rod for two minutes, and the increase in density observed after standing for a further four minutes. The added iron is 7 μg., which is sufficient to saturate an unsaturated iron-binding capacity of 700 μg. per 100 ml. of serum, that is, sufficient excess of iron is added to satisfy any unsaturated iron-binding capacity likely to be encountered.

The observed increase in density (△D) is converted to unsaturated iron-binding capacity expressed as μg. per 100 ml. of serum, by multiplying by K, the value of which was 91.9 from the calibration curve given in Fig. 4, and by 100.

All glassware and cells are washed with iron-free distilled water.

Comparison of the Present Procedure with Other Techniques

To establish the accuracy of the suggested procedure, the unsaturated iron-binding capacities of 10 different sera were determined according to four different methods. The results are given in Table II.

From Table II and Fig. 5 it may be seen that there is good agreement with Cartwright and Wintrobe's method. The advantage of the present procedure lies in its speedier mode of execution. Laurell's method, and that based on the intravenous injection of an easily ionized iron compound, sometimes give lower results. In one sample of serum, 10 determinations gave a coefficient of variation of 2.74%. It should be noted that, although the absolute increases in optical density are of the order of only 1 to 5% when

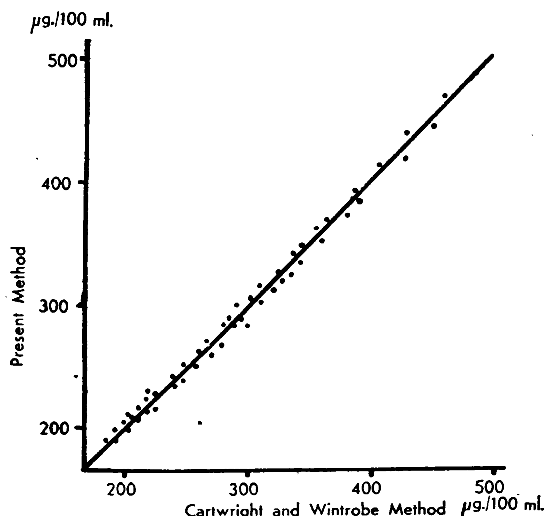

FIG. 5.—Scatter diagram comparing the values for the unsaturated iron-binding capacity of 50 serum samples determined by the method of Cartwright and Wintrobe (1949) and by the present method.

TABLE II

COMPARISON OF RESULTS OF DETERMINATION OF UNSATURATED IRON-BINDING CAPACITY OF SERUM BY VARIOUS METHODS

Case No.	Methods			
	Present Method	Cartwright and Wintrobe (1949)	Laurell (1947)	Intravenous Injections of Fe
1	230	217	206	198
2	470	458	405	417
3	371	364	360	352
4	216	225	214	—
5	229	225	240	201
6	326	337	305	299
7	317	309	311	—
8	269	278	239	247
9	198	205	200	—
10	325	319	296	305

the unsaturated iron-binding capacity of sera containing from 100 to 500 μg. per 100 ml. are saturated with iron, the readings are carried out with the same solution and cell, with consequent minimization of attendant experimental errors.

Summary

A method for determining the unsaturated iron-binding capacity of a 1 ml. sample of serum has been described. The procedure is carried out in the Beckman spectrophotometer.

The accuracy of the method compares favourably with other available methods, and has the advantages of speed and simplicity.

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