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Method for the determination of unsaturated iron-binding capacity of serum using radioactive iron and magnesium carbonate

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SYNOPSIS A method for measuring the unsaturated iron-binding capacity of serum using radioactive iron with magnesium carbonate as the adsorbing substance is described. The radioactive iron-magnesium carbonate method, compared with the methods of Caraway (1963), Herbert, Gottlieb, Lau, Fisher, Grevirtz, and Wasserman (1966), and Bothwell, Jacobs, and Kamener (1959), was shorter and simpler and equally reproducible.

The specific binding protein for iron in serum is a beta-1-globulin, siderophilin, or transferrin. The transferrin concentration in healthy persons is in the range of 200 to 320 mg/100 ml of serum, which corresponds to a total iron-binding capacity (TIBC) of 250 to 400 μg Fe/100 ml (Bothwell and Finch, 1962). Approximately one third of the transferrin is saturated with iron and the remainder represents the unsaturated iron-binding capacity (UIBC). A change in the concentration and saturation of transferrin in various diseases is well recognized and its significance has been reviewed in detail by Bothwell and Finch (1962). It is now generally acknowledged that the determination of total or unsaturated iron-binding capacity, together with the serum iron estimation, should be available as a routine procedure in hospital laboratories. The total or unsaturated iron-binding capacity is usually measured by using a colorimetric or a radioactive technique. Other techniques for the estimation of TIBC or UIBC have also been described, for example the immunodiffusion method (Jager and Gubler, 1952; Soothill, 1962), the gel filtration method (Barber, Dempster, and Anderson, 1963), and the atomic absorption spectroscopy method (Rodgerson and Helfer, 1966; Zettner, Sylvia, and Capacho-Delgado, 1966), but they are infrequently used in routine work.

The colorimetric determination of TIBC is carried out by saturating transferrin with iron and measuring the colour of the iron-transferrin complex (Rath and Finch, 1949; Ventura, 1952; Kaldor, 1955), or after removal of unbound iron, by estimating the total amount of iron left in the serum (Laurell, 1947; Ramsay, 1957; Peters, Giovanniello, Apt, and Ross, 1956). It is also possible to estimate UIBC, after saturation with iron, by separating and measuring the iron not bound to transferrin (Holmberg and Laurell, 1945; Schade, Oyama, Reinhart, and Miller, 1954; Ressler and Zak, 1958).

The successful use of radioactive iron for estimating UIBC was an elegant improvement which simplified the procedure. Transferrin was saturated with radioactive iron, excess iron was removed, and radioactivity of the supernatant counted. Unsaturated iron-binding capacity was calculated from radioactivity of a sample and a standard (Bothwell *et al.*, 1959; Tuaxe, 1961).

Different adsorbing substances have been used in colorimetric and radioactive techniques for the removal of excess iron after saturation of transferrin. Magnesium carbonate (Ramsay, 1957; Morgan and Carter, 1960; Caraway, 1963; Fischer and Price, 1964) and ion exchange resin (Peters *et al.*, 1956) have been used in colorimetric methods. In radioactive methods an ion exchange resin has been used (Bothwell *et al.*, 1959; Tauxe, 1961). More recently, Herbert *et al.* (1966) used haemoglobin-coated charcoal for the same purpose.

All these adsorbing substances have some disadvantages. Magnesium carbonate is a powder, and after centrifugation the light sediment and a number of particles on the sides of a tube might

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easily be stirred up by careless pipetting. The presence of particles in the supernatant considerably increases the optical density of a sample and affects the colorimetric measurement. A number of investigators have observed that the use of the methods involving ion exchange resin give falsely high results for UIBC (Charlton, Hardie, and Bothwell, 1965; Stojceski, Malpas, and Witts, 1965; Birdsall, Kok, and Wild, 1965). Charlton *et al.* (1965) suggested that the absorption of water by the dry resin, with a proportional increase in the concentration of all substances in the supernatant, results in increased UIBC, and that this can be overcome by counting the radioactivity in the supernatant. However, this means washing the resin which prolongs the procedure. The other modifications of the original method of Bothwell *et al.* (1959) by Stojceski *et al.* (1965) and Birdsall *et al.* (1965) proposed a change in the order of adding the reagents. These workers claim to have obtained good results in a comparison with the colorimetric method. The haemoglobin-coated charcoal is a good adsorbing substance but only removes approximately 98% iron from solution. It is said that this can be overcome by the application of a correction factor to the results. However, one disadvantage is that the coated charcoal has to be prepared every month.

It appeared possible that magnesium carbonate, which, in spite of the disadvantages referred to above, had been used successfully in some colorimetric methods for determination of UIBC, might be used in a radioactive method to which these disadvantages would not apply. Its use in such a method would overcome the difficulties experienced with resin and haemoglobin-coated charcoal.

The purpose of this paper is to present a simple method for the determination of UIBC using radioactive iron with magnesium carbonate as the adsorbing substance, and to compare it with three other methods for the determination of unsaturated iron-binding capacity.

MATERIAL AND METHODS

The determination of UIBC was carried out on sera from 30 patients suffering from various diseases.

COLLECTION AND STORAGE OF SAMPLES Using plastic syringes samples of venous blood were taken from the patients (Johnson's Ethical Plastics Ltd.) and placed in plastic tubes (Luckham Ltd.) made iron free by standard procedures. After separation of serum, samples were assayed immediately or deep frozen until the measurements were carried out. All measurements on one sample were done on the same day. Iron-free glassware was used throughout the experiment.

SERUM IRON Serum iron was estimated on duplicate samples by the method of Caraway (1963) as modified by Harriss (1963).

UNSATURATED IRON-BINDING CAPACITY This was estimated on duplicate samples using the method described in this paper and also by three other well known methods, details of which follow.

Using radioactive iron and magnesium carbonate To make up the stock solution, 9·6 g ferric chloride (Analar, Hopkin & Williams Ltd.) was dissolved in 100 ml 0·1 NHCl and 0·5 ml of this solution was diluted to 100 ml with 0·1 N HCl; for the working solution, to 5·0 ml of stock solution approximately 5 μc of radioactive iron (radioactive ferric chlorite in 0·1 N HCl, Radiochemical Centre, Amersham) was added and then diluted to 100 ml with distilled water. The solution contained approximately 5 μg Fe/ml in 0·005 N HCl, and the exact amount of iron was accurately measured by one of the existing methods for iron determination. The other reagent required is magnesium carbonate, anhydrous (Hopkin & Williams Ltd.) Adsorptive properties were checked before routine use.

ASSAY PROTOCOL Duplicate tubes were set up containing 0·5 ml of serum and 1·0 ml of working radioactive iron solution. The tubes were shaken and left to stand for 5 min at room temperature. Approximately 125 mg of magnesium carbonate was then added, the tubes were covered with parafilm, shaken vigorously for 10 to 15 sec, and reshaken four to five times during the next minute. After centrifugation (2,000 rpm for 10 min) 1·0 ml of supernatant was taken for radioactive counting. Duplicate standards were also made containing 0·5 ml of water and 1·0 ml of the working radioactive iron solution and 1·0 ml was taken for radioactive counting. The UIBC in μg Fe/100 ml of serum was calculated from:

$$\frac{\text{Radioactivity of sample}}{\text{Radioactivity of standard}} \times 2 \times \text{iron content of working solution } (\mu\text{g/ml} \times 100).$$

The colorimetric determination of TIBC using tripyridyltriazine and magnesium carbonate (Caraway, 1963) The method used was a modification by Harriss (1963). The volume of serum was reduced from 2·0 to 0·5 ml with corresponding reduction of the amounts of the reagents used. Unsaturated iron-binding capacity was obtained by subtraction of serum iron from the total iron-binding capacity.

Coated charcoal assay of UIBC using radioactive iron and haemoglobin-coated charcoal (Herbert et al., *1966)* The method was used as described by the authors.

The determination of UIBC using radioactive iron and an ion exchange resin (Bothwell et al., *1959)* The method was used as modified by Charlton *et al.* (*1965*).

RECOVERY OF TRANSFERRIN The recovery of transferrin was estimated using human transferrin (Behringwerke AG.) shown to be 100% pure on acetate cellulose electrophoresis. The transferrin was dissolved in saline to make a concentration of 6·0 mg/ml and 2·0 ml of this solution was added to 5·0 ml of serum. The control sample was made by adding 2·0 ml of saline to 5·0 ml of

the same serum. Unsaturated iron-binding capacity was measured on four samples of transferrin and serum mixture and four control samples by each method under investigation. The recovery was represented by the difference between UIBC values of samples with and without added transferrin, and expressed as a percentage of the expected increase of UIBC, calculated on the basis that 1 mg of transferrin was capable of binding 1·25 μg of iron.

APPARATUS A Unicam SP 600 spectrophotometer was used for colorimetric measurements and a Philips gamma automatic counter for radioactive counting.

RESULTS

SUITABILITY OF MAGNESIUM CARBONATE FOR USE IN A RADIOACTIVE METHOD FOR UIBC DETERMINATION

The use of magnesium carbonate in a colorimetric method for UIBC determination has two disadvantages. First, the recommended time for shaking magnesium carbonate suspension is at least 30 min (Ramsay, 1957; Morgan and Carter, 1960; Caraway, 1963). Secondly, if the particles of magnesium carbonate are left in the supernatant after centrifugation they may increase optical density of samples and thus give falsely high values for unsaturated iron-binding capacity. In order to see whether these disadvantages of magnesium carbonate affect a radioactive iron method for UIBC determination, the following experiments were carried out.

Magnesium carbonate was added to 12 tubes with 1·5 ml of radioactive ferric chloride in aqueous solution (3 μg of iron) and duplicate samples were in turn shaken for 30 sec, 1, 5, 10, 20, and 30 min respectively. After centrifugation, aliquot samples of supernatant were taken for radioactive counting. Although most of the iron was removed after 30 min the radioactivity removed from the supernatant of the samples taken from 1 to 20 min was higher than 99·8% (Fig. 1).

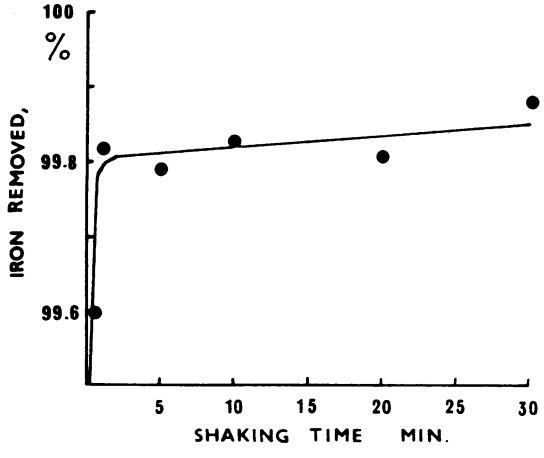

FIG. 1. *Shaking time and percentage of iron removed by magnesium carbonate from an aqueous solution containing 3 μg of iron.*

The possible effect of the traces of magnesium carbonate in the supernatant on the accuracy of UIBC determination is illustrated in the following experiment.

Magnesium carbonate was added to 10 samples of radioactive ferric chloride (3 μg of iron) in aqueous solution. Tubes were shaken for 1 min and then centrifuged for 10 minutes. After centrifugation tubes in duplicate were gently shaken so that different amounts of magnesium carbonate were suspended in the supernatant. Aliquots of these suspensions were pipetted, the optical density was determined in a spectrophotometer, and the radioactivity was measured. There was a good correlation when the radioactivity was plotted against optical density (Fig. 2). It can be seen that

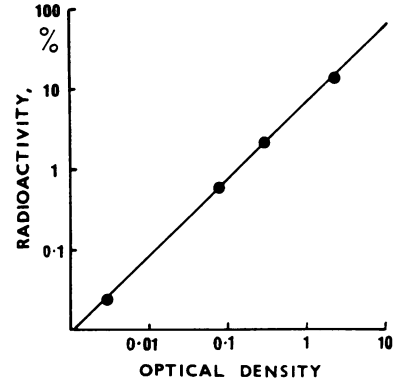

FIG. 2. *The relation between the optical density and the radioactivity of magnesium carbonate suspensions. Each circle represents the mean of a duplicate measurement.*

amounts of magnesium carbonate which would give an enormous increase of optical density did not affect the results of the radioactive assay.

COMPARISON OF THE ABILITY OF DIFFERENT ADSORBING SUBSTANCES TO REMOVE IRON FROM AQUEOUS SOLUTIONS

The ability of magnesium carbonate (anhydrous, Analar, Hopkin & Williams Ltd.), haemoglobin-coated charcoal (Charcoal, Norit A Neutral, pharmaceutical grade, Amend Drug and Chemical Co., Inc. New York, N.Y.), and ion exchange resin (Amberlite IRA 401 (HCl), Hopkin & Williams Ltd.) to remove 3 μg of iron from aqueous solutions of ferric chloride, ferrous ammonium sulphate, and ferric citrate, respectively, was compared. In all, 10 measurements in duplicate were made for each

adsorbent (Table I). Magnesium carbonate and resin removed more than 99% of iron from solution in every instance, while the amount of iron removed by haemoglobin-coated charcoal was slightly less and in the range from 95 to 99%.

TABLE I

COMPARISON OF ADSORBING SUBSTANCES IN THEIR ABILITY TO REMOVE 3 μg IRON FROM AN AQUEOUS SOLUTION (10 DUPLICATE MEASUREMENTS)

Adsorbing Substance	*Iron Salt*	*Iron Removed (%)*	
		Mean	*Range*
Magnesium carbonate	Ferric chloride	99·8	99·8-99·9
Haemoglobin-coated charcoal	Ferrous ammonium sulphate	97·4	94·6-98·7
Ion exchange resin Amberlite IRA-401 (Cl)	Ferric citrate	99·2	99·1-99·3

REPRODUCIBILITY OF METHODS USED FOR UIBC DETERMINATION The reproducibility of all methods for UIBC determination was tested by means of (1) the coefficient of variation obtained on 10 estimations on one sample; (2) the combined coefficient of variation between a number of duplicate samples with UIBC ranging from 50 to 550 μg Fe/100 ml; and (3) the recovery of transferrin added to serum samples.

These parameters, obtained for each one of four methods, were compared (Table II). It was shown that there was no substantial difference in the reproducibility of all four methods.

TABLE II

REPRODUCIBILITY OF THE RADIOACTIVE IRON-MAGNESIUM CARBONATE METHOD AND THE METHODS OF CARAWAY (1963), HERBERT *et al.* (1966), AND BOTHWELL *et al.* (1959)

	Method			
	Radioactive Iron-magnesium Carbonate	*Caraway* (1963)	*Herbert* et al. (1966)	*Bothwell* et al. (1959)
Coefficient of variation (%)[1]	3·1 (10) 3·2 (9) 1·9 (6)	4·7 (10)	3·0 (10)	5·6 (10)
Combined coefficient of variation between duplicate estimations (%)[2]	3·5 (27)	4·5 (19)	5·4 (19)	2·4 (8)
Transferrin recovery (%)[3]	98	97	98	—

[1]In brackets number of estimations on one sample.
[2]In brackets number of duplicate estimations.
[3]Estimated on quadruplicate samples.

CORRELATION OF UIBC MEASURED IN DIFFERENT SERUM SAMPLES BY FOUR METHODS The correlation of UIBC measured in different serum samples using the radioactive iron-magnesium carbonate method, the method of Caraway (1963), and the method of Herbert *et al.* (1966) is shown in Figure 3a, b, and c. There is an excellent correlation between measurements of UIBC done in duplicate, obtained by all three methods. However, when the method of Herbert *et al.* (1966) was correlated with the radioactive iron-magnesium carbonate method and the method of Caraway (1963) a slight displacement of the regression line from the ideal one was found.

There were insufficient samples measured by the method of Bothwell *et al.* (1959) to be included in the general statistical analysis. However, as can be seen from Table III, there is no obvious difference between UIBC determined by the radioactive iron-magnesium method and the method of Bothwell *et al.* (1959).

TABLE III

COMPARISON OF UIBC OBTAINED BY THE RADIOACTIVE IRON-MAGNESIUM CARBONATE METHOD AND BY THE METHOD OF BOTHWELL *et al.* (1959)

Unsaturated Iron-binding Capacity (μg Fe/100 ml)

Radioactive Iron-magnesium Carbonate Method	*Method of Bothwell* et al. (1959)
55	76
269	252
323	338
452	463
530	532
555	544

DISCUSSION

There was no striking difference in the ability of magnesium carbonate, haemoglobin-coated charcoal, and ion exchange resin to remove iron from solution. However, magnesiom carbonate had certain advantages over other adsorbing substances (Table IV),

TABLE IV

COMPARISON OF PROPERTIES OF MAGNESIUM CARBONATE, HAEMOGLOBIN-COATED CHARCOAL, AND ION EXCHANGE RESIN

	Magnesium Carbonate	*Haemoglobin-coated Charcoal*	*Ion Exchange Resin*
Preparation	Unnecessary	Necessary	Unnecessary
Quantity added	Approximate	Exact volume of suspension	Approximate
Shaking time	1 min.	10 min.	1 min.
Correction for radioactivity remaining in supernatant	Unnecessary	Necessary	Unnecessary
Storage	Indefinite at room temperature	1 month at +4°C	Indefinite at room temperature

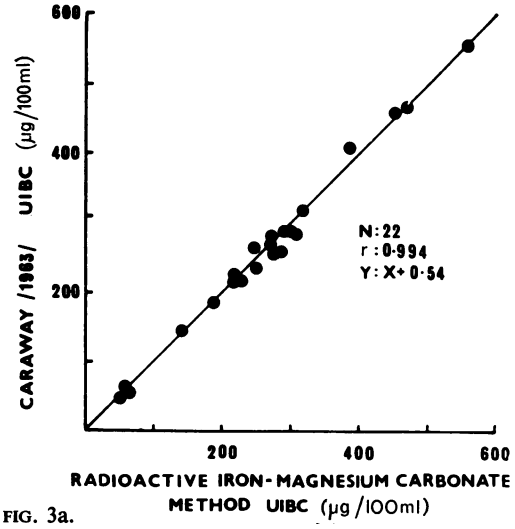

FIG. 3a.

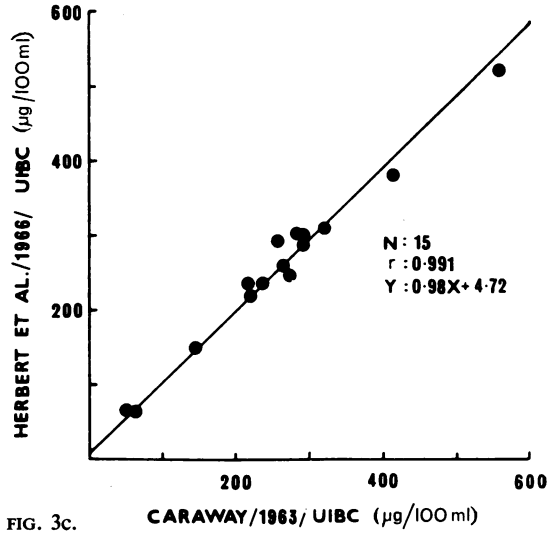

FIG. 3c.

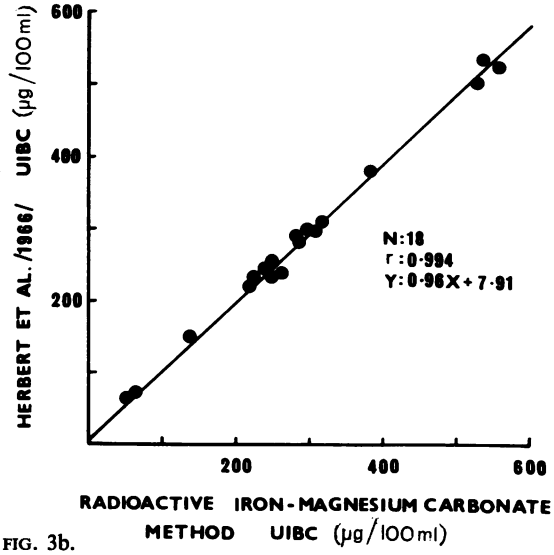

FIG. 3b.

FIG. 3*a, b, and c, The correlation of UIBC values measured by the radioactive iron-magnesium carbonate method, the method of Caraway (1963), and the method of Herbert* et al. *(1966). N=number of duplicate estimations, r=coefficient of correlation, y=regression line.*

viz., the fact that it was not necessary to add an exact amount of substance, the short shaking time required, and no need for the correction of results, no preparation and the indefinite shelf-life at room temperature make magnesium carbonate superior to haemoglobin-coated charcoal. In comparison to resin, magnesium carbonate appears to have an advantage because of the simplicity of the whole procedure.

The statistical analysis on results obtained by all four methods for determination of UIBC in serum (radioactive iron-magnesium carbonate, Caraway (1963), Herbert *et al.* (1966), Bothwell *et al.* (1959)) showed them to be equally reproducible (Table II). The coefficient of correlation also showed an excellent agreement between values for UIBC of different serum samples measured by three methods (radioactive iron-magnesium carbonate method, Caraway (1963) Herbert *et al.* 1966)). However, the position of the regression line indicated that the method of Herbert *et al.* (1966) gave slightly lower values for UIBC in the range from 400 to 600 µg Fe/100 ml when compared with the radioactive iron-magnesium carbonate method and the method of Caraway (1963). This could be the result of applying one correction factor as described by Herbert *et al.*

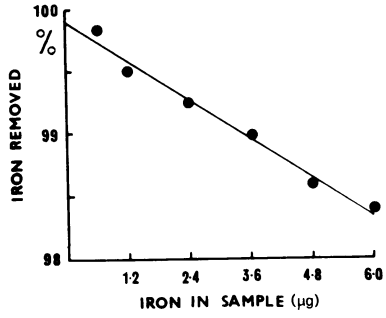

FIG. 4. *The relation between the amount of iron and the percentage of iron removed by haemoglobin-coated charcoal from an aqueous solution. Each circle represents the mean of a duplicate measurement.*

(1966) for correction of all values of UIBC. This is illustrated in the following experiment (Fig. 4).

Different concentrations of an aqueous solution of ferrous ammonium sulphate were prepared in duplicate and after the addition of haemoglobin-coated charcoal and centrifugation, the radioactivity of the aliquots of supernatant was counted. It was shown that the radioactivity remaining in the supernatant was dependent on the amount of free iron, and, therefore, a single factor could not be applied uniformly for correction of all UIBC values. Needless to say, this small error does not affect the validity of results in a routine procedure.

In a small number of sera, UIBC was measured by the radioactive iron and magnesium carbonate method and by the method of Bothwell *et al.* (1959), and no substantial difference was found. This is in agreement with reports in the literature that there is practically no difference in UIBC measured by a radioactive iron method using ion exchange resin and by a colorimetric or immunodiffusion technique (Birdsall *et al.*, 1965; Charlton *et al.*, 1965; Hillman, Morgan, and Finch, 1967; Stojceski *et al.*, 1965; Tauxe, 1961). On the other hand, Burrows (1967), Herbert *et al.* (1966), and Koepke (1965) found that UIBC values measured by the radioactive iron method with a resin were approximately 100 μg Fe/100 ml higher than measured by other methods. However, these authors used resin sponges (Irosorb-59, Abbott Laboratories, North Chicago, Ill.) instead of resin beads in their investigations. The discrepancy needs to be clarified.

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