Studies on the serum iron-binding capacity

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SYNOPSIS The total iron-binding capacity of serum has been estimated using a colorimetric method, a radioisotopic technique, and a method employing immunodiffusion. The modified isotopic technique described was considered to be the method of choice. The total iron-binding capacity starts to increase at an early stage of an iron-deficiency anaemia. It is decreased in infectious and malignant disease. Close correlation exists between the level of saturation of the total iron-binding capacity and the haemoglobin level in patients with uncomplicated iron-deficiency anaemia.

In patients with disease of the liver a difference was sometimes found between the total iron-binding capacity determined by the immunodiffusion method and by the two other techniques, the values with the immunodiffusion method being distinctly lower. This suggests the presence in the serum of iron which is not bound to transferrin. It is possible that ferritin was present in the serum in these cases.

Iron is carried in the plasma bound to transferrin which is a β_1 globulin. The total amount of iron which can be bound by transferrin has been used to measure the amount of the protein present in the plasma and has been termed the total iron-binding capacity. Earlier workers used colorimetric methods to determine the iron-binding capacity (Holmberg and Laurell, 1945; Kaldor, 1955; Laurell, 1947; Peters, Giovanniello, Apt, and Ross, 1956; Ramsey, 1957; Rath and Finch, 1949; Ressler and Zak, 1958; Ventura, 1952). More recently techniques have been developed using radioisotopes of iron (Bothwell, Jacobs, and Kamener, 1959; Feinstein, Bethard, and McCarthy, 1953) and a third approach has been to use an immunological technique (Goodman, Newman, and Ramsey, 1958; Jager and Gubler, 1952; Soothill, 1962; Wright, 1959).

The present investigation has been concerned with a direct comparison between three different types of technique for estimating iron-binding capacity in control subjects and patients with a variety of pathological conditions.

MATERIALS AND METHODS

All determinations were carried out with iron-free apparatus with serum obtained from fasting patients and stored at -20°C . in iron-free containers until required.

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THE SERUM IRON This was determined by a colorimetric method (Bothwell and Mallett, 1955). Iron was removed from the serum protein by adding 2 N hydrochloric acid. The proteins were then precipitated with 20% trichloroacetic acid and separated by centrifugation. The iron in the supernatant was estimated colorimetrically using α, α' -dipyridyl; reading of the optical density at 525 m μ was made on a Uvispeck spectrophotometer. The amount of iron present was read off from a previously constructed standard curve.

A COLORIMETRIC DETERMINATION OF SERUM TOTAL IRON-BINDING CAPACITY The method of Peters *et al.* (1956), also using α, α' -dipyridyl, was chosen. Ferric ammonium citrate, 0.1 ml., containing 5 μg , of iron was added to 1 ml. of serum. After standing for 10 minutes 0.5 g. of iron-free Amberlite I.R.A. 410 resin was added. Barbitone buffer, pH 7.5, 5 ml., was added after a further 10 minutes and then allowed to stand for an equal length of time. The mixture was then centrifuged, 4 ml. of the supernatant solution taken, α, α' -dipyridyl added, and the optical density was read at 525 m μ .

ISOTOPIC METHOD OF DETERMINATION OF TOTAL IRON-BINDING CAPACITY The reagents used in the radioisotopic determination and our technique for determining the unsaturated iron-binding capacity and consequently total iron-binding capacity, are similar in most respects to the method described by Bothwell *et al.* (1959). ^{59}Fe -labelled ferrous ammonium citrate was used and in the method described by Bothwell the buffer was added before the resin. The supernatant solution was counted in a small sodium iodide well type scintillation counter.

Initially a discrepancy was noticed between the results of the total iron-binding capacity determined by Peter's method and Bothwell's radio-isotopic method. In Bothwell's method the Amberlite resin is added to a relatively large total volume and sedimentation occurs rapidly. Inversion of the tube to prevent this results in some loss of the resin with its bound iron. When 0.5 g. of resin was added to the mixture of serum and radio-active ferric ammonium citrate before the buffer, mixing without inversion was possible and no resin was lost. After this the procedure was exactly that described by Bothwell. Results in close agreement with Peter's colorimetric method were then obtained.

IMMUNOLOGICAL METHOD FOR DETERMINING TRANSFERRIN
An immunodiffusion technique was used with a strong specific rabbit antihuman transferrin (Soothill, 1962). The diffusion was carried out in 1% Oxoid Ionager no. 2 gel with a veronal buffer pH 7.5. The gel was applied to microslides. As shown in Fig. 1, each 'antigen hole' was surrounded by an identical series of six 'antibody holes'. Antigen preparations were diluted. The unknown transferrin sera were used undiluted and diluted 1/8. The standards were diluted 1/2, 1/4, 1/8, and 1/16. The diffusion patterns produced by the known sera were matched with the patterns produced by the various dilutions of the standard. The concentration of the protein in the undiluted specimen can then be determined.

FIG. 1. Agar plate for the gel diffusion precipitin technique for estimating transferrin in serum. The antigen preparations are placed in the central holes. The unknown antigen undiluted and diluted 1/8 is placed in the middle column. The standard antigen diluted 1/2, 1/4, 1/8, and 1/16, is placed in the columns on either side. Antiserum in six dilutions is placed in the six holes arranged around the central hole. The dilutions used are 2/7, 1/5, 3/20, 1/10, 3/40, and 1/20.

HAEMOGLOBIN LEVEL The level of the haemoglobin was determined as oxyhaemoglobin using a photoelectrical method.

A COMPARISON OF DIFFERENT METHODS OF ESTIMATING RESULTS FOR TOTAL IRON-BINDING CAPACITY

The estimations of the total iron-binding capacity on the serum samples are shown in Tables I to V.

DETERMINATION OF THE TOTAL IRON-BINDING CAPACITY USING BOTHWELL'S METHOD AND THE MODIFICATION It can be seen from Table I that our modification of the method gave values for the total iron-binding capacity which were about 10% less in duplicate tests on the same sera than Bothwell's method. The modified radioactive method gives results much more in accord with the colorimetric method. This is shown in Table II.

TABLE I

COMPARISON OF BOTHWELL'S ISOTOPE METHOD AND PRESENT MODIFICATION FOR DETERMINING TOTAL IRON-BINDING CAPACITY

Number of Cases	Unsaturated Iron-binding Capacity ($\mu\text{g. \%}$)	
	Bothwell's Method	Modified Radcliffe Infirmary Method
1	217	203
	218	200.5
2	355	340.9
	361	338
3	212	177
	225	182
4	321	288
	313	279
5	425	370
	465	379
Mean values	301.5	268.3

TABLE II

DETERMINATIONS OF UNSATURATED IRON-BINDING CAPACITY BY TWO RADIOISOTOPE METHODS COMPARED WITH PETERS'S COLORIMETRIC METHOD ON EIGHT SERUM SAMPLES

Serum	Unsaturated Iron-binding Capacity ($\mu\text{g. \%}$)		
	Bothwell's Method	Modified Radcliffe Infirmary Method	Peters's Method
1	180	115	114
2	298	207	212
3	358	325	310
4	382	378	375
5	248	216	230
6	373	328	318
7	144	112	110
8	332	292	288
Mean values	289.3	246.6	244.6

DETERMINATION OF TOTAL IRON-BINDING CAPACITY USING THE MODIFIED ISOTOPE TECHNIQUE AND THE COLORIMETRIC METHOD The total iron-binding capacity was determined on stored pooled sera at twice-weekly intervals for a month. Successive estimations

with the modified radioisotope method agreed closely. The same series of determinations were done by the colorimetric method and again agreement was close (Table III). The same agreement was found between the isotope technique and the colorimetric method in 11 normal subjects and 73 patients with various conditions such as iron-deficiency anaemia, myeloid-sclerosis, haemochromatosis, and cirrhosis. Six patients with malignant disease showed a statistically significant difference in the results obtained by the two methods.

DETERMINATION OF TOTAL IRON-BINDING CAPACITY
COLORIMETRIC, ISOTOPIC, AND IMMUNODIFFUSION
METHOD Sera from three normal subjects and 29

patients were examined using all three methods. There was close agreement in the results from normal controls, iron-deficient patients, and patients with miscellaneous conditions, with the exception of one patient with pulmonary emphysema (Table IV).

DISCUSSION With the introduction of the modification in the isotopic method close agreement was obtained in a large number of determinations. If facilities for radioactive techniques are available the use of radioactive iron is a simple, rapid and satisfactory method of determining the total iron-binding capacity and would appear to be the method of choice. The immunodiffusion technique is time-

TABLE III
EIGHT DETERMINATIONS OF TOTAL IRON-BINDING CAPACITY AT TWICE WEEKLY INTERVALS IN POOLED SERUM
USING PETERS'S COLORIMETRIC AND THE RADIOISOTOPE METHODS

Serum No.	Peters's Method			Radcliffe Infirmary Method		
	Unsaturated Iron-binding Capacity	Total Iron-binding Capacity	% Saturation	Unsaturated Iron-binding Capacity	Total Iron-binding Capacity	% Saturation
1	246	343	28.2	251.5	348.5	27.9
2	248	345	28.1	253.1	350.1	27.7
3	247	344	28.1	245.5	342.5	28.4
4	252	349	27.7	219.7	316.7	30.6
5	252	349	27.7	245	342	28.4
6	252	349	27.7	242	339	28.6
7	252	349	27.7	262	359	27
8	252	349	27.7	248	345	28.1
Mean values	250	347.1	27.76	245.8	342.7	28.3

TABLE IV
COMPARISON OF COLORIMETRIC, RADIOISOTOPE, AND IMMUNODIFFUSION METHODS OF DETERMINING TOTAL
IRON-BINDING CAPACITY IN NORMAL CONTROLS AND PATIENTS WITH VARIOUS DISEASES

Case	Diagnosis	Serum Iron ($\mu\text{g. \%}$)	Total Iron-binding Capacity ($\mu\text{g. \%}$)		
			Peters's Method	Radcliffe Infirmary Method	Immunodiffusion Method
1	Normal	122	355	345	375
2	Normal	181	375	367	400
3	Normal	126	328	312	300
4	Iron-deficiency anaemia	66	307	317	300
5	Iron deficiency anaemia	32	342	351	350
6	Iron-deficiency anaemia	50	431	415	350
7	Iron-deficiency anaemia	48	350	352	325
8	Iron-deficiency anaemia	54	377	413	375
9	Iron-deficiency anaemia	84	294	308	300
10	Plummer-Vinson syndrome	92	399	405	400
11	Myeloid-sclerosis	53	260	257	225
12	Carcinoma of oesophagus	24	207	196	190
13	Refractory anaemia	46	201	188	250
14	Pulmonary sarcoidosis; haemothorax	64	160	145	175
15	Polycythaemia	64	291	279	275
16	Crohn's disease	20	190	195	225
17	Pulmonary emphysema	166	396	382	250

Statistical comparison of method

	Student's <i>t</i>	Value
Radioisotope-immunodiffusion	0.885	NS
Radioisotope-Peters's	0.617	NS
Peters's-immunodiffusion	1.045	NS

NS = not significant.

consuming and there has been insufficient experience for it to be used routinely.

No explanation was apparent for the differences observed between the colorimetric method and isotope method results for total iron-binding capacity in malignant disease.

RELATION OF THE TOTAL IRON-BINDING CAPACITY, THE LEVEL OF HAEMOGLOBIN, SERUM IRON, AND SATURATION OF THE IRON-BINDING CAPACITY TO EACH OTHER

RESULTS The results of the estimations of serum iron, unsaturated iron-binding capacity, total iron-binding capacity and percentage saturation in normal subjects and a variety of diseases are given in Table V. Iron-deficiency anaemia was defined as a haemoglobin level of less than 13.5 g. per 100 ml. in males and 11 g. in females, with a serum iron of less than 70 μ g. per 100 ml. and a percentage saturation of less than 28. Latent iron deficiency was defined as a percentage saturation of less than 28 in the absence of anaemia.

The other diagnoses were made on clinical grounds, supported where necessary by biochemical tests, barium studies, and biopsy. The relationship between the level of haemoglobin, serum iron values, total iron-binding capacity and percentage saturation in 36 patients with iron-deficiency anaemia and latent iron deficiency is shown in Figures 2 to 6.

DISCUSSION The measurement of the serum iron or total iron-binding capacity alone is not so helpful in assessing the state of the body's iron stores as a combination of the two investigations. The ratio of the serum iron to the total iron-binding capacity, the percentage saturation, can be shown to be closely correlated with the haemoglobin (Fig. 6), and our work confirms previous studies (Beutler, 1957; Cartwright and Wintrobe, 1949; Zizza and Block, 1961). There appears to be no correlation of the total iron-binding capacity with the level of haemoglobin

FIG. 2. The regression of total iron-binding capacity on serum iron is not significant.

FIG. 3. The regression of percentage saturation of total iron-binding capacity on serum iron is significant at the 0.1% level.

or serum iron (Fig. 2 and 5) and this also confirms previous studies (Antila, 1962; Beutler, Robson, and Bittenwieser, 1958; Zizza and Block, 1961).

TABLE V

SERUM IRON, UNSATURATED IRON-BINDING CAPACITY, TOTAL IRON-BINDING CAPACITY, AND PERCENTAGE SATURATION IN NORMAL CONTROLS AND IN A NUMBER OF DISEASES

Group	Diagnosis	Number of Cases	Mean Serum Iron (μ g. %)	Ranges	Mean Unsaturated Iron-binding (μ g. %)	Ranges	Total Iron-binding Capacity (μ g. %)	Ranges	% Saturation	Ranges
1	Iron-deficiency anaemia	24	44.4	16-70	337.4	260-484	381.8	315-520	11.6	3.6-19
2	Latent iron-deficiency anaemia	10	87	74-100	285.7	210-455	372	285-460	23.3	18.5-26.5
5	Malignancy and refractory anaemia	8	35.4	16-48	160	81-250	195.5	150-251	18.1	8-42
3	Ulcerative colitis and Crohn's disease	6	35.7	18-50	254	171-377	289	195-419	12.8	5.6-22.6
4	Myelosclerosis	4	58	46-68	242	217-271	292	260-330	19.6	17-22
5	Normal controls	11	124	89-188	173	151-195	297	275-319	41.6	34.6-48.6

FIG. 4. The regression of the serum iron on haemoglobin is significant at the 0.1% level.

FIG. 5. The regression of the total iron-binding capacity on haemoglobin is not significant.

FIG. 6. The regression of the percentage saturation of the total iron-binding capacity on haemoglobin is significant at the 0.1% level.

In our study there is a significant correlation between serum iron value and the level of haemoglobin, although this has not been considered to occur by previous workers (Antila, 1962; Beutler, 1957; Verloop, Meeuwissen, and Blockhuis, 1958).

Beutler maintains that the level of the total iron-binding capacity only starts to rise when the level of the haemoglobin falls below 9 g. per 100 ml. (Beutler *et al.*, 1958). In Fig. 5 the level of the total iron-binding capacity has risen above the upper limit of normal (350 μ g. per 100 ml.) before the haemoglobin has fallen to 11 g. In the absence of any complicating factor, therefore, it would seem that the level of the total iron-binding capacity rises at an early stage of iron deficiency.

Studies in the other pathological states confirmed previous findings. The depression of the total iron-binding capacity that occurs in malignant diseases

was noted (Laurell, 1947; Verloop *et al.*, 1958; Zizza and Block, 1961). The finding of a normal total iron-binding capacity in myelosclerosis confirmed that of Laurell (1952). In three patients with ulcerative colitis and three with Crohn's disease a low percentage saturation is similar to the result found by Zizza and Block (1961) in one patient with ulcerative colitis. In normal subjects the range of values closely paralleled those of previous studies (Antila, 1962; Hathorn, Gillman, Canham, and Lamont, 1960; Laurell, 1947; Mitchell, Halden, Jones, Bryan, Stirman, and Muirhead, 1960; Peters *et al.*, 1956; Ramsay, 1957; Rath and Finch, 1949).

THE TOTAL IRON-BINDING CAPACITY IN DISEASES OF THE LIVER

RESULTS The levels of serum iron and total iron-binding capacity in conditions other than disease

of the liver are shown in Table IV. There is in all cases except 6 and 17 close agreement between all three methods.

The results of the total iron-binding capacity estimations in patients with non-alcoholic and alcoholic cirrhosis, haemochromatosis and transfusional haemosiderosis, and one case of infectious hepatitis are given in Table VI. In six of the 15 patients studied the total iron-binding capacity of the serum sample is lower when estimated by the immunodiffusion method.

DISCUSSION The circulating iron in the serum of normal subjects is nearly all bound to the β -globulin transferrin. Iron not bound to transferrin will be present when ferritin is liberated in necrosis of liver cells.

Circulating ferritin is not found in measurable quantities in the serum of normal subjects but Mazur and Shorr (1948; 1950) showed its presence in shock, congestive heart failure, and cirrhosis of the liver. Reissman, Boley, Christianson, and Delp (1954) found that an increase in the serum iron occurred in animals subjected to liver toxins and that ferritin appeared in the serum in acute liver disease in man (Reissman and Dietrich, 1956). A few patients with cirrhosis and haemochromatosis were also found to have circulating ferritin, usually in the late stages of their illness (Reissman *et al.*, 1954).

In the present study three out of eight patients with cirrhosis of the liver and two out of four patients with haemochromatosis showed a discrepancy between the total iron-binding capacity found by the immunodiffusion technique and that estimated by the other two methods. This discrepancy can only be explained by the presence of iron not bound to transferrin. In one patient (E.R., Table VI) no discrepancy between the total iron-binding capacity determinations was noted on admission. Later, however, when she was deteriorating, iron not bound to transferrin appeared in the serum. The appearance of this iron not bound to transferrin in the terminal stages of liver disease would suggest that ferritin was present in this patient and possibly in the other patients with the discrepancy. Further work is being done to investigate this finding.

The presence of iron not bound to transferrin may lead to a false assessment of the percentage saturation of the total iron-binding capacity. It is possible that it may be responsible for the condition seen in some patients who have an apparently increased saturation of the iron-binding capacity and yet no evidence histologically of increased deposition of iron. In one patient reported by Conrad, Berman, and Crosby (1962) markedly increased saturation of the total iron-binding capacity was noted when determined by the colorimetric method. On phlebotomy, however, only a few grams of iron had been

TABLE VI
COMPARISON OF COLORIMETRIC, RADIOISOTOPE, AND IMMUNODIFFUSION METHODS FOR DETERMINING TOTAL IRON-BINDING CAPACITY IN LIVER DISEASE

Case	Diagnosis	Serum Iron ($\mu\text{g. } \%$)	Total Iron-binding Capacity ($\mu\text{g. } \%$)		
			Peters's Method	Radcliffe In- firmary Method	Immunodiffusion Method
V.H.	Non-alcoholic cirrhosis	258	258	269	275
E.B.W.	Non-alcoholic cirrhosis	144	—	244	275
E.W.	Non-alcoholic cirrhosis	184	—	322	350
R.A.	Non-alcoholic cirrhosis	104	331	345	300
E.R.	(i) Non-alcoholic cirrhosis	96	291	284	300
	(ii) In failure	212	270	278	200
P.Q.	Alcoholic cirrhosis	252	277	281	200
A.S.	Alcoholic cirrhosis	72	—	242	200
C.H.	Alcoholic cirrhosis	116	320	296	200
F.A.	Transfusion haemosiderosis	244	267	264	200
F.D.A.	Transfusion haemosiderosis	250	—	261	275
E.D.	Haemochromatosis	152	216	200	175
L.D.	Haemochromatosis	172	—	222	250
J.E.	Haemochromatosis	278	307	314	200
B.J.	Haemochromatosis and alcoholism	39	228	207	125
K.E.	(i) Haemochromatosis	213	250	263	275
	(ii) Haemochromatosis and alcoholism	246	270	300	200
J.T.	Acute infectious hepatitis				
	(i) When anaemic	23	—	—	—
	(ii) When jaundiced	110	321	333	225
Statistical comparison of method					
			Student's <i>t</i>	Value	
Radioisotope-immunodiffusion			3.026	S at 1%	
Radioisotope-Peters's			0.552	NS	
Peters's-immunodiffusion			3.854	S at 1%	
NS = not significant. S = significant.					

removed when the patient became iron-deficient.

In the case of our two cirrhotic patients who have high serum iron levels (E.R. and P.Q., Table VI) the percentage saturation of their total iron-binding capacity is lowest when calculated from the total iron-binding capacity obtained by the immunodiffusion method and when allowance is made for the presence of iron not bound to transferrin.

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REFERENCES

- Antila, V. (1962). *Acta med. scand.*, suppl., 393.
 Beutler, E. (1957). *New Engl. J. Med.*, **256**, 692.
 —, Robson, M. J., and Bittenwieser, E. (1958). *Ann. intern. Med.*, **48**, 60.
 Bothwell, T. H., Jacobs, P., and Kamener, R. (1959). *S. Afr. J. med. Sci.*, **24**, 93.
 —, and Mallett, B. (1955). *Biochem. J.*, **59**, 599.
 Cartwright, G. E., and Wintrobe, M. M. (1949). *J. clin. Invest.*, **28**, 86.
 Conrad, M. E. Jr., Berman, A., and Crosby, W. H. (1962). *Gastroenterology*, **43**, 385.
 Feinstein, A. R., Bethard, W. F., and McCarthy, J. D. (1953). *J. Lab. clin. Med.*, **42**, 907.
 Goodman, M., Newman, H. S., and Ramsey, D. S. (1958). *Ibid.*, **51**, 814.
 Hathorn, M., Gillman, T., Canham, P. A. S., and Lamont, N. M. (1960). *Clin. Sci.*, **19**, 35.
 Holmberg, C. G., and Laurell, C. B. (1945). *Acta physiol. scand.*, **10**, 307.
 Jager, B. V., and Gubler, C. J. (1952). *J. Immunol.*, **69**, 311.
 Kaldor, J. (1955). *Med. J. Aust.*, **2**, 886.
 Laurell, C. B. (1947). *Acta physiol. scand.*, **14**, suppl., 46.
 — (1952). *Pharmacol. Rev.*, **4**, 371.
 Mazur, A., and Shorr, E. (1948). *J. biol. Chem.*, **176**, 771.
 —, — (1950). *Ibid.*, **182**, 607.
 Mitchell, J., Halden, E. R., Jones, F., Bryan, S., Stirman, J. A., and Muirhead, E. E. (1960). *J. Lab. clin. Med.*, **56**, 555.
 Peters, T., Giovannelli, T. J., Apt, L., and Ross, J. F. (1956). *Ibid.*, **48**, 274.
 Ramsay, W. N. M. (1957). *Clin. chim. Acta*, **2**, 221.
 Rath, C. E., and Finch, G. A. (1949). *J. clin. Invest.*, **28**, 79.
 Reissman, K. R., Boley, J. O., Christianson, J. F., and Delp, M. H. (1954). *J. Lab. clin. Med.*, **43**, 572.
 —, and Dietrich, M. R. (1956). *J. clin. Invest.*, **35**, 588.
 Ressler, N., and Zak, B. (1958). *Amer. J. clin. Path.*, **30**, 87.
 Soothill, J. F. (1962). *J. Lab. clin. Med.*, **59**, 859.
 Ventura, S. (1952). *J. clin. Path.*, **5**, 271.
 Verloop, M. C., Meeuwissen, J. E. T., and Blockhuis, E. W. M. (1958). *Brit. J. Haemat.*, **4**, 70.
 Wright, S. T. C. (1959). *Nature (Lond.)*, **183**, 1282.
 Zizza, F., and Block, M. (1961). *Acta haemat. (Basel)*, **25**, 1.