CHEMICAL, CLINICAL, AND IMMUNOLOGICAL STUDIES ON THE PRODUCTS OF HUMAN PLASMA FRACTIONATION. XXXVIII. SERUM IRON TRANSPORT. MEASUREMENT OF IRON-BINDING CAPACITY OF SERUM IN MAN [1]

By CHARLES E. RATH [2] AND CLEMENT A. FINCH [3]

(From the Department of Medicine, Harvard Medical School, and the Medical Clinic, Peter Bent Brigham Hospital, Boston)

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Iron absorbed from the intestine, from destroyed erythrocytes, and from storage depots must be constantly redistributed via the blood stream to satisfy the needs of various body tissues. The studies of Heilmeyer and Plotner (1) and of Moore and his associates (2) indicate that the iron of the serum performs this function. It has been further established that this iron is protein-bound, since the iron is non-dialyzable (3), does not appear in the ultrafiltrate unless acidified (4) and is precipitable with the serum globulins (5).

Employing a micro-biological assay method, Schade (6) recently localized an iron-binding protein in Fraction IV–4 of Cohn and his associates. The crystallized protein (7) is a β_1 globulin [4] with a molecular weight of approximately 90,000, and binds two molecules of iron per molecule of protein. The globulin itself is colorless but when combined with iron, develops a salmon red color. This color reaction described by Schade has been utilized in the measurement of the iron-binding capacity of normal and pathological sera.

METHOD

Schade (6) has shown that a progressive development of red color occurs on the addition of iron to this β_1 globulin until the protein becomes saturated. At the point of saturation there is a sharp break in the color curve which corresponds to the exact point at which free iron may be demonstrated by bio-assay. The spectrophotometric absorption curve of this iron-protein combination has been described (8). A wave length of 525 mμ was arbitrarily chosen in our studies because of the greater color absorption of serum at shorter wave lengths. On each sample of serum a determination of serum iron and unsaturated iron-binding capacity was made.

Fasting venous blood is drawn without hemolysis into a syringe coated with mineral oil. The clotted blood is centrifuged at 2,000 r.p.m. for 15 minutes and the serum obtained is recentrifuged to remove all red cells. The Coleman Spectrophotometer, Model 11, and cuvettes of 1 cm. depth are used. One cuvette is filled with 5 cc. of 0.9 per cent saline, while in the other is placed 2 cc. of serum and 3 cc. of 0.9 per cent sodium chloride. Originally each cuvette was filled with serum, one serving as a blank. This was found to be unnecessary. Iron standard solution [5] was added in 0.05-cc. quantities to both cuvettes and a glass stirring rod used to mix the contents of the cuvette after each addition of iron. Readings of per cent of light transmission are made two or three minutes after each mixing. The iron solution is added until there has been no change in the per cent transmission after three successive readings. The data are plotted on graph paper and the point of intersection of the two slopes is taken as the amount of iron necessary to saturate the iron-binding protein (Figure 1). Serum iron determinations were made according to the method of Kitzes, Elvehjem, and Schuette (9). It is possible to determine the total capacity of each sample of serum by totalling the serum iron and the unsaturated binding capacity.

The blood is drawn in the morning with the patient in the fasting state. Lipemic serum, severe icterus, and serum over 24 hours old, were found unsatisfactory. All glassware is carefully cleaned with concentrated nitric acid and glass-redistilled water to render it iron-free. All reagents used are likewise iron-free. Figure 2 shows

[1] Supported by a grant-in-aid from the United States Public Health Service.

[2] Research Fellow in Medicine, Harvard Medical School.

[3] Associate in Medicine, Harvard Medical School; Associate in Medicine, Peter Bent Brigham Hospital.

[4] This β_1 globulin has been variously termed metal-combining globulin and iron-binding protein; the terms being used synonymously. *In vitro* evidence indicates that the protein is capable of combining with other metals (8). Since the only *in vivo* function of this protein thus far demonstrated is that of iron transport, reference to other metal binding will not be made in this paper.

[5] Iron standard is prepared by diluting 14 mgs. of ferrous ammonium sulfate plus 0.5 cc. of 1 N acetic acid to 100 cc. This represents 20 gamma of iron per cc. of standard. Each standard solution is checked by direct iron analysis. Therefore, each addition of 0.05 cc. to 2 cc. of plasma represents an increment of 50 gamma per 100 cc. serum.

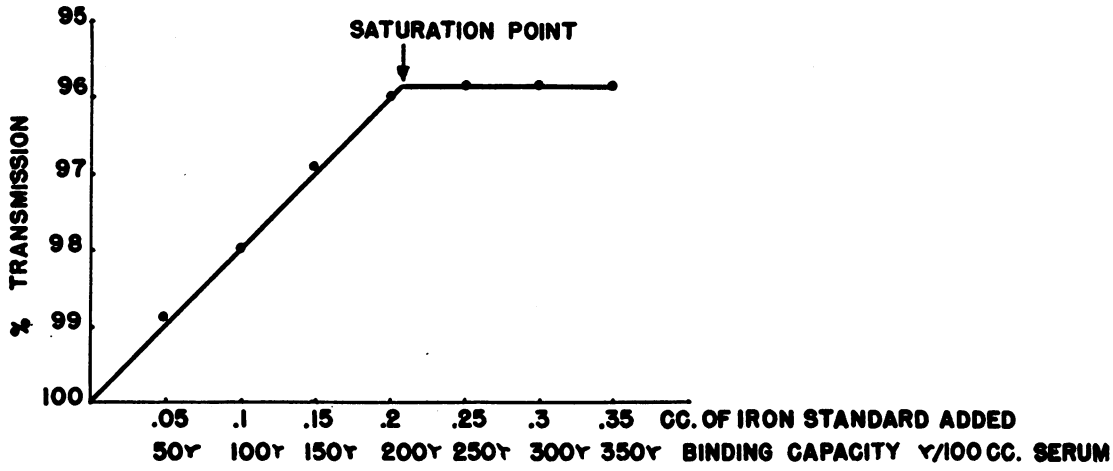

FIG. 1. DETERMINATION OF THE IRON-BINDING CAPACITY OF SERUM

the iron-binding capacity titration with known increments of crystalline iron-binding protein. Table I shows the measured as compared with the calculated increase in iron-binding capacity of serum upon the addition of increments of crystalline iron-binding protein. This method is readily adapted to a colorimeter with a filter of 525 mμ.

RESULTS

Measurements of serum iron, unsaturated iron-binding capacity, total capacity and per cent saturation on 30 normal subjects and 105 patients are shown in Table II, and the groups of particular interest are portrayed graphically in Figure 3. There was no significant difference between men and women. In the combined normal group, serum iron averaged 100 gamma, iron-binding capacity 200 gamma, and total capacity 300 gamma per 100 cc. of serum. Circulating iron-binding protein was 34 per cent saturated with iron. In iron

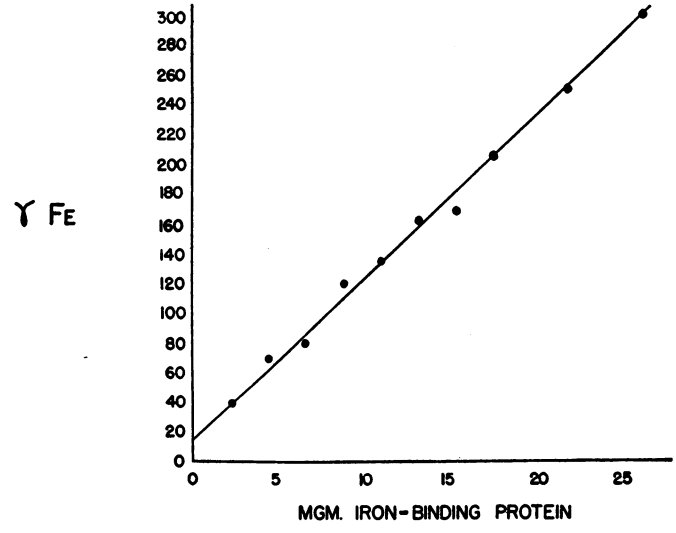

FIG. 2. IRON-BINDING PROTEIN TITRATION

The amount of iron required to saturate increments of the pure β_1 globulin was determined colorimetrically. Each point represents a single determination of iron-binding capacity. Each milligram of protein binds about 1.25 gamma of iron and the measurement is accurate within 25 gamma per 100 cc. When increments of β_1 globulin are added to plasma the same amounts are obtained with an accuracy of within 50 gamma per 100 cc.

TABLE I

Measurement of iron-binding capacity on addition of iron-binding protein to serum

	Capacity per 100 cc. of serum	Iron-binding capacity per 100 cc. of crystalline protein added	Iron-binding capacity per 100 cc.	
			Anticipated	Found
	gamma/100 cc.	*gamma/100 cc.*	*gamma/100 cc.*	*gamma/100 cc.*
I	100	30	130	140
	100	60	160	170
	100	90	190	180
	100	120	220	235
	100	150	250	265
	100	180	280	275
	100	210	310	300
	100	240	340	350
II	250	75	325	330
	250	150	400	400
	250	225	475	455
	250	300	650	630
	250	375	725	720

In the table above, increments of crystalline protein of known iron-binding capacity were added to serum with a measured capacity of 100 (I) and to serum with a measured capacity of 250 (II). The increased iron-binding capacity of the serum as determined by the method herein described is compared with the anticipated new binding capacity representing the sum of the capacity of the serum plus the capacity of the added crystalline protein.

deficiency, while the serum iron was lowered, there was an increase above normal in both the unsaturated iron-binding capacity and total carrying capacity of the serum. This is to be contrasted with infection in which serum iron was similarly reduced but where the iron-binding capacity and total capacity were reduced as well. It is of interest that the saturation was below 10 per cent in eight out of ten cases of iron deficiency, while it was above 10 per cent in all ten cases of infection. In general, in patients with iron deficiency who also had carcinoma or other debilitating disease, the per cent saturation was still in the vicinity of 10 per cent, but the total capacity was not significantly increased. In a variety of conditions having in common only general debility and reduction in circulating plasma protein (cancer, infection, liver disease, and renal disease), there was a reduction in the iron-carrying capacity of the serum. Ten normal pregnant women showed no significant deviation from the normal during the first, second, or third trimester of pregnancy. A high serum iron and high percentage saturation of the iron-binding protein were found in refractory anemia, pernicious anemia, hemochromatosis, transfusion hemosiderosis, and liver disease.

Preliminary work has been carried out with Fraction IV–7 processed from human plasma by Surgenor and his associates (8). This material bound *in vitro* 1 mg. of iron per milligram of protein. It has been administered intravenously to 22 individuals in amounts of 2.5–5.0 gms. over periods of 15 to 30 minutes. In two patients, the injection was repeated after two weeks and in neither case was there any reaction. In general, the injections produced a slight rise in serum iron during the first four to six hours, but the maximum rise occurred after a latent period of 12 to 24 hours after injection. The increase in serum iron was as much as 115 gamma per 100 cc. of plasma. This fell over the following two to six days un-

TABLE II

Measurements of serum iron, unsaturated iron-binding capacity, total capacity and per cent saturation on normals and patients

	S. I.	I. B. C.	Total	% Sat.	Hct.
	gamma/100 cc.	*gamma/100 cc.*	*gamma/100 cc.*		
Normal Male	147	285	432	34	48
	119	220	339	35	48
	98	200	298	33	40
	88	200	288	30	47
	115	190	305	38	47
	97	220	317	31	48
	107	200	307	35	47
	94	200	294	32	48
	87	200	287	33	43
	136	215	351	39	46
	97	222	319	30	51
	98	222	320	30	47
	104	150	254	41	45
	87	200	287	30	43
	121	150	271	44	46
Average	106	205	311	34	
Normal Female	110	190	300	37	45
	108	150	258	42	45
	120	280	400	30	40
	87	150	237	37	45
	85	250	335	25	45
	95	322	415	22	41
	130	165	295	44	43
	93	165	258	36	43
	84	144	228	37	41
	118	210	328	36	46
	76	148	224	34	40
	72	194	266	37	43
	74	200	274	27	41
	76	200	276	28	38
	82	150	232	35	40
Average	94	194	288	33	
Combined Normal Male and Female	100	200	300	34	

TABLE II—*Continued*

	S. I.	I. B. C.	Total	% Sat.	Hct.
	gamma/ 100 cc.	*gamma/ 100 cc.*	*gamma/ 100 cc.*		
Iron Deficiency Anemia					
Bleeding ulcer	37	395	410	9	29
Bleeding ulcer	60	210	270	22	37
Bleeding hemorrhoids	32	350	382	8	39
Pseudo hemophilia	26	145	171	15	35
Microcytic hypochromic anemia	27	475	502	5	27
Microcytic hypochromic anemia	11	325	336	3	18
Microcytic hypochromic anemia	15	320	345	4	25
Microcytic hypochromic anemia	29	330	359	8	31
Microcytic hypochromic anemia	32	320	352	9	36
Microcytic hypochromic anemia	22	320	340	6	19
Average	29	319	346	9	
Pernicious Anemia	129	165	294	44	20
	136	100	236	58	14
	160	50	210	76	18
	47	195	242	19	20
	164	100	264	62	23
	127	57	184	69	14
	136	60	196	69	19
Average	128	104	232	56	
Lymphoma and Leukemia					
Hodgkins	39	185	224	17	44
Lymphosarcoma	82	240	322	25	42
Chronic myelogenous leukemia	49	200	249	20	37
Chronic myelogenous leukemia	90	150	240	37	31
Subacute leukemia	64	61	125	51	23
Aleukemic leukemia	131	40	171	77	30
Acute monocytic leukemia	320	154	474	67	25
Hodgkins	46	169	215	21	29
Agnogenic myeloid metaplasia	80	100	180	44	25
Uremia	65	190	255	25	10
	77	110	187	41	20
	54	100	154	35	
	67	180	247	27	29
	35	150	185	19	20
	47	299	346	14	34
	50	157	207	24	27
	36	258	294		22
Transfusion Hemosiderosis	297	0	297	100	54
	305	0	305	100	30
	236	0	236	100	45
	207	0	207	100	11
Average	260	0	260	100	

TABLE II—*Continued*

	S. I.	I. B. C.	Total	% Sat.	Hct.
	gamma/ 100 cc.	*gamma/ 100 cc.*	*gamma/ 100 cc.*		
Infection					
Pneumonia	117	100	217	54	42
Pyrexia of unknown origin	27	170	197	14	29
Subacute bacterial endocarditis	26	220	246	11	29
Chronic pulmonary infection	21	140	161	13	54
Trichinosis	64	180	244	26	34
Subacute bacterial endocarditis	37	185	222	17	42
Pelvic inflammatory disease	42	165	207	20	41
Miliary tuberculosis	40	210	250	16	42
" "	41	195	236	17	48
Pyrexia of unknown origin	32	195	227	16	36
Average	44	176	220	20	
Hemochromatosis	233	0	233	100	
	170	50	220	77	
	235	50	285	82	48
	245	0	245	100	47
	250	57	307	82	42
	250	0	250	100	44
	204	0	204	100	45
	204	0	204	100	46
	220	50	270	81	47
Average	224	23	247	91	
Liver Disease					
Acute hepatitis	121	150	270	45	35
Cirrhosis	137	175	312	44	48
"	140	160	300	47	25
Cirrhosis with hepatitis	100	50	150	66	35
Cirrhosis	65	150	215	30	33
"	142	57	199	71	35
Cirrhosis with hepatitis	85	150	235	36	32
Cirrhosis	121	100	221	55	40
Cirrhosis, terminal	91	55	146	62	47
Average	111	116	227	50	

less the patient had hemosiderosis or hemochromatosis, in which case the elevation was maintained over a longer period. A second injection of globulin given to the same patient did not produce a rise in serum iron level except in cases of iron excess.

DISCUSSION

The validity of this measurement of the iron-binding protein capacity of human sera would seem to be established in a number of ways: (*1*) the increased capacity produced by the addition of

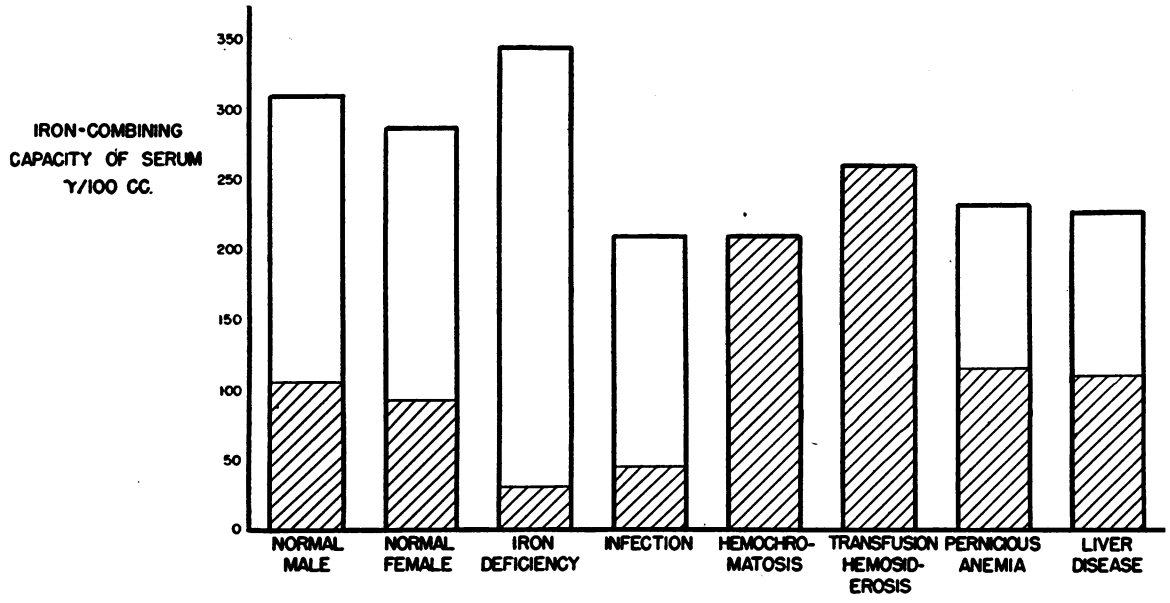

FIG. 3. IRON-BINDING CAPACITY OF SERA

The iron-binding capacity of human serum is represented in block diagram. The cross-hatched portion represents serum iron, and the clear area the unsaturated capacity of the iron-binding protein in gamma per 100 cc.

known amounts *in vitro* of pure iron-binding globulin to plasma may be measured with an error of less than 50 gamma per 100 cc.; (*2*) the intravenous injection of iron-binding protein results in an increase in the measured binding capacity proportionate to the amount of protein given; (*3*) injections of iron or of nonviable erythrocytes will result in increase in serum iron to the point of total binding capacity as previously measured but not beyond it; (*4*) those patients with saturation of their iron-binding protein show no significant increase in serum iron after oral or intravenous iron administration.

The observation that serum iron cannot be increased above the measured saturation point of the globulin confirms other evidence that iron cannot exist in a free state in the serum. The only exceptions to this are the injections of massive amounts of iron ascorbate (10) or iron ascorbate gelatin complexes and the serum of terminal hemochromatotic patients where very high levels of serum iron may be found. It would appear in both of these instances that the iron is bound to some other protein complexes. This complete protein binding of iron in the body, whether intracellular or within the blood stream, probably explains the inability of the body actively to excrete iron.

Holmberg and Laurell (11) have reported similar measurements of the iron-binding capacity of serum, employing a different method dependent on the color reaction between dipyridyl and unbound iron. In normal subjects the serum iron averaged 130 gamma per 100 cc. and the total capacity was 312 gamma per 100 cc. These figures are in good agreement with our data. As pointed out by these authors, the increase in serum iron and the maximum level attained following oral and intravenous iron tolerance tests are limited by the amount of iron-binding globulin in circulation. For example, the initial height of 291 gamma per 100 cc. observed by Waldenstrom (12) following intravenous injection of iron in normal subjects was very close to the total capacity as measured by Holmberg and Laurell and by us. The lower average level obtained by Wintrobe of 168 gamma per 100 cc. (13), in patients with infection after intravenous iron, again is consistent with the reduced binding capacity found in our group with infection.

The lack of response of serum iron in patients

with untreated pernicious anemia or hemochromatosis to oral iron administration is considered to be due to the pre-existing high degree of saturation of the β_1 globulin which will allow little or no increase in serum iron. The unaltered level of serum iron does not, however, preclude iron absorption, since the serum iron level is not an expression of the turnover rate of iron in the serum. This will be the subject of a later report.

The total binding capacity of normal sera of about 300 gamma per 100 cc. represents about 250 mgs. of iron-binding protein per 100 cc. of plasma.[6] Increases in iron-binding protein were observed by us in iron deficiency, and have been reported by Laurell (14) in pregnancy. These increases parallel the need of the body for more efficient iron absorption and transport. Further work is necessary to determine whether this increase in iron-binding protein is responsible for the increased iron absorption in these conditions. In other conditions decreases in total binding capacity to 50 per cent of normal have been observed, but it seems improbable that this reduction in any instance was capable of significantly impairing iron transport since there was an appreciable amount of unsaturated iron-binding protein still in circulation.

It would appear that the level of serum iron and the per cent saturation of the iron-binding protein are carefully regulated under normal circumstances. Conditions in which saturation of iron-binding protein is increased are those involving bone marrow block, iron excess, and severe liver disease. The important role of the liver in serum iron regulation is not unexpected since this organ is the chief iron storage depot of the body. Whether or not conditions of iron excess may be recognized without some degree of hepatic dysfunction is not yet clear. At any rate, it has been possible to make the diagnosis in nine cases of hemochromatosis by this technique and to separate these cases from simple cirrhosis. Depression of the per cent saturation occurs in iron deficiency and in infections. It seems reasonable to explain the former on the basis of depletion of body iron and the latter on an increased affinity of tissue storage depots for iron (15).

Laurell (14) in a very comprehensive and excellent study of iron transport has repeated measurements of the iron-binding protein in a variety of conditions. Although different methods were used, the results we have obtained are in perfect agreement with those of Laurell. We hesitate to accept the hypothesis that the degree of saturation may regulate iron transport and iron absorption. For example, in animals on diets which allow excessive iron absorption, the serum-binding protein becomes completely saturated with iron after about two weeks, yet iron absorption continues fully as rapidly over the following three to four weeks (16). Movement of iron within the body may be managed by the respective affinity of various tissues for iron, in which system the carrier protein of the serum would play a passive role. Injections of iron-binding protein exert only a very temporary effect on the serum iron level. It remains to be determined how much one may aid or interfere with iron transport by increasing or decreasing the amount of iron-binding protein.

[6] One milligram β_1 globulin binds about 1.25 gamma of tissue as demonstrated in Figure 1.

SUMMARY

A method is described for the measurement of the iron-binding capacity of human serum. Measurements of serum iron, total iron-binding capacity, and per cent saturation of this protein are reported in normal subjects and in a variety of diseases. The implications of these findings are discussed.

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