Determination of Iron in Serum or Plasma by Atomic Absorption Spectrophotometry

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The determination of serum iron has been shown to be feasible by the use of a modified single-beam atomic absorption spectrophotometer. The effect of interfering substances is eliminated by the use of standards prepared in physiological concentrations of protein. Average percentage recovery of added iron was 97.9 with an over-all coefficient of variation of 1.6%. Disparities between samples due to the effect of unequal viscosities and flow rates were overcome by the integration of the record curve generated by a measured volume of sample. Comparison between the method presented and a colorimetric procedure gave a higher mean value and normal range for plasma quantitated by atomic absorption spectrophotometry.

THE TECHNIC of atomic absorption spectroscopy has recently been adopted with increasing confidence in clinical biochemistry. Emphasis has largely been on the measurement of calcium and magnesium (*1–16*), although the trace metals zinc (*17, 18*), copper (*19, 20*), and lead (*18, 21*) have also received attention. Zettner (*22*) has recently reviewed the status of this technic in the clinical laboratory.

In the case of serum iron, attempts have already been made to circumvent the disadvantages of the colorimetric technics by using atomic absorption spectroscopy (*23*). However, optimum recoveries and operating conditions were not achieved. The purpose of this study was to devise a method which permits the rapid and accurate determination of total serum iron levels in the normal range without prior sample preparation. Dilution is required only for serum with an elevated level of iron.

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Method of Quantitation

Methods

The atomic absorption spectrometer used was a Jarrell-Ash series 82-360* (*11*) with a Zeiss Model 50-56-23 total combustion hydrogen-air burner.† The burner was positioned at maximum elevation above the optical bench so that it was just below the lower beam of the multiple-pass optical system. A Sargent Model SR strip chart recorder‡ was adapted for scale expansion by placing a zero suppression unit in the

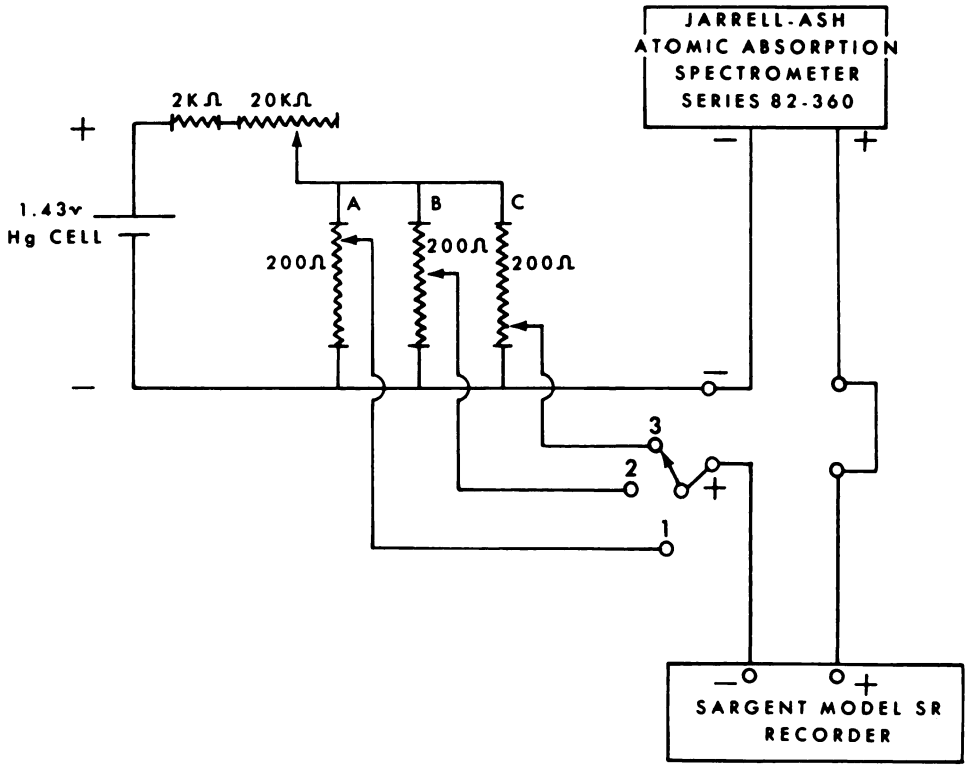
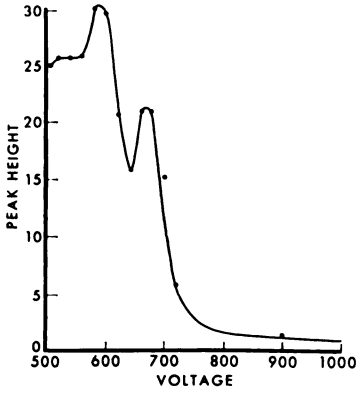


Fig. 1. Zero suppression circuit for adapting 10 mv recorder to atomic absorption spectroscopy. Variable resistances (A, B, and C) are adjusted so that bucking EMF is 5, 8, and 9 mv given a scale expansion of ×2, ×5, and ×10, respectively.

circuit (Fig. 1). The recorder was equipped with a Disc mechanical integrator§ to measure peak area rather than peak height so as to eliminate errors due to variable flow rate and sample viscosity (*24*).

*Jarrell-Ash Co., Waltham, Mass.
†Carl Zeiss, Inc., New York, N. Y.
‡E. H. Sargent Co., Chicago, Ill.
§Disc Integrator, Inc., Santa Ana, Calif.

One-milliliter samples were aspirated to completion directly into the metal capillary of the burner. The instrumental variables were optimized by examining each parameter in turn, while maintaining the others constant as shown in Fig. 2–4. Instrument settings chosen were as follows: air pressure, 10 psi; hydrogen pressure, 8 psi; current to hollow cathode lamp, 20 mamp.; voltage to photomultiplier tube, 590 v; absorption wave length, 2483 Å; and entrance and exit slits, 100 μ. The choice of 20 mamp. for the current to the iron hollow cathode lamp was made in relation to the plateau indicated in Fig. 3 and the finding that "noise" was reduced by operating at as high a current as was compatible with sensitivity.



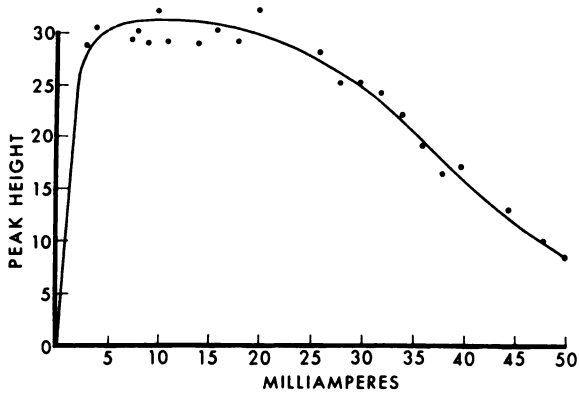
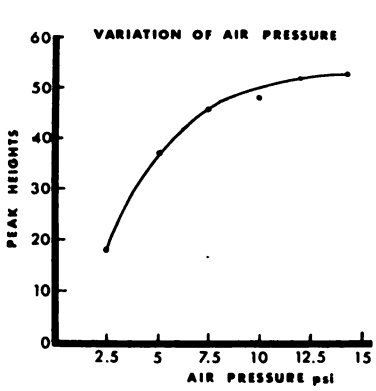


Fig. 2 (*left*). Effect of varying voltage to photomultiplier tube at optimum gain. Zero adjustment was made using gain control for each determination. Milliamps to hollow cathode = 20. **Fig. 3** (*right*). Effect of varying current to hollow cathode lamp.



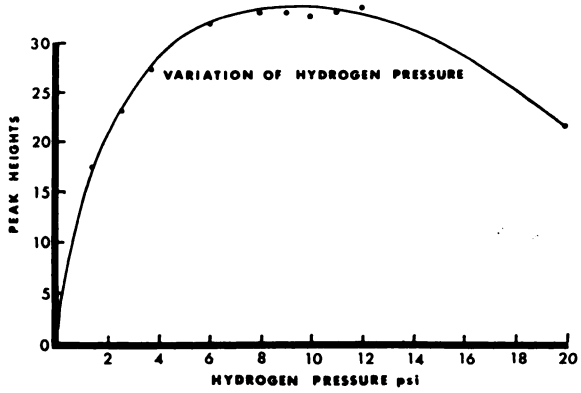


Fig. 4. Effect of varying hydrogen and air pressures. At left, hydrogen pressure is 8 psi. At right, air pressure is 7.0 psi.

Instrumentation

Recoveries of added iron, computed on the basis of the height of the peaks generated on the strip chart recorder, were unacceptably low (approximately 75%), and results tended to be erratic, which was undoubtedly due to variations in flow rate through the flame. Aspiration rates of 1-ml. samples of aqueous and protein solutions (6 gm./100 ml.), determined using a stopwatch, differed by 3.8 sec. (18 sec. for aqueous as against 21.8 sec. for protein solutions). Coefficients of variation for the aspiration rates were found to be 6.6% (18 samples) and 11.2% (21 samples), respectively. For this reason peak heights were discarded as a means of quantitation and recorder curve integration was substituted. Figure 5 shows the recorder presentation of results obtained for increasing increments of iron added to a serum of low iron content. The use of this means of quantitation depends on the hypothesis that, irrespective of the time taken for a sample to pass through the flame, the area beneath the recorder curve will be a constant, provided the sample

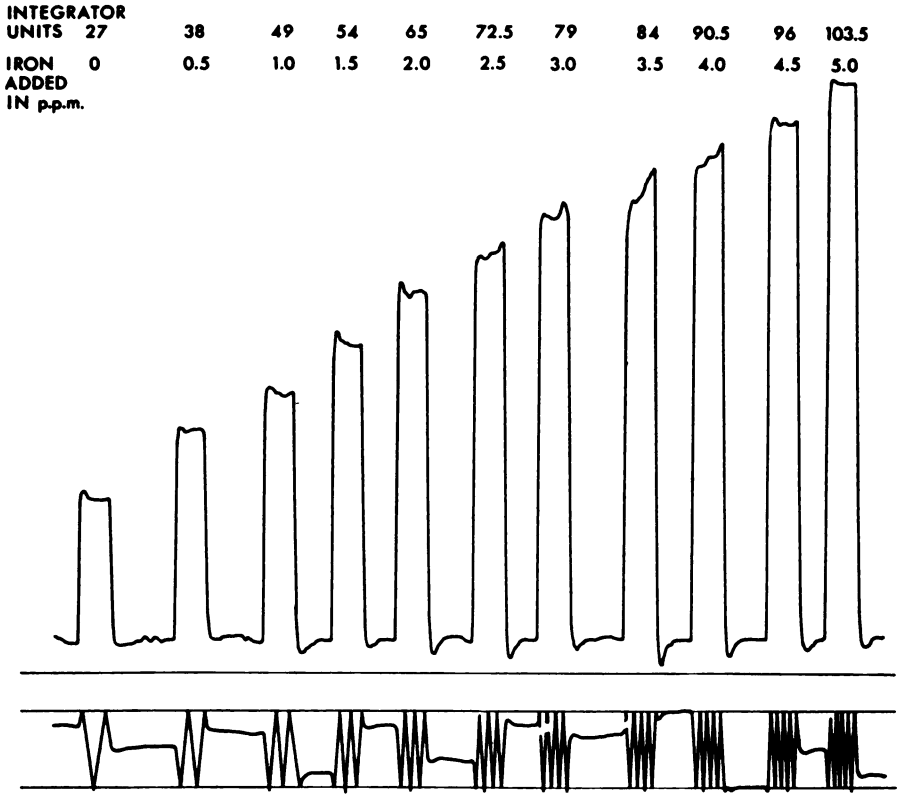


Fig. 5. Recorder curve with integrations generated by 1-ml. samples containing increasing increments of iron.

is not diluted below the limits of detectability. In order to test this hypothesis, a constant quantity of iron (5 μg.) was placed in volumes of diluent ranging from 0.5 ml. to 10 ml. The diluent in one experiment was de-ionized water and in the second experiment a solution containing bovine serum albumin (6 gm./100 ml.) which had been made iron-free. In each case the blank consisted of an identical volume of the same diluent. As can be seen (Fig. 6) the hypothesis proved only partially correct. At values above approximately 250 μg./100 ml. the areas deviated from the horizontal straight line that was expected. The explanation for this deviation must be that, under the conditions used, at concentrations of iron greater than 250 μg./100 ml., the flame became overpopulated with atoms, some of them passing through without absorbing light. While it is true that both standards and unknowns of concentrations above 250 μg./100 ml. will behave in the same manner, small variations from ideality will have a profound effect on the results obtained. It was, therefore, decided that serum specimens containing a concentration of iron greater than 250 μg./100 ml. would be diluted to concentrations below this figure.

Interfering Agents

Atomic absorption spectroscopy is known to be remarkably free of physical interferences (*22*). Chemical interference caused in particular by anions is troublesome (*3, 5, 7, 8, 11, 16, 25–30*). This interference is due to the formation of refractory salts between the metal and the anion, which reduces absorption. In addition to the anionic effect, protein has been shown to reduce absorption (*7, 8, 11, 22*). To ascertain if these

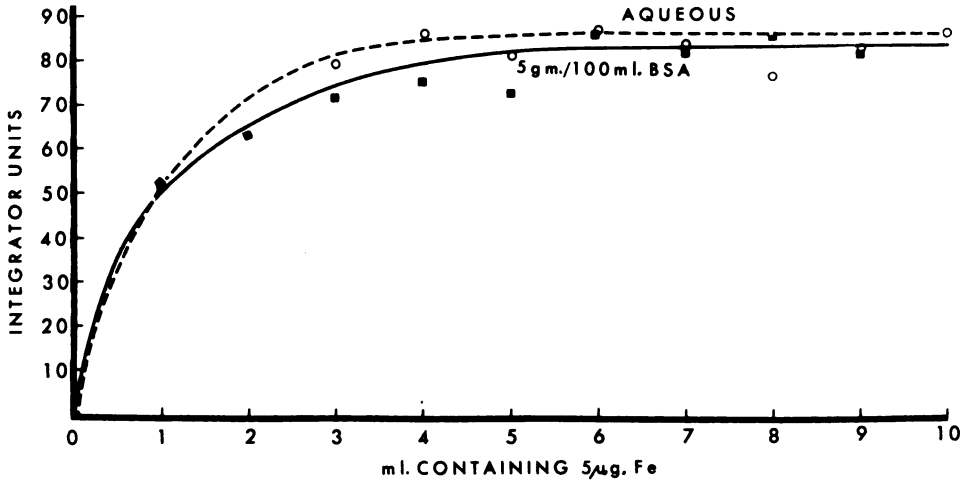


Fig. 6. Areas generated by 5 μg. of iron in varying volumes of diluent.

factors had an effect on absorption due to iron, various anions and protein were examined over a range of concentrations. Figure 7 shows the results of one such series of experiments. It is evident that phosphate (5 mg./100 ml.) produces a marked inhibition of absorption, which is counteracted over the working range of iron concentration by a physiological level of sodium chloride. The inhibition by phosphate was maximal at levels of phosphorus below 1 mg./100 ml. and remained constant between 1 and 10 mg./100 ml. The suppression of absorption caused by phosphate was not eliminated by the addition to the sample of lanthanum or strontium (*3, 7, 8, 11, 31*) or by protecting the iron with the specific chelator, desferrioxamine. Lanthanum produced a further depression in absorption, while the results obtained in the presence of desferrioxamine were unchanged. It was found that the interference of protein was similarly maximal at low concentrations and decreased

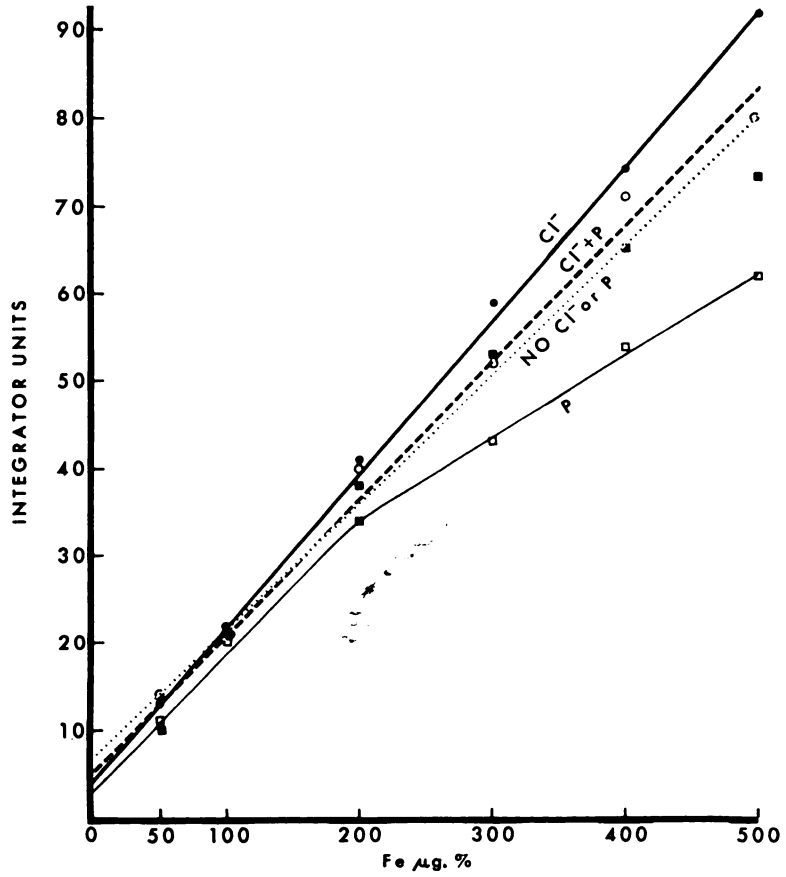


Fig. 7. Effects of chloride and phosphate on standard iron curve.

slightly with increasing concentration, but reached a plateau between 1.5 gm. and 10 gm./100 ml. (Fig. 8). The addition of phosphorus to protein solutions did not result in a cumulative effect, a further reason for not attempting to correct for the phosphate inhibition seen in aque-

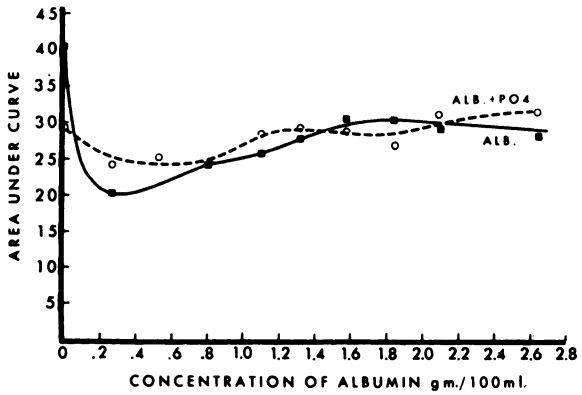


Fig. 8. Effect of increasing concentrations of albumin and phosphate on 200 μg./100 ml. iron standard.

ous solutions. In order to eliminate the possibility of other ionic interference, solutions containing physiological amounts of sodium, potassium, magnesium, calcium, chloride, bicarbonate, phosphorus, and 1 mg. of sulfate per 100 ml. were added to serum in sequence. No deviation from the original standard was observed.

Methodology

Preparation of Standards

Stock iron solution (100 μg./ml.) Ten milligrams of pure clean iron wire were dissolved in a mixture of 0.5 ml. 6 N HCl and 0.5 ml. 6 N HNO_3 and made up to 100 ml. with de-ionized water.

Working standards (50–500 μg./100 ml.) One to 10 ml. of stock standard were diluted to 100 ml. with 0.01 N HCl to give solutions with a range of iron concentration from 100 to 1000 μg./ml. An essentially iron-free solution (less than 20 μg./100 ml.) of bovine serum albumin was prepared by dialysis against repeated changes of de-ionized water. The protein solution was concentrated to approximately 15 gm./100 ml. by dialysis against Carbowax (Union Carbide Corp.). The protein concentration was determined by a biuret procedure (*32*) and the solution diluted to 12 gm./100 ml. Immediately before use, 0.5 ml. of protein solution (12 gm./100 ml.) was mixed with 0.5 ml. of each of the range of standards to give a series of solutions ranging in iron concentration from 50 to 500 μg./100 ml. in a 6 gm./100 ml. protein solution. As an

alternative to the use of bovine serum albumin, the preparation of which in an iron-free state is time-consuming, "ion-free" serum of high protein content (approximately 10 gm./100 ml.) may be used.*

Preparation of Unknowns

As has been previously stated, no dilution or preparation of sample is required provided that the concentration of iron in the serum does not exceed the level at which flame saturation (atomic overpopulation) becomes a factor in the variability of results. This level was empirically set at 250 μg./100 ml. on the basis of the results shown in Fig. 6. Samples with an iron concentration above this value are diluted into the range below 250 μg./100 ml. using an iron-free protein solution, or, should this not be available, "ion-free" serum or bovine albumin, after accurately determining the small amount of iron it contains. The results are then given by the expression:

$$\text{Iron in sample} = \left(\text{Result obtained} - \frac{\text{Concentration of iron in diluent}}{\text{Dilution factor of diluent}} \right) \times \text{Dilution factor of unknown}$$

One milliliter of sample is aspirated to completion and the area under the recorder curve determined by counting the number of integration

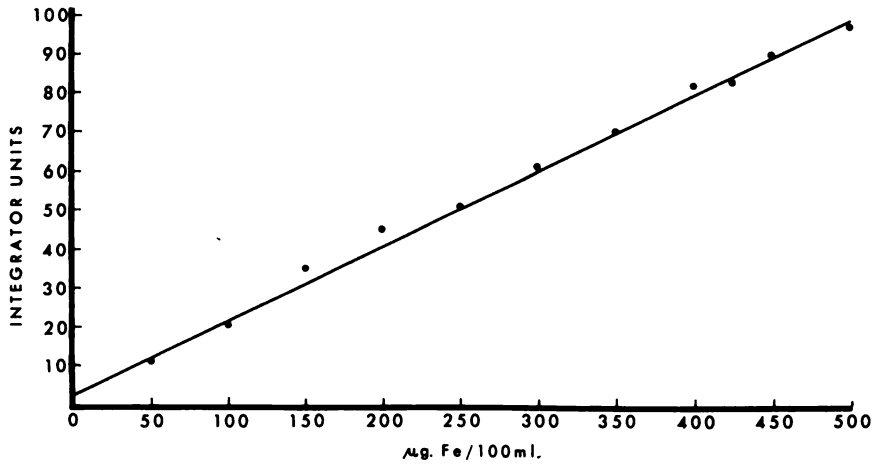


Fig. 9. Standard iron curve. Standards prepared in 6 gm./100 ml. iron-free albumin solution.

units. This figure is compared with a standard curve (Fig. 9), which in practice needs only to include readings between 0 and 250 μg./100 ml. It is important that a blank of exactly the same volume as the sample

*Available from Clinton Laboratories, Los Angeles, Calif.

be aspirated to completion in order to determine the apparent absorption due to light scattering and other causes. It was found that variation between the areas obtained for the same volumes of deionized water and a completely iron-free protein solution were small enough to be ignored; aqueous blanks were therefore used for the unknowns. Since it was more convenient to use protein solutions which were not completely iron-free for standards, a blank prepared by diluting 0.5 ml. of 12 gm./100 ml. protein solution with 0.5 ml. of de-ionized water was used in the preparation of the standard curve.

Results

In order to test the validity of the method, recoveries of iron added to a serum of low iron content were determined. The results of this study are shown in Table 1. Each result is the mean of 10 determinations. The average percentage recovery was 97.9 with a coefficient of variation of 1.6%. The coefficient of variation obtained for 10 standard solutions was 2.1% and the 95% confidence limits of the over-all procedure were 5.2%.

It must be emphasized that atomic absorption spectrophotometry will measure all iron in the serum or plasma including that contained in hemoglobin. Since it has been shown that levels of hemoglobin in plasma are markedly lower than in serum (*34*), it was decided that plasma would be the preferable matrix in which to measure iron.

A comparison between the present method and the colorimetric procedure of Fischer and Price (*33*) was made on plasma obtained from blood drawn from 12 healthy adult males. The blood was obtained by venipuncture using a heparinized "Vacutainer" without any special precautions to avoid hemolysis other than those normally taken. To determine the contribution of plasma hemoglobin to the iron levels obtained by atomic absorption spectrophotometry, plasma hemoglobin

Table 1. Recovery of Iron Added to Serum

Tube	*Mean reading (integrator units)*	*Measured concentration (μg./100 ml.)*	*Iron added (μg./100 ml.)*	*Iron recovered (μg./100 ml.)*	*Mean recovery (%)*	*Coefficient of variation* (%)*
1–10	8.5	30.0	0.0	0.0	—	1.3
11–20	13.5	60.0	33.3	30	90.0	1.6
21–30	20.3	95.0	66.6	65	97.6	1.5
31–40	26.1	125.0	100.0	95	95.0	1.2
41–50	33.6	163.5	133.3	133	99.5	2.2
51–60	40.6	200.0	166.6	170	102.0	0.9
61–70	47.0	235.0	200.0	205	102.5	1.8
71–80	51.6	260.0	233.3	230	98.5	2.4

*Coefficient of variation equals standard deviation/mean × 100.

levels were measured by a sensitive colorimetric method (*35*). The results obtained are shown in Table 2. The normal range (mean ±2 standard deviations) of 59–175 μg./100 ml. for the colorimetric method is in close agreement with that obtained by Fischer and Price (72–186 μg./100 ml.). The normal range for plasma quantitated by atomic absorption spectrophotometry without correction for hemoglobin iron was 102–292 μg./100 ml. Plasma hemoglobin content ranged from 0 to 9.8 mg./100 ml., which, on the basis of an iron content of hemoglobin of 0.34%, would contribute 0–29.2 μg. of iron/100 ml. of plasma. When the iron level of each plasma specimen was corrected for hemoglobin iron, the range was found to be 87–279 μg./100 ml. When plasma obtained from 13 adult females was assayed in the above manner a much greater scatter of results was obtained, indicating the difficulty, noted by other workers, of assessing the normal range of iron values for a female population.

Discussion

The range of values obtained by atomic absorption spectrophotometry is considerably higher than that obtained by the colorimetric method, but is compatible with values obtained by other workers (*36*). Since the atomic absorption technic eliminates precipitation and extraction steps, it would be anticipated that losses involved in these steps would account for the lower levels reported for most colorimetric procedures, a matter presently being investigated in our laboratory.

Table 2. Plasma Hemoglobin Levels in Relation to Total Plasma Iron, and Normal Ranges Obtained by Two Methods

Specimen No.	*Method of Fischer & Price (μg./100 ml.)*	*Atomic absorption spectrophotometry (μg./100 ml.)*	*Plasma hemoglobin (mg./100 ml.)*	*Plasma hemoglobin iron (μg./100 ml.)*	*"Corrected" atomic absorption spectrophotometry results (μg./100 ml.)*
1	133	275	5.0	17	258
2	132	255	3.2	11	244
3	121	255	9.6	33	222
4	143	270	3.6	12	250
5	110	175	3.0	10	165
6	115	210	3.0	10	200
7	75	150	3.6	12	138
8	180	190	3.6	12	178
9	74	130	4.9	14	116
10	80	160	6.6	19	141
11	124	180	9.3	32	148
12	112	150	3.8	13	137
Mean	117	200	4.9	16	183
S.D.	29	49	2.43	—	48
Mean ± 2 S.D.	59–175	102–298	0–9.8	—	87–279

Determination of the contribution of plasma hemoglobin to the total iron quantitated by atomic absorption spectrophotometry would appear to detract from the appeal of this method as a routine procedure. However, provided visibly hemolysed specimens are eliminated, the figures indicate that such is unnecessary for all but the most critical work. Should it be necessary to determine plasma hemoglobin levels in certain atomic absorption iron determinations, the time involved in determining both hemoglobin and iron is directly comparable with that required for most manual colorimetric methods.

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