CLII. THE ACTION OF CERTAIN REAGENTS ON THE "LOOSELY BOUND" IRON IN BLOOD.

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STUDIES of the iron in vertebrate blood have shown that the iron of the haemoglobin molecule is to be distinguished from the residual iron, which may reside partly in the corpuscles and partly in the plasma [see, especially, Barkan, 1925; 1927; 1933]. This important distinction has been established by at least three independent methods, supposedly giving similar values for non-haemoglobin iron. These are: (1) Colorimetric estimation of non-haemoglobin iron on an ultrafiltrate from blood which had been incubated with dilute hydrochloric acid [Barkan, 1925; 1927; 1933]. (2) Iodimetric estimation of iron in the trichloroacetic acid filtrate from blood which had been boiled with 5*N* hydrochloric acid [Starkenstein and Weden, 1928]. (3) Colorimetric estimation of iron in the trichloroacetic acid filtrate from blood which had been treated with thiolacetic acid [Tompsett, 1934, 1].

For whole blood or serum, Tompsett [1934, 1] has shown that 20 % trichloroacetic acid does not completely extract iron, added as iron alum, except in the presence of thiolacetic acid. Quantitative recovery of added inorganic iron in presence of thiolacetic acid however is not sufficient proof of the suitability of this reagent for the determination of non-haemoglobin iron in blood. Shorland and Wall [1936, 1] have shown in preliminary work that thiolacetic acid can liberate iron from haemoglobin, and this conclusion is now substantiated in this paper.

We have therefore examined sodium pyrophosphate [Tompsett, 1934, 2; 1935] as a possible alternative reagent and compared the results with those given by the Starkenstein and Weden [1928] method.

EXPERIMENTAL.

Experiments were made on samples of oxalated sheep's blood. Filter-papers were washed with dil. HCl and distilled water to remove traces of Fe and then dried. The trichloroacetic acid solution was prepared from freshly distilled acid. Periodic blank experiments showed the reagents to be practically free from Fe.

(1) *The action of thiolacetic acid on haemoglobin iron.*

In these experiments a method essentially similar to that recommended by Tompsett [1934, 1] was followed. 2 ml. of blood were diluted with an equal volume of water and treated with a specified quantity of thiolacetic acid. 4 ml. of 20 % trichloroacetic acid were immediately stirred in and the solution filtered after a given time through a no. 41 Whatman filter-paper. 1 ml. of filtrate was treated with 2 drops of conc. ammonia in a 1 ml. cup of a Duboscq colorimeter. The standard colour was prepared similarly from 2 ml. of a stock solution of

iron alum containing 2 mg. Fe in 100 ml. 1% H_2SO_4 . Comparison is made after dilution of the unknown to approximately the same depth of colour as the standard and the non-haemoglobin Fe evaluated.

Fig. 1 shows the variation of apparent non-haemoglobin Fe with time at two concentrations of thiolacetic acid. Fig. 2 shows the effect of varying the concentration of thiolacetic acid from 2 to 25 drops (1 drop = 0.05 ml. approx.) in a 48-hour reaction.

Fig. 1.

Fig. 2.

Fig. 1. Effect of time on the value obtained for non-haemoglobin iron by thiolacetic acid. Total Fe, 45 mg./100 ml. (A) 5 drops thiolacetic acid; (B) 1 drop thiolacetic acid.

Fig. 2. Effect of concentration of thiolacetic acid on the liberation of iron from blood. (A) Total Fe, 45 mg./100 ml. (B) Total Fe, 34 mg./100 ml.

Clearly, the time taken for the estimation, which is not specified by Tompsett [1934, 1], would materially affect the apparent value for the non-haemoglobin Fe. When the period of contact was short (3 min.), values of 0.83 to 1.09 mg./100 ml. were obtained for non-haemoglobin Fe. These compare with 0.96 to 1.56 mg./100 ml. for human blood [Tompsett, 1934, 1].

(2) *Sodium pyrophosphate as a reagent for the determination of non-haemoglobin iron.*

Method. Mix 2 ml. of oxalated blood and 2 ml. of 4% sodium pyrophosphate in a test-tube. After 15 min. stir in 2 ml. of 20% trichloroacetic acid solution and filter through a no. 41 Whatman paper. Add 2 ml. of filtrate to 1 ml. of 0.0012 *M* $\alpha\alpha'$ -dipyridyl in 5 *M* ammonium acetate. After developing the colour as described in Shorland and Wall's [1936, 2] modification of Hill's method [1931], compare with standard colour (prepared similarly from 2 ml. of a solution of iron alum containing 1 mg. Fe/100 ml. 1% H_2SO_4) in a Duboscq colorimeter.

Table I. *The determination by the sodium pyrophosphate method of inorganic iron added to blood.*

mg. Fe/100 ml.		
Non-haemoglobin Fe of sample	Added inorganic Fe	Reported non-haemoglobin Fe
0.72	1.00	1.70
0.33*	1.00	1.33
0.38*	1.00	1.35
0.87	2.00	2.86
0.86	2.00	2.85
0.51	2.00	2.51

* Determinations made on samples which had stood several days.

Table I shows that inorganic Fe, added as iron alum, is quantitatively recovered, affording an indication of the efficiency of the method. It is not, however, certain that non-haemoglobin Fe is fully determined by this reagent, as Barkan [1933] has shown that there is in blood the "easily-separable" Fe which can be distinguished from inorganic Fe by its failure to be adsorbed on aluminium hydroxide. Tompsett [1934, 2] has suggested the use of pyrophosphate for the liberation of Fe from such complexes.

The action of sodium pyrophosphate on the haemoglobin Fe was tested by allowing the reagent to stand in contact with blood for different periods prior to estimation of the liberated Fe (Table II).

Table II. *The effect of time of contact of the sodium pyrophosphate solution with blood on the reported non-haemoglobin iron.*

Time of contact (min.)	Reported non-haemoglobin Fe (mg. Fe/100 ml.)
Sample (A) 10	0.83
20	0.83
Sample (B) 5	0.70
20	0.70
50	0.70

Table III shows that fresh blood gives a higher non-haemoglobin Fe value by this method than blood which has stood several days.

Table III. *The effect of ageing of blood samples on their reported non-haemoglobin iron content.*

mg. Fe/100 ml.					
Fresh blood	3 days old	Fresh	8 days old	Fresh	15 days old
0.71	0.30	0.86	0.38	0.91	0.59
0.81	0.29	0.68	0.27	0.80	0.56
0.89	0.33	0.59	0.33	0.75	0.48
0.86	0.32	0.77	0.61	—	—
0.96	0.42	0.71	0.57	—	—

The actual rate of change of non-haemoglobin Fe as given by this method is shown for a particular sample as below:

Table IV.

Hours	mg. Fe/100 ml.
0	0.89
24	0.80
36	0.51
48	0.43

Barkan [1927] observed that by use of his method the apparent non-haemoglobin Fe increased if the blood were aged for several weeks prior to the determination. The reverse seems to be true of the present method.

Although, so far as the authors are aware, comparable figures for the non-haemoglobin Fe of sheep's blood are not available, Barkan [1927] finds for several species of vertebrates, including man, a mean value of approximately 1.7 mg. Fe per 100 ml. For human blood this has been confirmed by Starkenstein and Weden [1928], whilst Tompsett [1934, 1] gives lower values varying from 0.96 to 1.56 mg. Fe per 100 ml.

Even for fresh blood, the values given by the sodium pyrophosphate method appear to be somewhat low. The method was therefore compared (Table V)

with that of Starkenstein and Weden [1928]. Firstly, however, the statement of Starkenstein and Weden that boiling 5*N* HCl had no appreciable action on haemoglobin Fe was confirmed: boiling the sample for 1 min. with the 5*N* acid resulted in practically the same figure for non-haemoglobin Fe as boiling for 5 min.

Table V. *Reported non-haemoglobin iron.*

Mg./100 ml.	
Sodium pyrophosphate method	Starkenstein and Weden's method
0.79	1.22
0.86	1.52
0.76	1.69
0.71	1.25
0.87	2.00
0.89	1.73
0.97	1.79
0.89	1.64
0.80	1.88

The values for the non-haemoglobin Fe obtained by the sodium pyrophosphate method are much lower than those obtained with Starkenstein and Weden's reagent. Since both these reagents are without effect on the haemoglobin Fe under the experimental conditions used it seems that part of the non-haemoglobin Fe must be more labile than the rest.

SUMMARY.

The actions of thiolacetic acid and sodium pyrophosphate respectively on the iron in blood have been examined. The former reagent has a definite action on haemoglobin iron and is therefore not suitable for the estimation of non-haemoglobin iron. Good recoveries of inorganic iron added to blood are given by the latter reagent which is shown not to attack haemoglobin iron. The values obtained for the non-haemoglobin iron by the sodium pyrophosphate method, however, appear to be considerably lower than those obtained by means of Starkenstein and Weden's reagent (boiling 5*N* hydrochloric acid).

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