CCXXXVIII. STUDIES OF THE COMPLEXES OF IRON WITH VARIOUS BIOLOGICAL MATERIALS.

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IN a previous communication the present writer [1934, 1] has confirmed Hill's observation [1931] that all the iron of the hen's egg is in the inorganic form. It was found that when the proteins of a suspension of egg or egg-yolk were precipitated with trichloroacetic acid, no iron was detected in the filtrate. Iron was also absent from the filtrate after the addition of inorganic iron to the egg in the form of iron alum. When, however, thiolacetic acid was added before the precipitation of the proteins, iron was found in the filtrates.

The iron content of the filtrates was determined colorimetrically by means of the colour produced by iron and thiolacetic acid in alkaline solution [Lyons, 1927; Tompsett, 1934, 1]. The iron content of the egg as determined on the filtrates by this method was found to give the same value within the limits of experimental error as that obtained after ashing. It appears that iron forms very stable complexes with the proteins or other organic constituents of the hen's egg. Similar phenomena were noticed in the determination of the inorganic iron content of whole blood and serum. Copper did not appear to form such complexes. The writer has already shown [1934, 2] that, when the proteins of whole blood or serum are precipitated with trichloroacetic acid, the whole of the copper is present in the filtrate and added copper may be determined quantitatively.

Lintzel [1931] and Heubner [1926] have stated that ferric but not ferrous iron forms stable complexes with proteins and therefore suggest that iron must be reduced to the ferrous condition before absorption from the intestine. As thiolacetic acid liberates iron from its stable complexes and reduces ferric iron to the ferrous condition, in all probability complexes are not formed with ferrous iron.

This communication is an extension of earlier work instituted to determine the possibility of substances other than thiolacetic acid liberating iron from its complexes and to attempt to study the nature of the substances which apparently form the complexes. Sodium hydrosulphite was used owing to the ease with which it reduces ferric iron to the ferrous condition; also sodium pyrophosphate since it forms un-ionised complexes with iron and is an important constituent of muscle.

EXPERIMENTAL.

All filter-papers used in this investigation were washed with dilute acid and distilled water and dried, and the trichloroacetic acid was distilled *in vacuo*. The sodium pyrophosphate was found to be free from iron in the amounts used. The sodium hydrosulphite contained an appreciable amount of iron which was allowed for.

Egg-yolk was first studied. The yolk of one egg was diluted to 100 ml. Four 10 ml. samples were taken and treated as follows:

- (1) Control diluted to 15 ml. with distilled water.
- (2) 2 ml. thiolacetic acid added and distilled water to 15 ml.
- (3) 5 ml. of 4 % sodium pyrophosphate added and distilled water to 15 ml.
- (4) 2.5 ml. of 5 % sodium hydrosulphite added and distilled water to 15 ml.

After standing 5 minutes, 15 ml. of 20 % trichloroacetic acid were added, well shaken and filtered. To 5 ml. of filtrate 6 drops of thiolacetic acid were added followed by 1 ml. ammonia, sp. gr. 0.880. The colours were compared in a colorimeter with standards containing 0.015 mg. Fe similarly prepared. In the case of the controls negative reactions for iron were obtained. A precipitate of calcium phosphate appeared in the determinations containing pyrophosphate but this was separated by centrifuging without removing any of the colour. When sodium hydrosulphite was used the colour did not appear at once in either the test or the standard but only after vigorous shaking and standing. A precipitate of calcium phosphate appeared when sodium hydrosulphite was used to liberate the iron but could be removed by centrifuging without interfering with the estimation. When thiolacetic acid was used to liberate the iron no precipitate was ever obtained.

The results of these experiments are shown in Table I. It will be seen that within the limits of experimental error the iron content of egg-yolk is found to be the same whether thiolacetic acid, sodium pyrophosphate or sodium hydrosulphite is used to liberate the iron from its complex. Experiments were also carried out using sodium glycerophosphate and dithioglycollic acid but the filtrates gave negative tests for iron.

Table I. *The iron content of egg-yolk (expressed as mg. Fe per egg).*

	Thiolacetic acid	Sodium pyrophosphate	Sodium hydrosulphite	Control
1	1.28	1.24	1.22	Nil
2	1.24	1.18	1.26	Nil
3	0.96	0.99	1.00	Nil
4	1.10	1.12	1.17	Nil
5	1.16	1.12	1.10	Nil

In the second series of experiments ferric iron was added to various biological materials. Milk, sodium caseinogenate and egg-white gave negative reactions for iron within the limits of the amount used. Lecithin, gelatin and edestin contained appreciable amounts which were allowed for. The lecithin emulsion was prepared by pouring an ethereal solution of lecithin into boiling water and boiling off traces of ether. The sodium caseinogenate was made up in 2 % solution by dissolving a commercial preparation in boiling water and then cooling. The egg-white solution was prepared by diluting the white of a hen's egg to 100 ml. The edestin solution was prepared in a concentration of 2 % in 0.1N HCl. The gelatin solution was prepared in a concentration of 2 % in hot water and cooled. Ferric iron in the form of iron alum was added to these solutions and 10 ml. samples treated in the same way as the egg-yolk samples described in the previous experiments. The results are shown in Table II and are expressed in mg. Fe per 10 ml. solution or suspension. In the experiments with milk a precipitate of calcium phosphate appeared which could be removed by centrifuging without interfering with the depth of colour.

Table II. *Recovery of iron added to various biological materials.*

	Iron added mg.	Ferric iron. Iron recovered (mg.)			
		Control	Thiolacetic acid	Sodium pyro- phosphate	Sodium hydro- sulphite
1 % lecithin suspension	0.10	Nil	0.099	0.097	0.096
	0.05	Nil	0.052	0.054	0.051
2 % sodium caseinogenate	0.10	Nil	0.096	0.102	0.105
	0.05	Nil	0.052	0.053	0.051
Milk	0.10	Nil	0.106	0.105	0.107
	0.05	Nil	0.053	0.047	0.048
Egg-white	0.10	0.091	—	—	—
	0.10	0.081	0.102	0.106	0.098
	0.15	0.124	—	—	—
	0.10	0.078	0.106	0.107	0.103
2 % edestin	0.10	0.099	—	—	—
	0.15	0.145	—	—	—
	0.20	0.193	—	—	—
2 % gelatin	0.10	0.094	—	—	—
	0.15	0.144	—	—	—
	0.20	0.198	—	—	—

It will be seen that whereas no iron is present in the control filtrates of milk, lecithin or sodium caseinogenate, it is present in the filtrates after treatment of these materials with thiolacetic acid, sodium hydrosulphite or pyrophosphate, the iron being liberated quantitatively by these reagents. It appears that ferric iron forms complexes with these substances, similar to those formed with egg-yolk. In the case of egg-white however iron did appear in the control filtrates and in some cases almost quantitatively. Mixtures of egg-white and ferric iron were tested directly with $\alpha\alpha'$ -dipyridyl [*cf.* Hill, 1931] and were found to contain ferric iron, no signs of any reduction to ferrous iron being noted. In the cases of edestin and gelatin iron appeared in the control filtrates quantitatively. Gelatin did not appear to be completely precipitated with the concentration of trichloroacetic acid used in these experiments. It has been mentioned previously that the gelatin, edestin and lecithin contained appreciable amounts of iron. In the cases of edestin and gelatin this iron appeared quantitatively in the filtrates after precipitation of the proteins with trichloroacetic acid. With lecithin however no iron appeared in the filtrate until the phosphatide had been treated with thiolacetic acid, sodium hydrosulphite or pyrophosphate before precipitation with trichloroacetic acid.

Experiments were carried out to test the recovery of ferrous iron added to these biological materials. Using $\alpha\alpha'$ -dipyridyl it was found that under the conditions of the experiments ferrous iron was oxidised very quickly to the ferric condition in the presence of these substances. Under these conditions results identical with those obtained in Table II were obtained.

From the data which have been obtained it appears that ferric iron forms complexes with many biological substances. Since thiolacetic acid and sodium hydrosulphite reduce ferric iron to the ferrous state and also liberate ferric iron from its complexes, it seems that ferrous iron does not form such complexes. Although ferric iron forms complexes with sodium caseinogenate, lecithin, milk and egg-yolk it does not appear to form these with egg-white, edestin or gelatin. The slight loss of iron in the filtrates when the proteins of egg-white are precipitated with trichloroacetic acid is probably due to traces of lecithin which

egg-white invariably contains. This suggests that ferric iron forms complexes not with simple proteins but with non-diffusible phosphorus compounds, *e.g.* phosphatides and phosphoproteins. Of the non-reducing substances examined, pyrophosphate was the only one liberating ferric iron from its complexes. This is probably due to its property of forming un-ionised ferric pyrophosphate. The question naturally arises as to whether pyrophosphates play any part in the absorption and transport of iron in the human body.

The recovery of added copper.

Since iron and copper are closely related, the latter apparently influencing the formation of haemoglobin from the former, the recovery of copper added to milk, 2 % sodium caseinogenate, egg-yolk, egg-white and 1 % lecithin suspension was examined. Copper was added in the form of copper sulphate to these solutions or suspensions and the copper determined directly in the trichloroacetic acid filtrates by the method described by the writer [1934, 1] for blood. In the cases of egg-yolk and egg-white allowances were made for their initial copper contents but in the cases of the other substances negative reactions for copper were obtained in the amounts used. The results are shown in Table III where it will be seen that added copper could be determined quantitatively in the filtrates.

Table III. *Recovery of copper added to various biological materials.*

	Copper added mg.	Copper recovered mg.
Egg-yolk (1)	1.125	1.118
(2)	0.125	0.117
Egg-white (1)	0.100	0.095
(2)	0.100	0.093
10 ml. milk (1)	0.025	0.024
(2)	0.025	0.024
(3)	0.050	0.048
10 ml. 2 % sodium caseinogenate (1)	0.025	0.026
(2)	0.025	0.026
(3)	0.050	0.051
10 ml. 1 % lecithin suspension (1)	0.025	0.027
(2)	0.025	0.023
(3)	0.050	0.051

The initial copper contents of the two whole eggs were 0.06 and 0.08 mg. per egg respectively. These results are of the same order as obtained by Loeschke [1931] who obtained values of 0.055–0.095 mg. per egg, and McFarlane *et al.* [1930] who obtained an average value of 0.08 mg. per egg. The yolks contained slightly more copper than the whites.

The inorganic iron of serum and whole blood.

A method has been described for the determination of the inorganic iron content of whole blood [Tompsett, 1934, 1]. It has been found that, with blood also, pyrophosphate can be used in place of thiolacetic acid to liberate the inorganic iron from its complexes.

The condition of the inorganic iron of the plasma or corpuscles does not appear to have been studied. Any method involving several manipulations is unsuitable owing to the ease with which ferrous iron is oxidised to the ferric state. Any test used must be applied directly to the material. The most suitable

reagent appears to be $\alpha\alpha'$ -dipyridyl which gives a red colour with ferrous and no colour with ferric iron. It gives no reaction with haematin-like substances. The test was applied directly to serum in the manner used by Hill [1931] for egg-yolk but no colour appeared. On the addition of a reducing substance, *e.g.* sodium hydrosulphite or thiolacetic acid, a red colour appeared. It appears that the inorganic iron of serum is in the ferric state but if on the other hand it is in the ferrous state it is extremely doubtful whether it would remain in this condition long after the removal of the blood from the body. In the case of whole blood, after the addition of $\alpha\alpha'$ -dipyridyl sufficient alcohol was added to precipitate the proteins completely. A control was carried out at the same time but without the addition of $\alpha\alpha'$ -dipyridyl. The filtrate showed a decided reddish colour when compared with the control filtrate. This was obtained in every case examined and suggests that part at least of the inorganic iron of the corpuscles is in the ferrous state.

SUMMARY.

1. Ferric iron has been shown to form complexes with certain biological materials. It is liberated from these complexes by reducing substances such as thiolacetic acid and sodium hydrosulphite. Ferrous iron does not appear to form such complexes.

2. Ferric iron is also liberated from these complexes by sodium pyrophosphate. This does not involve a reduction but is more probably due to the formation of un-ionised ferric pyrophosphate.

3. Ferric iron appears to form complexes with the non-diffusible phosphorus compounds, *e.g.* phosphatides and phosphoproteins, and not with the simple proteins, *e.g.* egg-white, edestin and gelatin.

4. Copper does not form complexes with any of these substances.

5. Part at least of the inorganic iron of the corpuscles of blood is in the ferrous condition.

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