CCIV. THIOLACETIC ACID AS A REAGENT FOR THE DETERMINATION OF THE INORGANIC IRON CONTENT OF CERTAIN BIOLOGICAL MATERIALS.

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THE thiocyanate reaction for the determination of iron has been criticised by many writers.

However, if conditions are carefully controlled it gives accurate results but is not very suitable for the direct determination of iron in the inorganic form in extracts of biological material owing to the interference of many organic substances. Since certain of the analyses recorded in this paper were made by this method a discussion of the influence of various conditions is suitable at this stage. Pyrophosphates seriously interfere by depressing the reaction partly or completely. When dealing with biological material containing much phosphorus a wet ashing method in which an excess of sulphuric acid is present at the end of the reaction is essential. Elvehjem [1930] has employed a dry ashing method, after which the ash was boiled with alkali to convert pyrophosphates into orthophosphates. Such a technique appears to invite contamination due to the solvent action of hot alkali on glass. Kennedy [1927] and Elvehjem and Hart [1926] state that pyrophosphates are not formed during sulphuric-perchloric acid digestion but the present writer has noted that unless excess of sulphuric acid is present at the end of the reaction, pyrophosphates are liable to be formed. When 50 ml. of urine were digested with sulphuric-perchloric acid using only 1 ml. of sulphuric acid, no reaction with the thiocyanate reagent was obtained although iron was known to be present. When 5 ml. of sulphuric acid were used, satisfactory results were obtained. Marlow and Taylor [1934] however in determining iron in urine used 100 ml. urine and although they started with excess of sulphuric acid, namely 10 ml., digested to 0.5 ml. and claim to have obtained a reaction for iron with the thiocyanate reagent. Another important factor is the quantity of thiocyanate added. McFarlane [1932, 1] has shown that the quantity of thiocyanate added influences the depth of colour obtained very considerably. With increasing concentration of thiocyanate the depth of colour increases very rapidly, after which it remains constant. It is obvious from this work that the quantity of thiocyanate to be added should be sufficient to produce the maximum colour. Marlow and Taylor [1934] in their work on the iron content of urine failed to appreciate this and used such a concentration of thiocyanate that an error of a drop or two of the reagent would greatly influence the depth of colour.

Thiolacetic acid.

The colour produced when a solution of thiolacetic acid and iron is made faintly alkaline was first described by Andreasch [1879], who believed it to be due to the formation of a complex ferric thiolacetate, $\text{Fe}(\text{S} \cdot \text{CH}_2\text{COOH})_3$. It

was further stated that the colour faded after some time and was regenerated by shaking with air.

Lyons [1927] however has shown that the test is applicable to both ferrous and ferric salts. In the presence of alkali they give pink colours in very dilute solutions and reddish purple colours in more concentrated solutions. Lyons believes that when thiolacetic acid is added to a ferric salt in acid solution, a ferric salt, $\text{Fe}(\text{S} \cdot \text{CH}_2\text{COOH})_3$, is formed, which is immediately reduced to a ferrous salt, $\text{Fe}(\text{S} \cdot \text{CH}_2\text{COOH})_2$. This in alkaline solution gives an intensely coloured ion, $\text{Fe}(\text{S} \cdot \text{CH}_2\text{COO})_2^-$. The presence of oxidising agents prevents the formation of the colour probably owing to the formation of dithioglycollic acid which does not give the test. Lyons found that strong bases produced rapid colour fading and that ammonia was the most satisfactory to use. Michaelis and Barron [1929] believe that the colour in alkaline solution is not simply the ionised form of the ferrous complex which is colourless in acid solution but the oxidised form of the ferrous complex. More recently Hanzal [1933] and Burmester [1934] have applied the thiolacetic acid test to the determination of iron in biological material.

Lyons who first reported the reaction to be quantitative showed that it was uninfluenced by a large number of substances both organic and inorganic.

The present writer has attempted to apply the reaction to the direct determination of inorganic iron in biological material. In the first place the range of the reaction was studied. A standard iron solution was prepared containing 0.0855 g. of iron alum in 1000 ml. of 1 % sulphuric acid (1 ml. = 0.01 mg. Fe). Into a series of tubes were measured 0.001, 0.002, 0.003, 0.004, 0.005, 0.01, 0.02, 0.03, 0.04 and 0.05 mg. Fe. These were diluted to 5 ml. with distilled water, 6 drops thiolacetic acid added and then 0.5 ml. of ammonia sp. gr. 0.880. It was found that in the tubes containing 0.005 to 0.05 mg. Fe the colours were strong enough for comparison in a colorimeter and on comparison the depth of colour was found to be proportional to the concentration of iron.

Although distinct differences of colour were obtained in the tubes containing less than 0.005 mg. Fe yet they were too pale to compare in a colorimeter. In determining amounts of iron of this order it is best to use direct comparison with a set of tubes such as the above.

In the following work, all filter-papers used were washed with dilute HCl, distilled water and dried and the trichloroacetic acid was distilled *in vacuo*.

The iron of the hen's egg.

Nearly all the iron of the hen's egg is located in the yolk and many believe that it exists in the form of an organic complex with protein-like properties. This complex was first studied by Bunge [1884] who named it "haematogen." Hugounenq and Morel [1905] regarded haematogen as the prosthetic group in the vitellin molecule and as being the precursor of the haemoglobin formed by the chick during the incubation of the egg. They found that decomposition by acids breaks off a black pigment containing 2.6 % iron, which they called "haematovin."

Hill [1931] has submitted evidence to show that all the iron in egg-yolk is present as ferric iron, probably as a colloidal ferric hydroxide, and that no organic complex exists. He found that when egg-yolk is suspended in an acetate buffer and $\alpha\alpha'$ -dipyridyl added no colour is obtained. On the addition of sodium hydrosulphite a red colour similar to that obtained with ferrous salts was obtained. He found that the iron content of egg-yolk as determined by this simple procedure agreed with the iron content determined after ashing.

McFarlane [1932, 2] found that lecitho-vitellin prepared from egg-yolk contains a fair percentage of iron which appears to be present in very stable combination. He therefore concludes that part at least of the iron of the egg is present as an organic complex.

Additional evidence therefore appeared to be required to decide this point.

The contents of one egg were well mixed, treated with an equal volume of 20 % trichloroacetic acid, well shaken and filtered. To 5 ml. of the filtrate 6 drops of thiolacetic acid were added followed by 0.5 ml. of concentrated ammonia. No colour was obtained. A known volume of standard iron solution was added to another portion of the filtrate and the test repeated. The colour obtained was the same as that of a control, showing that none of the constituents of the filtrate interfered with the reaction.

To the contents of another egg some iron alum solution was added and the proteins precipitated with trichloroacetic acid as before. In this case also the filtrate gave a negative thiolacetic acid test. These reactions were repeated several times and found to hold in every case.

The following experiment was then carried out. The contents of one egg were treated with 5 ml. of thiolacetic acid and well mixed. Then an equal volume of 20 % trichloroacetic acid was added, well mixed and filtered. To 5 ml. of the filtrate 0.5 ml. of concentrated ammonia was added and the mixture shaken; a positive reaction for iron was obtained.

A series of experiments was then made in which the iron content of the hen's egg as determined in the protein-free filtrate directly with thiolacetic acid was compared with the total iron of the egg determined after ashing.

The contents of an egg were well mixed and diluted to 100 ml. 10 ml. of the mixture were treated with 2 ml. of thiolacetic acid, well shaken and 15 ml. of 20 % trichloroacetic acid added. The mixture was diluted to 30 ml. and filtered. 5 ml. of the filtrate were mixed with 1 ml. of concentrated ammonia. The colour so obtained was compared in a colorimeter with a standard prepared as follows. 2 ml. of a standard iron alum solution (2 ml. = 0.02 mg. Fe) were measured into a tube. 0.33 ml. of thiolacetic acid was added followed by 2.5 ml. of 20 % trichloroacetic acid and the whole diluted to 5 ml. 1 ml. of concentrated ammonia was added and the whole well shaken.

The total iron of the egg was determined as follows. 10 ml. of egg suspension were heated in a pyrex flask with 10 ml. of concentrated sulphuric acid and perchloric acid until all the organic matter was destroyed. On cooling, the mixture was diluted to 100 ml. To 10 ml. 2 ml. of 40 % potassium thiocyanate were added and the red ferric thiocyanate extracted with 5 ml. of amyl alcohol. The amyl alcohol layer was removed and compared in a colorimeter with a

Table I. *The iron content of the hen's egg expressed in mg. Fe per whole egg.*

A. Whole egg			B. Egg-yolk		
	Thiocyanate method after ashing	Direct deter- mination on trichloroacetic acid filtrate		Thiocyanate method after ashing	Direct deter- mination on trichloroacetic acid filtrate
1	0.92	0.90	8	1.15	1.07
2	1.26	1.25	9	1.28	1.31
3	1.00	0.96	10	1.28	1.22
4	1.27	1.21	11	1.30	1.26
5	1.25	1.17			
6	1.11	1.18			
7	1.27	1.19			

standard, which was prepared with the same amount of sulphuric and perchloric acids (the perchloric acid being boiled off) and contained 0.01 mg. Fe.

The results are shown in Table I. At the same time the recovery of iron added to egg was studied using the thiolacetic acid technique. The results are shown in Table II.

Table II. *The recovery of iron added to egg and determined by the thiolacetic acid method on trichloroacetic acid filtrates.*

	Initial iron content of sample mg.	Iron added mg.	Total iron content of sample mg.	Iron recovered mg.
1	0.0671	0.0878	0.153	0.0859
2	0.097	0.070	0.159	0.062
3	0.072	0.070	0.136	0.064
4	0.087	0.088	0.174	0.087
5	0.088	0.088	0.172	0.084
6	0.079	0.088	0.160	0.081

It will be seen that within the limits of experimental error, the iron contents of the egg as determined by the two methods are the same, and that iron (ferric) added to egg can be recovered quantitatively.

As a check the iron contents of the egg-yolks (Nos. 8–11 in Table I) after ashing were determined with the thiolacetic acid reagent as well as with the thiocyanate reagent. The maximum difference obtained between the two methods was 4 %. The figures obtained for the iron content of the hen's egg agree closely with those obtained by Cunningham [1931] whose average figure was 0.99 mg. Fe per egg.

The non-haemoglobin iron of whole blood and serum.

Many writers have determined what they describe as the "non-haemoglobin" iron content of the blood or serum. The results that have been obtained may be summarised as follows:

	Serum or plasma mg. Fe per 100 ml.
Riecker and Winters [1930]	1.0
Locke <i>et al.</i> [1932]	0.06–0.12
Marlow and Taylor [1934]	0.4–0.7
	Whole blood
McIntosh [1933]	0.5–1.0

In the above investigations the blood-serum or plasma was treated with trichloroacetic acid and the iron determined in the filtrates after ashing. Such methods however do not indicate the form in which the iron is present.

Barkan [1927; 1933] stated that inorganic iron could be determined in blood or serum by incubating with 0.4 % HCl at 37° for 24 hours. This was followed by ultrafiltration, after which the iron was determined in the ultrafiltrate with thiocyanate. He obtained values of 0.084 to 0.168 mg. Fe per 100 ml. human serum and a mean value of 1.7 mg. Fe per 100 ml. whole blood. Barkan found that none of the iron of serum was ultrafiltrable unless subjected to the above treatment.

Starkenstein and Weden [1928] state that all the inorganic iron and none of the iron in organic combination is extracted from tissue or blood by boiling with 5N HCl and then precipitating the proteins with trichloroacetic acid. They obtained values for whole blood of the order obtained by Barkan but higher values for serum.

The present writer has investigated the iron content of blood and serum using acid. When serum was treated with an equal volume of 20 % trichloroacetic acid and filtered, the filtrate was found to give no reaction for iron with the thiolacetic acid test. When known amounts of standard iron alum solution were added to serum and treated similarly, the filtrates were in some cases found to give negative reactions, in others only very faint reactions for iron. When whole blood was so treated, definite reactions were obtained for iron in the filtrates of the order of 0.5 mg. Fe per 100 ml., but recoveries of added iron were poor. It was decided therefore to apply the technique used for the determination of iron in eggs.

10 ml. of serum were treated with 24 drops of thiolacetic acid and well mixed. Then 10 ml. of 20 % trichloroacetic acid were added, well mixed and filtered. To 5 ml. of the filtrate, 0.5 ml. of concentrated ammonia was added and shaken. Using this technique a definite reaction for iron was obtained but was too faint to compare in a colorimeter. In these cases the colour was compared with a series of standard tubes that had been freshly prepared (0.004, 0.003 and 0.002 mg. Fe). The results obtained for the inorganic iron content of serum are shown in Table III and are of the same order as those obtained by Barkan. It

Table III. *The inorganic iron content of human serum (expressed as mg. Fe per 100 ml.).*

1	0.22	6	0.20
2	0.18	7	0.14
3	0.14	8	0.16
4	0.14	9	0.20
5	0.18	10	0.12

is not possible to compare these figures with figures obtained by other writers since the latter would possibly include organic iron as well. It will be seen that iron added in the form of iron alum to serum could be determined quantitatively using the above technique (see Table IV).

Table IV. *Recovery of iron (ferric) added to serum (expressed as mg. Fe).*

	Initial	Iron added	Total iron	Iron recovered
1	0.0035	0.01	0.0128	0.0093
2	0.004	0.01	0.0131	0.0091
3	0.005	0.015	0.0196	0.0146
4	0.004	0.01	0.0142	0.0102
5	0.005	0.015	0.0192	0.0142

In the case of whole blood the following technique was employed. 5 ml. of water were added to 5 ml. of oxalated blood followed by 18 drops of thiolacetic acid and well mixed. Then 5 ml. of 20 % trichloroacetic acid were added, mixed and filtered. To 5 ml. of filtrate 0.5 ml. of concentrated ammonia was added, well mixed, and compared in a colorimeter with a standard containing 0.015 mg. Fe similarly prepared. The results obtained for the inorganic iron content of whole blood are shown in Table V and it will be seen that they are slightly lower

Table V. *The inorganic iron content of human whole blood (expressed as mg. Fe per 100 ml.).*

1	1.14	5	0.96
2	1.06	6	1.08
3	0.93	7	1.40
4	1.36	8	1.56

than those obtained by Barkan. It appears that the corpuscles contain a very much greater amount of inorganic iron than the plasma. From results shown in Table VI it will be seen that iron added to whole blood in the form of iron alum can be determined quantitatively by this technique.

Table VI. *The recovery of iron added to whole blood (expressed as mg. iron).*

	Initial	Iron added	Total iron	Iron recovered
1	0.02	0.015	0.033	0.013
2	0.02	0.02	0.042	0.022
3	0.02	0.01	0.03	0.010
4	0.026	0.01	0.037	0.011
5	0.026	0.015	0.043	0.017
6	0.026	0.02	0.045	0.019

The iron content of urine.

Earlier writers have reported the presence of iron in normal human urine but more recent work has thrown doubt on this.

Lintzel [1929] and Lanyar *et al.* [1933] state that the iron content of urine does not exceed 0.01 mg. per litre. Henriques and Roland [1928] obtained values of 0.08 to 0.32 mg. *per diem* while Coons [1932] reports values of 0.06 to 0.48 mg. *per diem* in the urine of pregnant women. More recently Marlow and Taylor [1934] have obtained values of 0.03 to 0.8 mg. *per diem* in the urines of normal men.

Lanyar *et al.* found that the administration of iron salts by mouth produced no excretion of iron in the urine. Intravenous injection of iron salts into dogs, however, was followed by an excretion of iron into the urine. These writers were unable to detect iron in the urine in any condition other than nephrosis where there was gross albuminuria, and in these cases when the protein was coagulated by heat and filtered off the urine was found to be free of iron.

In the determination of small amounts of iron the greatest care must be taken to avoid contamination. Coons [1932] employed a method in which the urine was ashed and then heated with alkali to convert pyrophosphates into orthophosphates.

In the present study urines from males were used and were collected in paraffin-coated bottles. The following was the method employed.

50 ml. of urine were measured into a pyrex flask and 5 ml. of concentrated nitric acid added. The mixture was evaporated almost to dryness, and 5 ml. of concentrated sulphuric acid were added. The excess of nitric acid was boiled off and the residue then allowed to cool. 5 ml. of perchloric acid were added and the mixture heated until all the organic matter was destroyed and fumes driven off. A blank experiment was carried out at the same time. It was found that the reagents at present obtainable gave appreciable blanks when used in the above quantities. After cooling, the urine and the blank digest were each diluted to 20 ml., cooled, 4 ml. of 40 % potassium thiocyanate added and the red ferric thiocyanate extracted with 5 ml. of amyl alcohol. The amyl alcohol layers were separated and compared in a colorimeter with a standard prepared as follows. The standard containing 0.005 mg. Fe and 1 ml. of concentrated sulphuric acid was diluted to 10 ml. 2 ml. of 40 % potassium thiocyanate were added, and the ferric thiocyanate was extracted with 5 ml. of amyl alcohol.

The results are shown in Table VII. In the case of normal urines the colorimeter readings of the blanks and the urines were the same within the limits of experimental error, in fact these urines could not have contained more than 0.01 mg. Fe per litre. The urines from two cases of pernicious anaemia contained

Table VII. *The iron content of urine.*

Case										Iron in mg. per litre
1. Normal	...	...	...	...	...	...	...	...	...	<0.01
2. Normal	...	...	...	...	...	...	...	...	...	<0.01
3. Normal	...	...	...	...	...	...	...	...	...	<0.01
4. Normal	...	...	...	...	...	...	...	...	...	<0.01
5. Normal	...	...	...	...	...	...	...	...	...	<0.01
6. Normal	...	...	...	...	...	...	...	...	...	<0.01
7. Haemochromatosis	...	...	...	...	...	...	...	...	...	0.16
8. Pernicious anaemia	...	...	...	...	...	...	...	...	...	0.19
9. Pernicious anaemia	...	...	...	...	...	...	...	...	...	1.28
10. Nephritis (gross albuminuria)	...	...	...	...	...	...	...	...	...	1.72
Nephritis (after precipitation of protein with trichloroacetic acid)	...	...	...	...	...	...	...	...	...	1.46
11. Nephritis (gross albuminuria)	...	...	...	...	...	...	...	...	...	2.33
Nephritis (after precipitation of protein with trichloroacetic acid)	...	...	...	...	...	...	...	...	...	2.00

appreciable amounts of iron—this however is in contradiction to the findings of Lanyar *et al.* [1933] who found no iron in the urine of such cases. One case of haemochromatosis showed an appreciable amount of iron in the urine. In two cases of nephritis in which there was gross albuminuria, the urines contained very large amounts of iron. The urines gave negative tests for blood and, examined microscopically, contained no red cells. It will be seen from the results that precipitation of the proteins with trichloroacetic acid removed only a very small percentage of the iron.

DISCUSSION.

It has been shown that thiolacetic acid is a suitable reagent for the direct determination of iron in biological extracts.

It appears that although all the iron in the hen's egg is in the inorganic form yet it is in such firm combination as to appear to be part of some organic molecule. Since iron (ferric) added to egg behaves in the same way, this can hardly be the case. This will explain the presence of iron in the lecitho-vitellin prepared by McFarlane [1932, 2] and his view that iron was actually a part of the lecitho-vitellin molecule.

The whole of the inorganic iron of serum appears to be in a very stable combination also. On the other hand part of the inorganic iron of the corpuscles appears to be more loosely combined.

As has been previously mentioned, Barkan [1933] has shown that none of the iron of serum is ultrafiltrable; this will explain the almost complete absence of iron from normal urine.

Although iron and copper are closely related, the latter apparently influencing the formation of haemoglobin from the former, they seem to differ very considerably in their physical properties. The present writer [1934] has shown that filtrates prepared from either whole blood or plasma by precipitation of the proteins with trichloroacetic acid contain all the copper. Fairly large amounts of copper are also excreted in the urine. Rabinowitch [1933] has found human urine to contain from a trace to 0.41 mg. per litre and the present writer has obtained similar values.

The view of Hill [1931] that the iron of the hen's egg is present as a colloidal ferric hydroxide is however untenable in view of the results of this paper.

Although copper is more or less equally distributed between the corpuscles and the plasma [Tompsett, 1934], inorganic iron appears in much greater concentration in the corpuscles.

Lintzel [1931] and Heubner [1926] state that ferric but not ferrous iron readily forms complexes with proteins. It is believed therefore that iron is absorbed from the digestive tract as ferrous iron.

The views of Lintzel and Heubner are suggestive since thiolacetic acid reduces ferric iron to the ferrous condition and also releases the iron of the egg from its complex.

Whether the inorganic iron of the corpuscles and plasma is in the ferric or ferrous condition is not at present known.

SUMMARY.

1. The whole of the iron of the hen's egg is in the inorganic form.
2. Normal human urine contains less than 0.01 mg. Fe per litre.
3. Human corpuscles contain about twenty times as much inorganic iron as the plasma.

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