6. Methyleneaminoacetonitrile is much more active than glycine in accelerating the disappearance of free ascorbic acid in the presence of HCHO at pH 1.5. The possibility of errors in Lugg's method due to other substances behaving in a similar manner is emphasized.

7. A convenient procedure based on Lugg's formalin method is described.

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The Micro-volumetric Determination of Iron, with Particular Reference to Blood

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Modern research into the biochemistry of respiratory pigments demands iron determinations of an accuracy which is beyond that of the usual colorimetric procedures. Even such sensitive and specific reagents as $\alpha\alpha'$ -dipyridyl and thiolacetic acid cannot surmount the difficulties inherent in the visual matching of colours. It is surprising, therefore, that in this field so few attempts have been made to apply to biological materials any of the volumetric methods of modern inorganic chemistry. Dubnoff & Kirk (1936) reduced iron to the ferrous state with cadmium-amalgam and titrated with standard $\text{Ce}(\text{SO}_4)_2$. They claimed to be able to determine as little as 2–15 μg . Fe, but in this laboratory all reductions with pure metals and amalgams have led to 'blanks' of several μg ., which impair greatly the accuracy of the results. Pincussen & Roman (1931) and McFarlane (1932) titrated ferric iron with standard titanous salts, but both methods require about 1 mg. Fe for each estimation. This precludes their use for many biological purposes.

This paper describes a titrimetric technique which can conveniently be used for the determination of any quantity of iron not less than 8–10 μg .

METHOD

Principle of the method. Organic matter is destroyed by heating with H_2SO_4 , HClO_4 and HNO_3

in a small pyrex flask (10–12 ml. capacity, internal neck diameter 8 mm., over-all length 80–90 mm., kindly blown for me by Mr C. Bell, Department of Biochemistry, University of Edinburgh). The digest is diluted and titrated with standard $\text{Ti}_2(\text{SO}_4)_3$, using a burette modified from the design of Conway

Fig. 1. Modified Conway burette. A, pressure-regulating vessel. B, C, D, G and H, stop-cocks. E, the burette, standard bore capillary tubing against mm. scale. 10 cm. $\approx$ 0.05 ml. F, $\text{Ti}_2(\text{SO}_4)_3$ reservoir, capacity 200–250 ml. I, digestion and titration flask.

(1939) so as to permit the storage of the $\text{Ti}_2(\text{SO}_4)_3$ under hydrogen (see Fig. 1). KCNS is used as indicator, and CO_2 for stirring.

The method was originally designed for use with blood and solutions of haemoglobin pigments, but some of its limitations have been investigated with a view to its application to other biological material. It has been found that small amounts of copper cause the results to be irregular and high, but a modified technique has been worked out for use in the presence of Cu. This is based on the observation, believed to be original, that when a sulphuric acid solution of Fe^{+++} and Cu^{++} is saturated with NaCl or KCl and extracted with ether containing a little formic acid, the ferric iron alone passes into the ether layer.

Reagents. All reagents should be of A.R. quality unless otherwise stated.

Acids. H_2SO_4 , HNO_3 , HClO_4 (60%). The HNO_3 should be tested for iron, and distilled from glass if necessary.

Thiocyanate. KCNS, 50% solution in distilled water.

Titanous sulphate. $\text{Ti}_2(\text{SO}_4)_3$, $\text{N}/50$ to $\text{N}/20$ (depending on the quantity of iron and the bore of the burette). 200 ml. freshly boiled distilled water are poured into the bottle *F* (Fig. 1). The cocks *C*, *D* and *G* are closed, but *B* and *H* may be left open. An appropriate quantity of commercial 15% $\text{Ti}_2(\text{SO}_4)_3$ (or TiCl_3) is mixed with 10 ml. of water and 10 ml. conc. H_2SO_4 , and the mixture is boiled briskly for a full minute. The solution is at once poured into *F*, which should be immediately stoppered. The cock *D* is opened, and the apparatus is left overnight, so that a precipitate which sometimes forms in the titanium solution may settle. Next morning *D* is closed, *C* is opened, and hydrogen is allowed to flow briskly from the generator. When the gas is bubbling freely through *A*, *C* is again closed, and first *D* and then *G* are opened. The pressure of hydrogen should be sufficient to drive the $\text{Ti}_2(\text{SO}_4)_3$ over the siphon into the burette. The lubrication of the cocks *G* and *H* is important, since most greases allow a slight but serious leakage of the strongly acid reagent. W. Edwards & Co. (London), Ltd., have kindly supplied me with a sample of their 'Soft Vacuum Grease', which has proved more satisfactory than other commercial lubricants. The reagent, even if the most stringent precautions are taken to exclude air, still oxidizes slowly, and must be standardized daily by titration against a standard iron solution.

Standard ferric iron. A very accurate standard solution may be prepared from a weighed quantity of pure iron wire, but for all ordinary purposes it has proved sufficient to dissolve in 500 ml. of approximately 3N- H_2SO_4 such a weight of $\text{Fe}_2(\text{SO}_4)_3 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$ that 5 ml. of the solution require for reduction a volume of $\text{Ti}_2(\text{SO}_4)_3$ which alters the reading on the burette by 100–200 mm.

Reagents required only in the presence of copper

Sodium chloride. Solid, powdered.

Ether and formic acid. These need not be A.R., but must be redistilled from glass. They should be mixed only just before use.

Procedure

Digestion. This is described here for blood, but it has been shown by Levvy (1943), and confirmed in this laboratory, that the same reagents are satisfactory for the digestion of other tissues. 0.2 ml. of blood (a smaller volume may be used if great accuracy is not required) is measured into one of the flasks already described, followed by 0.2 ml. each of conc. HNO_3 and 60% HClO_4 , and 0.5 ml. conc.

H_2SO_4 . A small glass bead is added, and the flask is gently heated, with continuous shaking, over a micro-burner. The HNO_3 boils off first, the mixture darkens, and clears again as the boiling-point of H_2SO_4 is approached. Just before the last of the HClO_4 boils off, the mixture turns a clear deep greenish-yellow colour for 3–5 sec. Heating is continued for 30 sec. after the disappearance of this colour. The cooled digest is diluted with 5 ml. of water (unless Cu is present; see below), and is then ready for titration. The digestion of 0.2 ml. of blood usually requires about 6 min. It is, of course, easy to destroy all the organic matter of blood with H_2SO_4 and HNO_3 , but in the absence of HClO_4 some difficulty is experienced in getting rid of the last traces of HNO_3 . Moreover, if H_2SO_4 and HClO_4 are used without HNO_3 , it is much more difficult to prevent loss of material by bumping and splashing.

Separation of iron from copper (unnecessary in the analysis of blood). After the digestion the contents of the flask are diluted with 2 ml. of water and transferred as completely as possible to a narrow test-tube. The flask is rinsed with two successive portions of 1 ml. of water, each being transferred to the test-tube. 0.5 ml. of conc. H_2SO_4 is added, and the solution in the test-tube is saturated with NaCl. The tube is then shaken twice with 2 ml., and twice with 1 ml. of ether containing 5% (by vol.) of formic acid. The ether extracts are transferred back to the original flask, and the ether is evaporated over a warm water-bath. The formic acid has a tendency to form non-volatile polymers, so it is necessary to repeat the digestion, after the addition of 0.5 ml. of conc. H_2SO_4 and a drop or two each of HNO_3 and HClO_4 . Care must be taken to remove all the ether before the concentrated acids are added, or a minor explosion may result in the loss of most of the material. The digest is finally cooled and diluted with 5 ml. of water.

Titration. The titration technique is simple, but accurate results depend on attention to certain details. After the burette has been flushed out five or six times and the $\text{Ti}_2(\text{SO}_4)_3$ standardized (see below), the diluted digest, plus 1 ml. of 50% KCNS, is so placed that the tip of the burette dips just below the surface of the solution. The tube which delivers the stream of CO_2 should be 3–5 mm. longer. If it is lashed to the tip of the burette, the two fine tubes reinforce each other and are less liable to break. CO_2 is bubbled through the flask, the cock *H* is turned on, and $\text{Ti}_2(\text{SO}_4)_3$ is allowed to run out of the burette until the last trace of pink colour just disappears. The reaction between Fe^{+++} and Ti^{+++} is not instantaneous, even in the presence of a catalyst such as KCNS, and it is therefore necessary to regulate carefully the rate of flow from the burette. This is done, as with the simple Conway burette, by adjusting the height of the column of fluid in the tube leading from *A* to *B*, and the titration is finally done with the cock *C* closed and *B* open. The fluid should leave the burette at a rate of 0.01–0.02 ml./min. The last few μl . may be run out even more slowly if *B* is closed just before the end of the titration. The lighting used for the observation of the end-point is particularly important. The apparatus should be placed in a dull corner, a white card pinned behind the flask, and light from a 'daylight' blue bulb thrown on it from a few inches away. An aluminium reflector increases the illumination and shields the eyes of the operator. Under these conditions a perceptible change in colour (towards the end of a titration) is produced by the addition of 0.0005–0.001 ml. $\text{N}/25$ $\text{Ti}_2(\text{SO}_4)_3$.

Standardization is carried out by the titration of 5 ml. portions of ferric ammonium sulphate solution in exactly the same way.

RESULTS

The analysis of fourteen 0.2 ml. samples of the same blood, using the simple direct procedure, gave concentrations of 51.66, 52.05, 52.24, 52.10, 51.97, 51.93, 51.57, 51.75, 51.97, 51.36, 51.92, 51.95, 52.36, 52.00 mg. Fe/100 ml. blood. The standard deviation of a single observation from the mean was 0.25 mg./100 ml. This corresponds to a coefficient of variation of 0.48 %, which agrees well with the value of 0.45 % calculated from a large series of observations made in triplicate and quadruplicate. The error of the procedure modified for Cu is somewhat greater, for two reasons: the additional manipulation is contributory, and also consumes time, but more serious is the fact that all samples of NaCl hitherto examined contain significant traces of iron. This leads to blank titrations much higher and more variable than those encountered in the simple method.

Table 1 gives the results of some experiments on the recovery of known amounts of Fe added (as ferric ammonium sulphate) to 0.1 or 0.2 ml. samples of blood before digestion. Table 2 illustrates the separation of Fe from Cu by extraction with ether-formic acid.

Table 1. *Recovery of iron added to blood*

Fe added ($\mu\text{g.}$)	Fe re-covered ($\mu\text{g.}$)	Recovery (%)	Fe added ($\mu\text{g.}$)	Fe re-covered ($\mu\text{g.}$)	Recovery (%)
10.1	9.7	96.0	30.0	29.3	97.7
10.1	10.6	105.0	47.0	47.6	101.3
30.0	29.9	99.7	59.0	59.6	101.0
30.0	29.6	98.7	83.0	83.0	100.0
30.0	30.4	101.3	94.0	95.3	101.4
30.0	29.5	98.3	106.0	104.8	98.8
30.0	29.5	98.3	118.0	118.0	100.0

Table 2. *Separation of iron from copper by extraction with ether-formic acid mixture*

Material analyzed	Fe found ($\mu\text{g.}$)
0.2 ml. blood	101.6, 100.8
0.2 ml. blood + 15.3 $\mu\text{g.}$ Cu (not extracted)	119.6, 111.5
0.2 ml. blood + 15.3 $\mu\text{g.}$ Cu (extracted)	100.9, 100.4

DISCUSSION

The results quoted show that in the absence of copper the method is capable of giving highly accurate results. It has been in use in this laboratory for some time, and has given much more consistent results than $\alpha\alpha'$ -dipyridyl or thiolacetic acid. In theory, it is lacking in specificity, since titanous salts react with a variety of oxidizing agents. The digestion, however, destroys all organic

compounds, and in most biological material the ratio of iron to other inorganic redox systems is so high that the latter may, with certain reservations, be ignored. In the case of blood no difficulty arises, since the only other redox system surviving the digestion is copper, and the ratio of iron to copper is of the order of 300–400 to 1; but in other tissues, notably liver, the ratio may be 20 to 1 or even lower, with the result that the quantity of Ti^{+++} used in the titration may be considerably in excess of that required for the Fe present.

The observation that ferric iron in the presence of NaCl and H_2SO_4 is soluble in a mixture of ether and formic acid is obviously related to the fact that when FeCl_3 in exactly 6N-HCl is shaken with ether, the iron passes into the ether layer (Szaflka, 1929; cf. Vogel, 1939). The explanation in both cases lies no doubt in the formation of ether-soluble complexes. The presence of excess Cl^- should depress the ionization of FeCl_3 and facilitate the formation of the double molecule Fe_2Cl_6 . This can be regarded as the ferric salt of the hypothetical acid H_2FeCl_6 , analogous to $\text{H}_2\text{Fe}(\text{CN})_6$ and $\text{H}_2\text{Fe}(\text{CNS})_6$. The fact that the chlorides of Au^{+++} , Mo^{+++} and Ti^{+++} , which form complexes very readily, are also extracted into ether from 6N-HCl, tends to support this theory. The concentration of HCl in the experiments of Szaflka was critical, but in the experiments described here a good deal of latitude is permissible. Even with N- H_2SO_4 there is a perceptible passage of iron from the aqueous to the ethereal layer. The effect becomes pronounced at an acid concentration of about 3N, and approaches its maximum at about 5N. Increasing the concentration of acid, at least up to 9N, exercises no adverse influence. For practical purposes, also, the proportion of formic acid in the ether makes no difference. Equally efficient extractions have been obtained with ether containing 5, 10 and 20 % of formic acid.

SUMMARY

1. A titanometric method is described for the determination of 10–200 $\mu\text{g.}$ iron.

2. The coefficient of variation, when the method is applied to 0.2 ml. portions of blood, is 0.45 %.

3. Copper interferes, but may be separated from iron by saturating the solution with NaCl in dilute H_2SO_4 , and shaking with ether containing formic acid, when the iron passes into the ether layer.

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