70. A METHOD FOR THE MICRO-ESTIMATION OF IRON IN BIOLOGICAL MATERIALS

By R. H. THORP

*From the Wellcome Physiological Research Laboratories,
Beckenham, Kent*

(Received 23 April 1941)

A METHOD for the estimation of very small quantities of iron in biological materials has been in use in this laboratory for some years. It is based upon the reaction of ferrous Fe with $\alpha\alpha'$ -dipyridyl described by Hill [1930]. The method has proved so useful for the study of Fe in biological materials of all kinds, such as bacterial cultures, media and tissues of animal origin, that it seems worthy of wider use. When the concentration of the red dye produced by the reaction of ferrous Fe and $\alpha\alpha'$ -dipyridyl is measured colorimetrically it is possible to estimate 0.2 μ g. Fe per ml. of solution under examination. The sensitivity of the method is approximately the same as that of the thiocyanate method for the estimation of ferric Fe. The presence of Cu, Zn and other metals does not interfere with the test when the quantities of these elements are comparable with that of the Fe [Hill, 1930]. Cu gives a blue colour, but this does not interfere with the estimation of Fe, even when present in amounts up to 1 mg./ml., as a green light filter is used in the colorimeter and the green light is not absorbed by the blue colour due to the Cu. Zn forms a colourless compound with $\alpha\alpha'$ -dipyridyl, but this does not affect the Fe reaction when the reagent is present in excess. Although by theory three molecules of $\alpha\alpha'$ -dipyridyl combine with one atom of Fe (in molar equivalents, 468 g. of $\alpha\alpha'$ -dipyridyl to 56 g. of Fe), in practice it has been found best to use ten times more dipyridyl than the theoretical amount. Since the red complex produced in the presence of excess $\alpha\alpha'$ -dipyridyl is dissociated over a range of pH 3.5–8.5, it is satisfactory to estimate the colour at about pH 6. In our method the standard solutions have a pH of 6.3. Hill has shown that Fe may be estimated in many biological materials without previous ashing if precautions are taken to prevent the adsorption of the red iron dipyridyl upon insoluble proteins. We have preferred always to ash the materials by the usual wet ashing process, using perchloric acid, HNO_3 , or H_2O_2 , instead of selenium or catalytic copper methods, for oxidation. The red colour of the complex ion is formed only in the presence of ferrous Fe so that reduction is necessary after ashing the material. This is effected by sulphurous acid formed from sodium sulphite in the presence of HCl. At present $\alpha\alpha'$ -dipyridyl is not commercially available but it may be prepared in several ways. Blau [1888] prepared it first, from copper picolinate, and Hill describes a method based upon this reaction. Hein & Retter [1928] devised a simpler method of preparation by the oxidation of pyridine in a closed tube. We have found it convenient to use the method of Wibaut & Overhoff [1928], in which 2-bromopyridine is boiled with cymene in the presence of copper powder for 1½ hr. The cymene is then distilled off in steam, the residue made alkaline and finally the $\alpha\alpha'$ -dipyridyl is distilled. The product is purified by recrystallizing from 20 % alcohol.

METHOD

Reagents

Iron-free HCl. 20 % HCl is distilled in an apparatus made entirely of pyrex glass. Two strengths of the acid are prepared from the distillate, N and $N/10$; these are standardized and should be within 1 % of the nominal strengths.

$\alpha\alpha'$ -Dipyridyl reagent. An accurately weighed amount of the material is dissolved in sufficient 0.1 N HCl to make a 1 % solution. This solution is quite stable but should be stored in a white or pyrex glass bottle which has been filled with conc. HCl and allowed to stand for some days, and then repeatedly rinsed in glass-distilled water. Under these conditions the reagent should show no perceptible pink colour.

Sodium sulphite solution. A 20 % solution is prepared freshly as required from A.R. sodium sulphite crystals, $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$.

Standard iron solution. A stable stock solution of Fe is prepared from ferric ammonium sulphate by dissolving 0.4318 g. in a little water, adding 100 ml. of N iron-free HCl and making up to 1 l. with glass-distilled water. This solution contains 50 μg . Fe/ml.

Standard colours

Standard colours are prepared containing from 0.2 to 10 μg . Fe/ml. The standard solution when diluted five times contains 10 μg . Fe/ml. Appropriate dilutions are made from this by using a micrometer syringe or burette and the

Fig. 1. Spectrographic absorption curve of iron dipyridyl solution.

volumes adjusted to 1 ml. with distilled water. It is convenient to dilute the solutions in $3 \times \frac{3}{8}$ in. tubes calibrated at 1 ml. To each tube is now added 0.4 ml. of the dipyridyl reagent followed by 0.5 ml. of 20 % sodium sulphite solution and 0.1 ml. of N HCl. The red colour reaches a maximum in 15 min. and is permanent; it may be compared optically or photoelectrically. The peak of the

absorption of the red dye occurs at a wave-length of 5250 Å. In Fig. 1 is reproduced an absorption curve of the red colour of iron dipyrindyl, which was kindly furnished by Dr Foster of the Wellcome Chemical Works, Dartford. If a powerful light source is available a Wratten green filter no. 62 or Ilford 'Mercury Green' type of filter is most suitable, but if this is too dense a filter no. 61 is almost as efficient and transmits far more light. The usual convention of plotting the concentration against the logarithm of the percentage transmission is followed. A typical calibration is shown in Fig. 2, derived by using a photoelectric colorimeter employing a gas-filled caesium cell. If the filtered light is truly monochromatic, or nearly so, and is complementary in colour to the absorption band

Fig. 2. Calibration curve for photoelectric colorimeter.

of the solution, this curve will be linear. This is rarely the case unless the filter used is very dense or the solutions being compared are pale in colour. The calibration curve as shown in the figure is, however, quite satisfactory in practice. If the range of standard solutions of Fe used in the calibration extends only to 5 $\mu\text{g./ml.}$ the curve is virtually linear when plotted in the same way. Using a colorimeter employing a selenium cell the sensitivity is lower but a suitable calibration curve can be prepared in the same way over a wide range of Fe concentration. Although this curve is less reliable at lower values of Fe concentration it is equally practicable, and is particularly useful when the range of the concentrations of Fe is large.

Estimation of Fe in samples

For tissues or foodstuffs we have found it convenient to weigh out accurately an amount of material estimated to contain between 10 and 200 $\mu\text{g.}$ Fe and ash by the wet ashing method described by Eden & Green [1940] using H_2SO_4 and perchloric acid. With about 1 g. or less of tissue it is unnecessary to use HNO_3 as an additional oxidizing agent. The excess acid is neutralized with solid Na_2CO_3 (A.R.) until there is no further effervescence. The neutral solution

is then reacidified with HCl to about pH 4; the exact value is not vital, owing to the buffering effect of the sulphite in the subsequent stages of the estimation. The solution is then diluted to 50 ml. in a standard flask with glass-distilled water. For the preparation of the coloured solution 1 ml. of the liquid containing Fe is added to 0.4 ml. of the dipyrldyl reagent in one of the small tubes and this is followed by 0.5 ml. of sodium sulphite solution and 0.1 ml. of *N* HCl, as in the case of the standard colours. The mixture is then shaken and the red colour developed is read upon the colorimeter after an interval of 15 min. The concentration of Fe in the solution is then calculated from the calibration curve. In view of the sensitivity of the method it is necessary to prepare control tubes covering the chemicals used in the entire process. The value obtained for the blank is then subtracted from that obtained in the estimations. If the red colour developed is too deep for the range of the colorimeter the solution may be diluted with water until a convenient reading is obtained. Where the Fe content is known to be high by previous estimations it is more convenient to dilute the solution obtained after ashing and neutralization.

SUMMARY

1. A method of estimating small amounts of Fe in biological materials is described. The method is based upon the red complex formed when $\alpha\alpha'$ -dipyrldyl reacts with ferrous Fe.
2. Amounts of Fe as small as 0.2 $\mu\text{g./ml.}$ can be estimated colorimetrically or optically.
3. An outline is given of methods for preparing $\alpha\alpha'$ -dipyrldyl, since this is not available commercially.

I wish to take this opportunity of thanking Mr R. Sellar for the technical assistance he has given in the chemical work associated with the method.

REFERENCES

- Blau (1888). *Ber. dtsh. chem. Ges.* **21**, 1077.
Eden & Green (1940). *Biochem. J.* **34**, 1203.
Hein & Retter (1928). *Ber. dtsh. chem. Ges.* **61**, 1790.
Hill (1930). *Proc. roy. Soc. B*, **107**, 205.
Wibaut & Overhoff (1928). *Rec. Trav. chim. Pays-Bas*, **47**, 761.