Technical methods

We wish to thank Sister Stewart, Dr. Lowry, and Dr. Neely for their cooperation and help in the collection of specimens and examination of case histories, and Dr. S. Nelson for the animal inoculations.

REFERENCES


ADDENDUM

Since this paper was submitted for publication it is learned that Organon Laboratories have now reduced the sensitivity of their pregnancy test. A further series of tests have been carried out using this modified Pregnosticon. One false positive result was obtained from 115 non-pregnancy urines, and one false negative result from 78 pregnancy urines. These results indicate that Pregnosticon is now a satisfactory test for pregnancy diagnosis.

CORRECTION

The title of the paper by Jean Bernard, J. Lasneret, J. Chome, J. P. Levy, and M. Boiron (J. clin. Path., 16, 319) should read: 'A cytological and histological study of acute promyelocytic leukaemia'. Throughout, the nomenclature 'promyelocyte' should be used instead of 'premyelocyte'.

Standardization of haemoglobin solutions by iron determination

E. C. MASON and A. ADARRAGA-ELIZARAN From the Red Cross Blood Transfusion Service, Brisbane, Australia

Earlier evaluations of haemoglobin standards in this laboratory utilized a modification of the direct titanium sulphate microtitration of residual Fe in wet- or dry-ashed haemoglobin (McFarlane, 1932; Ramsay, 1944; O'Hagan 1957). In the spectrophotometric determination of a coloured complex formed by interaction of haemoglobin Fe with a sensitive reagent Fe is split from haemoglobin by wet-way oxidative procedures terminating in either partial destruction of the protein, requiring a subsequent filtration step (Wong, 1928; Sunderman, MacFate, MacFadyen, Stevenson, and Copeland, 1953; Dickenson, Crafts, and Zak, 1954), or in complete destruction of the protein followed by evaporation of residual volatile acids (Williams and Zak, 1957). Preliminary trials of the first alternative were not completely satisfactory in our hands, possibly because of partial irreversible absorption of Fe by the precipitated protein. On investigation of the second, certain modifications were introduced, including the elimination of the final evaporative step.

MATERIALS AND METHODS

REAGENTS All are made up in iron-free distilled water.

1 Buffered 2,2’-dipyridyl solution One gram of 2,2’-dipyridyl is dissolved with warming in about 700 ml. water, and to this is added 300 g. anhydrous sodium acetate (A.R.). When solution is complete, the whole is transferred to a one-litre volumetric flask and made up to the mark. This solution is stored in a dark glass bottle.

2 Mineral acids The concentrated acids used, H2SO4 (S.G. 1.83), HNO3, (S.G. 1.42) and HClO4 (72%), are each of A.R. quality.

3 Ascorbic acid One gram is dissolved in 100 ml. water for each standardization. The solution is stored in a refrigerator when not in use but is discarded after 24 hours.

4 Standard iron stock solution This contains 10 mg. Fe per ml.; 86.6 g. (Fe2(SO4)3(NH4)2SO4·24H2O (A.R.) is dissolved in 400 ml. water, 200 ml. 10% H2SO4 is added, and the solution is transferred to a one-litre volumetric flask and diluted to the mark. The iron content is accurately established by a standard oxidimetric procedure (Vogel, 1951).

5 Standard iron working solution Accurate 1/100 dilution of stock solution = 100 µg/ml.

APPARATUS All glassware must be free of iron. This is accomplished by cleaning with chromic acid, rinsing

Received for publication 26 March 1963
with iron-free distilled water, cleaning with 20% HNO₃, and finally rinsing with iron-free distilled water.

SPECTROPHOTOMETRY All measurements were made in a Hilger Uvispek spectrophotometer using the glass prism. The wavelength scale was calibrated against the hydrogen lamp (Gibson, 1950), and the extinction scales against the standard copper sulphate and cobalt sulphate solutions (Gibson, 1950).

METHOD

CALIBRATION GRAPH Aliquots of 1-0, 2-0, 3-0, 4-0, and 5-0 ml. respectively of the standard Fe working solution were pipetted into corresponding 100 ml. volumetric flasks. A blank was run in parallel. Then to each flask was added successively about 30 ml. Fe-free distilled water, 1-5 ml. concentrated H₂SO₄ and cooled, 5 ml. of ascorbic acid solution, 20 ml. of the buffered 2,2'-dipyridyl solution, and Fe-free distilled water to the mark. The absorbance of each solution was then measured in a 1 cm. cell in the spectrophotometer at 522 mλ. The colour is stable for 24 hours.

HAEMOGLOBIN IRON Blood or haemoglobin solution, 0-5 ml., was accurately measured into a 100 ml. micro-Kjeldahl digestion flask. This was followed by 0-5 ml. concentrated HClO₄, 0-5 ml. concentrated HNO₃, and 1-5 ml. concentrated H₂SO₄, respectively. A blank was run in parallel with each set of digestions. A small glass bead was added, and the flask was gently heated over a small flame from a micro-burner. The flask was occasionally gently swirled so that the digestion proceeded smoothly and losses of material by bumping were avoided. After the appearance of strong white fumes the flask was heated with the full flame of the burner and the digestion was continued for another five minutes. The cooled digest was diluted with about 30 ml. of Fe-free distilled water, again cooled, then 5 ml. of ascorbic acid and 20 ml. of buffered 2,2'-dipyridyl were respectively added. The contents of the flask were well mixed by gentle rotation and transferred quantitatively into a 100 ml. volumetric flask. The solution was diluted to the mark with washings and the final solution was measured in the same way as was the standard iron.

RESULTS

Preliminary investigation of the absorption curve showed that 522 mλ, the wavelength of maximum absorption (Fig. 1), was the most suitable wavelength for spectrophotometric measurements.

The pH of about 4-0 of the final solution is well within the required range for maximum colour development (Fig. 2), which occurs almost immediately, is very stable, and follows Beer's law (Fig. 3).

Two series of haemoglobin iron determinations were performed. The first was designed to test the reproducibility of the method when applied to a haemoglobin solution with and without graded additions of Fe (Table I). There were no discernible differences between replicates, and the recoveries of added Fe were essentially 100%.

In the second series four fresh citrated blood donations...
Temperature was found to have no effect on the absorbance of the Fe$^{2+}$-2,2'-dipyridyl complex within the testing range of 15°C to 30°C.

Errors due to handling are minimized because a washing procedure is applied to the washing of a precipitate but to effect the complete transfer of the Fe$^{2+}$-dipyridyl solution from the micro-Kjeldahl flask to the volumetric flask. The diminishing red colour of the iron chelate complex in successive washes is a good indicator of the completeness of transfer.

The time required for a single blood evaluation was less than one hour from the start of digestion; this indicates that the method can be applied also to obtain a rapid check on the calibration of a haemoglobinometer or spectrophotometer. The evaluation of the standard haemoglobin solution was found to be 0.45% higher than the value previously accepted.

The similarity of absorbance spectra for haemoglobin Fe and inorganic Fe, the minimal handling of sample, and the high recoveries obtained for added iron, provide adequate confirmation that this procedure is superior to methods involving protein precipitation. The quantitative expression of these advantages is in the low standard error of a mean result, namely, 0.2% of the group mean.

We wish to thank Dr. A. E. Shaw for advice and encouragement and Dr. J. E. O'Hagan for his helpful suggestions.

**SUMMARY**

A method is presented for the rapid colorimetric standardization of haemoglobin solutions by a wetting procedure. The iron is detached and evaluated as the Fe$^{2+}$-2,2'-dipyridyl complex.

**REFERENCES**


---

**TABLE I**

<table>
<thead>
<tr>
<th>Fe Added (µg.)</th>
<th>0</th>
<th>52.1</th>
<th>104.2</th>
<th>156.3</th>
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<tr>
<td><strong>Fe Found (µg.)</strong></td>
<td>Total</td>
<td>Total</td>
<td>Added</td>
<td>Added</td>
</tr>
<tr>
<td>-----------------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>1</td>
<td>250</td>
<td>302</td>
<td>52</td>
<td>354</td>
</tr>
<tr>
<td>2</td>
<td>302</td>
<td>250</td>
<td>52</td>
<td>354</td>
</tr>
<tr>
<td>3</td>
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<td>250</td>
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</tr>
<tr>
<td>4</td>
<td>302</td>
<td>250</td>
<td>52</td>
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</tr>
<tr>
<td>5</td>
<td>302</td>
<td>250</td>
<td>52</td>
<td>354</td>
</tr>
</tbody>
</table>

Results exactly duplicated those for replicate 1

---

**TABLE II**

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<tr>
<th>Blood Donor</th>
<th>Total</th>
<th>Total</th>
<th>Added</th>
<th>Added</th>
<th>Added</th>
<th>Added</th>
</tr>
</thead>
<tbody>
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<td>P.G.</td>
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<td>344</td>
<td>52</td>
<td>354</td>
<td>104</td>
<td>448</td>
</tr>
<tr>
<td>E.M.</td>
<td>255-2</td>
<td>307</td>
<td>52</td>
<td>359</td>
<td>103</td>
<td>412</td>
</tr>
<tr>
<td>R.S.</td>
<td>273-1</td>
<td>325</td>
<td>52</td>
<td>377</td>
<td>104</td>
<td>429</td>
</tr>
<tr>
<td>J.A.</td>
<td>292-2</td>
<td>344</td>
<td>52</td>
<td>396</td>
<td>104</td>
<td>448</td>
</tr>
</tbody>
</table>

---

1Figures in parentheses are corresponding haemoglobin concentrations in g./100 ml.

were evaluated, with and without graded additions of Fe (Table II).

On cooling the digested product a small amount of ferric sulphate is precipitated but this readily dissolves on the addition of the sodium acetate-2,2'-dipyridyl reagent. The final solution was always clear. The possibility that porphyrin or derivatives thereof could contribute to the absorbance at 522 mµ was tested by digesting 2-4 mg. of protoporphyrin dimethyl ester according to this method. The digest was found non-fluorescent to ultraviolet light indicating that the porphyrin had been decomposed; when the digest was treated in the same manner as for a blood sample and examined spectrophotometrically it was found that there was no absorbance at 522 mµ.

1Specimen kindly provided by Dr. J. E. O'Hagan, Princess Alexandra Hospital, Brisbane.
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*J Clin Pathol* 1963 16: 604-606
doi: 10.1136/jcp.16.6.604-b

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