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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

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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, H_2SO_4 (S.G. 1.83), HNO_3 (S.G. 1.42) and HClO_4 (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. $(\text{Fe}_2(\text{SO}_4)_3 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$ (A.R.) is dissolved in 400 ml. water, 200 ml. 10% H_2SO_4 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 $\mu\text{g./ml.}$

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

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with iron-free distilled water, cleaning with 20% HNO_3 , 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 H_2SO_4 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 $\text{m}\mu$. 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 HClO_4 , 0.5 ml. concentrated HNO_3 , and 1.5 ml. concentrated H_2SO_4 , 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 $\text{m}\mu$, 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

FIG. 1. Comparative absorption curves for Fe^{2+} -2,2'-dipyridyl complex from (O) standard Fe solution and (●) haemoglobin, showing a maximum at 522 $\text{m}\mu$.

FIG. 2

FIG. 3

FIG. 2. Relationship between pH and absorbance of Fe^{2+} -2,2'-dipyridyl complex. The absorbances were measured within 30 minutes of the addition of the complexing reagent.

FIG. 3. Iron calibration graph.

TABLE I
RECOVERIES OF FE ADDED TO REPLICATES
OF A HAEMOGLOBIN SOLUTION OF 14.8 g./100 ml.

Replicate No.	Fe Found (μg.)						
	Total	Total	Added	Total	Added	Total	Added
1	250	302	52	354	104	406	156
2	Results exactly duplicated those for replicate 1						
3							
4							
5							

TABLE II
RECOVERIES OF FE ADDED TO FRESH CITRATED
BLOOD SAMPLES

Blood Donor	Fe Found (μg.)						
	Total	Total	Added	Total	Added	Total	Added
P.G.	292 (17.3) ¹	344	52	396	104	448	157
E.M.	255.2 (15.1)	307	52	359	103	412	157
R.S.	273.1 (16.2)	325	52	377	104	429	156
J.A.	292.2 (17.3)	344	52	396	104	448	156

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 ester¹ 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.

Temperature was found to have no effect on the absorbance of the Fe²⁺-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 not to the washing of a precipitate but to effect the complete transfer of the Fe²⁺-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 absorption 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.

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SUMMARY

A method is presented for the rapid colorimetric standardization of haemoglobin solutions by a wet ashing procedure. The iron is detached and evaluated as the Fe²⁺-2,2'-dipyridyl complex.

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