made, and, using the formula $\sqrt{\frac{\Sigma d^2}{\Sigma n}}$, where d is the difference between pairs and n the number of pairs, it was found that the root mean square difference was 2·8 mg./100 ml.

METHOD

TEST Whole blood, 0·02 ml., is pipetted into 1·78 ml. of isotonic sodium sulphate-copper sulphate in a small ($3 \times \frac{1}{2}$ in.) Pyrex tube. Sodium tungstate, 0·2 ml., is added and the mixture is well shaken. The precipitated proteins and copper tungstate are centrifuged down (5 min. at 3,000 r.p.m.), and the entire supernatant is tipped, as completely as possible, into 2 ml. alkaline copper solution in a $6 \times \frac{3}{4}$ in. test tube.[1] One millilitre neocuproine is added, and the tube is plugged with cotton-wool and placed in a boiling water bath for six minutes. After immediate cooling the optical density is measured in a photoelectric colorimeter with a blue light filter (*e.g.*, Ilford 621). The colour is stable for at least one hour.

STANDARD AND BLANK Glucose standard, 0·02 ml., and 0·02 ml. water replace the whole blood in the test procedure.

HIGH BLOOD SUGARS For specimens containing over 200 mg. glucose/100 ml., a 3 ml. sample of the coloured solution is diluted with an equal volume of water and replaced in the boiling water bath for 2 min., and 3 ml. of the coloured solutions from the blank and from a 300 mg./100 ml. standard are similarly treated (Fig. 1, lines 1 and 2). For blood sugars higher than 500 mg./100 ml., the determination must be repeated using smaller volumes, *i.e.*, 0·5 ml., of supernatant from the test, from the 600 mg. standard and from the blank, plus 1·5 ml. water (Fig. 1, line 3).

$$\text{Blood glucose} = \frac{\text{Reading of test blank}}{\text{Reading of standard blank}} \times S$$

where S is concentration of standard in mg. glucose/100 ml.

REAGENTS

ISOTONIC SODIUM SULPHATE-COPPER SULPHATE A mixture of 320 ml. of 3% $Na_2SO_4 \cdot 10H_2O$ and 30 ml. 7% $CuSO_4 \cdot 5H_2O$.

10% SODIUM TUNGSTATE Ten grams dissolved in 100 ml. water and kept in a waxed bottle.

ALKALINE COPPER REAGENT Anhydrous Na_2CO_3, 40 g., and 7·5 g. tartaric acid are dissolved in 400 ml. water. $CuSO_4 \cdot 5H_2O$, 0·225 g., is then added and dissolved, and the solution made up to 1 litre.

0·12% NEOCUPROINE SOLUTION Neocuproine, 120 mg., is dissolved in a small volume of N-HCl and made up to 100 ml. with water.

[1]This 'tipping' technique, from small test tubes, is quicker and easier than pipetting. Results agree closely.

A new method for determination of the serum iron

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Measurement of the serum iron is a useful diagnostic test in many haematological disorders. The test is, however, tedious to perform and many laboratories do not regularly undertake the work. A methodological simplification was suggested by the work of Webster (1960), who used a detergent in the measurement of iron-dextran (Imferon) in the blood after intravenous injection. It has been found that this detergent (Teepol 710), together with ascorbic acid, dissociates iron from its protein linkage in serum and permits colorimetric measurement of the iron after reduction to the ferrous state with the reducing agent.

The purpose of this paper is to describe the new method using Teepol and ascorbic acid. This method does not require precipitation of the proteins, which is an essential preliminary step in most other methods (Kitzes, Elvehjem, and Schuette, 1944; Kaldor, 1953; Trinder, 1956). The results obtained by the method have been compared with those obtained by Kaldor's and Trinder's methods.

PROPOSED METHOD

IRON-FREE WATER Water for the preparation of all reagents and for the rinsing of all acid-washed apparatus is glass-distilled.

APPARATUS All glassware is washed in a detergent solution, rinsed in tap water and distilled water, soaked in 1 *N* hydrochloric acid for a minimum of 30 minutes, and finally rinsed in iron-free water.

Received for publication 24 August 1962

[1]This work has been supported by a grant from the National Health and Medical Research Council of Australia

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Colorimetric determination of glucose in 20 µl. of blood—concluded

STOCK GLUCOSE SOLUTION Pure anhydrous glucose, 2 g., is dissolved in 100 ml. saturated (0·3%) benzoic acid.

STANDARD GLUCOSE SOLUTIONS These are prepared by diluting 5, 10, 15, and 30 ml. of stock solution to 100 ml. with saturated benzoic acid, giving standards of 100, 200, 300, and 600 mg. glucose/100 ml.

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REAGENTS Two reagents containing Teepol were made up and one stock solution of iron.

Teepol solution Teepol is an anionic detergent made by Shell Chemical Company. Teepol 710 containing 40% active matter is used, 375 ml. being made up to 1 litre with distilled water. Traces of iron are removed by passing the dilute solution slowly through a column of the sodium form of Amberlite Resin 1R-120 (B.D.H.) or of Zeo-Karb 225 (Permutit). Ascorbic acid is added to the dilute Teepol stock solution to make a 0·75% solution of ascorbic acid. The dilute Teepol solution without added ascorbic acid is stable for an indefinite period; with ascorbic acid it is still suitable for use after storage for two weeks at 6°C.

Recently the Teepol solution has been prepared with 0·2 *M* sodium acetate-acetic acid buffer at a *p*H of 4·6 instead of water. A 39% solution of Teepol 710 in water is prepared. Traces of iron are removed as before, using a de-ionizing resin. Hydrated sodium acetate A.R. ($CH_3COONa.3H_2O$, 0·592 g.) and glacial acetic acid A.R. (2·5 ml.) are added to 67·5 ml. of this treated Teepol solution to give the required buffered solution. With this modification the ascorbic acid concentration has been reduced to 0·20% and all bound iron has been released from transferrin.

Orthophenanthroline solution Orthophenanthroline, 0·5 g., is dissolved in 32 ml. of Teepol 710 and the solution made up to 100 ml. with distilled water.

Standard iron solution A stock solution of 560 μg. Fe/ml. is prepared by dissolving 0·3922 g. ferrous ammonium sulphate A.R. ($FeSO_4.(NH_4)_2SO_4.6H_2O$) in 0·1 *N* sulphuric acid and making up to 100 ml. with the acid. This stock solution is diluted with distilled water as required.

PROCEDURE The procedure is shown in Table I. One millilitre of serum and 4 ml. of the Teepol-ascorbic acid solution are mixed in a cuvette (test solution). A blank is prepared in a second cuvette with water in place of serum (blank solution). The spectrophotometer is set with the blank solution at zero density and the optical density of the test solution is read at a wavelength of 510 mμ (optical density I). With a photoelectric absorptiometer an Ilford 623 green filter is used and the instrument adjusted to 100% transmission or zero density with the blank solution (density = log. 100 − log. transmission).

Orthophenanthroline solution (0·5%, approximately 0·05 ml.) is added to each cuvette, mixed, and allowed to stand for five minutes to permit full development of the iron-orthophenanthroline colour. The density of the test solution is read on the instrument set with the blank solution as before (optical density II).

The optical density of the iron-orthophenanthroline colour produced by the protein-bound iron in the serum is found by subtracting optical density I from optical density II. The iron content of the serum is read from a calibration graph prepared by mixing 1 ml. aliquots of standard iron solutions with 4 ml. of Teepol-ascorbic acid solution and 0·05 ml. of the orthophenanthroline solution. The optical densities of these solutions are read against the blank solution as before and plotted against the concentrations. The iron in the standards corresponds to serum iron values of 28, 56, 112, 280, and 560 μg./100 ml.

TABLE I

PROCEDURE FOR MEASUREMENT OF SERUM IRON VALUE

Reagents	*Blank Solution (Cuvette A)*	*Test Solution (Cuvette B)*
Water	1 ml.	—
Serum	—	1 ml.
Teepol-ascorbic acid solution	4 ml.	4 ml.
	Mix	Mix
	Set spectrophotometer on zero optical density	Read against Cuvette A (optical density I)
Orthophenanthroline solution	0·05 ml.	0·05 ml.
	Mix	Mix
	Allow colour to develop for 5 min. at room temperature	Allow colour to develop for 5 min. at room temperature
	Set spectrophotometer on zero optical density	Read against Cuvette A (optical density II)

INVESTIGATION OF PROPOSED METHOD

CONCENTRATION OF TEEPOL 710 Parallel estimations of serum iron values showed that the final serum to undiluted Teepol ratio should be approximately 1 to 1·5. If the ratio is 1 to 1 or less, a slow precipitation takes place, producing cloudiness after five to 30 minutes. Six different batches of Teepol 710 were tested on four sera in the new serum iron method and all batches proved satisfactory.

REDUCING AGENT (ASCORBIC ACID) The amount of ascorbic acid is important in relation to (a) the *p*H produced and its effect on the development of the iron phenanthroline complex; (b) the liberation of iron bound to transferrin in serum; and (c) the reduction of ferric iron to the ferrous state. These factors were investigated as follows:—

The standard solution of iron was diluted to give the same range of concentrations as previously and calibration iron-orthophenanthroline curves were prepared with different amounts of ascorbic acid added to Teepol as shown in Table II. Beer's law was not obeyed until the ascorbic acid concentration reached 0·2%.

The *p*H values of the iron solution with the Teepol solution containing differing amounts of ascorbic acid were measured, with the results shown in Table II. The effect of ascorbic acid on the *p*H value is obvious.

The Teepol-ascorbic acid solutions were used to determine the iron content of three different sera. The *p*H values of the serum and Teepol reagent mixtures are shown in Table II. The serum iron values were comparable when the ascorbic acid concentration was between 0·375% and 3·0%.

Even the solutions with 0·2% ascorbic acid or less (solutions 1 to 3) contained sufficient ascorbic acid to reduce 8·00 mg./100 ml. of ferric iron to the ferrous state. This was shown by mixing 1 ml. of a 0·023% ferric

TABLE II

EFFECT OF ASCORBIC ACID ON *p*H VALUES OF REAGENT MIXTURES

% Solution of Ascorbic Acid in Teepol	*Standard Iron-orthophenanthroline Calibration Curve*	*p*H *Values*	
		Iron Solution + Teepol-ascorbic Acid Solution	*Serum + Teepol-ascorbic Acid Solution*
(1) 0·025	Beer's law not obeyed	8·4	—
(2) 0·1	Beer's law not obeyed	7·4	—
(3) 0·2	Beer's law obeyed	6·0	—
(4) 0·375	Beer's law obeyed	4·3	6·3
(5) 0·75	Beer's law obeyed	3·7	4·9
(6) 1·125	Beer's law obeyed	3·6	4·5
(7) 1·50	Beer's law obeyed	3·4	4·2
(8) 3·0	Beer's law obeyed	3·0	3·7

chloride solution with 4·0 ml. of each of the three Teepol-ascorbic acid solutions and then adding orthophenanthroline.

As a result of these investigations it was decided to use Teepol containing 0·75% ascorbic acid in the serum iron method. This solution provides a *p*H value which completely dissociates iron from transferrin and permits full development of the iron-orthophenanthroline complex. It reduces ferric iron to ferrous iron, and it does not precipitate protein from the serum.

RELEASE OF HAEMOGLOBIN IRON Known amounts of haemoglobin iron were added to a serum with a low serum iron value and the serum iron values of these haemolysed samples were determined using the new method. The results are shown in Table III.

TABLE III

SERUM IRON VALUES MEASURED IN PRESENCE OF FREE HAEMOGLOBIN

Solution Tested	*Serum Iron Value (μg. Fe/100 ml.)*
Serum	18
Serum + 52 μg. Hb iron/100 ml.	17
Serum + 103 μg. Hb iron/100 ml.	15
Serum + 206 μg. Hb iron/100 ml.	21

Schade, Oyama, Reinhart, and Miller (1954) and Ramsay (1953) stated that 52 μg. of haemoglobin iron in 100 ml. of serum is clearly visible. The results in Table III show that the iron in haemoglobin is not liberated by the new method, even in samples containing 206 μg. of haemoglobin iron per 100 ml. of serum. Similar results have been obtained with samples refrigerated for approximately eight hours and samples frozen for two days, but increasing amounts of haemoglobin iron were released with increasing time of storage at room or refrigerator temperatures.

REPRODUCIBILITY A series of six estimations was performed on each of two sera to determine the reproducibility of the method. The values obtained are shown in Table IV. The standard deviations were 5·0 μg. and 4·7 μg. per 100 ml. and the standard errors of the means 2·2 μg. and 2·1 μg. per 100 ml.

TABLE IV

REPLICATE MEASUREMENTS OF SERUM IRON VALUE OF TWO SERA USING BUFFERED TEEPOL

	Serum A (μg./100 ml.)	*Serum B (μg./100 ml.)*
1	122	64
2	129	58
3	115	67
4	120	58
5	122	56
Mean	122	61
Standard deviation	±5·0	±4·7
Standard error of mean	±2·2	±2·1

FROZEN SERUM The serum iron values of 11 sera have been determined before and after the sera were frozen for 24 hours. The mean values before and after freezing were 111 μg. and 113 μg. per 100 ml. respectively, and the individual differences were not greater than the range found when replicate measurements were made on samples of fresh serum (Table IV).

RECOVERY EXPERIMENTS Recovery experiments, while not proving that all the iron originally present has been liberated, give some indication of the efficiency of the method. Three sera were used for these experiments with two concentration levels of added iron (45 μg. Fe^{+++} and 90 μg. Fe^{+++} per 100 ml. of serum). The serum iron value of each serum was measured before and after the addition of iron, which was in the form of a solution of ferric chloride. The results in Table V show satisfactory recoveries of the added iron.

TABLE V

RECOVERY BY TEEPOL METHOD OF FERRIC CHLORIDE ADDED TO SERUM

	Iron in μg./100 ml. Serum		
	Original Serum Iron Value	*Added as $FeCl_3$*	*Recovered*
Serum A	158	45	45
		90	90
Serum B	56	45	43
		90	90
Serum C	175	45	43
		90	95

COMPARISON WITH OTHER METHODS Results obtained with the Teepol method were compared with those obtained on the same sera by the methods of Kaldor (1953) and of Trinder (1956). The latter methods require

TABLE VI

COMPARISON OF THE TEEPOL WITH OTHER METHODS

No. of Parallel Estimations	*Mean Serum Iron Value (μg. Fe/100 ml.)*		
	Kaldor's Method	*Trinder's Method*	*Teepol Method*
38	129	—	133
18	—	114	124

precipitation of proteins from the serum, neither liberates iron from contaminating haemoglobin, and both have proved reliable in practice. The results by the three methods are shown in Table VI and indicate that the Teepol method is as satisfactory as the other methods. The sera used covered a wide range of iron values.

DISCUSSION

The method described depends on the release of iron from its complex with transferrin. This release is achieved by the combined action of Teepol and ascorbic acid. Teepol alone slowly liberates the iron, as shown by a gradual decrease of optical density at a wavelength of 470 mμ when it is added to serum. The addition of ascorbic acid, with consequent lowering of the *p*H value, accelerates liberation and the iron is completely released at a *p*H of 6·3. This is of interest, because without Teepol Schade, Reinhart, and Levy (1949) showed that the iron-transferrin complex is not completely dissociated at a *p*H of 6·3. Teepol also acts as an anionic detergent and prevents precipitation of plasma proteins.

In the described method the system of blanks is the same as that employed in the serum iron method developed by Beale, Bostrom, and Taylor (1961). Thus, only one blank (the water blank) need be set up regardless of the number of sera to be tested, because each test solution acts as its own 'internal' blank.

The Teepol method can also be used with bathophenanthroline instead of orthophenanthroline. It is not necessary to sulphonate the bathophenanthroline as is usually required, because it is soluble in buffered Teepol solution. The advantage of using bathophenanthroline is that its complex with iron has a more intense colour at equivalent concentrations of iron than the complex with orthophenanthroline. A less sensitive colorimeter can be used for measuring the optical densities, but bathophenanthroline is more expensive than orthophenanthroline.

The Teepol method has also been used for measuring iron in urine chelated with diethyltriaminopenta-acetic acid. It has been found that iron is not released from this chelate solely by lowering the *p*H value, and Trinder's (1956) method of measuring serum iron cannot be used. The only modification of the Teepol method necessary is that the tube or cuvette containing all reagents is placed for 20 minutes in a water bath at 96° to 98°C. to develop the colour with orthophenanthroline. The contents of the tube are cooled before the optical density is measured.

The Teepol method for measuring serum iron is simpler and more rapid for hospital laboratory use than other methods which have been described. No claims are made that it is more accurate than the methods currently in use, but it is believed that it is reliable for clinical purposes. The importance of treating glassware and other apparatus to remove contaminating iron must be emphasized.

SUMMARY

A rapid method for determining iron in serum is presented. The iron is extracted and measured without precipitation of the serum proteins, and only three reagents—a detergent (Teepol), ascorbic acid, and orthophenanthroline—are used. The method can be applied to both fresh and frozen sera and the results are not affected by haemolysis. Recovery of added ferric iron is quantitative. The new method has been compared with the methods of Kaldor (1953) and Trinder (1956).

I wish to thank Shell Chemical (Aust.) Pty. Ltd. for supplying the different batches of Teepol for testing.

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ADDENDUM

The Shell Chemical Company has replaced Teepol 710 with Teepol 610. The difference in these detergents appears to be solely in the concentration of 'active matter' present. Teepol 710 contains 40% 'active matter'; Teepol 610 contains 34%.

If Teepol 610 is used the reagents become:—

Teepol-acetate buffer solution 45·9% solution of Teepol 610 in water....

Orthophenanthroline solution 0·5% solution of orthophenanthroline in a 37·6% solution of Teepol 610 in water.