

Method using ortho-tolidine for the quantitative determination of haemoglobin in serum and urine

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SYNOPSIS Following a study of the spectrophotometric properties of ortho-tolidine and its oxidation products, with particular attention to variation brought about by change in the hydrogen ion concentration, a method for the quantitative estimation of haemoglobin in serum and urine in which ortho-tolidine is substituted for benzidine in a peroxidase system is described. The method is designed to measure haemoglobin value within the range 0-125 mg. per 100 ml. Evidence is presented showing that it gives results equal in accuracy and reproducibility to a benzidine method in common use and for reasons stated is a more satisfactory technique for estimating the haemoglobin content of urine.

Since the demonstration of a high incidence of carcinoma of the bladder amongst industrial workers who inadvertently ingest or inhale benzidine in small quantities over long periods (Scott, 1952; Barsotti and Vigliani, 1952; Baker, 1953; Douillet, 1956), the use of this substance in biochemical laboratories and clinical side rooms has been strongly discouraged.

As a consequence, during the past few years, the chemical ortho-tolidine has been substituted for benzidine² in tests designed to demonstrate qualitatively the presence of haemoglobin in faeces and urine. In spite of the similarity in chemical structure to benzidine, ortho-tolidine has not to date been reported as causing carcinoma in man. Its substitution for benzidine as a means of determining quantitatively the level of haemoglobin in serum and urine in some pathological states seems desirable.

THE ORTHO-TOLIDINE PEROXIDASE REACTION

CHEMICAL STRUCTURE OF ORTHO-TOLIDINE Ortho-tolidine (4,4'-diamino-3,3'-dimethyl-biphenyl) has the empirical formula $\text{NH}_2\text{CH}_3\text{C}_6\text{H}_3\text{C}_6\text{H}_3\text{CH}_3\text{NH}_2$. It is a colourless crystalline substance with a melting point of 126.5°C. It is slightly soluble in ethyl alcohol. It is prepared from o-nitro-toluene by reduction to the corresponding hydrazo compound by rearrangement in the same manner as benzidine is prepared from nitrobenzene (Vobes, 1959).

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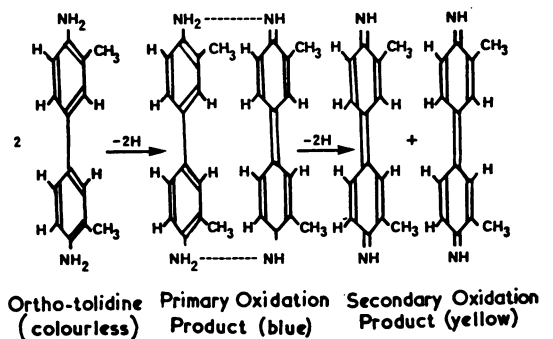


FIG. 1. *Ortho-tolidine and its oxidation products.*

The structural changes occurring on oxidation are represented by the formulae shown in Figure 1.

MATERIALS AND METHODS

All the spectrophotometric studies of the ortho-tolidine peroxidase reaction described below were carried out using a standard procedure.

REAGENTS All reagents were of 'Analar' or equivalent grade.

- 1 Ortho-tolidine-acetic acid reagent (see Appendix)
- 2 Hydrogen peroxide solution (see Appendix)
- 3 Haemoglobin standard solution (see Appendix)
- 4 Final diluents
 - 10% Acetic acid (see Appendix)
 - Acetate buffer pH 4.63
 - Citrate buffer pH 4.69
 (King and Wootton, 1956)

APPARATUS All readings were made using a Unicam S.P. 600 spectrophotometer.

PROCEDURE Of the haemoglobin standard, 0.02 ml. was washed into 1 ml. of the ortho-tolidine-acetic acid reagent. After thorough mixing, 1 ml. of the hydrogen peroxide solution was added and mixed by swirling. The reaction was allowed to proceed for 10 minutes (see 'Timing of the ortho-tolidine reaction' below), at the end of which 10 ml. of the selected buffer diluent was added. The test solution was then immediately read in the spectrophotometer.

RESULTS

SPECTROPHOTOMETRIC PROPERTIES Like benzidine, ortho-tolidine has two oxidation products. The primary oxidation product is blue, having a maximum absorption peak at 630 m μ . The secondary oxidation product is yellow and has a maximum absorption at 435 m μ (Fig. 2).

Table I gives a summary of the spectrophotometric properties of ortho-tolidine compared with those of benzidine.

EFFECT OF pH A, B, and C (Fig. 3) are the spectrophotometric absorption curves derived from three ortho-tolidine peroxidase reactions in which the quantities of a standard haemoglobin solution,

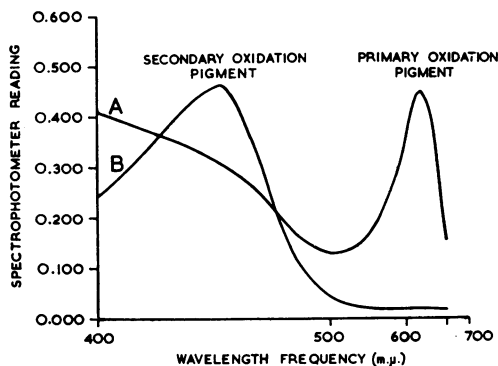


FIG. 2. *A* Ortho-tolidine peroxidase reaction with water as final diluent (final pH 4.6).
B Ortho-tolidine peroxidase reaction with glacial acetic acid as final diluent (final pH 1.50).

ortho-tolidine-acetic acid reagent and hydrogen peroxide were the same, but the final pH of the test solutions for reading in the spectrophotometer were made 3.75, 2.94, and 2.20 respectively by using three different diluting agents. It can be seen that a high pH favours the formation of the primary oxidation product whereas a low final pH favours the secondary oxidation product.

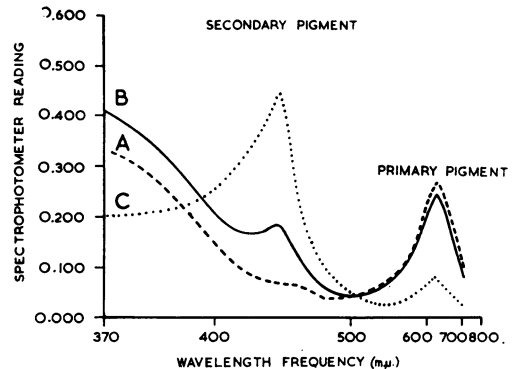


FIG. 3. *Effect of pH on the oxidation products of ortho-tolidine.*

A Final pH 3.75 (citrate buffer pH 4.96 used as diluent).
B Final pH 2.94 (acetate buffer pH 4.63 used as diluent).
C Final pH 2.20 (10% acetic acid used as final diluent).

Compared with benzidine, the primary oxidation product of ortho-tolidine is relatively stable, depending upon the pH of the medium in which it exists. The conversion of the primary to the secondary pigment is inhibited by raising the pH of the solution. This factor is of limited practical application because at high pH the primary oxidation product is rapidly reduced to a leuco derivative. This last change appears to be irreversible as far as re-oxidation by addition of more peroxidase (haemoglobin) is concerned. Lowering the pH favours the conversion of the primary pigment to the secondary form. This pigment when formed is quite stable but can be rendered colourless by the action of strong reducing agents such as ascorbic acid. This change is also irreversible.

Figure 4A shows the stability of the primary

TABLE I
COMPARISON OF SPECTROPHOTOMETRIC CHARACTERISTICS OF OXIDATION PRODUCTS OF BENZIDINE AND ORTHO-TOLIDINE

	Benzidine		Ortho-tolidine	
	Primary Pigment	Secondary Pigment	Primary Pigment	Secondary Pigment
Colour	Blue	Red	Blue	Yellow
Extinction maximum	600 m μ .	515 m μ .	630 m μ .	435 m μ .
Stability at pH 2.20	Very unstable	Stable	Stable	Stable
Stability at pH 3.75	—	—	Moderately stable	—

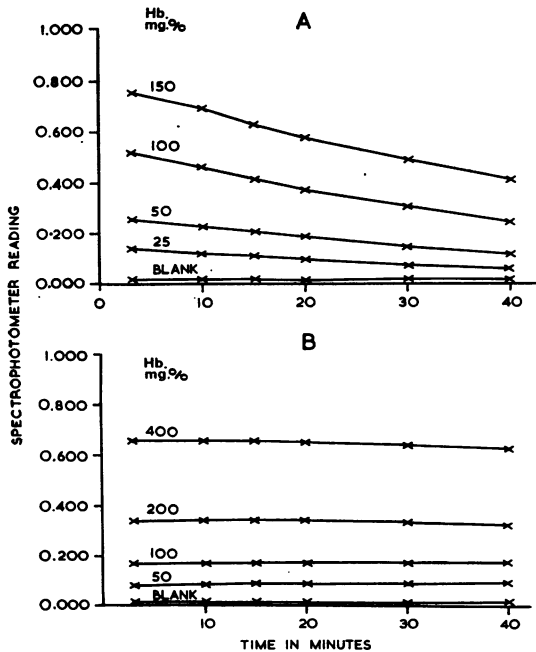


FIG. 4. Stability of the primary oxidation product of ortho-tolidine. A Using citrate buffer (pH 4.96) as diluent (final pH 3.75). B Using 10% acetic acid as diluent (final pH 2.20). The primary oxidation product of ortho-tolidine is stable at pH 2.20, although the intensity of colour development is appreciably less than when the citrate buffer, giving a final pH of 3.75, is used.

oxidation product of ortho-tolidine at varying concentration when made up with a citrate buffer diluent (pH 5.0) giving a final pH of 3.75. It can be seen that when this diluent is used a rapid fall-off in the colour intensity occurs. Spectrophotometric readings must, therefore, be made immediately following dilution if an accurate assessment of the maximum intensity of the reaction is to be obtained.

Figure 4B shows the results of similar experiments in which 10% acetic acid was used as the diluent making the pH of the final solution 2.20. The intensity of colour due to the primary pigment is less than when the citrate buffer diluent is used but the fall-off in pigment intensity is far less rapid, showing that at this pH the primary pigment is more stable.

In both the above instances the intensity of primary pigment colour was directly proportional to the concentration of peroxidase taking part in the reaction, Beer's law being obeyed to an extinction coefficient of at least 0.700 (Fig. 5).

TIMING OF THE ORTHO-TOLIDINE REACTION In order to determine the optimum time for the addition of the acetic acid diluent following the start of the

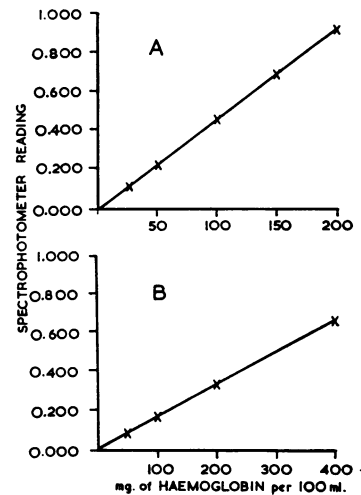


FIG. 5. The graphs show how the intensity of colour development in an ortho-tolidine peroxidase reaction read at 10 minutes following dilution is directly dependent upon the quantity of haemoglobin participating. Beer's law is obeyed to an extinction coefficient of at least 0.700. A Final pH 3.75 (citrate buffer diluent). B Final pH 2.20 (10% acetic acid diluent).

TABLE II
TIMING THE ORTHO-TOLIDINE PEROXIDASE REACTION¹

Reaction	Time (minutes \bar{p} H ₂ O ₂)										
	5	10	15	20	25	30	40	45	55	60	65
1	0.489	0.520	0.527	0.528	0.535	0.527	0.518	0.505	0.478	0.459	
2		0.538	0.554	0.550	0.552	0.530	0.525	0.510	0.485	0.481	
3			0.565	0.560	0.550	0.537	0.521	0.503	0.481	0.463	
4				0.583	0.581	0.569	0.543	0.523	0.505	0.483	
5					0.585	0.556	0.532	0.517	0.493	0.477	
6						0.573	0.560	0.553	—	0.508	
7							0.571	0.562	—	0.508	
8										0.492	0.478

¹The table shows how maximum colour development occurs 20 to 25 minutes following the start of the peroxidase reaction (reactions 4 and 5), but that the greatest stability of colour in the final test solution is obtained by allowing the reaction to proceed for only 10 to 15 minutes before diluting with 10% acetic acid and reading in the spectrophotometer (reactions 2 and 3).

ortho-tolidine peroxidase reaction, eight identical reactions were studied (Table II) but the time interval between the addition of the hydrogen peroxide solution and final dilution with 10% acetic acid was varied. All the initial spectrophotometric readings were made immediately following dilution of the reaction with acetic acid.

It will be seen that maximum colour intensity develops when the reaction is allowed to stand for 20 to 25 minutes before dilution (boxed numbers). Following dilution, a fall-off in colour intensity occurs (unboxed numbers).

Maximum stability is observed when the reaction is diluted 10-15 minutes after standing. The optimum time interval between the start of the reaction (following addition of hydrogen peroxide) and dilution is 10 minutes.

In the appendix to this paper is given a detailed description of a quantitative method for estimating the haemoglobin content of serum and urine using ortho-tolidine as the hydrogen donor in a peroxidase system. This method was evolved using the above information concerning the spectrophotometric properties of ortho-tolidine. Although technically similar to the benzidine method described by Crosby and Furth (1956), it differs in that first, the reaction is allowed to proceed for 10 instead of 20 minutes, and, secondly, spectrophotometric measurement of the intensity of the primary oxidation product of ortho-tolidine is made at a wavelength frequency of 630 millimicrons instead of at 515 millimicrons as used for measuring the intensity of the secondary oxidation product of benzidine.

COMPARISON OF BENZIDINE AND ORTHO-TOLIDINE METHODS FOR QUANTITATIVE ESTIMATION OF HAEMOGLOBIN IN SERUM Figure 6 compares the results of haemoglobin estimation using both the benzidine method (Crosby and Furth, 1956) and the ortho-tolidine technique described in detail in the appendix. The 160 sera examined were obtained from patients subjected to experimentally induced haemoglobinemia.

The benzidine method results are arranged in ascending order of magnitude on the left hand side of the serum haemoglobin axis, whilst the respective values obtained using the ortho-tolidine technique are arranged on the right.

It will be seen that the ortho-tolidine method results are within ± 5 mg. of haemoglobin per 100 ml. of the values obtained using the benzidine method.

ESTIMATION OF HAEMOGLOBIN IN URINE Crosby and Furth (1956) point out that when their method is used to measure the haemoglobin content of urine

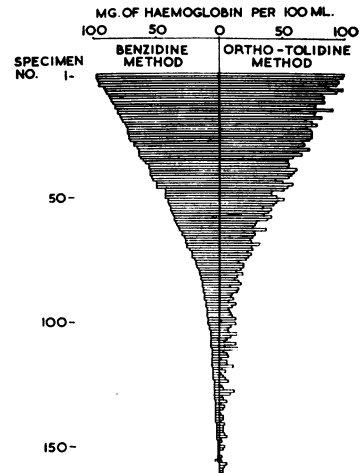


FIG. 6. Comparison of the benzidine and ortho-tolidine methods for the quantitative estimation of haemoglobin in serum. One hundred and sixty sera were studied. Results using the benzidine method are shown on the left hand side of the serum haemoglobin axis whilst the results obtained using the ortho-tolidine method are shown on the right.

samples, sulphates and urates normally present form insoluble complexes with benzidine and can cause it to 'salt out' of solution. This reduces the concentration of benzidine available for participation in the peroxidase reaction and also causes turbidity of the final test solution. To overcome this, they recommend overnight dialysis of the urine against normal saline so that the undesirable salts are removed. Following such a procedure, a correction factor needs to be applied for gain or loss of urine volume due to dialysis.

Ortho-tolidine has two advantages over benzidine when being considered for use for the estimation of the haemoglobin content of urine. First, it is more soluble than benzidine in 90% acetic acid and, secondly, weight for weight it gives a greater intensity of colour, thus allowing the strength of the reagent to be appreciably reduced. Both these factors decrease the ability of urinary sulphates and urates to 'salt out' ortho-tolidine. In fact, providing the concentration of the ortho-tolidine reagent is kept below 0.25 g. % and the quantity of urine used less than 0.02 ml., the method as described in the appendix can be applied without modification, and without the necessity of pre-estimation dialysis. When small levels of haemoglobin are to be measured enhancement of the ortho-tolidine colour development can be achieved by using the citrate buffer instead of 10% acetic acid, but measurement of colour intensity requires to be made immediately following dilution of the peroxidase reaction.

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APPENDIX

A METHOD FOR THE DETERMINATION OF SERUM HAEMOGLOBIN USING ORTHO-TOLIDINE

REAGENTS Glassware for storage of reagents must be cleaned with dilute HCl and washed with deionized water.

1 *Ortho-tolidine reagent* Ortho-tolidine (Analar), 0.25 g., is dissolved in 80 ml. of glacial acetic acid and 10 ml. of water added. After mixing, the volume is made up to 100 ml. with glacial acetic acid. The reagent tends to darken on keeping but this can be reduced to a minimum by storing in a refrigerator at 5°C. This reagent should be prepared freshly every eight to 12 weeks.

2 *Hydrogen peroxide* Two ml. of a 60 vol. % solution of hydrogen peroxide is made up to 100 ml. with distilled water. This reagent is prepared freshly every six to 10 days and is stored in a refrigerator when not in use.

3 *Standard haemoglobin solution* A fresh sample of 20 ml. of venous blood is mixed with 5 ml. of a 3% solution of sodium citrate. The red cells are separated from the plasma by centrifuging and the supernatant plasma removed with a Pasteur pipette. Then 0.9% saline is added to restore the volume to 20 ml. After mixing, the suspension of red cells is placed in a deep freeze refrigerator (-15°C.) for 12 hours so that haemolysis of the red cells is effected.

After melting, red cell debris is separated by centrifuging for one hour at a speed of 12,000 r.p.m. (M.S.E. high speed refrigerated centrifuge). The clear wine-coloured supernatant is then drawn off with a Pasteur pipette. This solution is then completely cleared of all remaining debris by passing through a Seitz filter. The precise haemoglobin content of this solution is then determined using the alkaline haematin method (Varley, 1962). By suitable dilution a 200 mg. haemoglobin standard is prepared from this stock solution; 0.5 ml. of this when mixed with 0.5 ml. haemoglobin-free serum or urine makes a haemoglobin standard of 100 mg. per 100 ml.

4 *Acetic acid diluent* Glacial acetic acid, 100 ml., is diluted to 1 litre with deionized water.

APPARATUS All glassware should be acid cleaned.

- 1 Spectrophotometer set at the wavelength 630 m μ .
- 2 Glass cuvettes with 10 mm. light path \times 3.
- 3 Interval timer with ten minute sweep.
- 4 Test tubes of 15 ml. capacity.
- 5 Sahli pipettes 0.02 ml. grade A, N.P.L. (one for each unknown plus one for the haemoglobin standard), 2 \times 5 ml. graduated pipettes, 1 \times 10 ml. graduated pipette or a 10 ml. automatic delivery pipette.

PROCEDURE For each unknown, 1 ml. of the ortho-tolidine reagent is placed in a test tube. Two other tubes are prepared in a similar manner, one for the haemoglobin standard and the second for the reagent blank and 0.02 ml. of the unknown is washed into the first tube. A similar quantity of the haemoglobin standard is washed into the second. The tubes are mixed by swirling. After the lapse of two minutes, 1 ml. of the hydrogen peroxide reagent is added to each tube. The contents are again mixed by swirling. They are then left to stand for precisely 10 minutes, using an interval timer. Then 10 ml. of the acetic acid diluting agent is added to each tube and after mixing by upending, the solutions are transferred to cuvettes and immediately read in the spectrophotometer. The reagent blank (ortho-tolidine, hydrogen peroxide plus acetic acid diluent) is used for adjusting the spectrophotometer to zero.

The optical density of the standard represents a haemoglobin concentration of 100 mg.%. The optical density of the unknown measures the concentration of haemoglobin in the sample serum under test.

The above procedure has been designed for measuring serum haemoglobin concentration within the range 0-125 mg.%. The method can be made more sensitive by: 1 Decreasing the volume of diluent added before reading in the spectrophotometer; 2 increasing the concentration of ortho-tolidine in the ortho-tolidine reagent; 3 increasing the quantity of serum used during the test to 0.04 ml.; 4 raising the pH of the final test solution by using the citrate buffer instead of the 10% acetic acid diluent but, when this is done, reading of the colour intensity in the spectrophotometer must be made immediately after dilution.

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