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A SAFER SUBSTITUTE FOR BENZIDINE IN THE DETECTION OF BLOOD

V. R. HOLLAND and B. C. SAUNDERS
University Chemical Laboratories, Cambridge, England
and
F. L. ROSE and A. L. WALPOLE
Pharmaceuticals Division, Imperial Chemical Industries Ltd., Alderley Park, Macclesfield, Chesire, England

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Abstract—The derivation and synthesis of 3,5,3',5'-tetramethyl benzidine are described. Prolonged subcutaneous administration to rats gave a negligible yield of tumours, unlike benzidine and o-tolidine with which it was compared. It is a sensitive and specific agent for the detection of blood, and could displace the use of these substances for that purpose.

Benzidine (1) has been used for many years as a reagent for the detection of blood.[1,2] The sensitivity and specificity of the test are both well illustrated by the fact that in spite of the extreme hazards associated with exposure to this compound, there has been a marked reluctance to abandon its use for any substitute hitherto found. In particular, the incidence of cancer among workers exposed to benzidine[3-6] has rendered the search for a satisfactory alternative of paramount importance.

A potential test for blood may be based on any property which blood is known to possess under the test conditions, but to be of practical value any procedure devised must satisfy several criteria. In a test designed for forensic purposes, in particular, the following are of importance.

(a) The test must be specific. The number of foreign materials which will give a positive result in the absence of blood must be minimal.

(b) The test must be able to detect blood under a wide range of conditions; even if, for example, the sample is not a fresh one.

(c) The test must be sensitive, being able to detect microgram quantities of blood.

(d) The test must be easy to apply i.e. it must not be either a laborious or dangerous procedure. It must give a clear and rapid indication of a positive or negative result. This is especially important, as the test may sometimes be in the hands of unskilled workers.

For many years, one of us (B.C.S.)[7] has had a considerable interest in the enzyme peroxidase which is widely distributed in animals and plants, and is known to give intense colours with certain amines and phenols in the presence of hydrogen peroxide. In many instances the nature and composition of the coloured products have been determined. For example, mesidine with peroxidase and hydrogen peroxide gives in quantitive yield a purple crystalline product.[8]

Neither hydrogen peroxide alone, nor peroxidase alone, has any action on mesidine, nor indeed have other oxidative enzymes such as indophenol oxidase, catechol oxidase or laccase. In short, this is a true peroxidase action. It has been known for some time that blood shows considerable peroxidase-like activity and that in the presence of hydrogen peroxide it will give highly coloured products with certain substrates, particularly amines, including benzidine. This reaction was investigated by Duijn[9] and the sequence of events is shown in Fig 1. The benzidine test for blood fulfils the requirements stipulated above, except for the fact that benzidine is highly carcinogenic. In searching for a non-carcinogenic substitute which also relies on peroxidase we must clearly retain all these advantages. The substitute must, in addition, be reasonably stable and not too expensive to manufacture. It should preferably be a solid of low volatility, so that even if it is relatively non-toxic, the possibility of human assimilation by inhalation or skin absorption is reduced to a minimum.

The factors which influence the carcinogenic activity of a compound must be considered. This subject has been reviewed by Lawley[10] and by Daudel and Daudel.[11] Miller *et al.*[12] have suggested that there is some evidence for the participation of *ortho*-hydroxy derivatives of aromatic amines in their carcinogenic action. 1-Hydroxy-2-naphthylamine has been detected as a metabolite of 2-naphthylamine, and 3,3'-dihydroxybenzidine as a metabolite of benzidine. Both are more potent carcinogens than the parent amines. Jacobs[13] has recorded that *o*-tolidine (3,3'-dimethylbenzidine) provides an equally sensitive and somewhat less hazardous test for blood. A logical extension of this approach is to employ 3,5,3',5'-tetramethylbenzidine (3) in which *ortho*-hydroxylation is impossible. We have prepared this compound according to the scheme described in Fig 2.

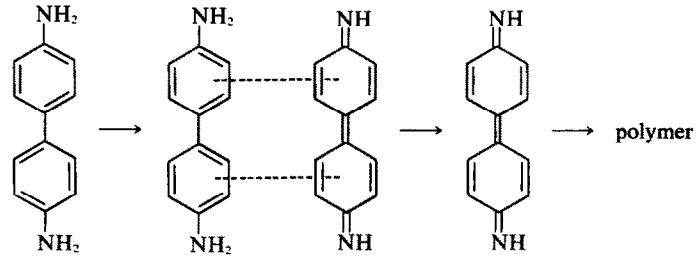

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Fig 1. Scheme for the formation of coloured products from the action of peroxidase/hydrogen peroxide on benzidine (after Duijn, 1955).

Attempts to prepare the intermediate hydrazobenzene (4) by reduction of the corresponding nitroxylene have failed. When reduction of the latter does occur, the xylidine (2) is always the isolated product. The overall yield by the route shown in Fig 2 is still only 15%, but work is in progress to improve this.

The tetramethylbenzidine (3) has been found to give a blood test approximately four times as sensitive as does the parent benzidine. Injected subcutaneously into rats, it produced no tumours specifically attributable to it, in doses greater than those in which benzidine or *o*-tolidine cause a high yield of neoplasms.

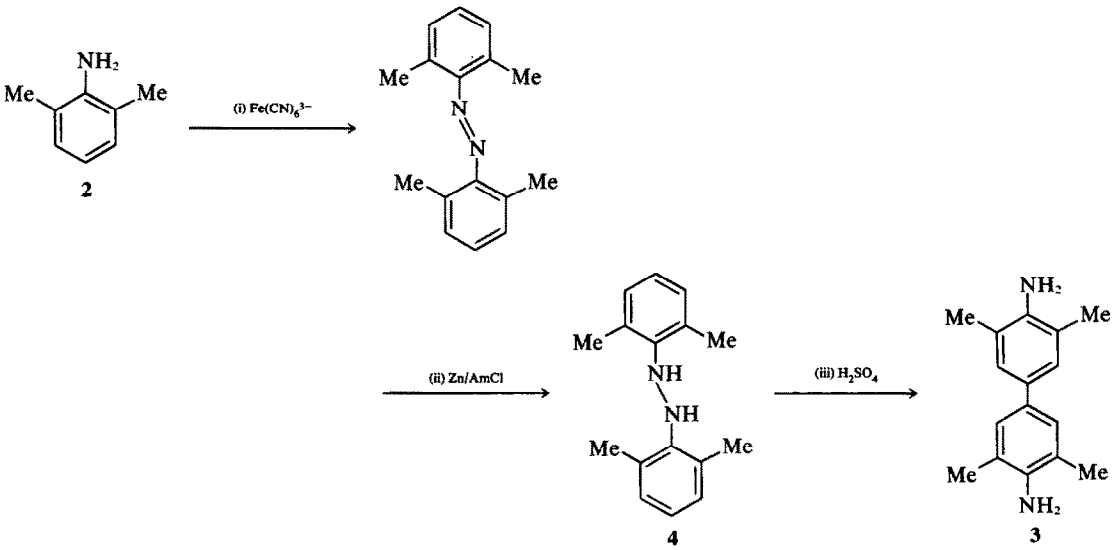

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Fig. 2. The route for the preparation of 3,5,3′,5′-tetramethylbenzidine (3).

Table 1. Comparative intensities of colours generated by three benzidine derivatives in a peroxidase blood test

Compound	Colour-ation	λ_{max} (mμ)	ϵ_{max}	S	Integrn.	S′
Benzidine	Blue green	602	1340	1	$1{\cdot}42 \times 10^5$	1
o-Tolidine	Blue green	632	1750	1·31	$1{\cdot}37 \times 10^5$	0·97
3,5,3′,5′-Tetramethylbenzidine	Blue green	655	5400	4·04	$4{\cdot}16 \times 10^5$	2·94

$$S = \frac{\epsilon_{max}\text{ (for compound)}}{\epsilon_{max}\text{ (for benzidine)}}$$

$$S' = \frac{\text{Integrn. (for compound)}}{\text{Integrn. (for benzidine)}}$$

(Integrn. = Area under absorption curve (400–750 mμ)).

Prof H. Lehmann F.R.S. of Addenbrooke's Hospital, Cambridge and one of us (B.C.S.), have carried out routine clinical tests with 3,5,3',5'-tetramethylbenzidine for occult blood in faeces and urine and are satisfied that the tetramethylbenzidine is at least as sensitive as benzidine.

It should be noted that leucomalachite green and leucophenolphthalein have been used in certain quarters as substitutes for benzidine. These substances are not true tests for peroxidase or peroxidase-like action and merely serve to indicate the presence of some undefined oxidising system.

Table 2. The incidence of tumours in groups of rats treated with 3,5,3',5'-tetramethylbenzidine

Group No.	Duration of dosing (days)	Cumulative dose (g/kg)	No. of rats	Day of autopsy (from first dose)	
				All rats	Rats with tumours
1	224	8·3	12	524–740	617, 690, 740, 740
2	224	4·15	12	538–744	538, 684, 743
3	224	0	12	443–746	609

Table 3. The incidence of tumours in groups of rats treated with benzidine and *o*-tolidine

Compound tested	Duration of dosing (days)	Cumulative dose (g/kg)	No. of rats	Day of autopsy (from first dose)	
				All rats	Rats with tumours
Benzidine	150	0·75	22	24–387	84 onwards (20 rats)
o-Tolidine	241	5·4	21	94–703	325 onwards (18 rats)

EXPERIMENTAL

2,6,2',6'-*Tetramethylazobenzene.* A soln of 2,6-dimethylaniline (20 g) in water (200 ml) and conc HCl (30 ml) was heated to 90° and added rapidly to a soln of potassium ferricyanide (300 g) in water (1·5 l.) and NaOH (42 g), itself pre-heated to 97°. The resulting suspension was stirred for a further 10 min, then cooled, and extracted with ether (500 ml). The ethereal extract was dried over Na_2SO_4, and the solvent was removed under reduced pressure. The residual deep red gum was chromatographed on silica gel (600 g), using 20% chloroform in light petroleum (b.p. 60–80°) as eluent. The fast-running red band was eluted and yielded dark red crystals (2·9 g) of 2,6,2',6'-*tetramethylazobenzene*, m.p. 47°. Sublimation gave a product m.p. 48°. (Found: C, 80·9; H, 7·3; N, 11·4. $C_{16}H_{18}N_2$ requires: C, 80·6; H, 7·6; N, 11·8%). IR spectrum (nujol) showed prominent bands at: 1585, 1410, 1290, 1280, 1250, 1200, 1165, 1090, 1035, 995, 975, 925, 900, 770 cm^{-1}. UV spectrum (EtOH) of *trans* isomer: λ_{max} 236 mμ, ($\log_{10}$ ε 3·848); 308 mμ, (3·985): 457 mμ, (2·906); λ_{min} 267 mμ, (3·757); 380 mμ (1·303).

3,5,3',5'-*Tetramethylbenzidine.* A soln of the above tetramethylazobenzene (4 g) in ether (200 ml) and EtOH (40 ml) was stirred vigorously for 3 hr at room temp (*ca* 20°) in a lightly closed vessel, with Zn dust (36 g), ammonium chloride (16 g), and water (200 ml), until the red colour was discharged. The organic solvent layer was separated, and the aqueous layer was then extracted with more ether (200 ml). The ethereal solns were combined and shaken for 1 hr with 3N H_2SO_4 (25 ml). MeOH was added to aid precipitation of the *sulphate* of the benzidine which was filtered off, washed with MeOH and dried over P_2O_5 (yield, 6·05 g). (Found: C, 52·6; H, 6·8; N, 7·7. $C_{16}H_{22}N_2$, SO_4, $1\frac{1}{2}H_2O$ requires: C, 52·8; H, 6·8; N, 7·8%). The free base, from treatment of the sulphate with NaOHaq and extraction into chloroform, sublimed (150°/0·5 mm) to form colourless crystals m.p. 168° (Found: C, 80·2; H, 8·3; N, 11·4. $C_{16}H_{20}N_2$ requires: C, 80·0; H, 8·3; N, 11·7%). IR spectrum (nujol) showed prominent bands at 3390, 3310, 1630, 1605, 1580, 1500, 1270, 1230, 1155, 1090, 1035, 1010, 985, 900, 885, 870, 840, 765, 745, 690 cm^{-1}. NMR spectrum ($CDCl_3$) showed a singlet (12 Me protons at 7·76 τ, singlet (broad, 4 N–H protons) at 6·47 τ; and singlet (4 aromatic protons) at 2·69 τ. UV spectrum (EtOH); λ_{max} 212 mμ ($\log_{10}$ 4·734) 287 mμ (4·387), λ_{min} 244 mμ (3·619).

The blood test. The compounds listed in Table 1 were examined in the blood test under two sets of conditions (A) those of the benzidine test in its original form, and (B) those of the peroxidase system. Precise conditions are tabulated below. The results listed in Table 1 are those obtained using the more satisfactory conditions found in each particular case.

(A) To the compound (100 mg) were added glacial AcOH (5 ml) and H_2O_2 (5 ml; 20 volume), followed by blood (0·025 ml).

(B) To the compound (100 mg) were added glacial AcOH (5 ml) and AcOH/acetate buffer (100 ml, 1M, pH 4·5). The soln was diluted to 250 ml and H_2O_2 (5 ml 20 volume) was added followed by blood (0·025 ml).

In each case the soln was allowed to stand 5 min before dilution with distilled water to a known volume suitable for analysis on a Perkin-Elmer 137UV visible recording spectrophotometer.

All the tests were carried out on human blood and under strictly comparable conditions of concentration, temperature and pH.

The intensity of colouration is often quantitatively assessed by measurement of the extinction coefficient a

the peak absorption wavelength in the visible absorption spectrum of the test soln and comparison of this value with that obtained using benzidine under the same conditions (S).

This has proved a reasonably reliable method for our investigations; but we have recently devised a more realistic method based on the fact that a visual colour test depends for its sensitivity on the absorption of the test soln over the whole visible range of the spectrum. A more representative value should therefore be obtained by measurement of the total area under the visible absorption spectrum curve in the range 400–750 mμ; and comparison of this value with the corresponding value for benzidine. The amended values (appearing in the column labelled S′) are shown in Table 1.

The amended figures show a diminuation in the sensitivity of the amines studied from the value (S) obtained by the previous method mainly because the absorption of the oxidised benzidine chromophore is considerable over the whole visible range. However, the values obtained indicate that the sensitivities of 3,5,3′,5′-tetramethylbenzidine is still very high, and is in excess of the sensitivity of benzidine.

Test for carcinogenic activity in rats. 3,5,3′,5′-Tetramethylbenzidine, dispersed in arachis oil, was given by repeated subcutaneous injection to 24 male albino rats (Alderley Park strain 1), about 200 g each in weight. At first the rats were dosed 5 days a week (Mon.–Fri.), 12 with 100 mg/kg (Group 1) and 12 with 50 mg/kg (Group 2). After 10 weeks, dosage was reduced to 50 mg/kg and 25 mg/kg respectively, given 3 times weekly (Mon, Wed, Fri.) for a further 22 weeks. Group 3 comprised 12 rats dosed in parallel with arachis oil alone (0.1 ml/100 g initially, reduced to 0·05 ml/100 g after 10 weeks).

In all three groups, most of the rats (9 or more) survived more than 600 days from the start of experiment. Whether killed *in extremis* (a majority)—or found dead, they were carefully examined at autopsy and the tissues sampled for histology. The results are shown in Table 2.

All the tumours found in the rats given tetramethylbenzidine were either benign tumours at the injection site (dermal fibromas (2) or lipoma) or tumours, benign or malignant, commonly found in untreated ageing rats of this strain (pituitary adenomas (2), a Leydig-cell tumour of the testis, and an islet-cell tumour of the pancreas).

These results are to be contrasted with those of similar studies carried out in these laboratories with benzidine and *o*-tolidine (Table 3).

With benzidine, the tumours found included hepatomas (19) and cholangiomas (18), intestinal tumours (7) and carcinomas of the acoustic sebaceous gland (4): with *o*-tolidine, tumours of the gastro-intestinal tract (11), hepatomas (7), tumours of bone and associated tissues (4) and carcinomas of the acoustic sebaceous gland (4). All these are regarded as having been induced.

In summary, the results obtained with 3,5,3′,5′-tetramethylbenzidine are consistent with the view that this compound is not carcinogenic. While it is, of course, difficult to prove a negative and more extensive tests are called for to substantiate this view, it is reasonable to conclude that the risk, if any, attending exposure to this compound would be far less than that to benzidine or even *o*-tolidine.

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REFERENCES

[1] O. Adler and R. Adler, *Zeitshr. f. Physiol. Chimie.*, **41**, 59–67 (1904)
[2] B. J. Culliford and L. C. Nickolls, *J. Forensic Sci.*, **9**, 175 (1964)
[3] A. Haddow, *New Scientist*, **25**, 348 (1965)
[4] C. E. Searle, *Chemistry in Britain*, **6**, 5 (1970)
[5] R. A. M. Case, J. M. Davis and G. M. Edwards, *Ann. Rep. Brit. Empire Cancer Campaign for Research*, **44**, 55 (1966)
[6] R. A. M. Case, *Ibid*, **45**, 90 (1967)
[7] B. C. Saunders, A. G. Holmes-Siedle and B. P. Stark, Peroxidase, Butterworths, London (1964)
[8] N. B. Chapman and B. C. Saunders, *J. Chem. Soc.*, 496 (1941)
[9] P. Duijn, *Rec. Trav. Chem.*, **74**, 771 (1955)
[10] P. D. Lawley, *Progress in Nucleic Acid Research and Molecular Biology*, **5**, 89 (1966)
[11] P. Daudel and R. Daudel, *Chemical Carcinogenesis and Molecular Biology*, Interscience, New York (1966)
[12] E. C. Miller, J. A. Miller and H. A. Hartmann, *Cancer Research*, **21**, 815 (1961)
[13] A. Jacobs, *Lancet*, **1**, 697 (1958)