TECHNICAL NOTE

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Extension of the Color Suite Available for Chemical Enhancement of Fingerprints in Blood

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ABSTRACT: The use of *ortho*- and *para*-phenylenediamine (OPD & PPD respectively) for the enhancement of fingerprints in blood has been investigated. Optimal pH conditions and H_2O_2 concentrations have been determined using UV/Vis spectroscopy. Both OPD and PPD are effective and less hazardous alternatives to the presently used 3,3′-diaminobenzidine (DAB) for the development of blood fingerprints, especially on porous surfaces. The orange color of OPD and the purple color of PPD offer alternative colors to the brown color of DAB and the light green color of ABTS for standing out against particular backgrounds. Both OPD and PPD can be used after ninhydrin treatment, but the reverse is not the case.

KEYWORDS: forensic science, blood, fingerprints, OPD, PPD, peroxidase, hemoglobin

In a recent paper (1) we reported on the use of 2,2′-azino-di-[3-ethylbenzthiazolinesulfonate] (ABTS) to enhance faint fingerprints formed in blood. In terms of its ability to enhance bloody fingerprints, ABTS was found to be as sensitive as the most effective alternative reagent (2), which is 3,3′-diaminobenzidine (DAB). However, unlike DAB, ABTS is not considered to be carcinogenic.

The other main difference between ABTS and DAB development of a bloody fingerprint is that, whereas DAB enhancement yields prints that are dark brown, treatment with ABTS results in light green prints. This result raises the prospect of being able to select the reagent on the basis of which of the two colors will provide the greatest contrast with a given background surface; generally, ABTS developed prints were more visible on dark surfaces than those developed with DAB, whereas the reverse is true for light surfaces.

The refinement of suites of fingerprint reagents that allow selection of a particular color, so as to provide maximum contrast with a given surface, was regarded by Pounds (3) as being a desirable target of fingerprint research.

Colors produced by DAB and ABTS are the colors of the oxidized products formed (which also happen to be less soluble after oxidation than they were before, thus forming a deposit). In both cases, the reagent involved is oxidized to its more colored form by hydrogen peroxide, and this reaction is catalyzed by the presence of the heme group in hemoglobin. The heme group does this by mediating the breakdown of hydrogen peroxide; it acts as a pseudo-peroxidase enzyme (4).

Dark brown and light green are not the only colors that are possible with such chemistry, and in this work we set out to add two further colors to those available: orange and purple. This was done by selecting two reagents that have been used in the biochemical area specifically because of their ability to form a colored product in the presence of a hydrogen peroxide and a peroxidase (or pseudo-peroxidase). These were then adapted and optimized for the specific enhancement of fingerprints in blood.

Ortho-phenylenediamine (OPD) and *para*-phenylenediamine (PPD) have been previously used as chromogens for the determination of plasma or serum hemoglobin. The structures of OPD and PPD are provided in Fig. 1. Like DAB and ABTS, both OPD and PPD can undergo oxidation to a colored form (orange and purple respectively) in the presence of hydrogen peroxide and hemoglobin.

PPD has been previously used in the identification of ceruloplasmin in blood serum (5). The colored, oxidized derivative of PPD is thought to be a molecule formed from diamine and diimine (6). OPD has been previously used to optically detect antigen-antibody associations occurring in a porous sol-gel (7); in this case the oxidation involved reaction of OPD with a peroxidase conjugate. A sensitive sandwich ELISA method has been developed for the determination of ABH antigens in bloodstains, in which visualization is through oxidation of OPD (8). Tyouichi et al. (9) have also made use of OPD in a sandwich ELISA method for identification of seminal stains; in this method oxidation is catalyzed by a horseradish peroxidase-labeled antibody.

In terms of human toxicology, OPD and PPD are both considered to be toxic by inhalation (may cause bronchial asthma), in contact with skin (may cause dermatitis; the skin becomes blackened), and if swallowed (10). The hazards however, seem to be somewhat less than those associated with 3,3′-diaminobenzidine (DAB). For example OPD has been referred to as a less toxic aromatic than DAB by Donlon et al. (11). Also, PPD was found to be only weakly mutagenic to Ames Salmonella strain TA98 with metabolic activation but was found to be nonmutagenic to the TA100 strain. However, PPD was found to induce a dose-related increase in chromosomal aberrations in Chinese hamster ovary cells (12).

Materials and Methods

Materials

Ortho and *para*-phenylenediamine (flakes), 3,3′-diaminobenzidine and 5-sulfosalicylic acid were purchased from BDH Labora-

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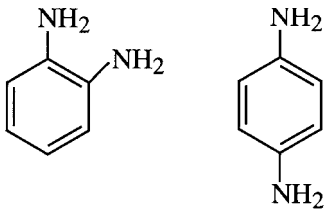

FIG. 1—*Chemical structures of ortho-phenylenediamine (OPD) and para-phenylenediamine (PPD).*

tory Chemicals, Aldrich Chemical Company, and M&B Laboratory Chemicals, respectively. Paper (A4, 80 gsm) was obtained from Copyright (Australian Paper, Ltd., Australia) and glass slides from Marienfeld.

Blood was supplied by one of the authors at the University of Waikato Medical Center in a 5 mL vaccutainer containing EDTA as an anti-coagulant. A 500 μL aliquot of this blood was then diluted with water to a final volume of 250 mL in a volumetric flask (500 times dilution). The remainder of the blood was used to deposit bloody fingerprints onto paper and glass slides for later use.

OPD and PPD stock solutions were prepared by dissolving 50 mg of each phenylenediamine in 100 mL distilled water.

Phosphate and phosphate/citric acid buffers were prepared from the following solutions according to the procedure of Dawson et al. (11): Na_2HPO_4 (0.2*M*), NaH_2PO_4 (0.2*M*), and citric acid monohydrate (0.1*M*).

Optimization of pH and H_2O_2 Concentrations

The optimal pH and H_2O_2 concentrations favoring best color development of OPD and PPD in a solution containing small amounts of blood were determined by UV/Vis spectroscopy, using a Varian 1 Cary UV/Vis spectrophotometer (SBW = 0.2 nm, Signal averaging time = 0.100 s). Solutions were held in a 10 mm quartz cuvette.

All solutions prepared contained 500 μL of OPD or PPD stock solution, 750 μL of the 1:500 blood solution, a specified volume of 2.7% H_2O_2, and a sufficient volume of buffer solution to give a final volume of 12.2 mL. Changes in the absorbance of these solutions with time at 434 nm (OPD) and 404 nm (PPD) were then determined on the UV/Vis Spectrophotometer against a blank containing 750 μL of the 1:500 blood solution, the same volume of 2.7% H_2O_2 as in the sample, and a sufficient volume of buffer solution to give a final volume of 12.2 mL. The H_2O_2 was always added last, with the time of the subsequent reaction being recorded from that point. Establishment of the absorbance maximums for oxidized OPD and PPD were carried out using sample and blank solutions containing 50 μL of 2.7% H_2O_2 and pH 5.4 buffer solution.

Experiments to determine the optimal concentration of H_2O_2 for OPD and PPD were carried out at a pH value of 5.4 (citric acid/phosphate buffer). A pH of 5.4 was chosen initially as it had resulted in optimal color development for ABTS (1), and so seemed a suitable pH to start with. Volumes of H_2O_2 added were varied from 10 to 500 μL for OPD and 10 to 1000 μL for PPD. Experiments to determine the optimal pH for OPD and PPD were then performed using a set volume of 50 μL 2.7% H_2O_2 and varying the pH from 4.4 to 7.4 (by use of phosphate and citric acid/phosphate buffers). All buffer solutions used were 0.1 to 0.2*M* in strength.

Fingerprint Trials

In order to assess the potential of OPD and PPD as alternatives to DAB for the enhancement of fingerprints in blood, a comparison trial with DAB was carried out. Various other trials were also performed in order to determine whether the optimized pH and H_2O_2 concentration conditions from the solution trials still applied to blood fingerprints on surfaces. Optimal conditions determined from the solution trials can be used as a starting point for trials of blood print development on surfaces, but specific surface tests are also necessary. This is because of a second factor that is important on surfaces, which is the ability of the oxidized chromophores to remain associated with the print, rather than dissolve back in to the solution. Various solution factors can impact on the oxidized chromophore's solubility.

All blood fingerprints (previously laid down on paper or glass microscope slides) were firstly fixed with 5-sulfosalicylic acid. This was done by soaking them for 3 min in a 20 g/L solution of the 5-sulfosalicylic acid, and is the method of fixing a blood fingerprint currently recommended by the New Zealand Crown Research Institute, ESR: Forensic (13). The prints were then rinsed in distilled water before being treated with either OPD, PPD, or DAB. Prints treated with DAB were immersed in 100 mL of DAB solution (1 g/L in buffer at pH 7.4) and 500 μL 27% H_2O_2 for 5 min (13). Prints treated with OPD or PPD solutions were initially immersed for 5 min in 50 mL of the solution involved (0.5 g/L OPD or PPD in citric acid/phosphate buffer at pH 5.4) to which had been added 1.0 mL of 27% H_2O_2. These were the conditions determined from the optimization of pH and H_2O_2 concentrations in solution. OPD, PPD, and DAB treatments were then followed by a further rinse in distilled water.

A number of trials were performed in order to determine (a) the most suitable conditions for development of blood prints using OPD and PPD on surfaces, (b) sensitivity of OPD and PPD compared with DAB, and (c) compatibility of the OPD and PPD with DAB and ninhydrin treatments. Experimental details of these trials are as follows.

Treatment on Porous Surfaces—Paper was used as the model porous surface. In all cases blood fingerprints were placed on unused white paper and each print was cut bilaterally down the center of the print. Comparison was made between treatment approaches by subjecting each half to a different treatment and then matching the corresponding halves afterwards.

1. Effect of treatment compared with no treatment: one half of a print was treated with OPD while the other half was left untreated. The procedure was repeated using PPD.
2. Effect of hydrogen peroxide concentration: sixteen bisected prints were used to compare the influence of H_2O_2 concentration on OPD treatment at five different strengths, specifically 50 μL, 100 μL, 0.5 mL, 1.0 mL, and 1.5 mL 27% H_2O_2. In a similar way, the effect of H_2O_2 concentration on PPD treatment was examined using eight bisected prints, at 200 μL, 1.0 mL, and 2.0 mL 27% H_2O_2. Four prints were then used to compare PPD treatment using either 200 μL or 1.0 mL 27% H_2O_2. Another four prints were used to compare PPD treatment using either 1.0 mL or 2.0 mL 27% H_2O_2.
3. Effect of pH: Sixteen prints were used to compare OPD treatment at pH 5.4 and pH 7.4, using citric acid/phosphate and phosphate buffers, respectively. The procedure was repeated using PPD.
4. Effect of reagent concentration: Four prints were used to compare treatment between 0.25 g/L and 0.5 g/L OPD solution. Another four prints were used to compare treatment between 0.5 g/L and 1 g/L OPD solution. The procedure was repeated using PPD.
5. Effect of soaking time: One print was used to compare a 2.5 min treatment with a 5 min treatment in OPD solution. Another print

was used to compare a 5 min treatment with a 10 min treatment in OPD solution. A third print was used to compare a 5 min treatment with a 30 min treatment in OPD solution. The procedure was repeated using PPD.

6. Effect of reagent age: Four prints were used to compare the effectiveness of a 48-hour-old PPD solution (dark purple) with a half-hour-old PPD solution (faint lilac color).
7. Comparison of OPD and PPD with DAB: Fifty prints were used to compare OPD treatment with DAB treatment. The procedure was repeated using PPD.
8. Compatibility of OPD and PPD with DAB: One print was used to compare the development obtained by treatment with DAB followed by subsequent OPD treatment with DAB treatment alone. Another print was used to compare OPD treatment followed by DAB treatment with OPD treatment alone. The procedure was repeated using PPD.
9. Compatibility of OPD and PPD with ninhydrin: Two strips of paper each containing a latent fingerprint and a blood fingerprint were used to see if OPD treatment could be followed by ninhydrin treatment and vice versa. The procedure was repeated using PPD.

Treatment on Nonporous Surfaces—Two glass microscope slides each with a fixed blood print were treated with OPD at pH 5.4 and pH 7.4. Two more glass microscope slides with a fixed blood print were then treated with PPD and DAB at pH 5.4.

Inherent Sensitivity of OPD and PPD Compared with that of DAB

The procedure used for determination of OPD and PPD sensitivity was based on the procedure used by Garner et al. (19), except that spot tests were carried out at the bottom of plastic microplate wells. A sample of the author's blood was diluted serially in isotonic saline. The following dilutions were prepared: 1×10^{-1}, 1×10^{-2}, 1×10^{-3}, 1×10^{-4}, 1×10^{-5} and 1×10^{-6}. A 10 μL aliquot of each dilution was then placed into the individual wells of the first row of the microplate. This was then repeated for the second and third rows. Three OPD solutions were then prepared (in distilled water) with the following concentrations: 0.1 *M,* 0.05 *M,* and 0.025 *M.* A drop of the 0.1 *M* solution was placed into each well of the first row of the microplate followed by a drop of 2.7% of H_2O_2. The 0.05 and 0.025 *M* solutions were then placed into the wells of Rows 2 and 3, respectively, of the microplate followed by 2.7% of H_2O_2. This procedure was repeated again on separate microplate wells for PPD and DAB.

A positive test was determined by color comparison with a control well containing only OPD, PPD, or DAB solution and H_2O_2. A negative test was assigned when the color in the test well was the same as that in the control well.

Results and Discussion

Solution Trials

Optimal pH and Peroxide Conditions for OPD Development in Solution—An absorbance maximum of 434 nm was chosen as an appropriate wavelength for monitoring of the oxidation of an OPD solution in a citric acid/phosphate buffer (pH 5.4) with 50 μL of 2.7% H_2O_2 added. This was the wavelength used for all subsequent analyses of OPD.

Experiments to determine the optimal concentration of H_2O_2 were performed at a pH value of 5.4 (citric acid/phosphate buffer). Volumes of 2.7% H_2O_2 added were varied from 10 to 500 μL. Results are presented in Table 1.

TABLE 1—*Effect of H_2O_2 volumes on color development of OPD (measured by absorbance at 434 nm) at pH 5.4.*

10 μL		50 μL (a)		50 μL (b)		100 μL		500 μL	
T (s)	Ab.	T (s)	Ab.	T (s)	Ab.	T (s)	Ab.	T (s)	Ab.
120	0.022	120	0.080	120	0.086	120	0.107	120	0.278
180	0.064	180	0.175	180	0.146	180	0.180	180	0.424
300	0.163	300	0.404	300	0.247	300	0.312	300	0.607
420	0.261	420	0.616	420	0.349	420	0.426	420	0.690
540	0.346	540	0.796	540	0.435	540	0.528	540	0.728
660	0.426	660	0.948	660	0.521	660	0.617	660	0.745
780	0.499	780	1.075	780	0.603	780	0.701	780	0.747
900	0.562	900	1.172	900	0.674	900	0.776	900	0.745
1800	0.876	1800	1.551	1800	1.093	1800	1.085	1800	0.849
2700	1.019	2700	1.654	2700	1.348	2700	1.258	2700	0.861
...	...	...	...	3600	1.503	6360	1.431	...	...
...	...	...	...	7200	1.659	...	...	...	...
...	...	...	...	10800	1.625	...	...	...	...
...	...	...	...	18000	1.624	...	...	...	...
...	...	...	...	26100	1.610	...	...	...	...
...	...	...	...	6 days	1.373	...	...	...	...

From these results it can be seen that an optimal absorbance was obtained with the use of 50 μL of 2.7% H_2O_2. At higher concentrations of H_2O_2, the rate of color development is similar at first, but the final absorbance values achieved are not so high. In other words, it is apparent that too much H_2O_2 will inhibit color development in the OPD/hemoglobin reaction. This effect was also seen for ABTS (1), and the most likely reason for it is that excess H_2O_2 is capable of inducing further oxidation of the product formed.

Experiments to determine the best pH were then performed using the optimal volume of 50 μL of 2.7% H_2O_2 and varying the pH from 4.4 to 7.4. Results are presented in Table 2 and Fig. 2. An extended time was used for pH 5.4 in order to determine the stability of the color development.

As is evident from the results (Table 2, Fig. 2), in solution, the maximum color development for oxidized OPD occurs at pH 5.4. Absorbance values grow significantly in moving from pH 7.4 to pH 5.4, and then decline slightly as the solution becomes still more acidic. Once developed, the oxidized product also appears to be fairly stable: at pH 5.4, only a small decrease in absorbance was observed over a period of six days.

TABLE 2—*Effect of pH on color development of OPD (measured by absorbance at 434 nm) with 50 μL 2.7% H_2O_2.*

pH 4.4		pH 5.0		pH 5.4		pH 5.8		pH 6.8		pH 7.4	
T (s)	Ab.	T (s)	Ab.	T (s)	Ab.	T (s)	Ab.	T (s)	Ab.	T (s)	Ab.
120	0.214	120	0.127	120	0.080	120	0.058	120	0.001	120	0.000
180	0.332	180	0.217	180	0.175	180	0.103	180	0.008	180	0.000
300	0.520	300	0.378	300	0.404	300	0.206	300	0.022	300	0.013
420	0.644	420	0.520	420	0.616	420	0.309	420	0.036	420	0.024
540	0.715	540	0.637	540	0.796	540	0.410	540	0.050	540	0.038
660	0.757	660	0.740	660	0.948	660	0.504	660	0.066	660	0.050
780	0.785	780	0.827	780	1.075	780	0.592	780	0.081	780	0.062
900	0.803	900	0.898	900	1.172	900	0.669	900	0.097	900	0.075
1800	0.822	1800	1.218	1800	1.551	1800	1.061	1800	0.214	1800	0.161
2700	0.827	2700	1.292	2700	1.654	2700	1.227	2700	0.317	2700	0.229

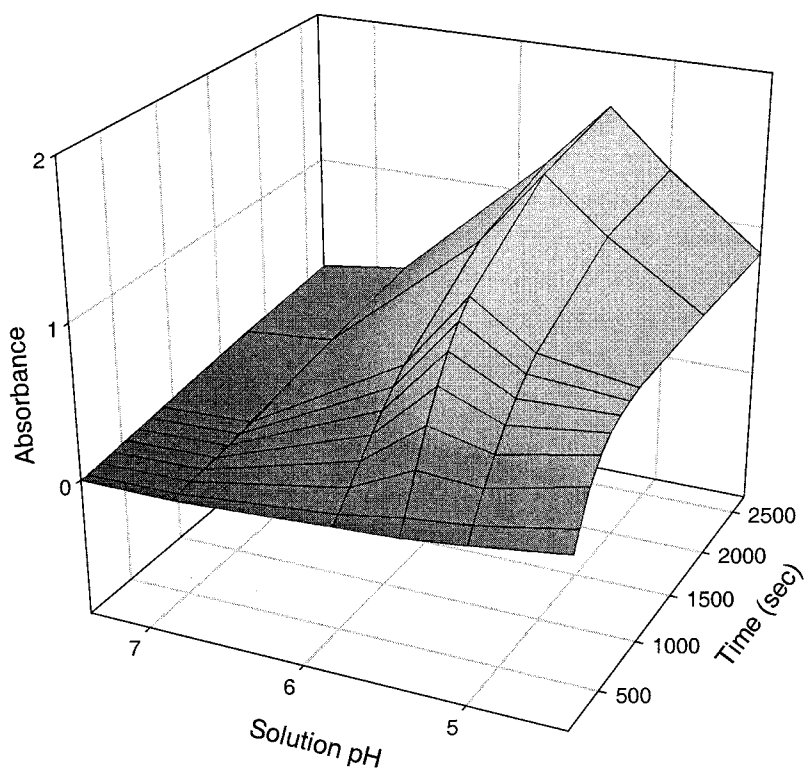

FIG. 2—*Effect of pH on color development of OPD (measured by absorbance at 434 nm) with 50 μL 2.7% H_2O_2.*

TABLE 3—*Effect of pH on color development of PPD (measured by absorbance at 404 nm) with 50 μL 2.7% H_2O_2.*

Time (m)	pH 4.4	pH 5.0	pH 5.4	pH 5.8	pH 6.0	pH 6.6	pH 7.0	pH 7.4	pH 8.0
1	0.161	0.198	0.092	0.178	0.110	0.102	0.119	0.124	0.123
2	0.202	0.246	0.130	0.217	0.134	0.121	0.135	0.131	0.123
3	0.219	0.288	0.166	0.253	0.160	0.139	0.149	0.140	0.127
4	0.228	0.325	0.203	0.292	0.184	0.155	0.162	0.150	0.134
5	0.232	0.354	0.241	0.328	0.206	0.171	0.174	0.159	0.141
6	0.234	0.374	0.275	0.362	0.227	0.184	0.186	0.168	0.148
7	0.234	0.391	0.307	0.390	0.248	0.198	0.198	0.177	0.154
8	0.234	0.406	0.334	0.416	0.266	0.210	0.208	0.185	0.160
9	0.234	0.418	0.357	0.437	0.284	0.222	0.219	0.193	0.167
10	0.233	0.428	0.378	0.454	0.300	0.232	0.229	0.201	0.173
11	0.233	0.436	0.397	0.470	0.315	0.243	0.238	0.207	0.178
12	0.236	0.442	0.414	0.481	0.329	0.252	0.250	0.214	0.184
13	0.237	0.448	0.428	0.491	0.341	0.262	0.254	0.221	0.189
14	0.235	0.453	0.441	0.498	0.353	0.270	0.259	0.227	0.194
15	0.242	0.458	0.453	0.504	0.363	0.279	0.264	0.234	0.199
16	0.241	0.461	0.463	0.508	0.373	0.286	0.269	0.239	0.203
17	0.251	0.463	0.472	0.512	0.382	0.293	0.275	0.240	0.208
18	0.250	0.465	0.477	0.515	0.390	0.301	0.281	0.245	0.212
19	0.256	0.466	0.484	0.516	0.397	0.307	0.283	0.247	0.216
20	0.263	0.466	0.490	0.518	0.403	0.309	0.285	0.248	0.220
21	0.266	0.467	0.496	0.519	0.408	0.312	0.302	0.252	0.224
22	0.275	0.470	0.506	0.519	0.413	0.317	0.307	0.256	0.228
23	0.280	0.471	0.508	0.519	0.417	0.322	0.317	0.261	0.231
24	0.281	0.470	0.515	0.519	0.421	0.326	0.320	0.259	0.234
25	0.281	0.469	0.517	0.519	0.424	0.329	0.321	0.259	0.237
26	0.282	0.470	0.518	0.519	0.427	0.332	0.324	0.263	0.239
27	0.282	0.470	0.521	0.518	0.430	0.335	0.327	0.263	0.242
28	0.283	0.471	0.523	0.518	0.432	0.338	0.323	0.268	0.244
29	0.283	0.470	0.524	0.518	0.434	0.341	0.326	0.271	0.247
30	0.283	0.469	0.525	0.517	0.435	0.343	0.325	0.274	0.250

Overall, the cuvette-based solution trials yield the following as optimal for development of color in the OPD/hemoglobin system: pH 5.4, and 50 μL of 2.7% H_2O_2, which corresponds to a ratio of 1 g OPD to 200 mL 2.7% H_2O_2 or 20 mL of 27% H_2O_2. These were taken as starting conditions for testing the activity of OPD on blood fingerprints.

Optimal pH and Peroxide Conditions for PPD Development in Solution—An absorbance maximum of 404 nm was chosen as an appropriate wavelength for monitoring of the oxidation of a PPD solution in a citric acid/phosphate buffer (pH 5.4) with 50 μL of 2.7% H_2O_2 added. This was the wavelength used for all subsequent analyses of PPD.

Experiments to determine the optimal pH were performed using 50 μL of 2.7% H_2O_2 and varying the pH from 4.4 to 7.4. The volume of 50 μL was chosen as a starting point for testing as it had been found to be the optimum H_2O_2 volume for OPD. Results for PPD are presented in Table 3, and reveal that once again, maximum color development occurs at pH 5.4.

Experiments to determine the optimal concentration of H_2O_2 were carried out at this pH value (citric acid/phosphate buffer). Volumes of 2.7% H_2O_2 were varied from 10 to 1000 μL. Results are presented in Table 4 and Fig. 3.

From these results, 100 μL of 2.7% H_2O_2 seems to give the optimum absorbance value. This is twice as much H_2O_2 as was required by OPD, with the difference likely to be a simple reflection of the ease with which each isomer is oxidized. (At the highest H_2O_2 concentration, the ultimate plateau stage is rather jagged; this was due to oxygen bubbles forming in the cuvette.)

Overall, the cuvette-based solution trials yield the following as optimal for development of color in the PPD/hemoglobin system: pH 5.4, and 100 μL of 2.7% H_2O_2, which corresponds to a ratio of 1 g PPD to 400 mL 2.7% H_2O_2, or 40 mL of 27% H_2O_2. As with OPD, these were taken as starting conditions for testing the activity of PPD on blood fingerprints.

Sensitivity of OPD and PPD Compared with DAB—Results for microplate well tests on DAB, OPD and PPD are presented

TABLE 4—*Effect of H_2O_2 volumes on color development of PPD (measured by absorbance at 404 nm) at pH 5.4.*

Time (m)	10 μL	25 μL	50 μL	100 μL	500 μL	1000 μL
2	0.209	0.228	0.211	0.270	0.219	0.113
5	0.286	0.341	0.328	0.401	0.247	0.113
6	0.306	0.372	0.361	0.417	0.304	0.118
7	0.322	0.399	0.386	0.428	0.343	0.162
8	0.336	0.420	0.406	0.433	0.351	0.185
9	0.346	0.436	0.424	0.439	0.421	0.160
10	0.354	0.448	0.438	0.443	0.437	0.171
11	0.359	0.459	0.452	0.451	0.445	0.244
12	0.364	0.466	0.463	0.498	0.455	0.289
13	0.367	0.472	0.473	0.504	0.454	0.321
14	0.369	0.476	0.480	0.506	0.471	0.342
15	0.370	0.477	0.487	0.509	0.473	0.335
16	0.371	0.479	0.492	0.515	0.481	0.356
17	0.371	0.480	0.493	0.519	0.499	0.337
18	0.371	0.481	0.497	0.520	0.530	0.328
19	0.371	0.482	0.500	0.522	0.537	0.332
20	0.369	0.483	0.494	0.523	0.529	0.319
21	0.368	0.481	0.498	0.525	0.536	0.367
22	0.367	0.480	0.500	0.527	0.525	0.349
23	0.366	0.478	0.505	0.527	0.530	0.402
24	0.365	0.477	0.514	0.526	0.523	0.414
25	0.364	0.477	0.514	0.525	0.526	0.406
26	0.362	0.477	0.515	0.525	0.535	0.391
27	0.362	0.478	0.516	0.526	0.538	0.417
28	0.361	0.478	0.515	0.527	0.541	0.404
29	0.360	0.478	0.516	0.524	0.526	0.405
30	0.359	0.477	0.515	0.524	0.530	0.424

in Table 5. In preliminary trials it was found that the controls became equally as dark as the positives after several hours. All assignments were therefore made within 10 min of H_2O_2 addition and a positive test was only assigned if it was darker than the control. The results indicate that, like ABTS, OPD and PPD are at least as sensitive as DAB, the conventional reagent of choice.

Surface Trials

Treatment on Porous Surfaces—The following results were obtained in trials examining OPD and PPD development of blood fingerprints on paper.

Effect of treatment compared with no treatment—In this control experiment, the treated half of the print was found to have much better ridge definition with more detail visible. In the case of OPD, the treated half was orange in color, and in the case of PPD it was purple/black in color. It was observed that blood prints on paper turn green first in the initial stages of PPD development, and this proceeds to the final black/purple color on continued immersion in the working solution.

Effect of hydrogen peroxide concentration—The optimized ratio of H_2O_2 to OPD determined from the cuvette trials translated to 0.5 mL (500 μL) of 27% H_2O_2 in 50 mL OPD solution (0.5 g/L). In the comparison trials of pairs of peroxide strengths, the 50 μL (27%) treatment resulted in slightly poorer OPD development compared with the 100 μL (27%) treatment, which produced a stronger color and better enhancement, with more ridge detail being visible. The 500 μL (27%) treatment resulted in better development than the 100 μL (27%) treatment, where 500 μL was the amount equivalent to the optimal value determined from the cuvette trials. Interestingly, on a surface there was still room for improvement, because the 1000 μL (27%) treatment resulted in still better development than the 500 μL (27%) treatment. However, increasing the peroxide concentration still gave no further improvement, there being no visible difference between the 1500 μL (27%) treatment and the 1000 μL (27%) treatment.

In the case of PPD, the optimized ratio of H_2O_2 to PPD from the cuvette trials already translated to 1 mL of 27% H_2O_2. Two out of four print halves treated with 1 mL 27% H_2O_2 resulted in better development compared with the 0.2 mL 27% H_2O_2 treatment. The other two print halves indicated no difference between the 1 and 0.2 mL 27% H_2O_2 treatment. There was no visible difference between the four print halves treated with 2 mL 27% H_2O_2 and the four corresponding print halves treated with 1 mL 27% H_2O_2.

It was decided therefore, that for both OPD and PPD, 1 mL of 27% H_2O_2 should be used for further fingerprint trials. In the case

of PPD, this figure is equivalent to that determined from solution trials; in the case of OPD, it was revised upward by a factor of two.

Effect of pH—In the case of PPD, treatment at pH 7.4 resulted in the same developed color (purple) as treatment at pH 5.4, but the more alkaline pH resulted in slightly more background staining. Treatment of the blood fingerprints with OPD at pH 7.4 resulted in development of a browner color (more like that of DAB) compared with treatment at pH 5.4, which resulted in development of the usual orange color expected. Another brown reagent would yield no particular additional benefit to forensic science, whereas orange could be useful against certain backgrounds. It was therefore decided that a pH of 5.4 should be retained for further work with both reagents.

Effect of reagent concentration—The development obtained for all four print halves using a concentration of 0.25 g/L PPD or OPD was lighter in color than that obtained using their respective 0.5 g/L strength solutions. Further increasing each reagent's con-

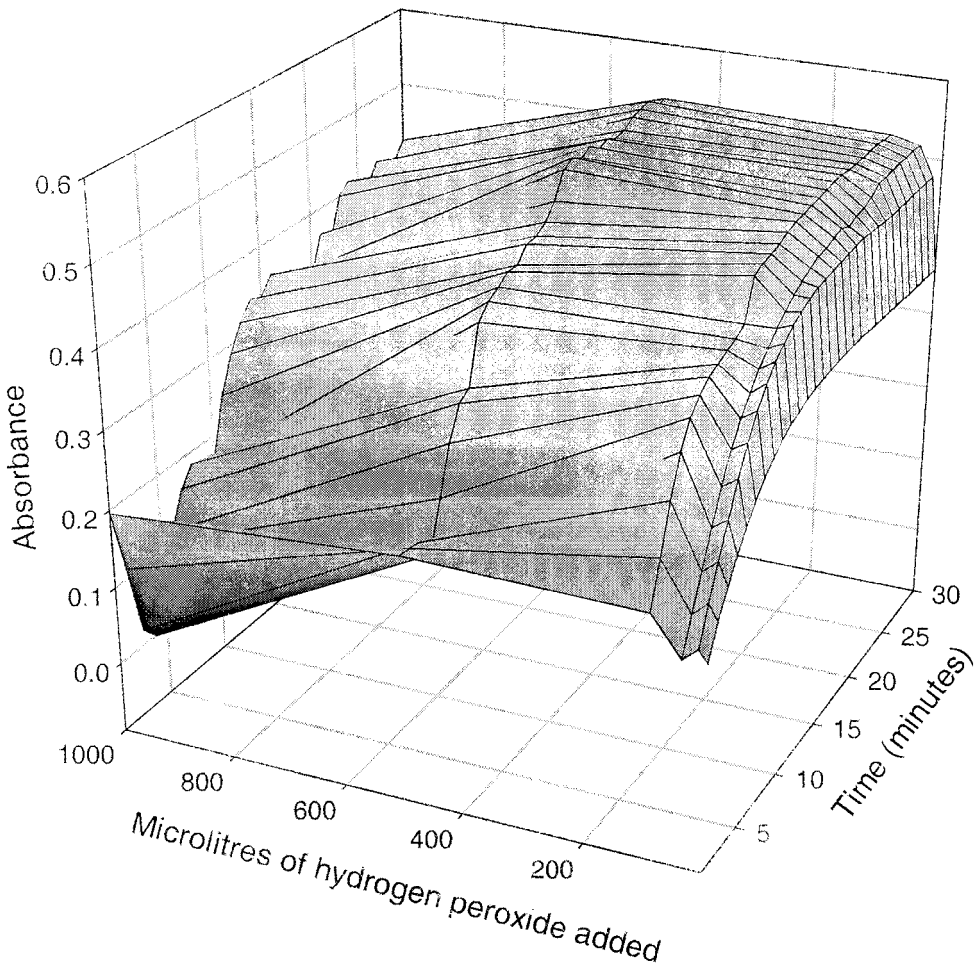

FIG. 3—*Effect of H_2O_2 volumes on color development of PPD (measured by absorbance at 404 nm) at pH 5.4.*

TABLE 5—*Determination of the sensitivity of OPD, PPD, and DAB using microplate wells (positive results are denoted by a + sign; other results are negative).*

		Blood Dilution Series					
Reagent	Concentration	1×10^{-1}	1×10^{-2}	1×10^{-3}	1×10^{-4}	1×10^{-5}	1×10^{-6}
OPD	0.1 *M*	+	+	+	—	—	—
	0.05 *M*	+	+	+	—	—	—
	0.025 *M*	+	+	+	—	—	—
PPD	0.01 *M*	+	+	+	—	—	—
	0.05 *M*	+	+	+	—	—	—
	0.025 *M*	+	+	+	—	—	—
DAB	0.1 *M*	+	+	+	—	—	—
	0.05 *M*	+	+	+	—	—	—
	0.025	+	+	+/−	—	—	—

centration to 1 g/L resulted in no further improvement in the quality of the developed prints. It was therefore decided that the OPD and PPD concentrations of 0.5 g/L would be retained for further trials.

Effect of soaking time—For OPD, there was no significant difference in the development obtained by treatment at 5, 10, and 30 min apart from greater background staining for the 30 min treatment. The 2.5 min treatment, however, was lighter in color and less visible.

For PPD, results were again similar. There was no significant difference in the development obtained by treatment at 2.5, 5, 10, and 30 min apart from greater background staining for the 10 and 30 min treatments.

A treatment time of 5 min appears to be the best soaking time to use for both reagents.

Effect of reagent age—Comparison was made between an "old" PPD solution (prepared 48 h earlier) with a new PPD solution (prepared 30 min earlier). The results were that one out of four print halves treated with the new solution was slightly better developed than the corresponding print half treated with the old solution. The other three print halves showed no difference. It is probably best, however, to prepare the PPD solution fresh each day, as is recommended for DAB.

Comparison with DAB—In this trial, 50 prints had been halved for each reagent. Fifty halves were treated with OPD while their corresponding partners were treated with DAB, and the halves were then "remarried" for comparison purposes. An identical trial was carried out comparing DAB with PPD developments.

In the OPD trial, 41 OPD treated halves were found to be of equivalent development (or indistinguishable) to their corresponding halves which had been treated with DAB. Two OPD treated fingerprint halves were better than their matching DAB treated halves, and the remaining seven OPD treated halves were of poorer development. In the PPD trial, seven PPD treated fingerprint halves were better than their corresponding DAB treated halves, 35 PPD treated halves were of equivalent development (or were indistinguishable), and eight PPD treated halves were of poorer development.

So overall, both OPD and PPD gave fairly equivalent performance to DAB for prints on paper, but with the resulting colors being orange and purple/black, respectively.

Compatibility with DAB—The print half treated with DAB first, followed by OPD treatment, resulted in the same development as the corresponding print half treated with DAB alone. The print treated with OPD first followed by DAB treatment resulted in the same development as the print treated with OPD alone.

The print half treated with DAB first followed by PPD treatment resulted in the same quality of development as the corresponding print half treated with DAB alone; however, the DAB/PPD treated print was purple/brown in color, and thus showed evidence of both reagents having been used. When the treatment sequence was reversed (PPD followed by DAB), the outcome was the same.

These results indicate that both OPD, PPD and DAB are similar in oxidizing strength. In our earlier work on the ABTS (1), we found that the developed light green color of oxidized ABTS was lost upon treatment with DAB, but that DAB could be used after ABTS treatment. (This effect also occurred in a mixed solution of the two reagents, suggesting that oxidation of DAB dominates the chemistry).

The compatibility of these reagents means that two or more of them could be used in series if necessary, with photographs being taken at each step. If ABTS is to be in a sequence, it is recommended that it come first; otherwise the order of application is optional depending on the background surface and the additive color obtainable. In order of lightness to darkness, the oxidized products would be ranked ABTS (light green) > OPD (orange) > DAB (brown) > PPD (purple/black).

Compatibility with ninhydrin—The fingerprint exhibit treated with OPD first, followed by ninhydrin, resulted in only the blood fingerprint developing. The latent fingerprint did not develop at all. The other fingerprint exhibit initially resulted in purple development of the latent fingerprint after the ninhydrin treatment (the blood print became grey in color with better visibility) followed by subsequent orange development of the blood fingerprint after OPD treatment. The ninhydrin developed latent print however, disappeared on immersion in the fixative solution.

Parallel behavior was exhibited by the PPD before and after ninhydrin treatment (with the exception that PPD development is purple/black).

Overall, prior use of ninhydrin will not inhibit OPD and PPD development. However, in the other direction, prior use of OPD or PPD will stop ninhydrin from working.

Treatment on Nonporous Surfaces

For OPD, the development obtained on glass at pH 5.4 was poor compared to that obtained at pH 7.4. At the latter pH, development is good and has a more reddish color. The reason for this shift in the optimal pH in moving from paper to glass is unknown. If oxidized, OPD is less soluble at pH 7.4 than at pH 5.4, the difference may be due to solubility, because the glass may be poorer at retaining the oxidized product than paper is (despite the fact that the oxidation itself is more efficient at pH 5.4). There is certainly scope for more work in this area.

In this case, PPD showed a significant difference to OPD in that it was better behaved. The development obtained on glass with PPD at pH 5.4 was equivalent to that obtained using DAB for both pairs of glass slides, but with a more purple/brown color for PPD.

Price Comparison

Ortho-phenylenediamine (OPD) and *para*-phenylenediamine (PPD) can be purchased as flakes from Aldrich Chemical Company in 1 kg quantities for $66 and $60, respectively. 3,3′-diaminobenzidine can also be purchased from Aldrich Chemical Company in 25 g quantities for $108. All Aldrich Chemical Company prices are from the Australian 1998–1999 price listings (prices converted by Australian/US exchange rate).

The price per 100 mg of OPD or PPD (used to prepare 200 mL of OPD or PPD working solution) when purchased from Aldrich Chemical Company will therefore be approximately $0.007. The price per 200 mg of DAB (used to prepare 200 mL of DAB working solution) will be $0.86.

Summary and Recommended Procedure

OPD and PPD, although both still toxic, represent less of a hazard than the carcinogenic risks of DAB for development of blood fingerprints, while still being as effective. OPD works well on

porous surfaces at pH 5.4 and on glass at pH 7.4. OPD development results in prints that are orange, which should show up more clearly than the dark brown of oxidized DAB on dark surfaces. PPD gives purple prints and works well on porous surfaces and on glass at pH 5.4. When ABTS (1) is included, this allows choice of four potential colors, the oxidized product of ABTS being bright green. OPD, PPD, and also ABTS are all "nothing-to-lose" reagents, because treatment with any of the three compounds can be followed by DAB treatment, with no loss in the latter reagent's performance. OPD and PPD themselves (but not ABTS) can also be used after DAB treatment. Whether they are used before or after DAB, the resulting color represents a mixture of the oxidized products of the OPD or PPD with that of DAB. Although OPD or PPD can be effectively used after ninhydrin, ninhydrin cannot be effectively used after OPD or PPD.

The recommended procedure for best visualization of fingerprints deposited in blood using OPD or PPD is as follows. The procedure is based on the combined results obtained from the solution and fingerprint optimization trials.

Fixative Solution

Dissolve 20 g of 5-sulfosalicylic acid in 1 L distilled water in a 2 L glass beaker. Transfer to a labeled, laboratory bottle with a screw top (use either a dark glass bottle or cover with silver foil). Store in dark at room temperature.

Citric Acid/Phosphate Buffer (pH 5.4)

1. Dissolve 71.64 g of $Na_2HPO_4.12H_2O$ or 35.61 g $Na_2HPO_4.2H_2O$ in distilled water and make up to the mark in a 1 L volumetric flask (0.2 *M*), shaking vigorously to ensure that all solids are dissolved.
2. Dissolve 21.01 g of citric acid monohydrate in distilled water and make up to the mark in a 1 L volumetric flask (0.1 *M*), shaking vigorously to ensure that all solids are dissolved.
3. Measure out 223 mL $Na_2HPO_4.12H_2O$ or $Na_2HPO_4.2H_2O$ solution (0.2 *M*) and 177 mL of citric acid monohydrate (0.1 *M*) into a labeled laboratory bottle with a screw top, and mix well.

OPD and PPD Stock Solutions

Dissolve 50 mg of OPD or PPD in 100 mL of citric acid/phosphate buffer solution (pH 5.4) in a labeled, laboratory bottle with a screw top. Unaided, OPD and PPD take a while to dissolve. This process can be facilitated by shaking vigorously or placing in an ultra-sonic bath for 10 min. Store in fridge (away from light) for up to a week for OPD and use on the day for PPD.

Immersion Method

- Place blood fingerprint exhibit in a clean, shallow, glass dish.
- Pour out sufficient fixative solution into the dish to cover exhibit. Leave for about 3 min before removing exhibit and rinsing in distilled water.
- Place exhibit in a clean, shallow, glass dish. Pour out 50 mL of the OPD or PPD stock solution into a laboratory bottle with a screw top followed by 0.5 mL of 27% H_2O_2 and shake to ensure thorough mixing (if 50 mL is not enough to cover exhibit then add more and adjust the H_2O_2 volume accordingly).
- Pour the activated working solution over the exhibit and leave to develop for 5 min. Remove the exhibit and rinse in distilled water.
- Leave exhibit to air dry in a dark place.

Reservoir Method

- Lay a piece of clean, dry filter paper over the area of the blood fingerprint exhibit to be treated.
- Saturate the filter paper with fixative solution, using a Pasteur pipette, and keep the paper saturated with solution for three minutes. Remove the paper and wash the area under treatment with distilled water.
- Lay a piece of clean, dry filter paper over the area of the blood fingerprint exhibit to be treated.
- Saturate the filter paper with activated working solution (previously mixed), using a Pasteur pipette. Keep the paper saturated with solution for 5 min. Remove the paper and wash the area under treatment with distilled water.
- Leave the exhibit to air dry in a dark place.

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