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ABTS: A Safe Alternative to DAB for the Enhancement of Blood Fingerprints

REFERENCE: Caldwell JP, Henderson W, Kim ND. ABTS: a safe alternative to DAB for the enhancement of blood fingerprints. J Forensic Sci 2000;45(4):785–794.

ABSTRACT: The use of 2,2′-azino-di-[3-ethylbenzthiazolinesulfonate(6)] diammonium salt (ABTS) 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. ABTS is an effective and safe noncarcinogenic (though more expensive) alternative to the presently used 3,3′-diaminobenzidine (DAB) for the development of blood fingerprints, especially on porous surfaces. The bright green color of the oxidized ABTS is an advantage on certain colored surfaces where the dark brown color of DAB does not stand out well from the background. Development with ABTS does not interfere with subsequent DAB treatment, making ABTS a "nothing-to-lose" reagent. ABTS can be used after ninhydrin treatment, but the reverse is not the case.

KEYWORDS: forensic science, fingerprints, ABTS, blood, peroxidase, hemoglobin

Current approaches to latent fingerprint development, including the range of methods that have been used to enhance bloody fingerprints, have been reviewed by Lee and Gaensslen (1,2). More recently, approaches to developing bloody imprint evidence have been reviewed at forensic science gatherings by Lee (3) and Messina et al. (4).

A number of reagents can be used for the enhancement of fingerprints in blood. Some of the more commonly used reagents are 3,3′-diaminobenzidine (DAB), luminol and *ortho*-tolidine. Of those currently in use, DAB provides the best results in visualizing impressions made in blood (5). The sensitivity and specificity of the test are both well-attested to by the fact that in spite of the hazards associated with exposure to this compound, there has been a marked reluctance to abandon its use for any substitute found until now. However, the incidence of cancer among workers exposed to DAB has rendered the search for a satisfactory alternative of paramount importance (2,6).

The enhancement of blood fingerprints using DAB involves an oxidation reaction in which the oxidant, hydrogen peroxide, oxidizes DAB to a dark brown product. The heme group of hemoglobin in blood exhibits a peroxidase-like activity, which acts to catalyze the breakdown of hydrogen peroxide (7).

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Received 18 June 1999; and in revised form 21 Sept. 1999; accepted 21 Sept. 1999.

2,2′-Azino-di-[3-ethylbenzthiazolinesulfonate(6)] diammonium salt (ABTS), the chemical structure of which is shown in Fig. 1, has been used previously as a chromogen for the determination of plasma or serum hemoglobin and has proven sensitive and accurate (8,9). ABTS, although structurally unrelated to DAB, also undergoes oxidation to a colored form (green) in the presence of H_2O_2 and hemoglobin. However, unlike DAB, ABTS is considered to be nontoxic and safe to use (8,10).

Materials and Methods

Materials

ABTS®-$(NH_4)_2$, 3,3′-diaminobenzidine, and 5-sulfosalicylic acid were purchased from Boehringer Mannheim (BM) Laboratories, Aldrich, and M&B Laboratory Chemicals, respectively. Paper (A4, 80 gsm) was obtained from Copyright (Australian Paper, Ltd.) 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, glass slides, and ceramic tile for later use.

An ABTS stock solution was prepared by dissolving 125 mg ABTS in 50 mL distilled water (2.5 g/L).

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 Concentration

The optimal pH and H_2O_2 concentrations favoring best color development of ABTS in a solution containing small amounts of blood in a 10 mm quartz cuvette were first determined by UV/Vis spectroscopy on a Varian 1 Cary UV/Visible Spectrophotometer (SBW = 0.2 nm, signal averaging time = 0.100 s). For oxidized ABTS the peak maximum occurs at 415 nm.

All solutions prepared contained 100 μL of ABTS 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 11.8 mL. Changes in the absorbance of this solution with time at 415 nm 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 11.8 mL. The H_2O_2 was always added last, with the time of the subsequent reaction being recorded from that point.

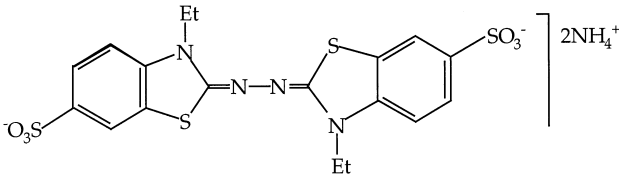

FIG. 1—*Chemical structure of ABTS diammonium salt.*

Experiments to determine the optimal concentration of H_2O_2 were initially carried out at a pH value of 7.4[2] (using a phosphate buffer). Volumes of H_2O_2 added were varied from 5 to 100 μL. Experiments to determine the optimal pH were then performed using a set volume of 10 μL 2.7% H_2O_2 and varying the pH from 4.4 to 8.0. Further experiments to determine the optimal concentration of H_2O_2 at a pH value of 5.4 (citric acid/phosphate buffer) were carried out with H_2O_2 volumes ranging from 1 to 1000 μL. All buffer solutions used were 0.1 to 0.2 *M* in strength.

Effect of Anti-coagulants

Collection of blood using an anti-coagulant helps matters considerably. However, normal fingerprints laid down in blood (in real-life situations) do not contain an anti-coagulant, so it was of interest to establish whether the presence of such a compound in our trials was likely to influence the ABTS/hemoglobin reaction in any way. For this purpose, three 10 mL samples of ovine blood were collected into three separate vaccutainer tubes, each containing one of three different anti-coagulants. These were EDTA(K_3), sodium heparin, and potassium oxalate/NaF. A fourth 10 mL sample of blood without anti-coagulant was also collected. The tubes were inverted a few times to ensure thorough mixing, and 500 μL was immediately taken from each tube and diluted up to 250 mL in volumetric flasks (giving a dilution factor of 500).

Aliquots of the blood solutions (1:500) were then diluted by two times to give final dilutions of 1000 times. UV/Vis absorbance spectra of these four blood samples were then obtained for comparison purposes.

A 500 mg/100 mL (5 g/L) ABTS stock solution was prepared for use in testing for possible effects from the anti-coagulants on the ABTS–oxidation reaction. A UV/Vis spectrophotometer was used to investigate any effect on the oxidation of ABTS. For each run, a subsample of the following was added to the 10 mm quartz cuvette: 10.99 mL citric acid/phosphate buffer (pH 5.4), 0.75 mL blood solution (1:500), 50 μL ABTS stock solution (the final concentration of ABTS in the cuvette was therefore the same as that used in previous trials), and 10 μL 2.7% H_2O_2. This solution was run against the following blank: 11.04 mL citric acid/phosphate buffer (pH 5.4), 0.75 mL blood solution (1:500), and 10 μL 2.7% H_2O_2.

Absorbance values for each solution at 415 nm were recorded with increasing time, starting from the point where H_2O_2 was added to each solution.

ABTS Fingerprint Trials

All blood fingerprints (previously laid down on paper, glass microscope slides, or ceramic tiling) were first fixed with 5-sulfosalicylic acid (3 min soak in 20 g/L of aqueous 5-sulfosalicylic acid). This is the method of fixing a blood fingerprint currently recommended by the New Zealand Crown Research Institute, *ESR:Forensic* (12). The prints were then rinsed in distilled water before being treated with either ABTS or DAB. Prints treated with ABTS were immersed for 5 min in 10 mL of ABTS solution (5 g/L in buffer at pH 5.4) to which had been added 200 μL of freshly prepared 2.7% H_2O_2. These were the conditions used unless otherwise stated and were the same proportions of reagents as determined from the optimization of pH and H_2O_2 concentrations in solution. A number of variations on these conditions were trialed in order to test whether they were still optimal for development of blood prints on surfaces. This is because of a second factor which might be important on surfaces—the ability of the oxidized chromophore to remain associated with the print (rather than be dissolved back into the solution). Prints treated with DAB were immersed in 100 mL of DAB solution (1 g/L in buffer at pH 7.4[3]) and 500 μL 27% H_2O_2 for 5 min. This is the method currently recommended by the New Zealand Crown Research Institute, *ESR:Forensic* (12). Both ABTS and DAB treatments were then followed by a further rinse in distilled water. The following trials were carried out.

Treatment on Porous Surfaces—Blood fingerprints were placed on unused white paper and each print was cut bilaterally down the center of the print, so that a comparison could be made between treatment methods by treating the two corresponding halves differently.

1. Treatment compared with no treatment: One half of a print was treated with ABTS while the other half was left untreated.
2. Varying H_2O_2 concentrations: Four prints were used to compare treatment between 20 μL and 200 μL 2.7% H_2O_2. Another four prints were used to compare treatment between 200 μL (2.7%) and 200 μL (27%) H_2O_2.
3. Varying ABTS concentrations: Four prints were used to compare treatment between 2.5 g/L and 5 g/L ABTS solution. Another four prints were used to compare treatment between 5 g/L and 10 g/L ABTS solution.
4. Varying soaking times in ABTS solution: One print was used to compare a 2.5 min treatment with a 5 min treatment in ABTS solution. Another print was used to compare a 5 min treatment with a 10 min treatment in ABTS solution. A third print was used to compare a 5 min treatment with a 30 min treatment in ABTS solution.
5. Varying fixative times: Four prints were used to compare fixative treatment at 3 min and 6 min soaking times.
6. Comparison of ABTS method at pH 5.4 and pH 7.4: Sixteen prints were used to compare ABTS treatment at pH 5.4 and pH 7.4, using citric acid/phosphate and phosphate buffers, respectively.
7. Comparison of ABTS with DAB: Fifty prints were used to compare ABTS treatment with DAB treatment.
8. Compatibility of ABTS with DAB: Ten prints were used to compare the development obtained by using a mixture of ABTS and DAB with that obtained by using ABTS alone. One hundred milliliters of ABTS solution (5 g/L) and 100 mL of DAB (1 g/L) solution, both prepared in buffer pH 5.4 with 500 μL of added H_2O_2 (27%), was used for the mixture. A further seven prints were used to compare the development obtained by treatment with DAB

[2] This is the pH commonly used for treatment using DAB.

[3] The optimization of pH was carried out for DAB as well and an optimum pH of 5.0 in solution was discovered, but development on paper at pH 5.0 was no different to that at pH 7.4. Therefore, no alternation to the DAB procedure was necessary.

followed by subsequent ABTS treatment, with DAB treatment alone. Four more prints were used to compare ABTS treatment followed by DAB treatment, with DAB treatment alone.

9. Compatibility of ABTS with ninhydrin: Two strips of paper, each containing a latent fingerprint and a blood fingerprint, were used to see if ABTS treatment could be followed by ninhydrin treatment, and if ninhydrin treatment could be followed by ABTS treatment.

10. Heat treatment: In one literature reference (9), incubation temperatures between room temperature and 50°C were tested on the ABTS oxidation reaction. The ABTS oxidation reaction produced greater absorbances at higher temperatures but was more unstable. It was therefore decided that ABTS treatment at 50°C should be compared to treatment at room temperature. The ABTS treatment at 50°C involved pre-warming the solution (apart from the 200 μL of 2.7% H_2O_2) by placing it in a laboratory oven at 50°C for 20 min. The halves of four blood fingerprints (previously fixed with 5-sulfosalicylic acid) were immersed in the heated solution with 200 μL 2.7% H_2O_2 for 5 min in the oven. The other four corresponding blood fingerprint halves were treated with ABTS solution at room temperature in the usual way.

11. Cold treatment: Because the ABTS solution is stored in the fridge it was decided that the effectiveness of the ABTS solution at 12°C (approximate temperature of solution after it has been taken out of the fridge and been prepared for use) should be compared with the effectiveness of the solution after it has been allowed to warm up to 20°C (room temperature). The halves of four blood fingerprints (previously fixed with 5-sulfosalicylic acid) were immersed in the 12°C solution with 200 μL 2.7% H_2O_2 for 5 min. The other four corresponding blood fingerprint halves were treated with ABTS solution at 20°C for 5 min.

12. DNA compatibility: During the course of this research, a sample of ABTS with instructions on its use for treatment of latent fingerprints was sent to another laboratory in order to assess its compatibility with subsequent DNA treatment.

Treatment on Non-porous Surfaces

1. Glass: Four prints were placed onto glass microscope slides and subsequently fixed with 5-sulfosalicylic acid before being treated with ABTS. Another print was placed over two slides set side-by-side so that two halves of the same print could be obtained. The print was used to compare ABTS treatment with DAB treatment.

2. Ceramic tile: Twelve prints were placed on a white ceramic bathroom tile and subsequently fixed with 5-sulfosalicylic acid before being treated with ABTS.

Sensitivity of ABTS Compared with DAB

Spot Tests on Filter Paper—The procedure for the determination of ABTS sensitivity was based on the procedure used by Garner et al. (13). 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}, 1×10^{-6}. A 10 μL aliquot of each dilution was placed onto a separate strip of filter paper and left to dry. This procedure was repeated twice so that there were three sets of filter paper strips each with the same series of dried, diluted blood spots. Three ABTS 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 onto the blood spots on each strip of filter paper of the first set of filter paper strips followed by a drop of 2.7% of H_2O_2. The 0.05 and 0.025 *M* solutions were then placed onto the second two sets of filter paper strips, respectively, followed by 2.7% of H_2O_2. A control was also used with just ABTS and H_2O_2 on filter paper with no blood spots.

A positive test was assigned when the green spot produced on reaction with the blood migrated out across the paper but left a darker green spot in the center. A negative test was assigned when the color of the green spot was no darker than that obtained from a control.

The whole procedure was then repeated but using 0.1 *M*, 0.05 *M*, and 0.025 *M* DAB solutions in place of ABTS.

Microplate Well Tests—The procedure used for the spot tests above was repeated again for ABTS and DAB, but, instead of using filter paper, spot tests were carried out at the bottom of plastic microplate wells with a negative being assigned for wells producing the same result as a control (based visually on color). The following dilutions were prepared: 1×10^{-1}, 1×10^{-2}, 1×10^{-3}, 1×10^{-4}, 1×10^{-5}, 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. The three ABTS or DAB 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.

A positive test was determined by color comparison with a control well containing only ABTS 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.

Crystal Structure of ABTS

Blue/green irregular crystals of dimensions 0.56 by 0.13 by 0.07 mm were grown from an aqueous solution (0.1 *M*) of ABTS-$(NH_4)_2$. Intensity data and accurate cell parameters were collected at the University of Auckland on a Siemens SMART CCD system diffractometer with monochromated M_0-K_α X-rays ($\lambda = 0.71073$ Å). The data collection nominally covered over a hemisphere of reciprocal space, by a combination of three sets of exposures; each set had a different f angle for the crystal and each exposure covered 0.3° in *w*. The crystal to detector distance was 5.0 cm. The data sets were corrected empirically for absorption using SADABS.

Results and Discussion

Optimization of pH and H_2O_2 Concentration

The results from the trials to determine the optimal concentration of H_2O_2 at a pH value of 7.4 (using a phosphate buffer) are presented in Table 1. From these results, it was established that 10 μL of H_2O_2 (2.7%) produces optimal color development. This corresponds to 1 g of ABTS per 40 mL of 2.7% H_2O_2.

At pH 7.4, color development is not particularly sensitive to the amount of H_2O_2 added but use of the minimum volume which works (10 μL) compared with the higher volume of 25 μL was felt reasonable due to the observation of color loss at higher concentrations (and long times). 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 ABTS/hemoglobin reaction. This could occur by H_2O_2 causing further oxidation of the ABTS to the point of destroying the com-

TABLE 1—*Effects of H_2O_2 volumes on color development of ABTS (measured by absorbance at $\lambda = 415$ nm) at pH 7.4.*

Volume of 2.7% H_2O_2 Added																	
5 μL		10 μL (a)		10 μL (b)		25 μL		37 μL		50 μL		60 μL		75 μL		100 μL	
T (s)	Ab.	T (s)	Ab.	T (s)	Ab.	T (s)	Ab.	T (s)	Ab.	T (s)	Ab.	T (s)	Ab.	T (s)	Ab.	T (s)	Ab.
129	0.04	124	0.06	110	0.04	115	0.06	107	0.08	110	0.11	99	0.08	108	0.08	80	0.08
197	0.08	196	0.10	182	0.08	188	0.12	180	0.13	180	0.16	173	0.15	180	0.13	140	0.12
275	0.11	265	0.14	256	0.11	262	0.17	256	0.18	258	0.21	248	0.20	256	0.17	240	0.16
352	0.15	351	0.19	332	0.15	336	0.22	350	0.23	332	0.25	321	0.24	369	0.21	320	0.18
451	0.19	459	0.25	411	0.19	476	0.29	423	0.26	415	0.28	437	0.28	486	0.25	398	0.20
526	0.22	571	0.29	488	0.22	570	0.32	523	0.29	485	0.31	511	0.29	606	0.27	472	0.21
641	0.27	719	0.34	646	0.28	644	0.35	598	0.30	562	0.33	628	0.30	726	0.28	543	0.23
820	0.32	848	0.37	721	0.30	765	0.38	730	0.33	633	0.35	778	0.31	799	0.29	616	0.22
953	0.36	926	0.40	794	0.32	907	0.40	880	0.34	918	0.39	851	0.31	967	0.30	727	0.22
1974	0.46	1977	0.47	867	0.34	971	0.41	1964	0.36	2270	0.43	1949	0.29	1987	0.31	866	0.23
…	…	…	…	942	0.36	1920	0.46	…	…	…	…	…	…	…	…	2110	0.21
…	…	…	…	1978	0.45	…	…	…	…	…	…	…	…	…	…	…	…

TABLE 2—*Effect of pH on color development of ABTS (measured by absorbance at $\lambda = 415$ nm) with 10 μL 2.7% H_2O_2.*

pH 4.4		pH 5.0		pH 5.4		pH 5.8		pH 6.8		pH 7.4*		pH 8.0	
T (s)	Ab.	T (s)	Ab.	T (s)	Ab.	T (s)	Ab.	T (s)	Ab.	T (s)	Ab.	T (s)	Ab.
107	0.29	108	0.91	126	0.83	107	0.55	106	0.13	117	0.05	103	0.01
180	0.53	180	1.25	198	1.18	184	1.04	197	0.27	189	0.09	180	0.03
254	0.75	254	1.25	273	1.25	259	1.24	287	0.41	260	0.13	286	0.05
327	0.92	363	1.23	349	1.25	331	1.23	377	0.54	341	0.17	402	0.06
455	1.10	484	1.23	426	1.23	409	1.22	466	0.65	474	0.23	483	0.07
576	1.17	602	1.22	546	1.22	529	1.20	556	0.74	609	0.29	587	0.07
696	1.19	722	1.21	664	1.21	607	1.20	636	0.80	720	0.32	679	0.08
816	1.19	842	1.20	845	1.20	727	1.19	791	0.87	857	0.36	827	0.08
936	1.18	962	1.20	1025	1.19	899	1.17	927	0.91	934	0.38	947	0.08
1981	1.15	2158	1.13	1986	1.14	2127	1.06	1986	0.92	1977	0.46	1984	0.10
…	…	…	…	4455	1.03	3526	0.98	…	…	…	…	…	…
…	…	…	…	…	…	5955	0.86	…	…	…	…	…	…
…	…	…	…	…	…	67745	0.58	…	…	…	…	…	…

* An average of two trials at pH 7.4.

pound. By extension, it is possible that this effect may also occur in the similar DAB/hemoglobin system (as was observed in DAB trials).

Trials to test the most appropriate pH were then carried out using the optimal volume of 10 μL 2.7% H_2O_2 found for pH 7.4. Results are presented in Table 2 and Fig. 2.

Absorbance values grew significantly as the pH was lowered, appearing to reach a maximum at about pH 5.4. Raising the pH from 7.4 to 8.0 had the opposite effect, with absorbance values for ABTS falling substantially. Thus, in solution, the effect of pH on ABTS color development is quite pronounced, and maximum development occurs at pH 5.4. This effect is illustrated graphically in Fig. 2. During these trials it also became apparent that absorbance values started to decrease again if solutions were allowed to stand for long periods. This could also be due to over-oxidation (or "bleaching") by H_2O_2 in solution, and was not an effect observed in the case of ABTS-developed fingerprints, where H_2O_2 is rinsed off after development.

Once the optimum pH was determined, it was felt prudent to establish whether the optimum H_2O_2 volume of 10 μL still applied at the higher acidity. The results for different H_2O_2 volumes at pH 5.4 (citric acid/phosphate buffer) are presented in Table 3.

From these results it can be seen that 10 μL 2.7% H_2O_2 (H_2O_2:ABTS ratio of 40 mL:1 g) was still the best volume to use at the lower pH. It gives the smallest absorbance decrease with time after the initial absorbance maximum is reached. Use of 1 μL leads to a lower rate of initial color development, whereas use of 100, 500, and 1000 μL lead to increasingly greater rates of color loss after development. This latter observation lends support to the idea that unreacted H_2O_2 is involved in the color loss.

Overall, the cuvette-based solution trials yield the following as optimal for development of color in the ABTS/hemoglobin system: pH 5.4, ratio of 1 g ABTS to 40 mL 2.7% H_2O_2, and a development time of 4 to 8 min. These were taken as starting conditions for testing the activity of ABTS on blood fingerprints. Unknowns in moving from solution work to development of blood on surfaces were (a) whether the concentration of ABTS would have any effect on final color development, and (b) the extent to which the oxidized ABTS would be able to deposit on the surface-bound blood and remain there (rather than move back into solution). This second parameter is quite important; in an earlier trial involving phenolphthalein, it was found that although this reagent provides a strong, magenta color immediately on reaction with a fixed print, within a few seconds this color rises from the print surface and disperses

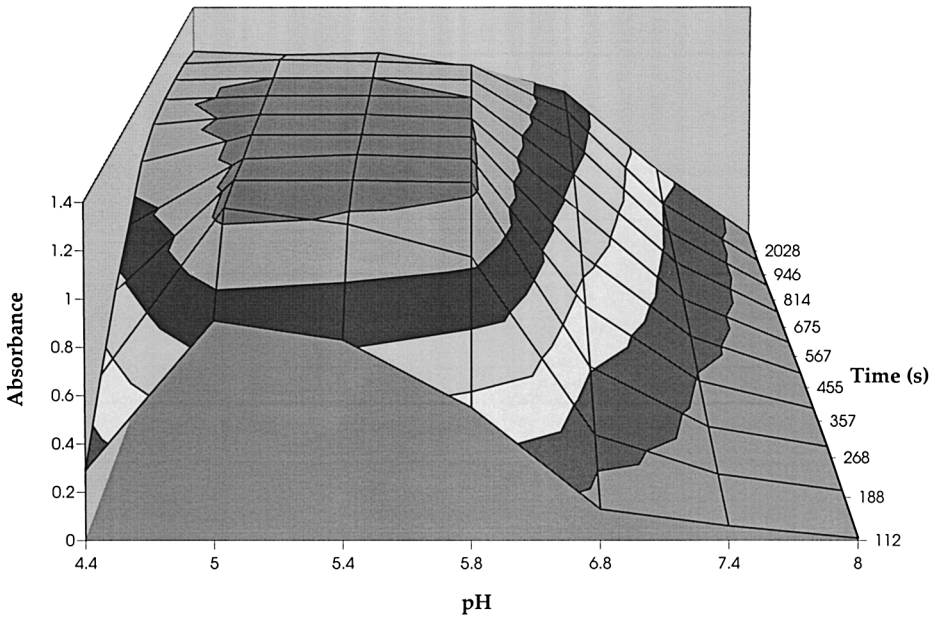

FIG. 2—*Effect of pH on color development of ABTS (measured by absorbance at λ = 415 nm) with 10 μL 2.7% H_2O_2.*

TABLE 3—*Effect of H_2O_2 volume on color development of ABTS (measured by absorbance at λ = 415 nm) at pH 5.4.*

Volume of 2.7% H_2O_2 Added									
1 μL		10 μL		100 μL		500 μL		1000 μL	
T (s)	Ab.	T (s)	Ab.	T (s)	Ab.	T (s)	Ab.	T (s)	Ab.
99	0.13	126	0.83	86	1.26	90	1.20	99	1.16
173	0.23	198	1.18	173	1.25	165	1.20	178	1.13
252	0.36	273	1.25	275	1.22	240	1.20	262	1.10
328	0.48	349	1.25	362	1.20	314	1.18	380	1.05
446	0.66	426	1.23	481	1.17	405	1.17	500	0.99
566	0.82	546	1.22	601	1.14	525	1.15	621	0.95
686	0.97	664	1.21	721	1.11	645	1.12	741	0.90
806	1.09	845	1.20	841	1.09	765	1.10	837	0.85
926	1.18	1025	1.19	962	1.07	945	1.06	960	0.81
2125	1.18	1986	1.14	1985	0.87	1986	0.86	1982	0.44
…	…	4455	1.03	…	…	…	…	…	…

back into solution. Evidently, part of the reason DAB is an effective reagent is that the oxidized form is less soluble in aqueous solution than the reduced form and will therefore precipitate and settle out on the fingerprint surface.

Effect of Anti-coagulants

A proper comparison of the three different anti-coagulants, EDTA(K_3), sodium heparin, and potassium oxalate/NaF, along with the absence of an anti-coagulant necessitates the determination of the absorbance at a specific time for each blood sample in order to establish whether there is any influence on the ABTS/hemoglobin reaction. The time chosen was 300 s. The UV/Vis results previously obtained from the pH/H_2O_2 optimization using the original blood solution with no anti-coagulant at pH 5.4 and with 10 μL 2.7% H_2O_2 were also used for purposes of comparison. Results from the trial of the effect of different and no anti-coagulants are presented in Table 4 and Fig. 3.

TABLE 4—*Comparison of absorbances at 300 s for different and no anti-coagulants.*

Anti-coagulant	Absorbance at 300 s
EDTA(K_3)	1.36
Sodium Heparin	1.30
Potassium Oxalate	1.30
No anti-coagulant	1.29
No anti-coagulant*	1.25

* Result obtained from an earlier blood sample used in pH/H_2O_2 optimization trials.

From these results it can be seen that the presence of anti-coagulants in the blood do not significantly suppress the reaction between ABTS, H_2O_2, and hemoglobin, with absorbance values being no smaller, and in fact, largely indistinguishable from those obtained without use of anti-coagulants.

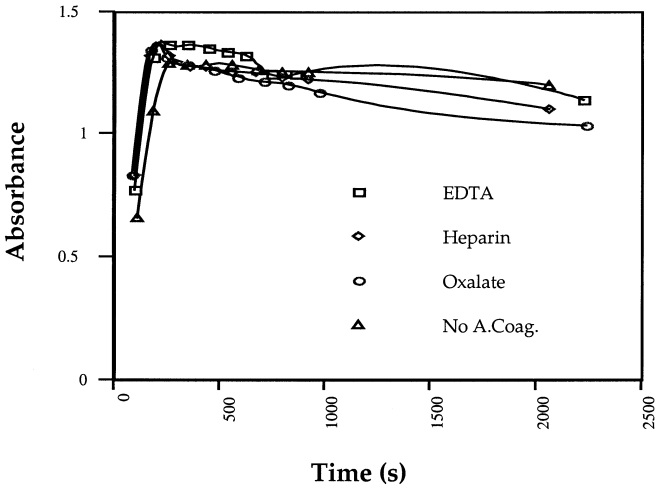

FIG. 3—*Comparison of effect of anticoagulants on oxidation of ABTS with time for 10 μL H_2O_2 and pH 5.4 (absorbance measured at λ = 415 nm).*

ABTS Fingerprint Trials

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

1. Treatment compared with no treatment: Treatment of a blood fingerprint with ABTS results in a much better bright green print with good ridge definition.

2. Varying H_2O_2 concentrations: The optimized ratio of H_2O_2 to ABTS from the cuvette trials translated to 200 μL of 27% H_2O_2 in these trials. However, on the print, treatment with ten times less H_2O_2 (200 μL 2.7%) resulted in equivalent development to 200 μL of 27% H_2O_2. Treatment with one hundred times less H_2O_2 (20 μL 2.7%) resulted in undeveloped patches compared with 200 μL (2.7%) treatment, which produced a distinctly stronger color, better enhancement, and with more ridge detail visible.

3. Varying ABTS concentrations: Treatment using ABTS at a concentration of 2.5 g/L resulted in prints which were lighter in color than those obtained from ABTS at a 5 g/L concentration. However, further increasing the ABTS concentration to 10 g/L resulted in more background staining with less distinct ridge detail compared with the 5 g/L solution.

4. Varying soaking times in ABTS: There was no significant difference in the development obtained by treatment at 2.5, 5, and 10 min. The 30 min treatment, however, resulted in greater background staining with less distinct ridge detail.

5. Varying fixative times: There was no detectable difference in the final development for the fingerprint halves fixed with 5-sulfosalicylic acid for 3 or 6 min prior to ABTS treatment.

6. Comparison of ABTS treatment at pH 5.4 and pH 7.4: Treatment at pH 7.4 resulted in a brown development color compared with treatment at pH 5.4, which resulted in the usual bright green development color expected. The treatment at pH 5.4 resulted in slightly more background staining but this actually proved to be an advantage in helping to better define the ridge detail (there seemed to be more shades of color).

7. Comparison of ABTS with DAB: Nine ABTS-treated fingerprint halves were better developed than their corresponding DAB treated halves (Fig. 4*a*), nine ABTS-treated halves were of poorer development (Fig. 4*b*) and 32 ABTS-treated halves were of equivalent development, or were indistinguishable (Fig. 4*c*). Overall, ABTS gives equivalent performance to DAB for prints on paper (but with the resulting color being bright green) with the added advantage of being a nontoxic option. It should be noted that the preparation of the ABTS stock solution is easier than preparation of the DAB stock solution, because ABTS is very soluble in water compared with DAB. It was necessary to use an ultrasonic bath in order to help dissolve the DAB. In terms of general readability, faint bloody prints developed with ABTS were judged to be (on average) equivalent to those developed with DAB.

8. Compatibility of ABTS with DAB: For the prints treated with a mixture of ABTS and DAB the development was the same as for those treated with DAB alone. The bright green color of the ABTS solution totally disappeared after the DAB solution was added to it, indicating that the DAB was "masking" or deactivating the ABTS in some way. This effect may be because DAB is a stronger reducing agent than ABTS. It is conceivable that oxidation of DAB causes reduction of ABTS when the two are in close proximity. The prints treated with DAB followed by ABTS treatment were indistinguishable from the prints treated with DAB alone. In fact, the bright green color of the ABTS solution slowly disappeared after placing the DAB-treated prints into the ABTS solution for 5 min. The prints treated with ABTS first, followed by DAB treatment, resulted in the same development as the prints treated with DAB alone. The bright green color of the ABTS treated prints slowly faded to a light gray color before changing to the dark brown color consistent with DAB treatment.

9. Compatibility of ABTS with ninhydrin: The fingerprint exhibit treated with ABTS 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 gray in color with better visibility) followed by subsequent green development of the blood fingerprint after ABTS treatment. The ninhydrin-developed latent print, however, disappeared on immersion in the fixative solution.

10. Heat treatment: There was no visible difference between ABTS treatment at 50°C and ABTS treatment at room temperature, and therefore no advantage in heating.

11. Cold treatment: There was no visible difference between ABTS treatment at 12°C and ABTS treatment at 20°C. Therefore, it is not necessary to warm the ABTS solution to room temperature before use.

12. DNA compatibility: Boyd (16) found that ABTS treatment (and DAB treatment) resulted in only partial DNA profiles. Further research, however, is probably required for confirmation of this.

13. Comment on effect of print age: In these trials, development of bloody fingerprints on paper after two weeks was found to be as good as development on fresh prints. However, at this stage, trials have not been carried out to determine how old a bloody fingerprint can become before ABTS fails to work. Similarities in the mode of action of this chemical with DAB suggest that as a bloodstain ages, ABTS should continue to function for at least as long as DAB. The critical component in whether both reactions work is likely to be the ability of the heme groups in the blood to maintain their integrity as catalytic sites; these function by enhancing the breakdown of peroxide, with the concurrent oxidation of the ABTS or DAB.

Treatment on Non-porous Surfaces

1. Glass: On this surface, reasonably good visual enhancement was obtained with no background staining. It was necessary to hold

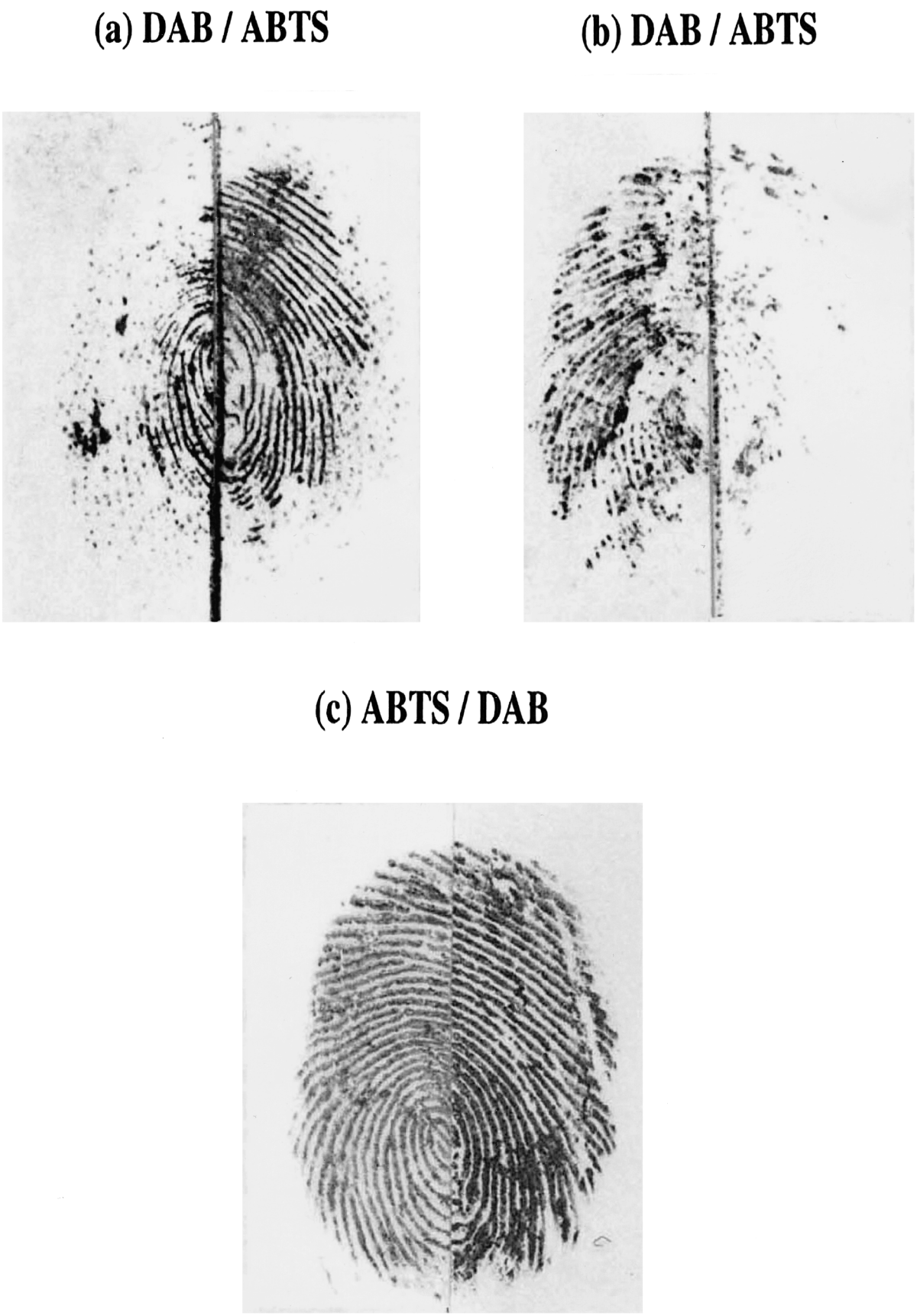

FIG. 4—*ABTS treatment compared with DAB treatment.* (a) *ABTS treatment is better than DAB treatment.* (b) *DAB treatment is better than ABTS treatment.* (c) *ABTS and DAB treatment are equivalent.*

the slide at the correct angle to the source of light for good visualization, due to reflection from the surface. The DAB-treated half-fingerprint on glass was better developed and was easier to see on the glass than the ABTS-treated half. Reflection of light from the glass surface seems to cause more of a problem in visualizing the ABTS-treated prints than those treated with DAB. Visualization under UV light (254 nm and 366 nm) did not improve the print resolution.

2. On ceramic tile: Reasonable enhancement was obtained using ABTS, but this was not as good as on glass, and certainly not as good as that achieved on paper.

Sensitivity of ABTS Compared with DAB

Results for the ABTS and DAB spot tests on filter paper are presented in Table 5 and results for ABTS and DAB using microplate well tests are presented in Table 6.

These sensitivity results are not very accurate as a positive or negative test is assigned purely on the basis of color matching with a control by eye (however, it is worth noting that indicative tests for blood using DAB are also reliant on visual inspection). Even the controls became as equally 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 do indicate, however, that ABTS is at least as sensitive as DAB, which is confirmed by a previous study (14).

Crystal Structure of ABTS

A crystal structure of ABTS was obtained just prior to publication of the first ABTS crystal structure by Mousty et al. (15). It was felt, however, that it was still worth reporting the crystal structure determined in this study, which compares favorably with that of Mousty et al. (15).

Formula: $C_{18}H_{28}N_6O_8S_4$
Mr = 584.70
Crystal class = monoclinic
Space group = $P2_1/c$
Unit cell dimensions: a = 8.7066 (2) Å, b = 36.7362 (9) Å, c = 8.2111 (2) Å
β = 97.103 (1)°
U = 2606.1 (1) $Å^3$
Z = 4
F(000) = 1224
$\mu(M_0\text{-}K_\alpha)$ = 0.419 mm^{-1}
D(calc) = 1.490 g cm^{-3}
Transmission factors: 1.000, 0.913

A total of 15 080 reflections at $2\theta = 53°$ were collected at 203(2) K, of which 5272 were independent [R(int) = 0.0321].

The structure was solved by direct methods and routinely developed. The asymmetric unit consists of two independent halves of two centrosymmetric molecules (Fig. 5). All nonhydrogen atoms were anisotropic, the aryl and ethyl protons were included in calculated positions, but the NH_4^+ and H_2O protons were located from difference maps and included in the refinement without constraint.

The refinement based on F^2 (SHELXL-97) converged with R_1 = 0.0425 for 4198 data with $I > 2\sigma(I)$, 0.0612 for all data; wR_2 = 0.0933 and GooF = 1.099. After the final least-squares refinement the final difference map showed no peaks or troughs greater than ± 0.34 e$Å^{-3}$.

Price Comparison

2,2′-Azino-di-[3-ethylbenzthiazolinesulfonate(6)] diammonium salt (ABTS) can be purchased from Aldrich Chemical Company in 1 g quantities for U.S.$26.23. Alternatively, ABTS diammonium salt can be purchased from Boehringer Mannheim (BM) Laborato-

TABLE 5—*Determination of the sensitivity of ABTS and DAB using spot tests on filter paper (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}
ABTS	0.1 *M*	+	+	+	−	−	−
	0.05 *M*	+	+	+	−	−	−
	0.025 *M*	+	+	+	−	−	−
DAB	0.1 *M*	+	+	+	+	−	−
	0.05 *M*	+	+	+	−	−	−
	0.025 *M*	+	+	−	−	−	−

TABLE 6—*Determination of the sensitivity of ABTS 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}
ABTS	0.1 *M*	+	+	+	−	−	−
	0.05 *M*	+	+	+	−	−	−
	0.025 *M*	+	+	+	−	−	−
DAB	0.1 *M*	+	+	+	−	−	−
	0.05 *M*	+	+	+	−	−	−
	0.025 *M*	+	+	+/−	−	−	−

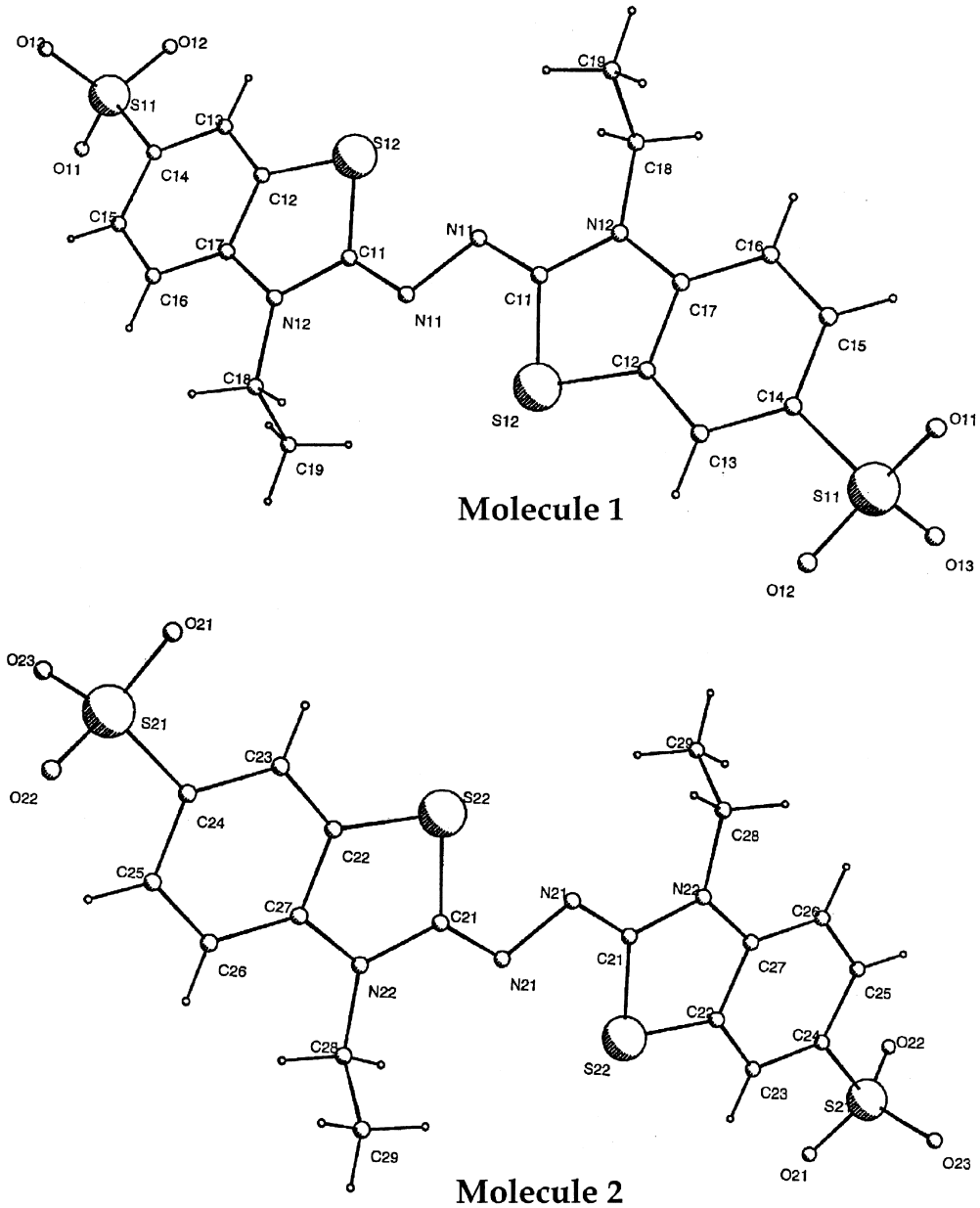

FIG. 5—*Crystal structure of 2,2′-azino-di-[3-ethylbenzthiazolinesulfonate(6)] diammonium salt (ABTS).*

ries in 2 g quantities for U.S.$17.46 (1996 NZ price listings). 3,3′-Diaminobenzidine can be purchased from Aldrich Chemical Company in 25 g quantities for U.S.$122.39. All Aldrich Chemical Company prices are from the Australian 1998–99 price listings (prices converted by Australian/U.S. exchange rate).

The price per 1 g of ABTS (used to prepare 200 mL of ABTS working solution) when purchased from Boehringer Mannheim (BM) Laboratories in New Zealand will therefore cost U.S.$8.73. The price per 0.2 g of DAB (used to prepare 200 mL of DAB working solution) will cost U.S.$0.98.

Summary and Recommended Procedure

ABTS is an effective, noncarcinogenic alternative to DAB for development of blood fingerprints, especially on porous surfaces. Prints developed with ABTS are bright green in color, which show up more clearly than the dark brown of oxidized DAB on certain surfaces. ABTS is a "nothing-to-lose" reagent, because subsequent DAB treatment after ABTS treatment is as good as DAB treatment by itself. Although ABTS treatment can be used after ninhydrin treatment of any latent prints, ninhydrin treatment cannot be used after ABTS treatment. The effect of ABTS or DAB treatment on subsequent DNA analysis is equally detrimental, although further research is probably required for confirmation of this. On the negative side, ABTS treatment is approximately ten times more expensive than DAB treatment; however, it is still relatively cheap in the context of laboratory operations. The structure of ABTS as the diammonium salt has been determined by XRD and confirmed in this work as consisting of two independent halves of two centrosymmetric molecules with the inclusion of two water molecules of hydration.

The recommended procedure for best visualization of fingerprints deposited in blood using ABTS is provided 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)

Dissolve 71.64 g of $Na_2HPO_4 \cdot 12H_2O$ or 35.61 g $Na_2HPO_4 \cdot 2H_2O$ 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. Transfer the solution to a labeled laboratory bottle with a screw top. 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. Transfer the solution to a labeled laboratory bottle with a screw top. Measure out 223 mL $Na_2HPO_4 \cdot 12H_2O$ or $Na_2HPO_4 \cdot 2H_2O$ 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.

ABTS Working Solution

Completely dissolve 1.25 g of ABTS in citric acid/phosphate buffer solution (pH 5.4) in a 250 mL volumetric flask. Invert a few times to ensure that all ABTS is dissolved. Transfer to a labeled, laboratory bottle with a screw top. Store in fridge (away from light) for up to a week.

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 ABTS, working 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 the solution for 3 min. 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 exhibit to be treated.
- Saturate the filter paper with activated working solution (previously mixed). Keep the paper saturated with the 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.

ABTS-treated fingerprints should be photographed as soon as possible, and preferably within two weeks of development, as noticeable fading in color was observed over a one-month period (developed fingerprints could also be stored in the dark so as to slow down the fading process).

Acknowledgments

We thank the following people for their contributions to this work: Associate Professor Peter Molan for suggesting ABTS, and Mr. Godfrey Shouler and Mr. Marc Barlow from the Fingerprint Department at the Hamilton Police Station for their expert advice on comparison of fingerprints. One of us (JPC) also acknowledges financial support in the form of a doctoral scholarship received from the University of Waikato.

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