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POLICE SCIENCE

SIMPLIFIED PRELIMINARY BLOOD TESTING An Improved Technique and a Comparative Study of Methods*

Morris Grodsky, Keith Wright, and Paul L. Kirk

Paul L. Kirk, Professor of Biochemistry and Criminology, University of California, has undertaken a series of studies on blood tests. This is the first report on these studies. With the importance of accurate identification of blood stains in criminal investigation, we are pleased to publish this important paper which develops new field techniques and evaluates the various tests when used in combination.

Assisting Dr. Kirk with this study are Morris Grodsky, a graduate student in Criminology, and Keith Wright, a recent graduate of the University of California.

—EDITOR.

The location of faint and invisible blood stains and the preliminary identification of visible stains have long been performed by means of the benzidine test, supplemented less frequently by other catalytic color tests such as the leuco-malachite green and the phenolphthalin tests, and the luminescent 3-aminophthalhydrazide (luminol) test. Studies of the various tests have shown that there is a degree of interference with some of them that effectively prevents their use as proof of the presence of blood. Pinker (1), particularly, has made an excellent study of the substances which interfere with the three color tests listed. Other investigators also list such interferences, and we have extended this study somewhat. Less is known of the materials interfering with the leuco-malachite green test (2) or with the phenolphthalin test (3) than with the benzidine test. Specht (4), who originated the luminol test for blood, claimed a virtual lack of interference with it. This assertion was essentially confirmed by Proescher and Moody (5) who also believed it to be completely specific for blood when properly performed. That such is clearly not the case is shown by the similar use of luminol as a test for copper ions (6) and other uses of a similar nature (7). It is probably true that materials interfering with the benzidine test do not interfere with the luminol test and vice versa. It can also be shown that the phenolphthalin test is relatively specific for blood, and that the leuco-malachite green test also shows less interference than does the benzidine test. It can be further shown that materials interfering with the phenolphthalin, benzidine, and luminol tests are different in nature in all cases so far tested.

Another important consideration which has been treated less completely than is desirable in the literature is the absolute and relative

* Aided by a grant from the Research Board of the University of California.

sensitivity of the tests. The quoted sensitivity of the benzidine test is quite variable, ordinarily being given as about 1 in 300,000. In most statements of the sensitivity, the conditions of determination are omitted completely. It is clear that a dried blood stain is not comparable with the same amount of blood dissolved in a solution, and many of the discrepancies reported are probably due to the fact that in some instances the reagent is added to a dilute solution of the blood, thus also diluting the reagent, while in others the blood is dried first and tested with full strength reagents. Even more important, the sensitivity of the reagent is greatly influenced by its composition. Some comparable results of sensitivity tests are reported in this paper.

Most investigators restrict themselves largely to the benzidine test, partly because it is well known and simple to apply. This is unfortunate because it is unquestionably inferior in several respects to all of the other tests discussed here. Reluctance to utilize the better tests may be due in part to unfamiliarity with the tests and in part to technical difficulties in obtaining exactly the correct reagent concentrations. That this is not a negligible difficulty follows from the fact that all of them require the use of hydrogen peroxide in a carefully determined concentration. This reagent is quite unstable, and it is almost always impossible to determine the exact reagent concentration without prior analysis. For this reason, two sets of blood tests run with what is presumed to be identical reagents often yield quite diverse results. It will be shown in this paper how this difficulty can be conquered through utilization of sodium perborate as a substitute for peroxide in all of these tests. Sodium perborate has been employed frequently with the benzidine test (8). So far as can be ascertained, it has not been utilized frequently with the other color tests or with the luminol test.

EXPERIMENTAL

Study of the mode of preparation of reagents in such a manner as to simplify their use will be described first. It is easily possible to adjust the solid reagents so that they may be packaged in capsules in predetermined amounts and proper ratios. The mixed or simple solids are added to the correct amount of the solvent and dissolved to yield a reagent ready for use and of reproducible sensitivity. This is especially useful for field application because it involves no measurements or adjustments, nor is it ordinarily necessary to test the sensitivity at the time of use. All reagents described have been tested for stability and found to remain as sensitive as they would if stored in a bottle in the laboratory. The solvent, water or acetic acid, is carried separately

and may be measured approximately by filling a container to a predetermined line or other mark. The details of the reagent quantities follow.

Benzidine Test. As stated previously, sodium perborate has been used and described for use with the benzidine test in several publications. It does not appear to have been added to the benzidine in a uniform mixture and stored in predetermined quantities in the dry state. With proper mixing of weighed quantities, the amounts of the reagents can be standardized, and the constant uncertainty as to the strength of hydrogen peroxide is eliminated. The reagent is made with the following composition based on a 10 ml. quantity: Sodium perborate, 0.2 g. and 0.1 g. benzidine, added to 10 ml. of glacial acetic acid.

The use of benzidine dihydrochloride solution as a substitute for a solution of benzidine in glacial acetic acid as employed by many investigators (9) may also be followed but with loss of some of the advantages of the reagent described above. Because of the very slow rate of solution of the benzidine dihydrochloride, the solution must be made in advance and carried as such instead of transporting the solvent alone. A suitable reagent is prepared as follows: A 1 per cent aqueous solution is prepared by shaking the proper quantity of benzidine dihydrochloride at intervals of about 2 hours over a period of 1 to 3 days. The solution is filtered and stored in a dark bottle. For field use, 10 ml. of the reagent is carried in a suitable small bottle, and before use 0.2 g. of sodium perborate is added and dissolved, followed by 0.1 g. of sodium acetate crystals. Two capsules of dry reagent are required as well as the stock solution. The latter is stable for at least 3 months when properly stored but is not as stable as the dry benzidine. It is, therefore, much more difficult to utilize for field investigations which may be necessary at irregular and unpredictable intervals.

Leuco-Malachite Green Test. The use of perborate with leuco-malachite green does not seem to be recorded in the literature. Basically, the reaction is in every way similar to the benzidine test in that the reagent is oxidized by peroxide or perborate under the catalysis of the hemoglobin. An entirely satisfactory reagent is made as follows: Leuco-malachite green (4,4'-tetramethyldiaminotriphenylmethane), 0.1 g. and 0.32 g. sodium perborate mixture are dissolved in 10 ml. of a 2:1 mixture by volume of glacial acetic acid and water.

Phenolphthalin Test (3). The reagents for this test are not ordinarily made in such a manner as to be suitable for preparation at the scene from dry reagents. That it is possible to do so will be shown. It is, however, less satisfactory in terms of sensitivity and convenience. For

this reason, both the excellent method described by Pinker (1) for making the reagents and a modification to use in the dry state will be described. In either case, the very high sensitivity and comparative specificity of the reagents recommend them for general use. The standard method of preparing the stock solution is as follows: To 200 ml. of water is added 20 g. of sodium or potassium hydroxide and 2 g. of phenolphthalein. The solution is heated to boiling, and zinc powder is added in a quantity sufficient to keep a good excess present at all times. Boiling with excess zinc is continued until the reagent is completely free of red color. The stock solution is stored over a little powdered zinc.

To make the reagent for use, 0.14 g. of sodium perborate is added to 10 ml. of the stock solution. This reagent is the most satisfactory one so far described for laboratory use. It may be employed in the field only by carrying the solution, freshly removed from the stock bottle, along with the proper dry reagent in a capsule. The reagent becomes quite rapidly tinged with pink unless a little zinc is added to the bottle. The resulting slow generation of hydrogen gas makes storage in a sealed container impractical for extended periods.

A modification which may be employed for convenient field use is as follows. The stock solution prepared as above is chilled in a refrigerator and to it is added cold glacial acetic acid in the proportion of 1 part acid to 5 parts of stock. A dense flocculent precipitate of the phenolphthalin is formed. It is filtered as rapidly as possible through a chilled Buchner funnel and dried. The dry powder is suitable for transport in a field kit. The reagent to be placed in capsules is made as follows: Phenolphthalin powder, 10 mg., and 40 mg. sodium carbonate are placed in one capsule. To a second capsule is added 0.1 g. of sodium perborate. The contents of the perborate capsule are added first to 10 ml. of water and dissolved, followed by the contents of the phenolphthalin capsule. The resulting reagent is far less sensitive than the stock reagent described above, but is highly specific and reasonably convenient to use.

Luminol Test. No record has been noted of the use of perborate with the luminol reagent. Its adoption is quite advantageous because this reagent must be made fresh immediately before use and applied as rapidly as possible. The sensitivity and stability of the reagent are highly dependent on the concentration of oxidizing agent which is quite difficult to control with hydrogen peroxide. Too little of the peroxide gives an insensitive reagent, while too much leads to rapid luminescence in the reagent itself. A suitable reagent is made as follows: Sodium perborate, 0.07 g. is completely dissolved in 10 ml. of water. To this

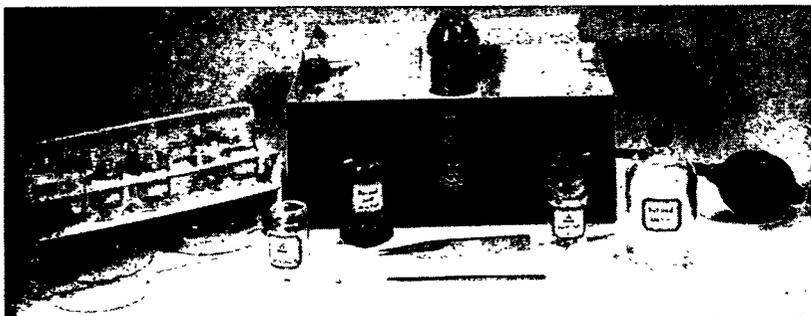


Figure 1
Blood Test Kit

is added a mixture of 0.01 g. 3-aminophthalhydrazide and 0.5 g. of sodium carbonate which is dissolved with stirring or shaking. The reason for the addition in this order is that sodium perborate dissolves only slowly in sodium carbonate solution, and better in water, while the 3-aminophthalhydrazide dissolves rapidly only in sodium carbonate solution. If all solids are added simultaneously, the rate of solution is slow, and autoluminescence may develop in the solution before the last of the solid is dissolved. If the reagent is sprayed before all the solid is in solution, the spray will be plugged.

Field Kit. Advantage of the use of dry reagents was taken by constructing a small but complete field kit for blood testing.* the kit was assembled in a metal box about 9 inches in length by 5 inches in breadth and 4 inches high. A rack allowed storage of a series of small plastic capped bottles of uniform size in which solvents and capsuled dry reagents could be carried and reagents mixed. Accessories included plastic and glass sprays used directly with the bottles, filter paper for removal of blood stains by soaking, a larger reserve bottle of distilled water, a flash light to allow mixing of luminol reagent in the dark, and plastic containers for storing and transporting transferred blood stains or other small evidence. The complete outfit is shown partially disassembled in Figure 1. All tests described here could be conveniently carried out in the field with this kit without any measurements being required. All reagents were premixed and placed in capsules which are commercially available in correct amounts and proportions. The kit is not only convenient for use in the field, but may be immediately adapted for laboratory use by removing the rack and its contents to the laboratory table where all bottles and accessories are compactly stored and kept in order. The unique advantages of the system are its

* Available from Microchemical Specialties Co., Berkeley, California.

convenience and the complete reproducibility of tests without the necessity of pretesting each reagent in advance.

Sensitivity of Reagents. As mentioned previously, the statements regarding sensitivity of the various catalytic oxidative blood tests in the literature are usually ambiguous and in many cases do not agree. For this reason, tests were run in a completely comparable manner with the four reagents discussed. Blood was diluted to various low concentrations with distilled water to a final dilution of 1:5,000,000. The tests were made in the depressions of a porcelain spot plate with the solutions. Because the luminol reagent is normally added by spraying, this reagent was applied to filter paper soaked with the diluted blood and not allowed to dry. It is realized that this does not duplicate the conditions of use in that dry blood stains ordinarily are being tested. However, it will lead to an increasing concentration of the blood and should give higher rather than lower sensitivity for the reagent. In separate tests, this was found to be essentially correct, at least insofar as the dry stain from the very dilute solutions always gave at least as strong a test as when diluted. Further, the age of the blood is a factor since old blood reacts more strongly with luminol reagent than does fresh blood.

The results of the tests were not entirely uniform, apparently because of some slight aggregation of clotted pigment with some samples which then gave tests in a slightly higher dilution than samples in which such aggregates were absent. Table I shows the highest dilutions at which definite positive tests were obtained with the four reagents under the conditions stated.

Table 1
Sensitivity of Blood Tests

<u>Test</u>	<u>Dilution of Blood</u>	<u>Time for Positive Reaction</u>	<u>Duration</u>
Benzidine (in glacial acetic acid)	1:300,000	10 seconds	10 min.
Benzidine	1:100,000	1 second	
Leuco-Malachite Green	1:100,000	15 seconds	7 min.
Phenolphthalin (original stock)	1:100,000	3 seconds	30 min. or more
	1:5,000,000	20 seconds	
Phenolphthalin (dry reagents)	1:10,000	20 seconds	10 min.
Luminol	1:5,000,000*	5 seconds	15 min. or more

*Proescher and Moody (5) give a sensitivity of 1:100,000,000 for decomposed blood. They recommend preliminary spraying with hydrochloric acid to decompose hemoglobin and increase the sensitivity of the test.

The data of Table I shows clearly the advantage in terms of sensitivity of the phenolphthalin and luminol tests as compared with the more commonly employed benzidine, and the leuco-malachite green test. The luminol test has the added advantage that it may be easily applied to original stained surfaces, while with the color tests it is preferred technique to soak off the suspected stain on filter paper wet with saline solution. The reagent is then added only to the paper. The spraying of a surface, e.g., an automobile or the walls and floor of a room, with the luminol reagent does not damage the surface nor does it interfere with subsequent blood tests of other types or with precipitin tests or grouping reaction. It is for this reason and because of its high sensitivity, by far the most advantageous known test for preliminary search for occult blood. Its only serious disadvantage, other than interference, is the fact that it must be applied in the dark in order to perceive the chemilluminescence.

From the standpoint of sensitivity and specificity, the phenolphthalin test is also the most useful follow-up test because it can be expected to detect blood in amounts not perceptible by either the benzidine or the leuco-malachite green tests.

Interference. Because of interference commonly possible with certain of the color tests for blood, notably the benzidine test, most authors list such tests as "presumptive" or "preliminary." It is generally agreed in practice that much of the emphasis placed on such interference is a ruse of defendants and their attorneys to avoid the consequences of these tests. However, it must be admitted that the benzidine test is subject to definite interference from a number of materials, notably those that are high in peroxidase content. With few exceptions, such false positive tests are fainter and more fleeting than with blood and can ordinarily be distinguished from the latter by the experienced investigator. Some exceptions must be noted to this also. Horse-radish, for example, gives a benzidine test almost as strong, rapid, and persistent as does blood. False positive tests are given with the benzidine reagent by some fruits such as the white pulp (but not the juice) of oranges, lemons, certain melons, and persimmons. Apples, berries, grapes, pomegranates, and olives do not give any test within 10 seconds. The inner part of the skin and network of fibers of the tomato give a positive benzidine test, as does the flesh of the carrot, radish, and turnip. The reticulated structure of the lettuce leaf gives the benzidine test, but the main fleshy portion of the leaf does not. The almond gives a test when the reagent is applied directly to the kernel. Beets, tobacco, dry leaves, milk, urine, perspiration, saliva, semen, and many other possible

materials do not give such tests. Pinker (1) notes few biological materials which interfere and mentions the ease of avoiding such interference by proper procedures. More important was his finding that certain fairly uncommon chemicals interfered with not only benzidine but with the leuco-malachite green and phenolphthalin reagents. For the benzidine reagent, these were ammonium persulfate, calcium phypochlorite, gold chloride, lead peroxide, potassium dichromate, potassium permanganate, sodium chromate, and certain lead oxides. *Of 95 chemicals tested by Pinker, not one was found which gave true positive tests with all three of the color reagents.* Fifty common sources of stains, including many food materials, dyes, pharmaceuticals, and physiological materials other than those containing blood, were tested and none of them produced positive tests with all three reagents, nor did any considerable number produce tests with any of the reagents. Phenolphthalin, because it is in basic solution as contrasted with the other reagents, showed no interference from those materials that cause most trouble with benzidine and leuco-malachite green. Highly significant was his failure to locate even one common material that gave a true positive reaction with phenolphthalin reagent, a result confirmed by extensive search for such interference in the author's laboratory. Every available material known to interfere with the benzidine reagent has failed to react with the phenolphthalin reagent with the exception of solid horse-radish which gave a transient color easily distinguishable from a true blood test. Extract of horse-radish failed consistently to yield any test.

Pinker did not claim that positive tests with all three color reagents constituted proof of blood, but proved that if there is any substance which can be mistaken for blood with all three, it remains to be discovered. Were all evidence offered in the courtroom as satisfactory at this, the work of the criminalist would be considerably simplified!

No test was made by Pinker of the luminol reaction with respect to interference. Extensive tests made by Specht (4), Proescher and Moody (5), and others indicate similar findings. In general, no interference is encountered from common sources of stains other than blood though fresh potato juice has been listed as a possible source of false positive reactions. Certain uncommon chemicals are listed as causing interference (7). Our experience with this reagent indicates that it has never yielded a positive reaction with a dry stain of common materials other than blood with one exception, viz., copper salts. Luminol has been listed as a reagent for the detection of copper salts (6). We find that it reacts strongly on brass, bronze, and similar copper alloys which include most door handles, locks, valves, and numerous other

metal objects that might well be contaminated with blood. This finding is in contradiction of other statements in the literature (5), but has been uniform and almost invariable. Copper containing materials such as those listed have not been found to react with any of the color reagents. Luminol has failed to react on any metal free of copper. Aside from the remarkable utility of luminol in the preliminary discovery of occult blood stains, this reagent is both highly specific and highly sensitive. It does not interfere with any other test made subsequently, and it allows rapid survey of large areas, a most important advantage. Used in conjunction with the color tests, it appears that the combination of these four preliminary blood tests yields stronger proof of the presence of blood than is true of almost any other set of testing methods available for criminal investigation. It is time that this fact be recognized by experts and jurors alike, and that an end be put to intimidation by attorneys who have "read the books." It is possible that some material not contaminated by blood may sometime be found that will give definite positive reactions with all of the four tests discussed. It is also possible that a gun may be found sometime that will accurately reproduce the markings of another gun, or that duplicate fingerprints may sometime be found. The fact that none has been found to date is the best proof of the significance of firearm and fingerprint evidence. Why, then, should we continue to insist that "preliminary blood tests" may not be used to produce proof of blood when properly used? Certainly, in spite of the continued and extensive search already made, there is no case on record in which any substance other than blood has given positive reactions with the four color and chemilluminescence tests described, nor is there any significant probability that this will ever occur if the tests are made by experienced personnel, by proper technique, and with application of both alkaline and acid reagents.

It is not suggested that color and chemilluminescence tests should replace crystal or spectroscopic tests which are considered specific for blood when the amount of blood available is sufficient for their application. It is proposed that positive and virtually conclusive results may be obtained when the amount of blood is below the visible range as often occurs, and that the ease and rapidity of obtaining these results may save much effort expended in attempting the "confirmatory" tests when conditions are not favorable for them. It is obvious that neither type of test can substitute for precipitin tests for species determination since all hemoglobin-containing bloods react similarly with both the "preliminary" and "confirmatory" tests, which show presence of blood only, with no indication of source, either by species or by groups.

SUMMARY

There is described a simple blood test kit which eliminates most of the liquid reagents normally used for preliminary blood testing and simplifies and standardizes the method of making reagents so as to obtain reproducible conditions under either field or laboratory conditions.

A comparative study of sensitivity of the three most common blood color tests and the chemilluminescence test for blood is presented. It is found that the phenolphthalin and the luminol tests are of approximately equal sensitivity and much greater than the other color tests studied. Consideration of the question of specificity of the various tests is given along with certain experimental results and the suggestion is made that the four tests described, properly applied, can virtually eliminate the necessity of running confirmatory crystal tests for the presence of blood.

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