

BLOOD DETECTION BY CHEMICAL METHODS

The presence or absence of blood stains often provides important information for those investigating criminal cases. For this reason, forensic scientists are often called on to determine whether or not a particular stain is blood, and if so, whose. The detection of blood is usually based on one of three classes of methods.

Crystal tests

Haem forms crystals when reacted with certain reagents. The most common such reagent is pyridine, which forms characteristic pink crystals.

Catalytic tests

These tests rely on the fact that haem can catalyse the breakdown of hydrogen peroxide. As the H_2O_2 breaks down, another substance in the reaction mixture is oxidised, producing a colour change. It is important to note that a positive test does not mean that a given stain is blood, let alone that it is human blood, as various enzymes and certain metals can also give positive results.

Instrumental methods

Chromatography can be used to identify the presence of haemoglobin.

These tests are used practically for several different purposes. These include both the confirmation of the nature of visible stains (i.e. that they probably are or definitely are not blood), the detection of non-visible stains (e.g. on plants or washed clothing) and the enhancement of hard to see stains. Stain enhancement is useful for situations where a footprint, handprint, fingerprint etc. is faintly outlined in blood, as chemical methods can enhance that stain so that the print can be measured and matched with suspects. In all of these tests it is important to ensure that the chemical reactions do not prevent later tests being done to help to identify who the blood belongs to.

INTRODUCTION

Forensic scientists are often asked to determine, both in the field and in the laboratory, whether a particular stain is or is not blood. This is a surprisingly difficult question to answer with certainty.

This article describes some of the approaches which have been taken to answering this question over the years.¹ The discussion is confined to chemical methods and therefore does not consider biological methods such as antigen - antibody reactions. The biological methods are generally slower than chemical methods but more specific. One of the requirements of forensic science is for methods which can be used in the field, and generally only chemical methods possess sufficient speed for this.

CHEMICAL METHODS USED TO DETECT BLOOD

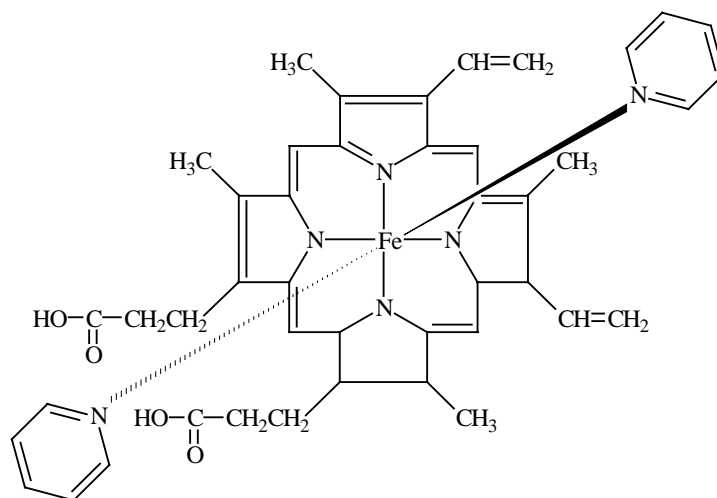
Chemical methods can be divided into 3 categories:

- crystal tests
- catalytic tests
- instrumental methods

All of the methods are in some way dependent on the presence of haemoglobin, and will therefore give positive results for both animal and human blood.

Crystal Tests

The crystal tests, which are now rarely used, are all based on the formation of haemoglobin derivative crystals such as haematin, haemin and haemochromogen. The test is carried out on a microscope slide, with the reagents being added to the stain under a cover slip, and crystal formation observed microscopically. Probably the best known of the crystal tests is that developed by Takayama about 80 years ago.² An alkaline solution of pyridine is added to the stain and, if blood is present, pink crystals of a complex between pyridine and haem form as the slide is warmed. The structure of the complex is shown in **Figure 1**. As well as pyridine, a number of other nitrogenous bases, including nicotine, methylamine, histidine and glycine have been used in variations of this test.

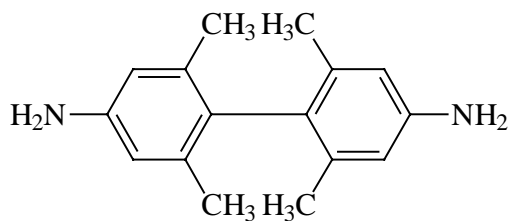


**Figure 1 - Pyridine ferroprotoporphyrin
(the complex formed in the Takayama test)**

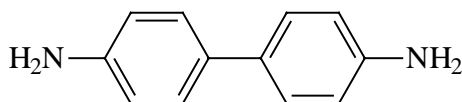
It is generally accepted with the crystal tests that a positive result confirms the presence of blood. The sensitivity is about 0.001 mL of blood or 0.1 mg of haemoglobin. A negative result does not necessarily show that blood is absent - it may, for example, indicate faulty technique - and a positive control should always be run for comparison. Bloodstains up to 20 years old have given positive results in crystal tests.

Catalytic Tests

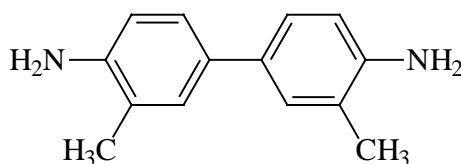
These methods depend on the fact that the haem group of haemoglobin possesses a peroxidase-like activity which catalyses the breakdown of hydrogen peroxide. The oxidising species formed in this reaction can then react with a variety of substrates to produce a visible colour change. Among substrates in common use are benzidine and various substituted benzidines, ortho-tolidine, leucomalachite green, leucocrystal violet and phenolphthalein - the last of these being known as the Kastle-Meyer test. The reaction with 3-amino-phthalhydrazide (luminol) to form a luminescent rather than a coloured product is also a catalytic test. A derivative of ortho-tolidine is used in the "Sangur" test sticks manufactured by Boehringer Mannheim. These are intended for the detection of blood in urine in clinical situations but are equally useful as a screening test for dried bloodstains.



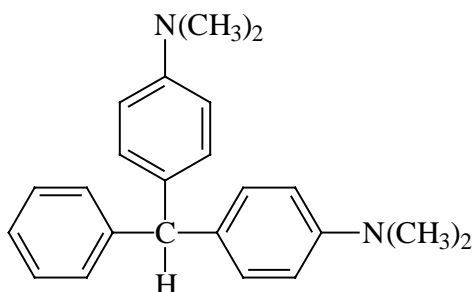
Tetramethyl benzidine



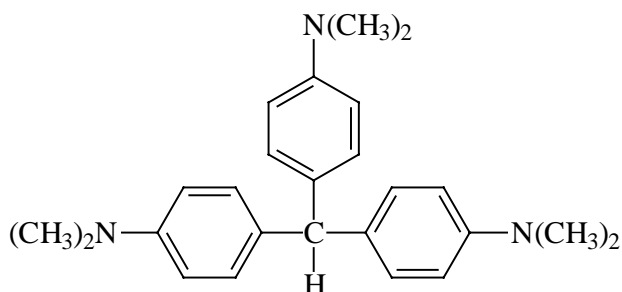
Benzidine



Ortho-tolidine



Leucomalachite green



Leucocrystal violet

The catalytic tests are extremely sensitive (blood can be detected to dilutions of about 1 in 100,000), but are subject to a number of interferences and are therefore not totally specific for blood. Substances which can interfere include enzymes such as catalase and peroxidases (which can occur in both plant and animal materials), oxidising chemicals and metals - in particular copper and iron. There has to be an awareness of this when results are interpreted, particularly when testing outdoors, where many types of plant material can be present, or testing in vehicles, where metal surfaces can interfere. The general principle is that if the test is negative, blood is absent, but that if the test is positive, blood is probably, not definitely present. For this reason the tests are often described as "presumptive" tests.

An interesting example of possible interferences occurred with the testing of the car belonging to Michael and Lindy Chamberlain after the disappearance of their daughter Azaria at Ayer's Rock, Australia, in 1980. The Chamberlains lived in Mt Isa, a copper mining town, with a high concentration of copper-containing dust in the atmosphere. The car was later tested for the presence of blood with *ortho*-tolidine. Some positive results found were subsequently attributed to the presence of copper.³

Instrumental methods

High performance liquid chromatography (HPLC) can be used to confirm the identity of blood using the absorbance of haemoglobin for detection. This method can also be used to identify the species of origin from variations in the globin chains⁴, to distinguish foetal haemoglobin from adult haemoglobin⁵, and to give an estimate of the age of a bloodstain⁶.

APPLICATIONS

Confirmation that visible stains are (probably) blood

This is largely carried out using either the "Sangur" sticks mentioned earlier, or using the Kastle-Meyer test. The Sangur sticks, in which the detecting reagent is in an immobilised form, require only to be rubbed gently on the stain and moistened. An immediate change in colour from pale yellow to an intense greenish blue indicates the probable presence of blood. The test is very sensitive but because of the way it is set up is not easily modified to check for possible interferences.

In the Kastle-Meyer test the reduced phenolphthalein is kept in alkaline solution in the presence of zinc. This solution is colourless. Oxidation with haemoglobin and peroxide causes an instant colour change to the well known bright pink. **Figure 2** shows the reaction. The test was originally used in one step, but many of the potential interferences can be eliminated by carrying it out in two steps.

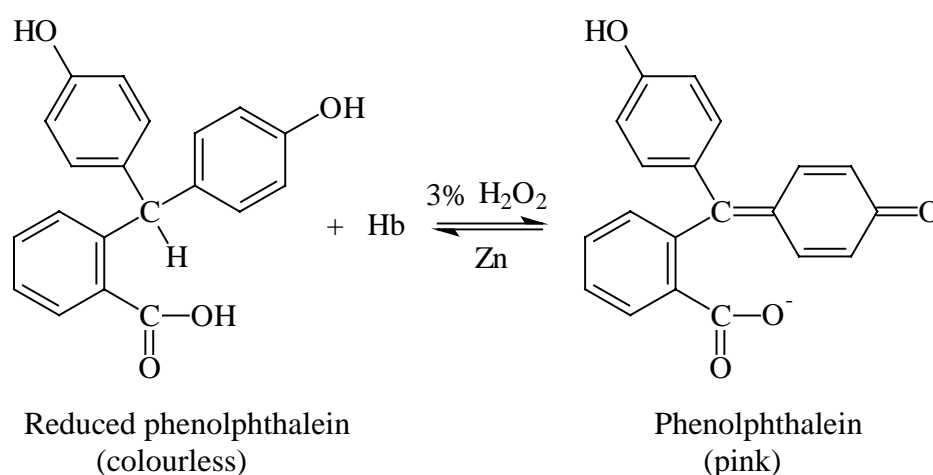


Figure 2 - Oxidation of reduced phenolphthalein by haemoglobin and peroxide

In the original form, a small amount of the Kastle-Meyer reagent as prepared is mixed with equal volumes of 95% ethanol and 10% hydrogen peroxide solution. The suspect stain is rubbed gently with a small piece of filter paper and a drop of the mixed reagent added to the paper. The development of a pink colour is indicative of the presence of haemoglobin, which has catalysed the breakdown of hydrogen peroxide to an oxidising species. However, used in this form, the test will give an apparently positive result with other oxidising materials.

In the 2-step version of the test, the Kastle-Meyer reagent is mixed only with an equal volume of 95% ethanol. This solution is first added to the stain on the filter paper. If a pink or red colour develops at this point, that is without the addition of hydrogen peroxide, the stain in question is not blood. If there is no reaction at this point, a drop of hydrogen peroxide solution is added, and the presence of a pink colour indicates the likely presence of blood.

A sample giving a positive result in both the Sangur and Kastle-Meyer tests would be reported as probable blood. Unless a positive result was subsequently obtained with a biological test known to be human-specific, the presence of blood could not be confirmed. Tests which could be used for confirmation would include antigen-antibody reactions such as Ouchterlony double diffusion, the presence of an enzyme such as alpha-2-HS-glycoprotein known to be human specific, or the presence of a DNA sequence specific to humans.

Detection of non-visible bloodstains.

This has traditionally been carried out using luminol. Luminol's major application is in areas where blood may be present but is difficult to see, such as outdoors among vegetation, or where attempts have been made to clean up blood and traces are still present. A positive reaction can also sometimes be given by bloodstained clothing which has been washed.

Luminol is made up in alkaline solution (pH 10.4-10.8) using sodium carbonate, and sodium perborate ($\text{NaBO}_3 \cdot \text{H}_2\text{O}$) rather than hydrogen peroxide is used as the source of the oxidising species. Hydrogen peroxide can be used but yields a shorter-lived luminescence than sodium perborate. The solution is applied as a spray and the presence of blood produces a bluish luminescence which persists for about 45 seconds. The luminescence can be restored by additional spraying but this needs to be done carefully as the stain will lose definition if too much liquid is added to it. The luminescence can be photographed in either black and white or colour but requires some specialised techniques. **Figure 3** (adapted from reference 8) outlines the chemistry of the reaction.

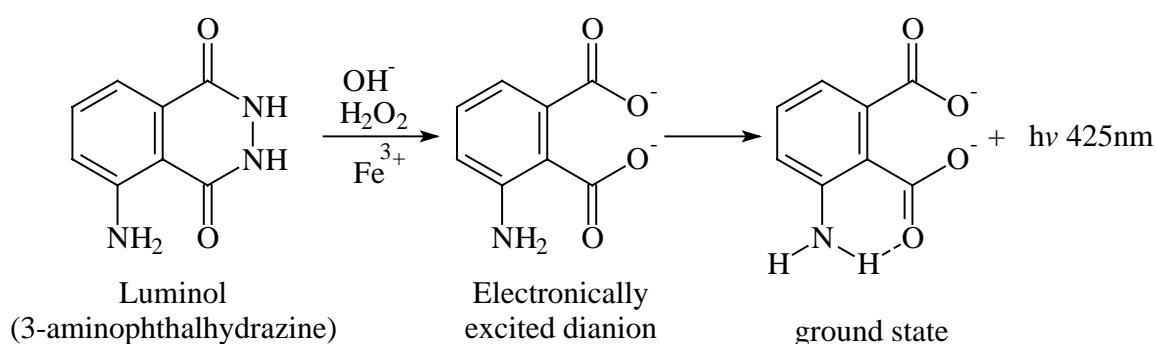


Figure 3 - The reaction of luminol

As with other catalytic tests, luminol is not specific for blood and can also give a positive reaction with some plant enzymes, oxidising agents and metals. An experienced user of luminol can distinguish these reactions from those given by blood by the colour of the luminescence, how long it persists for, and in the degree of "sparkle" of the luminescent product. Blood tends not to sparkle, but produces a steady luminescence, whereas some metals tend to give a definite sparkling luminescence.

Luminol needs to be used with care as there are uncertainties relating to its safety. Although it is described in some literature as non-mutagenic, its structure suggests that non-mutagenicity or non-carcinogenicity cannot be assumed. In addition, some users find the other contents of the solution irritating if the spray is inhaled during use.

Enhancement of blood stains

A further area in which blood detection reagents have proved useful has been in the enhancement of existing bloodstains. In cases of partial shoeprints or fingerprints in blood there is often more of the print present than can be seen, and treatment of the print with a chemical which reacts with blood can often produce a much more detailed print. This can then be photographed and subsequently compared with a suspect's shoe or fingerprint. Luminol has been used for this purpose but, as noted earlier, detail can be lost by excessive spraying of the stain, and photography is often difficult.

Leucocrystal violet is now being used extensively for shoeprint enhancement with considerable success. Its major disadvantage is that the stains are indelible, so it cannot be used in situations where the surface is required to be left clean. It is extremely easy to use, as a light spray with the reagent solution produces a purple stain almost immediately. This is very easily photographed. There are a number of other reagents such as amido black which can also be used for shoeprint enhancement. Some of these are specific for protein rather than for blood.

Subsequent reactions of stains treated with blood detecting reagents

Once it is determined that a stain is probably blood, the next question one asks is "whose?" It is therefore necessary to ensure that the presumptive tests for blood do not interfere with subsequent tests used to "type" or "group" the blood. The Sangur or Kastle-Meyer tests use only a small part of the stain and the major part remains for further testing. Problems can arise when an entire stain is treated with a reagent which can affect subsequent tests. It is well known, for example, that the use of amido black on bloodstains removes any possibility of subsequent blood grouping, while stains treated with leucocrystal violet can still be typed in some systems. If typing of the stain is likely to be required then enhancement reagents known not to interfere must be used. Several compilations of such results have been published^{7, 8, 9}. Forensic biology is moving towards almost exclusive use of DNA polymerase chain reaction (PCR) methods for individualisation of blood. Because these methods can work with degraded DNA, it is likely that most current detection reagents can continue in use.

In a recent case, a pair of apparently washed jeans with no visible blood was treated with luminol and showed the probable presence of blood on both knees. The stain from one knee gave a positive human antigen-antibody test (this is unusual with an invisible stain) and subsequently a positive DNA PCR result in one system. This result identified the stain as not being excluded as the blood of an assault victim but excluded it from coming from the owner of the jeans.

Compiled by Dr R V Winchester (ESR:Forensic) with summary box written by Heather Wansbrough