SECTION 4. CRYSTAL TESTS

4.1 Structure and Nomenclature of Porphyrins and Hematin Compounds

The discussion of crystal tests and of spectrophotometric and spectrofluorimetric methods which follows will involve many terms which refer to porphyrin and hematin compounds. The history and development of nomenclature of these materials is somewhat complex and can lead to considerable confusion. Interested readers are referred to the specialized works by Lemberg and Legge (1949), Falk (1963) and Marks (1969). Most standard biochemistry texts also carry a discussion of the subject (e.g. White *et al.*, 1973; Pritham, 1968; Mahler and Cordes, 1971). The present discussion is designed primarily to provide an outline of the nomenclature, and give some indication of the different meanings that may be associated with various terminology.

Porphyrins are derived from the cyclic ring compound *porphin*, a structure with four pyrrole-like rings linked by methylene bridges. Represented in Figure 4.1 in two different ways, the structures (a) and (b) are identical, neither is "correct" or "incorrect", and neither is preferable for any particular reason. Both are encountered in the literature.

The porphyrins which occur in nature are all compounds in which side chains are substituted for some, or all, of the hydrogens at positions 1 through 8 in Figure 4.1. It is convenient in giving structural representations of these compounds to use a "shorthand" notation for the porphyrin nucleus, omitting structural detail, but allowing the different side chains to be shown in their correct positions. Figure 4.2 (a) and (b) shows the shorthand representations corresponding to the structures in Figure 4.1 (a) and (b), respectively.

Introducing side chains into the molecule gives rise to a large number of different structural isomers. If, for example, the eight numbered hydrogen atoms are substituted with four methyl- and four ethyl- groups, four isomers are possible. These are shown in Figure 4.3 according to both "shorthand" conventions, corresponding to Figure 4.2 (a) and (b), respectively. Only compounds deriving from structures I and III, Figure 4.3, are found in nature, those from III being more important. In representing the isomers according to the shorthand notation, it is usual to number the positions as has been done in Figure 4.2. Not all authors number the positions in the same way, however, and while any sequentially numbered system is interconvertible to any other by rotation in the plane of the paper, it eliminates confusion, in my opinion, if the numbering convention is clearly stated. Some of the different, naturally occurring porphyrins are indicated in Table 4.1. Only the type I and type III isomers (Figure 4.3) are given. If the number of types of substituent side chains is increased, then clearly the number of possible structural isomers increases as well. In the case of protoporphyrin, for example, with three substituent types, there are fifteen structural isomers. All the porphyrins derived from hemoglobin and naturally occurring hematin compounds are of protoporphyrin type III, Table 4.1. The molecule is more commonly referred to as protoporphyrin IX, since it was the ninth in a series of isomers listed by H. Fischer (White *et al.*, 1973). Protoporphyrin IX is shown in shorthand notation in Figure 4.4.

Porphyrins possess the ability to combine with many metals, the most important biologically active molecules being those in which the porphyrin is combined with Fe or Mg. These compounds are collectively referred to as metalloporphyrins. The hematin compounds, which are the only metalloporphyrins of major interest to this discussion, are all iron-protoporphyrin compounds. The iron atom in an iron-protoporphyrin complex is coordinated to the four pyrrole nitrogen atoms in a planar configuration (Figure 4.5), replacing the two dissociable hydrogens of the porphyrin nucleus. The detailed properties, including the nature of the coordinate binding and thermodynamic stability, or iron and other metal porphyrins were discussed by Phillips (1963). The iron complexes readily add two additional ligands, which coordinate to the metal forming an octahedral structure and in which the metal is then hexacoordinated.

The valence of the iron atom is specified by the prefixes *ferro-* (for Fe^{2+}) and *ferri-* (for Fe^{3+}) in naming the various compounds. *Heme* is *ferroprotoporphyrin*. *Heme* is spelled *haem* in some countries, the spelling variation carrying over to other terms derived from the word *heme*, e.g. hemoglobin/haemoglobin, hematin/haematin, hemochromogen/haemochromogen, etc. *Ferriprotoporphyrin*, obtained as the chloride, is called *hemin chloride*, or *hematin chloride*. *Ferriprotoporphyrin hydroxide* is simply call *hematin*. The use of the term *hemin* is restricted by some authors to ferriprotoporphyrin halides, especially the chloride, (Lemberg

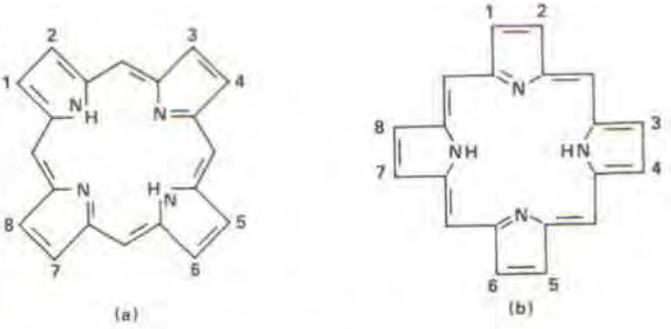


Figure 4.1 Porphin

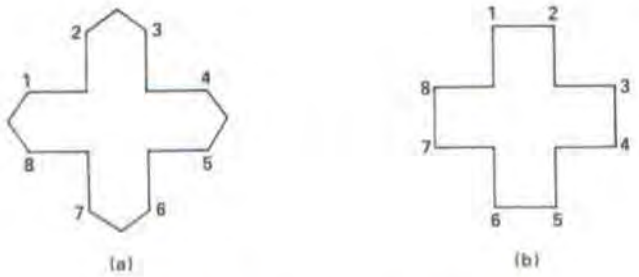


Figure 4.2 Porphin - Shorthand Notation

chromogens, and the terms *hemichrome* and *parahematin* have been applied to *ferrihemochromes*. Table 4.2, a modification of Table I, Chapter V, of Lemberg and Legge (1949), gives a comparison of some of the different nomenclatures.

Another important consideration, which is not really a matter of nomenclature, but which may be worthy of brief discussion here, is that of the interconvertibility of the various hemoglobin derivatives. Both the crystal and spectral tests for the presence of blood in stains rely on these conversions. There are, in addition, a number of methods designed to determine the age of bloodstains which rely on the

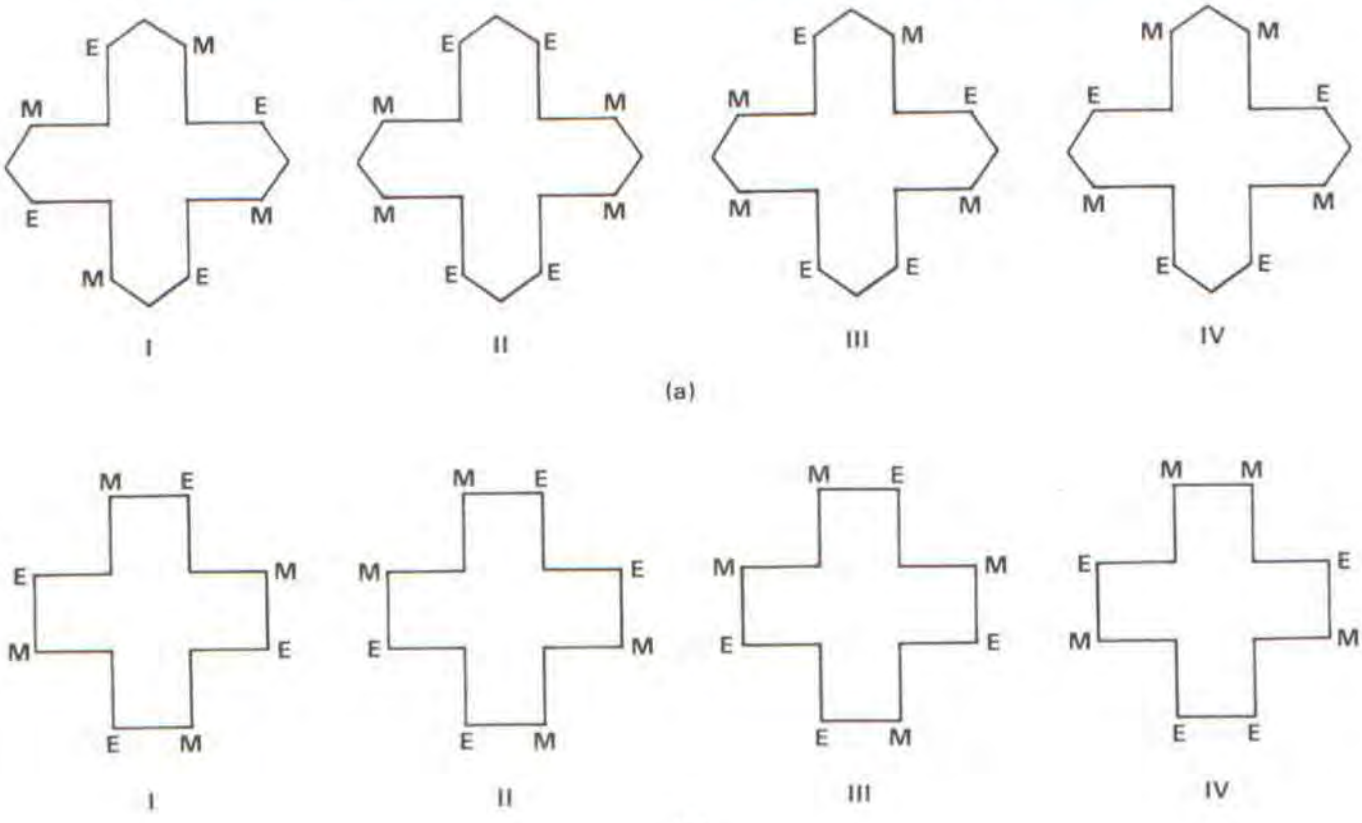


Figure 4.3 Structural Isomers of Etioporphyrin - Equivalent Representations

M - methyl E - ethyl

and Legge, 1949; Phillips, 1963; White *et al.*, 1973). Marks (1973) seems to be suggesting that the term *hemin* be reserved for ferriprotoporphyrin halide crystals (see Teichmann, 1853), and that it should not be used in place of the term *hematin*. *Ferroprotoporphyrin* may be called *ferroheme*, as *ferriprotoporphyrin* may be called *ferriheme* (or *ferrylheme*). In compounds in which the fifth and sixth liganding molecules are nitrogenous bases, the term *hemochromes* is often applied. The names *ferrohemochrome* and *ferrihemochrome* may be used to specify the valence of the iron atom. *Ferrohemochromes* have long been called *hemo-*

hemoglobin-methemoglobin interconversion. These are discussed in a later section.

The structure of hemoglobin will not be discussed here, but in a later section dealing with the determination of genetically-determined hemoglobin variants. Suffice it to say that native human hemoglobin is a tetrameric molecule, consisting of two α and two β polypeptide chains, having one heme per peptide chain, or four in the intact molecule, and a molecular weight of about 68,000. The iron atom is divalent in hemoglobin. Oxidation of the iron atom to the ferric state gives rise to methemoglobin (hemiglobin; fer-

Table 4.1. Side Chain Structures of Some Porphyrins

Porphyrin	Substituents	Type I	Type III
Etioporphyrin	4 M, 4 E	1,3,5,7-M 2,4,6,8-E	1,3,5,8-M 2,4,6,7-E
Mesoporphyrin	4 M, 2 E, 2 P	1,3,5,7-M 2,4-E 6,8-P	1,3,5,8-M 2,4-E 6,7-P
Protoporphyrin	4 M, 2 V, 2 P	1,3,5,7-M 2,4-V 6,8-P	1,3,5,8-M 2,4-V 6,7-P
Coproporphyrin	4 M, 4 P	1,3,5,7-M 2,4,6,8-P	1,3,5,8-M 2,4,6,7-P
Uroporphyrin	4 A, 4 P	1,3,5,7-A 2,4,6,8-P	1,3,5,8- A 2,4,6,7-P
Deuteroporphyrin	4 M, 2 H, 2 P	1,3,5,7-M 2,4-H 6,8-P	1,3,5,8-M 2,4-H 6,7-P
Hematoporphyrin	4 M, 2 HE, 2 P	1,3,5,7-M 2,4-HE 6,8-P	1,3,5,8-M 2,4-HE 6,7-P

Abbreviations used in the table: Numbering corresponds to Fig. 3.2. M- methyl E- ethyl P- propionic acid V- vinyl HE- hydroxyethyl A- acetic acid H- hydrogen

rihemoglobin). Methemoglobin does not bind oxygen, but will bind a number of other ligands, such as hydroxide, cyanide, azide and nitrite (Kiese, 1954). Figure 4.6 summarizes the interrelationships between the various derivatives (Lemberg and Legge, 1949; Pritham, 1968).

4.2 Crystal Tests

4.2.1 Introduction.

Parkes (1852) reported that, in examining microscopically the residual matter in a bottle which had contained partially putrefied blood, had been rinsed with water, and allowed to stand for a time, needle-like crystals could be observed in abundance. These crystals were insoluble in water and in strong acetic acid, but soluble in what I presume to be KOH. The crystals could be reprecipitated with strong acetic acid, but were less satisfactory and less abundant than the original crystals. Parkes noted that around this same time Funke had reported similar crystals from blood-water mixtures using horse spleen blood and fish blood. Drabkin (1946) has suggested that Funke may have been looking at hemoglobin crystals. Kölliker (1853-1854) reported that he had observed crystals in dog, fish and python blood in 1849. These were soluble in alkali and in acetic and nitric acids, and he said they were identical to Funke's crystals. Parkes subsequently attempted to prepare crystals similar to those which he had discovered by accident, but did not again obtain them in the same quantity. He did note that a number of different types of crystals are obtainable from putrefying blood, but could not identify them. He did not think they were identical to the hemoglobin crystals of Virchow nor to the albumin crystals of Reichert.

The preparation, microscopical and spectroscopic examination of crystalline forms of hemoglobin derivatives have occupied a great deal of attention in the development of methods for the medico-legal identification of blood. Many methods have been devised, and most are based on the preparation of either hematin or hemochromogen crystals. Many authorities have regarded crystal tests as methods of certainty in the identification of blood in stains (Beam and Freak, 1915; Brunig, 1957; Bertrand, 1931; Casper, 1861; Chiodi, 1940; Derobert and Hausser, 1938; Gonzales et al, 1954; Guarino, 1945; Lopez-Gomez, 1953; Lucas, 1945; Mueller, 1975; Olbrycht, 1950; Rentoul and Smith, 1973; Schleyer, 1949; Smith and Fiddes, 1955; Sutherland, 1907). Others who have considered the crystal tests in detail have been less explicit about the issue of whether the tests should be considered certain or not (Ziemke, 1938; Kirk, 1953). Dalla Volta (1932) took the position that the microscopical methods used to examine the crystals in routine forensic practice were inadequate to insure proof. Rather more sophisticated crystallographic analysis than would be routinely practical would be needed, in his view, to establish with certainty the presence of blood by these methods.

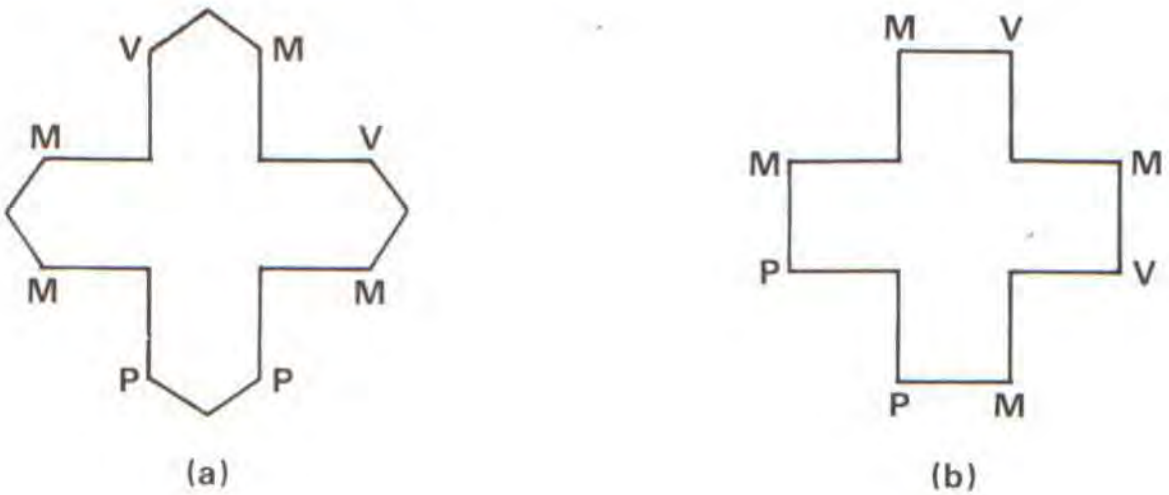


Figure 4.4 Equivalent Representations of Protoporphyrin IX

M - methyl V - vinyl P - propionic acid

4.2.2 Hematin crystal tests

In 1853, Teichmann published an extensive study of the formation of crystals in blood; this paper, still occasionally cited, formed the basis of the use of crystal tests for identification of blood in bloodstains. Teichmann's name has subsequently become synonymous with the hematin crystals which he obtained as well as with his own, and various modifications of the method for doing so. Teichmann's crystals are obtained by treatment of blood with glacial acetic acid in the presence of small quantity of salt and gentle heating. He called these crystals hemin, but they are now known to be hematin chloride. He suggested that the ability to form them reliably would provide the basis for medico-legal identification of blood in stains, as indeed it has done. Sutherland (1907) regarded a positive Teichmann crystal test as *"a sure proof of the presence of blood in the suspected stain"* (emphasis his own).

The extensive literature which developed on the subject has had mainly to do with the following matters: (a) development of different (hopefully improved) reagents for obtaining the crystals, including the use of halides other than chlorides, and of acids other than acetic; (b) variations in the way the test is carried out; and (c) experiments to determine the different conditions and substances that could interfere with the test, cause it to fail, or yield a result in the absence of blood. Casper (1861) in his Handbook mentions the test, noting that it is of value only when positive. The older literature on hematin crystals and crystals from blood in general was reviewed by Bojanowski in 1862. Table 4.3 is a modified version of one which was prepared by Lewin & Rosenstein (1895) in their review and reproduced by Sutherland (1907).

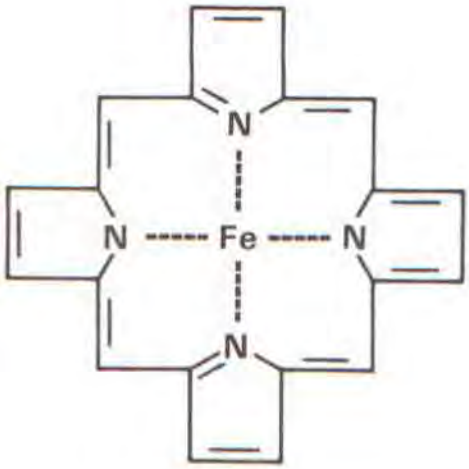


Figure 4.5 Iron Protoporphyrin

From it, one can get an idea of the different variations of the hematin crystal test in the older literature. It should be noted that some authorities felt that it was unnecessary to add salt because blood contains salts, which are present in sufficient quantity to yield crystals.

Henocque (1875) reported that an investigator named M. C. Husson had prepared hematin iodide crystals from the blood of a number of species. Sarda (1910) noted that Husson's work was reported in 1875, and that it involved both the iodide and the bromide of hematin. He reported very good results of his own with the potassium and ammonium salts of bromide and iodide.

Table 4.2. Comparison of Nomenclature of Hematin Compounds

Coordinating Ligands in iron protoporphyrin IX	Charge on over-all coordinate complex	Valence of Fe	Old Names	Nomenclature of Pauling & Coryell (1936) and Guzman Barron (1937)	Nomenclature of Clark (1939), Clark et al. (1940) and Drabkin (1938 and 1942a)	General Nomenclature
four pyrrole N	0	2	reduced hematin; heme	ferroheme	ferroporphyrin	heme
four pyrrole N, two additional N of nitrogenous base	0	2	hemochromogens; reduced hemochromogens; reduced hematin	ferrous or ferro-hemochromogens	base (e.g. dipyridine—, nicotine—) ferroporphyrin	hemochromes
four pyrrole N, water and OH^-	0	3	hematin; hydroxyhemin; oxyhemin	ferriheme hydroxide	ferriporphyrin hydroxide	hematin
four pyrrole N	yes ★	3	hemins, e.g. chlorhemin, bromhemin	ferriheme chloride, bromide etc.	ferriporphyrin chloride, bromide etc.	hemins (Cl, Br, etc.)
four pyrrole N, two additional N of nitrogenous base	yes ★	3	parahematins; oxidized hemochromogens	ferri- or ferric hemochromogens	base (e.g. dipyridine—, nicotine—, etc.) ferriprotoporphyrin	hemichromes

★ Charge depends on pH.

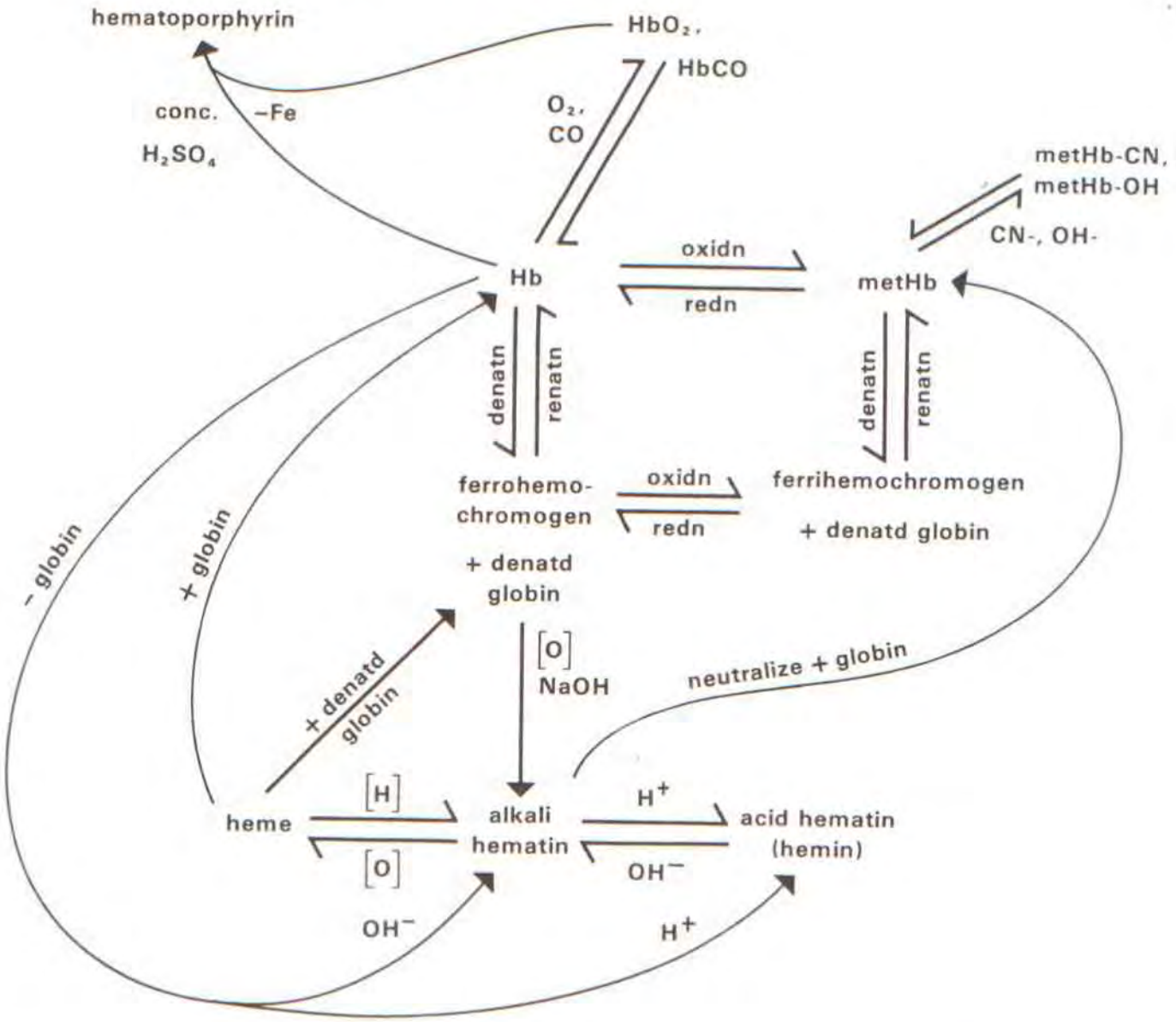


Figure 4.6 Interrelationships Among Hemoglobin Derivatives

Abbreviations used: Hb = hemoglobin HbO_2 = oxyhemoglobin Hb CO = carboxyhemoglobin metHb = methemoglobin metHb-CN = cyanomethemoglobin metHb-OH = hydroxymethemoglobin denatn = denaturation renatn = renaturation denatd = denatured [H] = reducing agent [O] = oxidizing agent oxidn = oxidation redn = reduction H^+ = acid OH^- = base conc = concentrated

Table 4.3 Some Modifications of the Hematin Crystal Test (modified from Lewin and Rosenstein, 1895)

Blood Preparation	Acetic Acid	Other Acid	NaCl	Other Salt	Heat or Temperature (°C)	Reference
dried,	much	oxalic, tartaric citric lactic	–	–	25 - 62.5	Teichmann (1853)
dried, fluid	slight excess	–	only if none in blood	–	cold or 40 - 60	Büchner and Simon (1858)
dried	to fill space under cover slip	–	yes	–	heat in flame	Virchow, 1857
–	yes	–	no	–	gentle heat or spontaneous evaporation	Morache, 1881
dried	few drops	–	no	–	heat until bubbles appear	Janert, 1875
dried, fluid	yes	–	few drops salt soln	–	heat in H_2O bath	Brücke, 1857
dried	yes	oxalic, tartaric in alcohol	yes if blood Cl free	NaBr, KBr NH_4Br, NaI KI	–	Bikfalvi, 1886
sediment prepared by copper sulfate pptn and extraction with alcoholic sulfuric acid	yes	–	yes	$BaCl_2$, SrCl, KCl LiCl, $CaCl_2$, NH_4Cl $MnCl_2$, $SnCl_2$, $FeCl_2$ $MgCl_2$	–	Teichmann, 1856
dilute solutions of blood pigment, add ammonia, tannic acid, then acidify with acetic acid, get hematin tannate precipitate	yes	–	no	NH_4Cl	–	Struve, 1880
alcoholic extract of dried precipitate by sodium carbonate pptn of defibrinated blood	yes	–	yes	$CaCl_2$	–	Gwosdew, 1886

According to Oustinoff (1929), who advocated the iodide crystals, Strzyzowski had prepared hematin iodide in 1902. Guarino (1945) employed iodoform in alcohol for the preparation of the crystals, and Lopez-Gomez (1953) reviewed, in some detail, the use of bromide and iodide salts in hematin crystal test reagents. Lopez-Gomez and Cantero (1942) are said to have carried out systematic studies on bromide and iodide crystals. Gouillart (1939) studied the formation of hematin iodide crystals in great detail. There is some confusion in the literature regarding the preparation of hematin fluoride crystals, Welsch and Lecha-Marzo (1912a) seeming to advocate their use, while Leers (1910 and 1912) believed that other halogens were greatly preferable. In any case, there does not seem to have been much subsequent use of fluoride-containing reagents. A number of authorities have recommended solutions containing 0.1g each of KCl, KBr and KI in 100 mℓ glacial acetic acid for the production of Teichmann crystals (Nippe, 1912; Kirk, 1953; Smith and Fiddes, 1955). Fiori (1962) said that there is no particular advantage to preparing hematin crystals from halogens other than chloride.

An extremely thorough study of the formation of both hematin and hemochromogen crystals was published by Mahler in 1923. He studied bloods from different species, including human, mostly as dried stains under a variety of different conditions and using a number of different methods. In these expriments, Mahler compared among other things the method of Wachholz (1901), utilizing alcoholic solutions of strong acids, with that of Nippe (1912) which called for a solution that is 0.1% (w/v) in KI, KBr and KCl in glacial acetic acid. Mahler got his best results from a combination of the two methods, wherein the blood-stained fragment was first warmed with alcoholic glacial acetic acid, and then warmed again following the addition of the Nippe reagent.

A similar set of comparative experiments was done by Kerr and Mason (1926). Some of the methods they studied were the same ones that Mahler had considered, but there were some differences. They preferred the method of Sutherland (1907), according to which a drop of saline is evaporated by heating on a clean slide, the stained fragment then being placed on the residue, and a drop of glacial acetic acid added. After applying a cover slip, the preparation is heated gently until bubbles just appear. It is then set aside and crytallization allowed to proceed.

Blood or bloodstains heated to temperatures in excess of 140° to 145° will not yield Teichmann crystals (Katayama, 1888; Hammerl, 1892; Wood, 1901). Bell (1892) discussed the hematin test in his review, and described the techniques then being used in this country by Formad (Formad, 1888) and by Prof. Tidy. Wood (1901) discussed his own experiences with the test in a paper read to the Massachusetts Medico-Legal Society. Any substance or condition which causes hemoglobin or hematin to form its decomposition product, hematoporphyrin (iron free hematin), he said, will interfere with crystal formation. Heat, long exposure to sunlight, and some organic solvents often cause difficulty. It is to be noted, nonetheless, that Muller *et al.* (1966) reported a positive Teichmann test from bloodstains on clothes that had been dry cleaned. Schech (1930), in his studies of the effects of ironing bloodstains on cloth on the subsequent ability to detect and analyze the bloodstain, noted that hematin crystals might be obtained if the iron had not been applied directly, but through a wet cloth for example. Rust and exposure, and especially the combination of these, interfere with the test (Sutherland, 1907).

Among the many modifications of the test that have been proposed, a few others will be mentioned. Oustinoff (1929) thought that the incorporation of gum arabic into the reagent improved the results. The heating step in the procedure, if done, is very critical. Bertrand (1931) discussed this point, noting that it is possible to err either in the direction of overheating or of underheating. He recommended a solution containing glycerol, apparently to lower the volatility of the reagent. Wachholz (1901) recommended a solution of a concentrated acid (lactic, sulfuric or acetic) in 95% alcohol because this boils easily, and reduces the chances of overheating. Sottolano and DeForest (1977) have described a technique utilizing Kirk's (1953) solution for hematin crystals which involves placing the test slides on a rack within a pressure cooker. Crystal formation is facilitated by the increased pressure, and the danger of total evaporation is overcome. The technique is applicable to the formation of hemochromogen crystals using Takayama's solutions (see Section 4.2.4) as well. Beam and Freak (1915) proposed a technique which, they stated, rendered crystal formation much more certain. The essential ingredient of this modification is very slow evaporation. The material to be examined is placed in the bottom of a flat, arsenic sublimation tube. A few drops of glacial acetic acid, containing 0.01 to 0.1% NaCl, are added, and a fine cotton thread is placed in the tube such that its lower end contacts the solution and its upper end is near the top of the tube. The thread is moistened if necessary to insure that it is everywhere in contact with the side of the tube, and evaporation is allowed to proceed at its own rate, without heating. The process which may require from 12 hours to more than a day, is accompanied by capillary movement of the solution within the thread, the crystals forming along the thread's length. This technique, recommended by Lucas (1945), is capable of giving large crystals suitable for crystallographic analysis (Fiori, 1962), but may not be very practical for routine work because of the investment of time required.

Although a number of materials and conditions interfere with the formation of hematin halide crystals, the age of the stain alone does not seem to be deleterious. Haseeb (1972) got a positive Teichmann test on a 12-year old stain kept on the laboratory bench in the Sudan. Beam and Freak (1915) obtained crystals from 10-year old human and 12-year old ovine bloodstains, as did Mahler (1923) from human stains over 20 years old. Dervieux (1911) mentioned that he had obtained hematin-iodide crystals from a 4000 year old bloodstained cloth from a mummy.

4.2.3 Acetone chlor-hemin crystal test

In 1935 Wagenaar recommended the preparation of acetone chlor-hemin crystals. A few drops of acetone are added to a fragment of bloodstain, followed by a drop of dilute mineral acid. Crystals form quickly at room temperature, even when the stains are old or the blood partially putrefied (Wagenaar, 1937). Dérobert and Hausser (1938) discussed this technique in their reference book. Chiodi (1940) carried out extensive studies on it with human, and different animal bloods, and stains exposed to adverse conditions. He recommended that it replace the Teichmann test. In 1949, Schleyer showed that the test could detect as little as 2 to 8 μg hemoglobin, and that methemoglobin and putrefied blood would give a positive test. Hematoporphyrin and blood heated above 200° do not give the test. Apparently, crystals are obtained from bile as well (due perhaps to the bilirubin content), but not from the urinary or fecal pigments (probably stercobilin, urobilin and urochrome) (Schleyer, 1949). Stassi (1945) reported that the test did sometimes fail in the presence of blood.

While the Teichmann and Wagenaar crystal tests are at least valuable, if not conclusive, tests for the presence of blood in stains, the crystals are not always easy to obtain. Even in experienced hands, these tests sometimes fail in the undoubted presence of blood (Sutherland, 1907; Corin, 1901; Dalla Volta, 1932; Olbrycht, 1950; Stassi, 1945; Mahler, 1923).

4.2.4 Hemochromogen crystal test

Hemochromogens are those compounds of ferroporphyrin (i.e., Fe^{2+}) in which the fifth and/or sixth positions of the hexacoordinate complex are occupied by the N atom of an organic base, such as pyridine, histidine, pyrrolidine, or various amines.

Hemochromogen was first prepared by Stokes in 1864. He obtained this material, which had a very characteristic spectrum by treating hematin with a reducing agent in alkaline solution. Stokes called the substance "reduced hematin." Hoppe-Seyler took up a number of further studies on the pigment finding, among many other things, that it bore as close a resemblance to hemoglobin as it did to hematin. He proposed that it be called "hemochromogen" (Hoppe-Seyler, 1877, 1879), this name having essentially supplanted that given the compound by the original discoverer. In 1889, Hoppe-Seyler prepared crystals by exposing hemoglobin to 100° temperatures in basic solution. These crystals have often been referred to in the literature as the first example of hemochromogen crystals. Leers (1910) noted that Hoppe-Seyler's student, Trasaburo-Araki reported obtaining similar crystals in 1890. Gamgee (1898) said that Hoppe-Seyler had not in fact obtained hemochromogen crystals, as had been reported in the textbooks for a number of years:

> It is quite erroneous to state, as is asserted in all textbooks, that Hoppe-Seyler succeeded in separating haemochromogen in a crystalline condition. He only succeeded (*at most*) in obtaining crystals of the CO-compound, and concluded that haemochromogen itself must be a crystalline body, but he never asserted that he had actually obtained the crystals, and a promise made in 1889 to describe the assumed crystalline haemochromogen, though implying that he had already obtained the body in this condition, was never fulfilled. Moreover, in the last systematic account of haemochromogen which he published in 1893 [Hoppe-Seyler and Thierfelder, 1893] Hoppe-Seyler does not refer to its being crystalline, but, on the contrary, speaks of it (as he had done in 1889) as separating in the form of a violet-grey powdery precipitate.

Copeman (1890) observed that hemochromogen crystals form from hemoglobin crystals upon long standing. Menzies (1895a) noted that allowing blood to stand in a water bath for some days in the presence of the chloride, bromide or iodide salts of potassium gave rise to a substance which could be converted to hemochromogen upon addition of a reductant, $(NH_4)_2S$. He further showed that ammonium sulfide would convert acid hematin to hemochromogen (Menzies, 1895b). Hüfner (1899) used hydrazine hydrate to convert alkaline hematin to hemochromogen.

Donogany (1893a) working in Budapest was the first investigator to note that pyridine reacted with hemoglobin to form hemochromogen crystals, and that these formed within a few hours if the material was placed on a microscope slide and sealed with a cover slip. He showed that this reaction occurred with dried blood, and suggested its application to the problem of medico-legal blood identification. In a subsequent paper (1897), Donogany suggested that the pyridine hemochromogen test would be useful for the determination of blood in urine, noting at the same time that he had first published his observations on pyridine hemochromogen in the Hungarian literature four years earlier (Donogany, 1893b).

Hemochromogen crystals may be obtained from either acid or alkaline solutions. Fiori (1962) stated that crystallization from acid solution was first carried out using acetic acid, pyridine and pyrogallol (1,2,3-benzenetriol), quite possibly a reference to the papers of Welsch and Lecha-Marzo (1912) or of Lecha-Marzo (1907), the latter of which was cited by Gisbert Calabuig (1948) who proposed the use of an improved solution: 0.5 mℓ glacial acetic acid, 1.5 mℓ pyridine and 1 mℓ 2% ascorbic acid.

It is far more common in the literature, however, to find descriptions of crystallization from alkaline solution. The older methods consisted of treating the stained material with pyridine and ammonium sulfide (Bürker, 1909; De Dominicis, 1902 and 1911), following upon the work of Menzies (1895a). Lochte (1910) suggested a modified version of the reagent containing NaOH and alcohol. Kürbitz (1909) and apparently Lecha-Marzo (1908) recommended extraction of the stain with alcoholic iodine solution prior to adding the pyridine-ammonium sulfide reagent. Leers (1910) used this method, but apparently employed an aqueous iodine solution. Alkaline solutions of pyridine containing hydrazine hydrate (Mita, 1910) or hydrazine sulfate (De Dominicis,

1909; Puppe, 1922) as reductants were described as well, being logical in view of Hüfner's earlier observations (1899). Nitrogenous bases other than pyridine will participate in hemochromogen crystals formation. Cevidalli (1905) successfully employed piperidine solutions, and Lochte (1910) noted that piperidine or picoline could be substituted for the pyridine.

The most comprehensive study of hemochromogen in the early literature is almost surely that of Dilling, published in 1910 in both German and English. This volume records the results of Dilling's extensive experiments carried out in Prof. R. Kobert's laboratory. Hemochromogen crystals were prepared from blood, hematin and other derivatives of hemoglobin utilizing pyridine, piperidine and a number of other nitrogenous compounds, with ammonium sulfide, hydrazine hydrate, and ammonium sulfide in NaOH as reductants. In each case the microscopic and spectral characteristics of the crystals obtained were described, as well as any problems encountered in the course of applying a particular technique. As nitrogenous bases, Dilling tested pyridine, piperidine, nicotine, methylpiperidine, ethylpiperidine, coniin (2-propylpiperidine), conhydrine (2-(α-hydroxypropyl) piperidine), and its isomer pseudoconhydrine, 2-methyl- and 3-methyl-pyridine (α- and β-picoline, respectively), α-dimethylpyridine (α-lutidine), as well as trimethylpyridine (collidine)† and tetramethylpyridine (parvoline), the two last mentioned probably consisting of mixtures of the isomers. Pyridine and piperidine were found to be the most satisfactory of all these compounds for crystal formation from blood. In addition to presenting his results, Dilling gave a good, comprehensive review of the pre-1910 literature. Somewhat less comprehensive reviews have been given by Kalmus (1910) and by Kurbitz (1910).

A great many authorities since 1912 have preferred to prepare hemochromogen crystals using the reagents described by Takayama in that year (Akaishi, 1956; Brünig, 1957; Gonzales *et al.*, 1954; Greaves, 1932; Hunt *et al.*, 1960; Kerr and Mason, 1926; Kirk, 1953; Lopez-Gomez, 1953; Mahler, 1923; Olbrycht, 1950; Rentoul and Smith, 1973; Thomas, 1937; Ziemke, 1924). Takayama's name has come to be used to describe not only the reagent he devised, but also the procedure and the hemochromogen crystals thus obtained, in much the same way as did Teichmann's name in the case of hematin halide crystals. The original paper in 1912 is cited in several different ways in the literature‡ and some difficulty was encountered in locating it. The paper, written in Japanese, appeared in *Kokka Igakkai Zasshi.*

Curiously, Takayama's paper was published in 1912 in Japan, but it does not appear to be mentioned in the European literature until Strassmann's paper appeared in 1922. It would be of interest to know how the information got from Japan to Germany. Takayama was in Germany around the turn of the century, but before 1912 (see in Unit IX, Translations). We had some correspondence on this point with Prof. Dr. Hiroshi Hirose of Kyushu University in Fukuoka, Japan. After some extensive searching in the early literature, Prof. Hirose discovered the paper by Strassmann (1922), and kindly shared the fruits of his search with us. His letter to me, in which the historical details are given and fully documented, has now been published (Hirose, 1979). Strassmann learned of the Takayama test from Prof. Fujiwara who was a student of Takayama's, and who was in Europe from 1920–1923. Takayama had noted that Reagent I required that the preparation be warmed for best results, while Reagent II did not require warming. Perhaps for this reason, many authorities preferred the second reagent, even in Japan where the test was widely used before its introduction in Europe. As mentioned in the footnote, the citation of the original paper was not correct in the German and English literature. Neither we nor Prof. Hirose could find any journals with the titles given in the incorrect citations. Takayama proposed two solutions, II being a sort of improved version of I.

Solution I:		Solution II:	
10% dextrose	5 mℓ	Saturated dextrose solution	3 mℓ
10% NaOH	10 mℓ	10% NaOH	3 mℓ
pyridine	10–20 mℓ	pyridine	3 mℓ
water	65–75 mℓ	water	7 mℓ

The crystals, obtained by treating a small amount of blood or stain fragment with these solutions are shallow rhomboids, salmon-pink in color. Gentle heating was needed with the first reagent, but not with the second.

As mentioned previously, Mahler's (1923) extensive study of blood crystals included hemochromogen crystals as well as hematin crystals. He compared a number of different methods and solutions for obtaining the crystals from a large number of different types of dried blood specimens. Kerr and Mason (1926) discussed the test in some detail as well, concluding, as had Mahler, that Takayama solution II was the most reliable reagent for obtaining hemochromogen crystals. The speed with which the crystals appear depends on temperature, being almost immediate if the material is heated, somewhat slower at room temperature (1 to 6 minutes), and slower yet if in the cold (e.g. if the reagent has been kept in the refrigerator). The reagent is stable for 1–2 months, crystals taking somewhat longer to form with older reagent. Greaves (1932) recommended the use of Takayama solution II for hemochromogen crystals as the method of choice for blood identification. He preferred not to heat the material, and suggested waiting up to several hours if necessary before deciding that the result is negative. Puppe (1922) also noted that good results were obtained without heating. Oustinoff (1930) suggested that gum arabic be included in the reagent, 1 part to 3 parts pyridine.

There are a number of advantages to the hemochromogen

† 2,4,6-trimethylpyridine is now called γ-collidine, while α-collidine is 4-ethyl-2-methylcollidine and β-collidine is 3-ethyl-2-methyl-collidine.

‡ Kerr and Mason (1926) and Greaves (1926) cited the paper as having appeared in the Japanese Journal of Toxicology. Ziemke (1924) and Wagenaar (1929) cited it as having appeared in Japan. Zeitschrift für Staatsarzneikunde, this last having probably been taken from Strassmann (1922).

test, as compared with the hematin test. A technical advantage is that heating is not required to obtain results within a reasonable amount of time; and even if one does prefer to apply heat, the test is not subject to being ruined by overheating. The test also yields positive results under some of the circumstances where the Teichmann test fails. Thus, Mahler (1923) obtained a positive Takayama test in cases of various 22 year old bloodstains on linen and of stains on rusty knives up to 23 years old, which failed to give Teichmann crystals. Similarly, Kerr and Mason (1926) showed that stains on linen and glass which had been heated to 150° for 30 minutes, stains (relatively fresh) washed in hot water, and stains on rusty metal surfaces up to 45 years old, all yielded hemochromogen crystals but did not give hematin crystals by the Sutherland technique.

According to Kirk (1953), both the specificity and sensitivity of the hemochromogen test are about the same as those of the hematin crystal test. Kerr and Mason (1926) and Greaves (1932) say that the hemochromogen test never failed in their hands in the known presence of blood. Mahler (1923) got some failures, but with solutions other than the Takayama II. The proof value of a positive hemochromogen test, with spectroscopic confirmation of the identity of the product, is not widely disputed. A negative crystal test, however, should not necessarily be interpreted as meaning that blood is absent (Kirk, 1953; Olbrycht, 1950). That bloodstains which have been exposed to heat, or are old or weathered, become increasingly insoluble has been known for a long time (Katayama, 1888; Hammerl, 1892). In cases such as these, solubility can be a problem in itself; if it is not possible to solubilize any hemoglobin or hemoglobin derivatives, it will obviously not be possible to obtain a positive crystal test.

Comparisons of sensitivity present some difficulty because various different authors express sensitivities in different ways. It is not always possible to convert one set of units or reference frame to another. This problem is encountered in many of the identification and serological and biochemical tests used in this field.

As to hemochromogen crystal tests, Greaves (1932) mentions only that the fragment of stain or stained material should be very small, only just large enough to be seen and manipulated onto a slide. Antoniotti and Murino (1956) noted that the test is still positive with 1 $\mu\ell$ of blood or about 0.1 mg hemoglobin, while Hunt *et al.* (1960) could obtain crystals from a stain fragment containing only 0.2 $\mu\ell$ of blood. Akaishi (1965) stated that a positive test could be obtained from a stain made from a 1:30 dilution of whole blood, but did not state how much stain was taken for the test. Miller (1969) thought that the Takayama test was considerably more sensitive than the Teichmann test. He could obtain Takayama crystals from 5 $\mu\ell$ of a 1:1000 dilution of whole blood, provided that the material was dispensed onto a slide in 10 separate 0.5 $\mu\ell$ aliquots in order to keep the area occupied by the test material as small as possible.

Not long after Kerr and Mason (1926) published their article on the Takayama test, Dilling (1926), in a letter to the Editor of the *British Medical Journal*, suggested that the hemochromogen test should not supplant the hematin test, but rather be used as to supplement it. He brought up two other points: (1) Kerr and Mason had said that the only discussion of hemochromogen tests in English prior to their paper was Sutherland's discussion, and Dilling correctly pointed out that he had published an extensive study of the subject in 1910; and (2) he believed that the purpose of the sugar in the Takayama reagent was to decrease the solubility of hemochromogen, and not, as Kerr and Mason had suggested, to serve as a reductant. Kerr (1926a) replied to the letter, saying that he agreed with Dilling's interpretation of the mechanism of action of the sugar. He further said that there was no prior account of the Takayama method in English, and that he still believed it to be much superior to the hematin test for medico-legal work.

Recently, Blake and Dillon (1973) have investigated the question of false positive hemochromogen crystal tests. They correctly note that the question has received virtually no attention in the medico-legal literature. Of particular interest was the issue of whether other iron-protoporphyrin containing substances, such as the enzymes catalase and peroxidase, would give misleading false positive crystal test reactions. Three presumptive tests were also carried out on the material, including the benzidine and phenolphthalin catalytic tests (sections 6.3 and 6.4) and the luminol test (section 6.7). A number of microorganisms were tested, since these are known to be particularly rich in catalase and peroxidase activities. Pure samples of both the enzymes gave Takayama crystals, those formed with catalase being virtually indistinguishable from the crystals obtained with blood. These materials also gave positive results with all three presumptive tests. The effectiveness with which different bacteria reacted with benzidine, in a two stage test, was directly related to the catalase content of the cells. One drop (about 0.05 mℓ) of a suspension of Citrobacter having a cell concentration of 400 $\times$ 10^6/mℓ gave a positive benzidine reaction, and several microbial suspensions which had been dried as spots on filter paper gave positive benzidine reactions after 2 months. Heating a number of different bacteria at 100° for 30 min or at 150° for 15 min did not abolish the benzidine reaction. Although pure catalase and peroxidase could give a positive crystal test, none of the bacteria tested contained sufficient concentrations of these enzymes under the test conditions to give a false positive Takayama test. Blake and Dillon cautioned that great care should be used in the interpretation of catalytic and crystal tests, and in combinations of them, since it is not only possible, but even likely in some situations, that case material will be contaminated with bacteria.

and aniline, and methylene blue. Neutrophil granules are stained purple-red, eosinophil granules, lighter red, and basophil granules, a deep purple. The nuclei are stained dark blue and the cytoplasm light blue. It is said that the monocyte granules become peroxidase negative in bloodstains in about a month, about 8–12 months being required for neutrophil granules to be negative, while eosinophil granules may be positive in up to 5-year old stains (Undritz and Hegg, 1959). Environmental influences can greatly influence these values. Undritz and Hegg also noted, as did Ziemke (1938), that tissue and organ cells could be histologically differentiated in blood stains, such as in cases on blood crusts on a knife which had been used in a stabbing, or where stains may contain nasal or epithelial cells. Le Breton did not share the same level of confidence in or enthusiasm for the microscopical methods (Fiori, 1962).

De Bernardi (1959) suggested paraffin embedding, thin sectioning and staining with hematoxylin preparations containing alum for bloodstains in deeply absorbed in wood. Däubler (1899) had employed a similar procedure much earlier. If blood crusts are detached from rusty surfaces by the celloidin technique, the film may be fixed and soaked in a saturated solution of oxalic acid containing a bit of uranium nitrate, then exposed to light to dissolved the rust, prior to histological staining (Romanese, 1930).

Some of the more recent texts in forensic medicine do not mention microscopical methods for identification of blood at all, e.g. *Glaister's Medical Jurisprudence and Toxicology*, 13th ed. (Rentoul and Smith, 1973). It should be kept in mind that these techniques require a comparatively large amount of material, and that a certain degree of skill with histological technique is doubtless required if good results are to be expected. In some instances, however, some workers prefer these methods. If whole (liquid) blood is encountered, microscopical identification might well be the method of choice, since a smear could be quickly and easily prepared. Fiori (1962) said emphatically, however, that microscopical methods should never be employed *in place of* other, more reliable procedures, such as spectral, chromatographic and immunological ones. Discussions of microscopical methods are, in any case, of more than purely historical interest, because of the newer methods for the cytological determination of sex of bloodstains (Section 48).

5.3.2. Biological stains and dyes

The histological stains employed in this work have known, and often differential, staining affinity for blood cells. Most of the stains commonly employed in histological work have been in use since the last century. Considerable information about the structure, properties and mode of action of biological stains is available (Clark, 1973; Conn, 1933; Gurr, 1960; Gurr, 1962; Lillie, 1977). In 1922 a Commission on Standardization of Biological Stains was set up in this country to devise standards of uniformity for commercially available products and techniques, and to disseminate information. The handbook of biological stains, published under the Commission's auspices, has gone through nine editions (Lillie, 1977). Conn's *History of Staining* (1933) was authorized by the Commission, and a guide to recommended staining procedures, now in its 3rd edition, is published as well (Clark, 1973). A brief discussion of the more commonly used stains is given here. Readers interested in histological stains should consult the above-cited specialized works on the subject.

Hematoxylin was first employed as a stain in 1863, apparently without success. It was successfully employed two years later by Böhmer (Clark, 1933). Hematoxylin itself is a naturally occurring glycoside from logwood, and is not a dye. It is easily oxidized to the dye form, however, by oxidizing agents or exposure to air. The oxidation product, which is the dye, has the unfortunate name *hematein*, and should *not* be confused with the hemoglobin derivative *hematin*. Fig. 5.4 (a) and (b) shows the structures of hematoxylin and hematein. Eosin, also called eosin Y, is a tetrabromofluorescein (Fig. 5.5), and was discovered in 1871. Giemsa stain, which came into use in 1902, is a mixture of methylene blue and its oxidation products, the azurs, in combination with eosin Y. May-Grünwald stain (1902) is a mixture of eosin Y and unoxidized methylene blue (Fig. 5.6), and is equivalent to Jenner stain (1899), after the latter of whom it should no doubt have been named. Wright's stain is the result of heating methylene blue in the presence of $NaHCO_3$, adding eosin, collecting the precipitate which forms and dissolving it in methanol. The so-called Feulgen reaction for staining of nuclei relies on the acid hydrolysis of the purine residues from DNA, and reaction of the liberated aldehyde groups with Schiff's fuchsin-sulfurous acid reagent to form red-purple complexes. Basic fuchsin is a mixture of pararosanilin and related compounds.

Biological stains and dyes will come up again in this book in other contexts, for example in the visualization of spermatozoa under the microscope (Section 10.2.1) and as coupling reagents for the visualization of the naphthols liberated when naphthyl phosphates are employed as substrates for acid phosphatase (Section 10.3.2). These dyes and stains have frequently had a number of names over the years. It is, thus, not always apparent to the uninitiated that two very different sounding names may refer to the same material. This problem has existed for a long time. There have been standardized lists of dyes and stains prepared over the years, but different lists have not been consistent with one another, nor has there always been consistency in the same list as it was revised and re-revised over time. Dye manufacturers and trade associations have usually designated products by number. There is a lengthy history to the various dye indexes (see Lillie, 1977), but for purposes of this book, suffice it to say that there is now a fairly widely accepted numbering system which is derived from the *Colour Index* in its most recent edition. Most stains and dyes are assigned a five digit "CI number".

In Table 5.3 are listed a number of stains and dyes which come up in medico-legal biology. The preferred name is given, along with synonyms and CI number.

Table 5.3 Biological Stains and Dyes

Preferred Name	Synonyms	C.I. Number	Chemical Name or Nature
Amido Black 10B	Naphthol Blue Black; Naphthalene Black 10B	20470	Acid Diazo Dye
Aniline Blue WS	Water Blue I; Cotton Blue; China Blue	42755	Acid Triphenylmethane Dye
Biebrich Scarlet	Scarlet 3B of B of EC; Croceine Scarlet	26905	Diazo Dye
Brilliant Indocyanin G	Coomassie Brilliant Blue G-250; Supranolcyanin G;	42655	Arylmethane Dye
Carmine; Carminic Acid	Cochineal	75470	Derivative of Anthraquinone Glycoside
Crystal Violet	Methyl Violet 10B; Gentian Violet	42555	Hexamethylpararosanilin
Eosin B, BMX	Eosin BN, BA, BS, BW or DHV; Saffrosin; Eosin Scarlet	45400	Dinitro derivative of dibromofluorescein
Eosin	Eosin Y or G;	45380	Tetrabromofluorescein
Erythrosin	Erythrosin R or G; Pyrosin J	45425	Diiodofluorescein
	Erythrosin B; N or JN; Pyrosin B	45430	Tetraiodofluorescein
Fast Blue B	Diazo Blue B; Dianisidine Blue; Fast Blue Salt BN; Naphthanil Blue B; Brentamine Fast Blue B	37235	See Fig. 10.3
Fast Blue RR	Blue RR; NRR; Diazo Blue RR	37155	Diazonium Dye
Fast Red AL	Naphthanil Diazo Red AL; Red AL; ALS	37275	See Fig. 10.2
Fast Red RC	Red RC; RCS; Red Salt I; Diazo Red RC; RS; Fast Red 4CA	37120	See Fig. 10.4
Hematoxylin; Hematein	Logwood	75290	See Fig. 5.4

Table 5.3 (cont'd)

Preferred Name	Synonyms	C.I. Number	Chemical Name or Nature
Kernechtrot	Nuclear Fast Red; Calcium Red	60760	Anthraquinone Derivative
Malachite Green	Solid Green, O; Victoria Green, B; Malachite Green BXN	42000	See Fig. 6.6
Methyl Blue	Helvetia Blue; Cotton Blue; Soluble Blue; Ink Blue; Sky Blue	42780	Aminotriarylmethane Dye
Methylene Blue	Methylene Blue Chloride	52015	A Thiazin Dye
Methylthiazolyldiphenyl Tetrazolium (BS8)	MTT; Chelating Tetrazole MTT	——	3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyl tetrazolium bromide
∝-Naphthylamine	Fast Garnet B	37265	See Fig. 10.1
Nitro Blue Tetrazolium (BS8)	NBT; Nitro BT; Ditetrazolium Chloride	——	3,3'(4,4'-di-o-anisylene)-2,2'-di(p-nitrophenyl)-bis(5-phenyl)
Pararosanilin	Magenta O; Basic Fuchsin	42500	Triaminotriphenylmethane chloride
Ponceau S	Fast Ponceau 2B	27195	Polyazo Dye
Procion Blue HB	Cibachron Blue F.3GA	61211	Aminoanthraquinone Derivative
Procion Brilliant Red M-2B	Mikacion Brilliant Red 2BS	18158	A Monoazo Dye
Pyronin Y	Pyronin G	45005	Xanthene Derivative
Remazol Brilliant Blue R	Remalan Brilliant Blue R; Ostazin Brilliant Blue VB; Primazin Brilliant Blue RL	61200	Aminoanthraquinone Derivative
Rhodamine B	Brilliant Pink B; Rhodamine O	45170	Xanthene Derivative

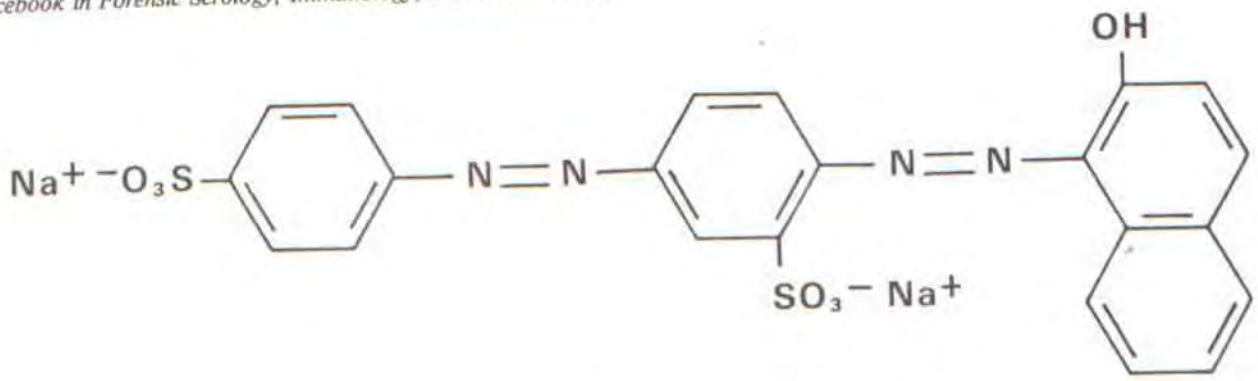
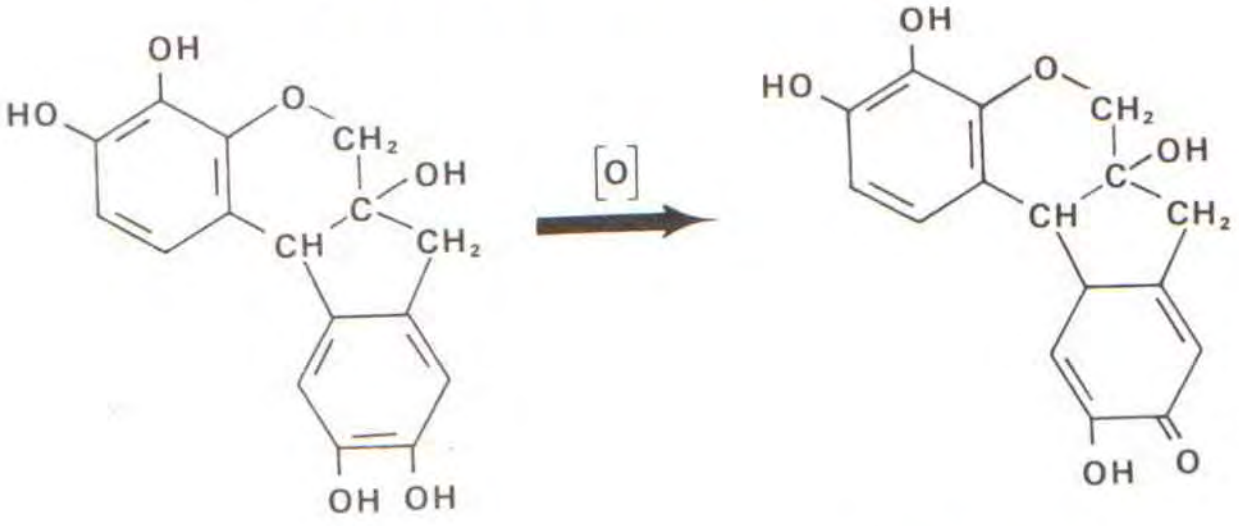


Figure 5.3 Biebrich Scarlet



(a) hematoxylin

(b) hematein (colored dye)

Figure 5.4 Hematoxylin and Hematein

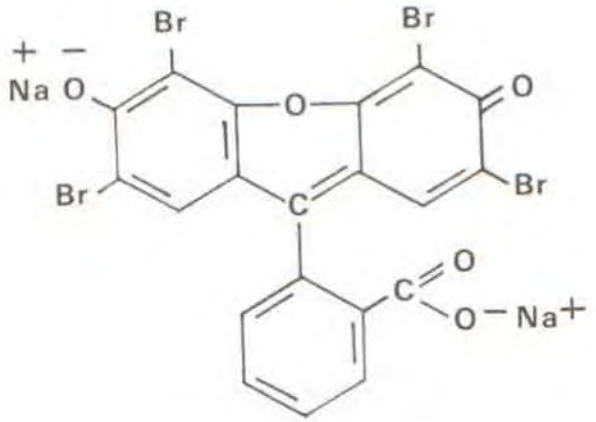


Figure 5.5 Eosin or Eosin Y

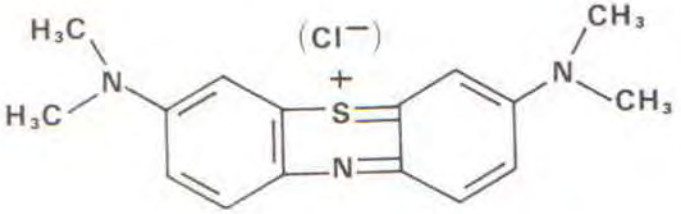


Figure 5.6 Methylene Blue

SECTION 6. CATALYTIC TESTS

Catalytic tests for blood are all based on the fact that hemoglobin and a number of its derivatives exhibit a peroxidase activity. If enzymes are defined as proteins which have *in vivo* catalytic functions, then hemoglobin is not an enzyme. It behaves as an enzyme in these tests, however, as do some of its derivatives, in that they catalyze the oxidation by peroxide of a number of organic compounds to yield colored products. Enzymes which catalyze the peroxide-mediated oxidation of organic compounds *in vivo* are called peroxidases; hemoglobin and the other derivatives which show this catalytic property are thus said to have "peroxidase activity". The "tests" based on the property are generally named after the compound undergoing oxidation (e.g. the guaiacum test, the benzidine test, the phenolphthalin test), or after the discoverer(s) (e.g. van Deen's test, Adler's test, the Kastle-Meyer test). The majority of tests which have been devised for the medico-legal identification of blood are based on the peroxide mediated oxidation of guaiacum, aloin, phenolphthalin, benzidine, the leuco base of malachite green, p-phenylenediamine, eosin hydrate, rhodamine, o-tolidine, o-toluidine, o-dianisidine or tetramethylbenzidine. Some of the tests have enjoyed wide use and great popularity, while others have been used by only one or a few workers. Likewise, some are very much in use today, while others have become a part of the historical archives of this field.

The generalized peroxidase reaction may be written

$$AH_2 + ROOH \rightarrow A + ROH + H_2O$$

where AH_2 is the donor and ROOH is the peroxide. Hydrogen peroxide is frequently used, in which case R = H, and the products would be $A + 2H_2O$. The AH_2 donor can be any oxidizable substrate yielding a detectable (usually colored) product A. In the special case of the catalase reaction, $AH_2 = H_2O_2$ and $2H_2O_2 \rightarrow O_2 + 2H_2O$. Additional information on the peroxidase and catalase reactions may be found in Mahler and Cordes (1971).

6.1 The Guaiacum Test

The subject of the peroxidase activity of blood was opened by Schönbein in 1857. He had shown that ozone and certain inorganic peroxides would cause guaiacum to turn blue, but that H_2O_2, "ozonized turpentine", and "ozonized ether" would not do so by themselves (Schönbein, 1848a, 1848b). The last three mentioned substances were called "antozones", this terminology being based on a theory of oxidation he held which said that "ozone" represented oxygen in a "positive polar state" while "antozone" represented it in a "negative polar state". Substances which behaved like ozone itself were called "ozonids" or "ozonides" while those behaving like "antozone" were called "antozonid(e)s". It was thought that the two forms of "polarized" oxygen reacted in some way to give oxygen. Ozone and "ozonides" would cause guaiacum to turn blue by themselves, while the so-called "antozonides" would not. The addition of a catalytic quantity of platinum black, however, would bring about the bluing reaction with H_2O_2, and the "antozones", and it was soon found that this same catalytic activity was possessed by blood and wheat gluten, neither of which worked by themselves (Schönbein, 1857). The catalytic principle in blood was demonstrable in red cell lysates, in the absence of fibrin and serum, and was not destroyed by boiling or drying.

Guaiacum is a resin isolated from *Guaiacum officinale* and *Guaiacum santum*, trees indigenous to Mexico, South America and the West Indies. It consists of a mixture of substances, one source saying that "guaiacum" is 70% guaiaconic acid, 10% guaiaretic acid and 15% resin (Snell and Snell, 1962). Guaiaconic acid has the formula $C_{20}H_{24}O_5$ according to Grant (1969) and Kastle and Loevenhart (1901), and can be substituted for guaiacum in the reaction (Mitchell, 1933; Buckmaster, 1907). Guaiacum is also sometimes called "guaiac".

In 1858, Schönbein showed that $FeSO_4$ catalyzed the guaiacum reaction. His colleague, Prof. Hiss, had shown that the catalytic activity of the red cells was proportional to their iron content, and he believed, therefore, that the catalytic principle in blood depended more on its iron content for activity than on any specific structural or organizational feature. In 1863, Schönbein suggested that the power of blood to blue guaiacum might serve as the basis of a medico-legal identification test. Van Deen (1862) extended the studies on the guaiacum reaction with blood, and was the first to suggest it as the basis of a medico-legal test. He showed that it occurred with minimal amounts of old, putrefied samples, as well as with blood which had been dried or boiled. Dilutions of whole blood of up to 1:40,000 in water still gave positive reactions. A number of inorganic salts gave positive reactions too, but could be eliminated using other methods. Van Deen used oil of turpentine as oxidizing agent. This so-called "ozonized turpentine", as well as "ozonized ether" could serve as oxidants because of the presence of organic peroxides which form upon exposure to air. According to Taylor (1868), not much notice was taken of Van Deen's work until Liman's extensive experiments on the subject were published in 1863. Liman thought that a negative reaction could be taken as an indication of the absence of blood without further testing. A positive result, however, did not constitute proof that blood was present, since various vegetable gums, milk casein, tanned sheep leather and some inorganic salts gave "false positive" reactions. Dr. Day in

Geelong, Australia, confirmed most of the results of earlier workers in 1867. He employed "ozonized ether" for the test, and correctly surmised that it worked because of the peroxides which formed when it was exposed to the air. In what may well have been the first reported use of the test in a medico-legal case, Dr. Day reported that he had detected blood on the trousers of a Chinese man suspected of a murder in a place called Scarsdale, Australia, on October 19, 1866. The trousers had been washed by the time they were taken as evidence, and the Government's forensic chemist could find no traces of blood on them by microscopical examination. Day wrote of this case to Dr. Taylor in London, and even enclosed a portion of the trousers on which he said he had detected blood. Dr. Taylor re-examined the cloth, some months old by then, by means of the guaiacum test, and confirmed Day's findings (Taylor, 1868). He reported this result in a rather lengthy study of the guaiacum test, prompted apparently by Day's communication. Taylor also noted that he gave the bloodstained cloth from the trousers in Day's case to Mr. Sorby, who was unable to confirm the presence of blood using his microspectroscopic method (see section 5.1). Taylor regarded the test as a useful one, to be used in conjunction with microscopical and microspectroscopic methods. Negative results were conclusive in his view, while a positive result ". . . enables a chemist to speak with reasonable certainty to the presence of blood . . .". The "false positive" results caused by oxidants which blued guaiacum in the absence of peroxide were easily eliminated by applying the guaiacum tincture first, and the peroxide after a short time if no reaction had taken place.

The guaiacum test was the first catalytic test devised for forensic blood identification, and, except for the aloin test which enjoyed very little popularity, was the only one in use for about 40 years. It is often referred to in the literature as Van Deen's test. Some English authors have called it Day's test. The old literature on this test was extensively and excellently reviewed by Kastle (1909). Kastle and Loevenhart (1901) stated that the product of the reaction, the so-called "guaiacum blue", had the formula $C_{20}H_{20}O_6$ and that guaiaconic acid, $C_{20}H_{24}O_5$, was the component of guaiacum resin which underwent oxidation. Guaiaconic acid can be separated, as it turns out, into α and β compounds (Richter, 1906), having the formulas $C_{22}H_{24}O_6$, and $C_{21}H_{45}O_5$, respectively. Guaiacum blue was said to be the oxidation product of the α compound, and to have the formula $C_{22}H_{24}O_9$. The guaiacum test has been applied to the detection of blood in urine (Schumm, 1909) and feces (Messerschmidt, 1909) for clinical purposes.

Medico-legal investigators were divided as to the relative value of the test as proof of the presence of blood. Buckmaster (1907) believed that, if the test were carried out on boiled samples (to eliminate vegetable peroxidases), a positive result was meaningful. Negative results indicated the absence of blood with certainty. Jenne (1896) thought that a positive result in a carefully performed test, using proper controls, warranted the conclusion that the stain was "surely blood". Siefert (1898) stated that a positive guaiacum test indicated a "high probability" of the stain being blood, while a negative result insured that it was not. Hemphill (1875) thought the test was a good and useful one, but did not clearly state that a positive result constituted conclusive evidence of the presence of blood. Schumm (1907) thought the test was trustworthy with certain precautions.

A larger number of authorities did not believe that a positive result was to be taken as proof of blood, but they did think the test had value as a preliminary or sorting technique (Chapman, 1892; Delearde and Benoit, 1908; Dérobert and Hausser, 1938; Ewell, 1887; Macnamara, 1873; Marx, 1905; Sutherland, 1914; Wood, 1901). Other workers believed that the primary value of the test was in eliminating stains that were not blood. They stressed the importance of negative results, which warranted the conclusion that blood was absent (Liman, 1863; Mialhe *et al.*, 1873; Mecke and Wimmer, 1895; Palleske, 1905a; Siefert, 1898; Whitney, 1909). A few investigators believed that the test was virtually worthless (Alsberg, 1908; Dervieux, 1910). Breteau (1898) said that very great caution should be used in interpreting results because of the large number of substances that would give a positive test.

The objections to the value of the test were based on the relatively large number of substances other than blood which had been reported to give positive results (Kastle, 1909). A number of inorganic elements and compounds, vegetable extracts, milk, gelatin, bile, gastric secretions, nasal mucus, saliva, pus, leather, soap, and certain types of papers have all been reported to give false positive reactions. It must be said that the way the test is carried out, the nature of the guaiacum, and of the oxidant used all make a difference in this regard. Carrying out the test on boiled samples (to eliminate vegetable peroxidases), addition of the guaiacum and peroxide in two steps (to eliminate inorganic oxidants), and the use of guaiaconic acid and H_2O_2 (to eliminate variability in the resin, ether and/or turpentine preparations) excludes the possibility of most, but not all, of the interfering substances. Workers who believed in the value of positive results recommended all these precautions, in addition, of course, to substratum controls.

Various claims have been made for the sensitivity of the test, it being difficult to compare the values in some cases because of the different ways in which they were expressed. Van Deen (1862) reported a positive test with a 1:40,000 dilution of whole blood in water. Mitchell (1933) and Kastle (1909) both say that Liman (1863) confirmed this result, but I have quite the opposite impression from Liman's paper. I understand him to say that he got a positive result with a 1:6,000 dilution of fresh blood in water, but that the reaction failed at dilutions of 1:40,000. However that may be, Schumm (1909) reported 1:40,000 to 1:100,000 dilutions of blood in water and 1:20,000 to 1:40,000 dilutions of blood in urine as the limits of sensitivity. Nicolesco (1934) gave 1:20,000 as the value. The most extravagant claim is that of Vitali (1903) who said that a $1:10^{11}$ dilution of dessicated blood in water still gave a positive result; this value is greater by six orders of magnitude than any other published figure.

Expressed as a dilution of whole blood in water, the range most often quoted for sensitivity is 1:20,000 to 1:100,000. The measurements are obviously affected by the reagents used.

The guaiacum test is now no longer employed as a catalytic test for the presence of blood in the forensic practice, having been supplanted by benzidine, phenolphthalin, etc. Schwarz (1936) used guaiacum as a substrate for testing the peroxidase activity of bloodstains in a series of experiments designed to correlate the color intensity with the age of the stain. In more recent times, guaiacol, which is o-methoxyphenol, and component of guaicum resing, has been employed as a staining substrate for haptoglobin (as the hemoglobin complex) in gels following electrophoresis (Reich, 1956; Queen and Peacock, 1966).

6.2 The Aloin Test

The aloin test for identification of blood in stains (and in urine and feces) is, like the guaiacum test, of primarily historical interest. In many ways, it is quite similar to van Deen's test. Aloin is a mixture of pentosides in the extracts of aloes, a genus of plants of the Family Lilaceae. Barbaloin, the major ingredient, is a hydroxyanthroquinone derivative of glucose. The structure is shown in Fig. 6.1 (Merck Index, 1968). Hemoglobin and some of its derivatives catalyze the oxidation of this material by H_2O_2 to yield a bright red product. Klunge (1882 and 1883) first noted that this test could be employed as a test for blood. A number of studies concentrated on chemical reactions of aloin, and the analogy between these and comparable guaiacum reactions (Neuberger, 1899; Schaer, 1900). Rossel (1901) noted that the test could be used for the detection of blood in urine. Buckmaster (1907) found that the test was less sensitive than the guaiacum test, but that the oxidation product (i.e. the color) was quite a bit more stable. The test was never as widely used as van Deen's, and not as much was written about it. Sutherland (1907) regarded it as a good confirmatory negative test.

6.3 The Phenolphthalin Test

In 1901, Kastle and Shedd showed that preparations of cellular "oxidases" would catalyze the oxidation of phenolphthalin to phenolphthalein in slightly alkaline solutions. At the time, the cellular enzymes responsible for the catalysis had not been purified to any great extent, nor had there been any attempt to systematize the enzyme nomenclature. The crude enzymatic preparations were often referred to as "oxidizing ferments". Phenolphthalein is, of course, pink to red in alkaline solution, while phenolphthalin is colorless. Thus, the latter was an excellent artificial substrate for assaying the "ferments" because the colored oxidation product was soluble and readily quantitatable colorimetrically. Meyer (1903) utilized phenolphthalin to detect the "oxidases" in leucocytes. In particular, he found differences in this activity between normal and leukemic samples. He noted further that this test could be used for the qualitative and quantitative determination of blood in urine. The first unequivocal suggestion that the test be applied to medico-legal blood identification was made later in 1903 by Utz. He reported that the test served well on bloodstains up to 1½ years old, gave a negative reaction with rust, but, not surprisingly, gave "false positive" reactions with pus and other leucocyte-containing secretions. The test became known as the "Kastle test", the "Meyer test" or the "Kastle-Meyer test."

It was soon quite clear that the test relied on the peroxidase activity of hemoglobin. Kastle and Amoss (1906) showed that the catalytic activity of blood toward the peroxide oxidation of phenolphthalin in alkaline solution was directly proportional to the hemoglobin content. The reagent, phenolphthalin, was prepared from phenolphthalein by reduction in the presence of Zn and strong NaOH or KOH. Kastle (1909) recommended the precipitation of the phenolphthalin by acidifying the reaction mixture, and collection of the precipitate. This material was recrystallized several times from minimal alcohol by cold water, and stored as a solid. Liquid solutions of the compound were stable for a matter of weeks if kept dark, and stability was greatly increased by the presence of a small quantity of zinc dust. One of the advantages of this test, in comparison to guaiac and aloin, was that the reagent was a pure compound. Kastle, who was very partial to this test for blood, discussed its many aspects in great detail in his review in 1909.

Deléarde and Benoit (1908a) studied the phenolphthalin test and showed that it was positive with hemoglobin, methemoglobin, hematin chlorhydrate, reduced hemoglobin, and old, putrefied blood. They got a positive test on a control bloodstain 26 years old, and believed that the test, properly controlled, was both sensitive and specific (1908b), in addition to its usefulness in detecting blood in urine, feces and gastric juice. Boas (1911) indicated that the test was useful for occult blood. Pozzi-Escot (1908), however, thought that no value should be attached to the test for blood because saliva, pus, malt extract, vegetable extracts and the salts of heavy metals such as Co, Mn, Pb and Fe could give false positive reactions. Dervieux (1910) agreed with this view, suggesting that the test had no value at all, positive or negative.

The phenolphthalin to phenolphthalein oxidation reaction, and the structures of the latter compound in both acidic and basic solution, are shown in Fig. 6.2. Because phenolphthalein is colorless in acidic solution but pink to red in basic solution, it has been widely employed as a pH indicator. Many have noted that the phenolphthalin test is more sensitive than either the guaiac or aloin tests. Deléarde and Benoit (1908a) and Nicolesco (1934) have indicated positive reactions with blood diluted $1:10^6$, as has Girdwood (1926). Gettler and Kaye (1943) reported a sensitivity of $1:10^7$ dilution of whole blood, but of $1:10^6$ dilution for old, decomposed blood. Glaister (1926a) noted that saline extracts of 1 year old stains reacted at 1:212,000 dilutions but that a 1:800,000 dilution of a water extract of the stain gave a positive result. Kastle (1909) did sensitivity experiments by dissolving

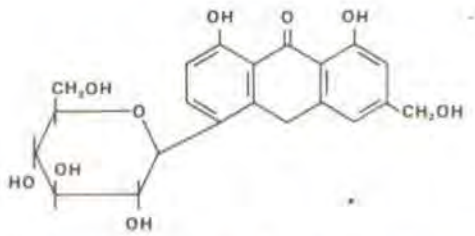


Figure 6.1 Barbaloin (1,8 dihydroxy - 3 - hydroxymethyl - 10 - (6 - hydroxymethyl - 3,4,5 - trihydroxy - 2 - pyranyl) - anthrone)

3.8 mg of blood in 100 mℓ water as a first dilution and making three serial ½ dilutions in addition. These, he denoted solutions (1), (2), (3) and (4), and they contained 38μg, 3.8 μg, 0.38 μg and 0.038 μg of blood per mℓ, respectively. One mℓ of each solution was tested by the addition of 2 mℓ reagent. Solution (3) which contained 0.38 μg/mℓ could be readily distinguished from the control colorimetrically. Solution (4), the weakest, could not be, but Kastle noted that two independent observers (he, presumably, being one of them) were able to distinguish the difference between solution (4) and the control by eye. In terms of dilutions of whole blood, therefore, since the assays were carried out in a total volume of 3 mℓ, solutions (3) and (4) correspond to about $1:8 \times 10^6$ and $1:80 \times 10^6$ parts blood to parts water. Kirk (1953) noted that $1:10^5$ dilutions of blood gave a positive reaction within 3 sec while $1:5 \times 10^6$ dilutions required 20 sec to do so.

Glaister (1926a) tested a variety of substances and body fluids, including rust, urine, saliva, semen, perspiration and milk, and got negative results with the phenolphthalin test. Kerr (1926b) took exception to Glaister's confidence in the method, noting that feces from patients taking aspirin gave a false positive test. Glaister (1926b) replied that, while he did not question the need for corroboration of the presence of blood, his own experience with the test had convinced him of its value in medico-legal cases. Girdwood (1926) noted that he did not think the test should be relied upon by itself as an indication of blood in stains, nor of occult blood in stool samples. Gettler and Kaye (1943) thought the test was more specific than guaiacum, benzidine or o-tolidine, and Gradwohl (1956) said that he preferred this test to benzidine. More recently, Higaki and Philp (1976) re-evaluated the test for blood in terms of sensitivity, reagent stability and specificity. The reagent was prepared essentially as recommended by Camps (1968), which follows almost exactly the original method of preparation used by Kastle (1909) except that the product is not isolated and recrystallized. Phenolphthalein (2 g) is dissolved in 100 mℓ water containing 20 g KOH and boiled with a reflux condenser in the presence of 20 g zinc powder until colorless. The resulting solution is kept in a brown bottle with some Zn dust present. The test was carried out in a number of different ways, with and without ethanol or methanol, and with peroxide or perborate. A so called one-stage test amounted to the addition of the combined reagents to the sample; a two-stage test consisted of the successive addition of reagent and either peroxide or perborate; a three-stage test involved the addition of the alcohol, the reagent, and the peroxide or perborate successively. Benzidine was employed as a control, because the experiments were designed to evaluate phenolphthalin as a substitute for benzidine in routine practice. It may be noted here that Camps (1976) recommended that the phenolphthalin test be substituted for the benzidine test. Dilutions of whole blood as well as stains prepared from them were tested. Stains were tested by applying reagents to a stained thread on filter paper. All results were recorded after 5 sec of observation. With liquid dilutions, ethanol or methanol, reagent and perborate, used in a one-stage test, proved to be most sensitive (in excess of $1:10^6$). A one-stage phenolphthalin-perborate test was sensitive to about $1:10^5$-$1:10^6$ dilutions, there being no advantage to a two-stage test with these reagents, nor to a three-stage test involving either alcohol. Reagent-peroxide combination, with or without alcohol, regardless of the number of stages, gave

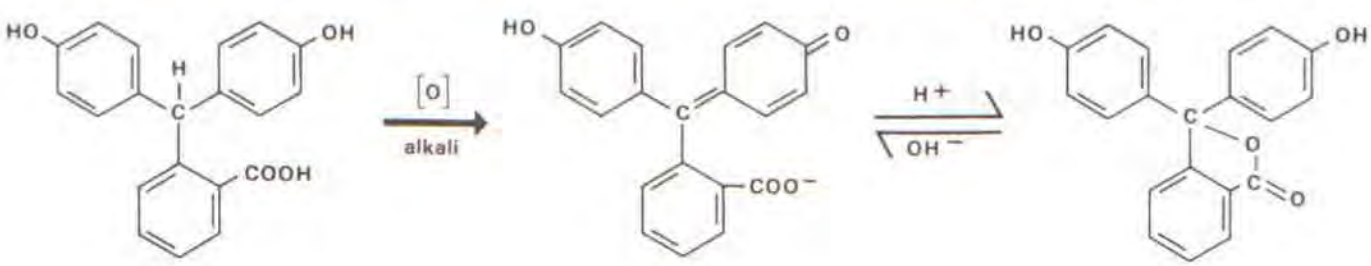


Figure 6.2 Phenolphthalin Oxidation and Phenolphthalein

sensitivities of about $1:10^4$-$1:10^5$ dilutions, fairly comparable to the benzidine control. With bloodstains, the one-stage methanol-reagent-perborate test was most sensitive, to about $1:10^3$ with the benzidine control being about $1:10^4$. Reagent, with peroxide or perborate, was considerably less sensitive in a one-stage test (neat-$1:10^2$ dilutions), and was unsatisfactory in a two-stage test, giving weak reactions at all dilutions to $1:10^2$. From the standpoint of stability, all perborate and one-stage reagents were relatively unstable. A three-stage test with peroxide, reagent and either alcohol, stored separately, was most desirable from the point of view of reagent stability. This test was recommended for screening, in spite of the fact that its sensitivity was of the order of $1:10^3$, because if greater sensitivity were required, the stain could be extracted, and the extract tested. Under these conditions, the sensitivity is increased by one to two orders of magnitude. The tests were performed on a number of vegetable extracts as well, the phenolphthalin test being found to be more specific than benzidine, particularly if the three-stage test with ethanol and peroxide was employed. Hunt *et al.* (1960) found that phenolphthalin gave positive reactions at blood dilutions of $1:10^7$, but that the reagent was useful for the examination of bloodstains only if extracts were made. The results obtained directly on stains, or on filter paper rubbings, were found to be less than satisfactory. These investigators noted further that the test appeared to be more specific for blood than some of the other catalytic tests, false positives having been noted only with copper salts, as had been noted much earlier by Glaister (1926a).

6.4 Benzidine Test

This test may well be the most familiar and widely used of the catalytic tests for blood. Its employment for that purpose began with a quite extensive series of experiments by Rudolf and Oscar Adler, published in 1904. A large number of oxidizable organic compounds were tested for their ability to form colored products in the presence of H_2O_2 and a $1:10^5$ dilution of rabbit blood in water. Among the substances tested were: aniline, its monomethyl-, dimethyl- and diphenyl- derivatives, p-toluidine, xylidine, the o-, m- and p-isomers of phenylenediamine, dimethyl-p-phenylenediamine, tetramethyl-p-phenylenediamine, phenol, p-amidophenol, the cresols, thymol, catechol, resorcinol, guaiacol, hydroquinone, pyrogallol, benzoic and salicylic acids, benzidine, tolidine, α- and β-naphthols, α-naphthylamine, the leuco bases of malachite green, brilliant green and acid green, methyl violet, crystal violet, a number of triphenylmethane dyes, eosins and rhodamines. The leuco base of malachite green (section 6.5) was used to test bloodstains, and this reagent, as well as benzidine and the leuco base of crystal violet, were recommended for the identificaiton of dilute blood in aqueous solutions. Benzidine and leucomalachite green were also recommended for testing for the presence of blood in urine and feces.

Benzidine is p-diaminodiphenyl (Fig. 6.3). The blue oxidation product obtained with peroxide in the presence of blood is often called "benzidine blue". The course of benzidine oxidation by peroxide is generally carried out at acid pH; a possible mechanism for the reaction is indicated in Fig. 6.4. The benzidine blue is an intermediate in the reaction. Eventually, the diimine compound forms, and it is brown. This latter may, in turn, polymerize. For histochemical work, the blue intermediate is more desirable than the brown end product and there have been efforts to find conditions which render it more stable. It is known that the stability is maximized at pH 4.5, and the blood test is usually carried out in acetic acid to achieve approximately this condition. The structure of benzidine blue is given by Feigl (1966) as consisting of one mole of amine, one mole of imine and one mole of whatever acid is present. Van Duijn (1955) looked into this question. He found that if milk peroxidase is used to form benzidine blue from benzidine and H_2O_2 in the presence of 5% NH_4Cl, the product had the composition $C_{24}H_{24}N_4Cl{\cdot}2H_2O$. The ammonium chloride had been found to stabilize the benzidine blue intermediate, a desirable goal in histochemical staining work. The amounts of time involved in the transition from benzidine blue to the brown diimine, which are of the order of minutes at acid pH, are too long to be of any practical concern for the medico-legal blood test, which occurs instantaneously or within a few sec.

This test quickly gained favor, and has become widely used. Messerschmidt (1909) preferred the test for detecting occult blood in feces, and recommended the method of Schlesinger and Holst (1906). Lyle *et al.* (1914) conducted experiments to determine the optimal concentrations of reagent, acetic acid and H_2O_2, primarily for the clinical test. They could achieve positive results with blood diluted $1:5 \times 10^6$ under optimal conditions, but were willing to allow up to 5 min for color development. In testing for the presence of blood in medico-legal exhibits, where little is known of the history of the article, the benzidine test has suffered the same criticisms levelled against other catalytic tests concerning specificity. Michel (1911) addressed the specificity problem in regard to metals. He reported that iron, copper and certain of their oxides, will oxidize guaiac, benzidine and leucomalachite green in the presence of H_2O_2. He noted, however, that treatment of the colored product obtained in the test with 2,4-diaminophenol would cause formation of a red-colored product if the original oxidation had been catalyzed by metals, but no such change would result if the original catalyst had been blood or pus. He added, however, that chloride or permanganate can oxidize the testing reagents, but are not subsequently affected by the 2,4-diaminophenol. Glemser (1939) noted that a variety of iron oxides cause the oxidation of benzidine, and concluded that the test could not be regarded as specific in the presence of rust. In 1973, Eisele tested approximately 50 substances for false positive benzidine reactions, and confirmed many of the observations of earlier workers. In addition, he confirmed the fact that fruit and vegetable extracts which contain peroxidases can give the reaction. Hunt *et al.* (1960) in their extensive studies noted that feces often gave a false positive,

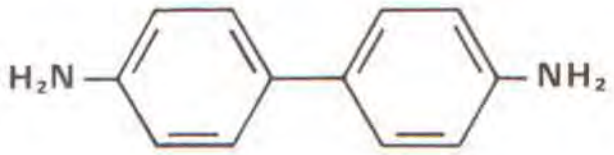


Figure 6.3 Benzidine

as did green leaf material smeared on filter paper. Smears of shoe polish (or leather) from used shoes gave a false positive reaction in one case of 30 tested. The fingernail scrapings from a grocer who handled fruits and vegetables gave the test, as did axillary wipings from normal men. These workers decidedly regarded the test as presumptive, and unequivocally stated so:

> . . . occasions do occur when a garment which is expected to be contaminated with blood gives a positive presumptive test for blood, although the deposit is insufficient or unsuitable for more specific tests. However tempting it may be to use this as evidence, it is scientifically and morally incorrect to do so, for it is clearly recognized that such tests are not specific and their introduction into evidence may well mislead. It is no argument that the evidence can be challenged, for to do so implies scientific advice which is not always available.

This strong remark was prompted in part by a case, which the authors review in the paper, in which a suspect willingly submitted to scientific investigation. His clothes and a leather container, alleged to have been used to transport the murder weapon (which was not recovered), were examined using the benzidine test. Material was insufficient for further tests. The Crown's expert testified that the benzidine test was presumptive, but upon being questioned, said that in skilled hands, it could be accepted as proof of blood's presence. That judgment, he said, would be based in large part on the time taken for the test to come up, but would not commit himself to a specific time period. In the estimation of Hunt *et al.*, the impression was left with the jury that blood had indeed been found, and there was an unstated implication that it was human. An opposite point of view on this question is discussed below.

In 1951, Grodsky *et al.* did a study of the catalytic tests and the luminol test (see Section 6.7). In addition to a number of important points discussed below, they noted that the use of sodium perborate instead of H_2O_2 for the benzidine, phenolphthalin and leucomalachite green tests represented a great improvement for the simple reason that the concentration of the perborate could be accurately determined, once reagent concentrations had been optimized. It should be noted that sodium perborate had been recommended previously as a substitute for H_2O_2 in the benzidine test (Lucas, 1935), but that Grodsky *et al.* (1951) were the first to suggest its use with phenolphthalin (Cf Higaki and Philp, 1976) and leucomalachite green. Grodsky *et al.* did not deny that a positive benzidine test alone should not be interpreted as positive evidence of blood without corroboration, but they did suggest that the use of several catalytic tests along with a luminol test provided far more convincing evidence than any one test alone (see Section 6.8).

There is no doubt that the majority of authorities have regarded the benzidine test as a sorting, or presumptive, test (Camps, 1968; Dérobert, 1974; Fiori, 1962; Gonzales *et al.*,

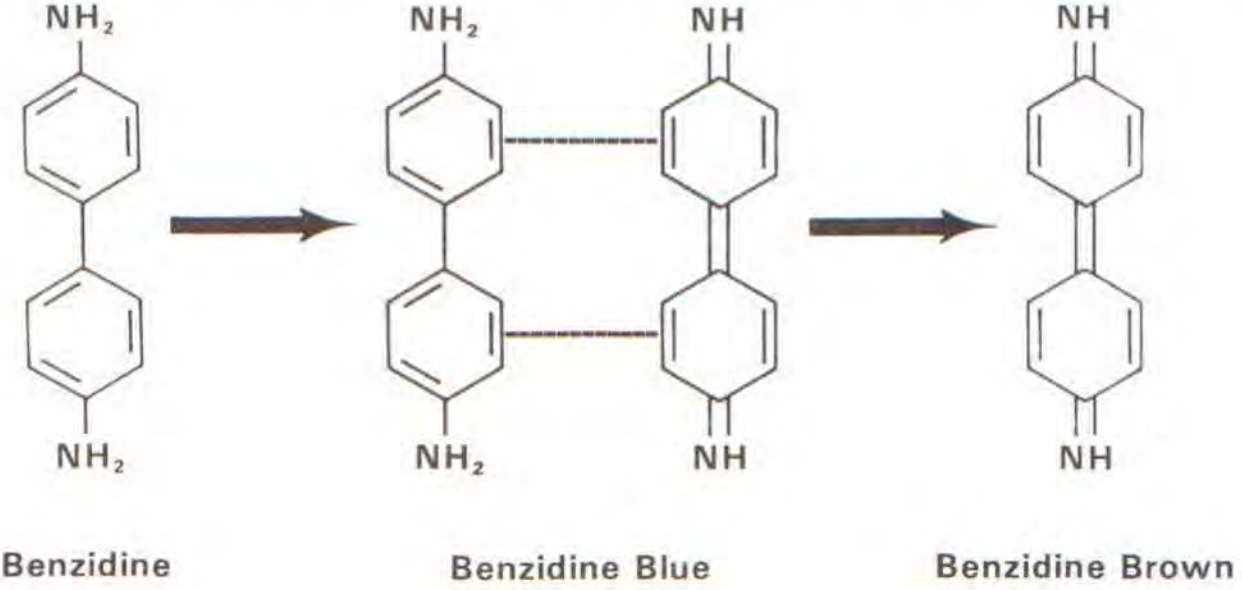


Figure 6.4 Course of Benzidine Oxidation by Peroxide

1954; Hunt *et al.*, 1960; Kerr, 1954; Lucas, 1935 & 1945; Mikami *et al.*, 1966; Prokop, 1966; Simpson, 1965; Thomas, 1937). In 1964, the benzidine test was critically examined by Culliford and Nickolls. They noted that by 1931, the test had fallen into disfavor, at least in some quarters, as a certain test for blood, Glaister having written in that year in the 5th edition of *Medical Jurisprudence and Toxicology:*

> While some employ this test, it has the disadvantage that, like the Guaiacum test, it can only be of value as a negative test, in that if no colour reaction occurs—blue or green—on applying it to a stain, it indicates the absence of blood. Should the colour reaction take place, it only suggests the presence of blood, since gluten, many plant juices as horseradish, and hypochlorites will give the blue colour reaction, although these may give the reaction either before or without the addition of ozonised ether. We do not put our trust in this test.
>
> We have abandoned completely the Guaiacum and Benzidine tests for the reason chiefly that the reaction obtained in the presence of minute amounts of known blood is uncertain and doubtful, and also because a reaction may be produced by it by substances other than blood. These objections do not apply to the Kastle-Meyer test.

On the other hand, Gradwohl (1954) wrote in his book, *Legal Medicine*, that positive reactions with benzidine (or phenolphthalin), assuming properly negative controls, do indicate "the presence of blood." This statement suggests that many laboratories regarded the test as considerably more valuable than did Dr. Glaister. Culliford and Nickolls point out, as did Grodsky *et al.*, (1951), that the judgment made about the test must be evaluated in the context of a number of variable parameters, such as the precise way in which the test was done (i.e., one-stage, two-stage, what controls were used, testing stains directly, testing extracts, etc.), the concentration and purity of the reagents used, whether peroxide or perborate was used, and the experience and judgment of the person carrying out the procedure. The major sources of "false positives" were categorized by these workers as: (1) blood contamination; (2) chemical oxidants or catalysts; and (3) vegetable or fruit peroxidases. Contamination should not be a problem in practice if the reagents are pure, the glassware clean, and the examination area kept scrupulously uncontaminated. The test is exceedingly sensitive, and contamination does not have to be very great to be a serious problem. Chemical oxidant interference is readily dispensed with by adding the reagent and the peroxide in successive steps; if color develops upon the addition of reagent alone, the presence of a chemical oxidant is indicated. Chemical catalysts, which work only in the presence of the peroxide, are not eliminated by the two-stage procedure, but Culliford and Nickolls argued that reactions caused by these materials appear quite different to the experienced eye than do those caused by blood. The plant peroxidases are heat-labile, and testing samples that have been heated to 100° for a few minutes serves to differentiate them from blood, which still reacts readily after the heating step. It was further shown that the majority of plant peroxidases which would give the reaction were quite labile in the dried state. These gave weak to negative benzidine reactions after three days in the dried state, whether the test was done directly on cloth, on a rubbing, or on an extract. Finally, these investigators described a simple electrophoretic procedure, carried out on 1% agar gels, for the differentiation of a great number of substances which could give misleading results in the benzidine test. The procedure is also described by Culliford (1971). It should be noted that these authors took strong exception to the statements of Hunt *et al.* (1960) above, to the effect that the reporting of a positive presumptive test in a case where there was no additional material available for testing would be scientifically and morally incorrect, because it could be misleading. Failure to report such a result, Culliford and Nickolls argued, would be to usurp the prerogatives of the Court, and in such a case as the one discussed by Hunt *et al.*, the result should be reported with a suitable explanation of its meaning.

Reports of the sensitivity of the benzidine test vary in the literature. Adler and Adler (1904) originally reported a sensitivity limit of $1{:}10^5$ dilution of whole blood in water. Nicolesco (1934), Dérobert and Hausser (1938) and Thomas (1937) all cite 1:200,000 dilutions as the limit. Grodsky *et al.* (1951), Hunt *et al.* (1960) and others have noted that the sensitivity quotations can be misleading, because the results depend on so many different parameters. Unless the technique and the reagents used are fully described, it is not at all certain that the results will be able to be duplicated exactly. Indeed, while it is easy enough to compare the sensitivities of reagents in terms of the maximal dilutions of whole blood which still give positive tests, it is not always clear how such values translate in their applicability to bloodstains. Hunt *et al.* (1960) noted great variability in different lots of commercially obtained benzidine. Expressed as the highest dilution of blood, dried onto filter paper, which gave the test, the sensitivity ranged from 1:20,000 to over 1:150,000, and was even more variable if the amount of time required for the color to develop were taken into account. Grodsky *et al.* (1951) noted that stains made from 1:300,000 dilutions of blood came up within 10 sec while those made from 1:100,000 dilutions came up within 1 sec. Akaishi (1965) reported a sensivity of only 1:12,800 for benzidine in stains made at that dilution, and noted that 20 sec was allowed for color development.

It may be noted here that Alavi and Tripathi (1969) recommended that blood testing in the field (e.g. at scenes etc.) could be done with benzidine-impregnated filter papers. The suspected material was moistened with water, the benzidine paper then pressed against it, and peroxide added to the paper from a sealed vial. The papers were said to be stable for up to 1 year and could be regenerated by soaking again in benzidine solution and drying.

The foregoing discussion of the benzidine test, without which the sourcebook would obviously be incomplete, may nevertheless be almost purely academic from a practical point of view. That benzidine was a chemical carcinogen has

apparently been known for some time. Hunt *et al.* (1960) mention that the manufacture of Analar Benzidine was discontinued in 1951 for that reason. Camps (1976) notes that phenolphthalin has replaced benzidine in routine practice. Rentoul and Smith (1973), in the 13th edition of *Glaister's Medical Jurisprudence and Toxicology*, suggest a saturated solution of amidopyrine in 95% ethanol as a benzidine replacement. (See Section 6.6.8). Apparently, therefore, the use of benzidine has been largely discontinued in England. Higaki and Philp (1976) carried out their study of the phenolphthalin test (see Section 6.3) primarily to check its applicability as a substitute for the benzidine test, suggesting that benzidine has been abandoned in Canada as well.

In this country, the use and manufacture of benzidine has become subject to extremely stringent restrictions and controls, according to regulations issued by the Occupational Safety and Health Administration of the Department of Labor (Code of Federal Regulations, 1976). While manufacture and use have not been ordered to cease, the regulations and restrictions are prohibitively involved for a laboratory doing routine work. Regardless of the merits of the test, or the qualifications that should or should not be place on interpretation of the results obtained with it in medico-legal cases, it is probable that the substance will shortly be unavailable. Supervisors will probably be increasingly unwilling to place their examiners at risk by continued use of the reagent, and the OSHA Regulations may also be sufficiently constraining that manufacturers will consider them prohibitive as well. It is likely, therefore, that the benzidine literature will shortly become a part of the archives of this field.

6.5 Leucomalachite Green and Leucocrystal Violet Tests

The use of the leuco base of malachite green as a blood testing reagent was first reported by Adler and Adler (1904), as noted above. The term "leuco compound", or in this case, "leuco base", comes from the literature of biological stains and dyes (Lillie, 1969). Compounds to be employed as stains or dyes obviously have to be colored. Although they differ greatly from one another chemically, all contain a chromophore group, a structure which renders them colored. They all share in common additionally the property of being reducible and reduction alters the chromophore group rendering the compound colorless. These colorless reduction products are referred to as "leuco compounds". Clearly, the leuco compounds are oxidizable to the dye forms. The "leuco bases" are particular types of leuco compounds, usually carbinols, and characteristic of the triphenylmethyl derivatives.

In the original work, the Adlers used leucomalachite green and leucocrystal violet, in addition to benzidine, for blood detection in aqueous solution. The structures of crystal violet (hexamethylpararosanilin) and malachite green are shown in Figs. 6.5 and 6.6, respectively. Only the leuco base of the latter has been widely used in forensic practice. The leucomalachite green test had a sensitivity limit, like

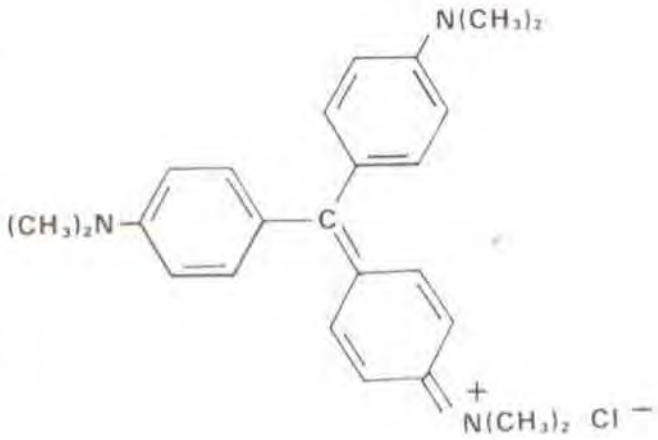


Figure 6.5 Crystal Violet

benzidine, of 1:10^5 dilution of blood. Michel (1911a) recommended this test and said that it was more sensitive then phenolphthalin. Von Fürth (1911) utilized the test on bloodstain extracts prepared by digesting the bloodstained material with 50% KOH in ethanol, and extracting that solution with pyridine. The test was then performed on a piece of filter paper, moistened with the pyridine extract. Medinger (1933) strongly recommended the reagent, and tested various physiological fluids, plant extracts and inorganic compounds, all with negative results, provided the peroxide was added in a second step after no color had developed in the presence of reagent alone.

White (1977) showed that leucocrystal violet was as sensitive in detecting iron (III) mesoporphyrin IX as a spot on filter paper as tetramethylbenzidine, guaiacum and aminodiphenylamine.

Alvarez de Toledo y Valero (1935) review the test rather extensively, and tested a large number of organic and inorganic compounds for false positive reactions. He found that there are many chemical oxidants that will give the reaction in the absence of peroxide, as well as some that catalyze the

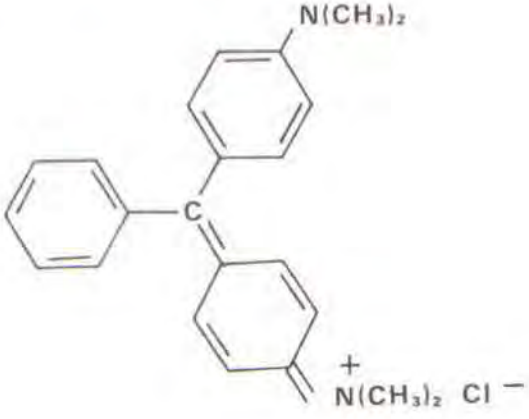


Figure 6.6 Malachite Green

reaction in its presence in much the same manner as does blood. The test is, therefore, not more specific for blood than most of the other catalytic tests.

The sensitivity of the test was originally reported to be $1:10^5$ dilution of blood by Adler and Adler (1904). Alvarez de Toledo y Valero (1935) reported the same value, but Nicolesco (1934) quoted the sensitivity as a 1:20,000 dilution of blood. Alvarez de Toledo y Valero noted that more dilute solutions of blood required longer times for color development, 1:1000 dilutions being instantaneous, 1:2,000 dilutions requiring 15–20 sec, and so on until 55 sec was necessary at $1:10^5$ dilutions and 25 min was needed for $1:2 \times 10^5$ dilutions. Grodsky *et al.* (1951) reported that stains made from $1:10^5$ dilutions of blood came up in 15 sec, using a reagent prepared by dissolving 0.1 g leucomalachite green and 0.32 g sodium perborate in 10 mℓ of 2:1 (v/v) glacial acetic acid in water. The benzidine test on the same sample, by contrast, came up in 1 sec. Hunt *et al.* (1960) similarly reported leucomalachite green to be less sensitive than benzidine, and apparently more specific. But the apparent increase in specificity was attributed to the lower sensitivity, and was thus not considered an advantage.

6.6 Other Catalytic Tests

Over the years a number of catalytic tests have been proposed for the detection of blood in stains, or of blood in feces or urine, which have enjoyed only limited use, or about which there is not a great deal of literature. All these tests are briefly discussed together in this section.

6.6.1 Peroxide

It should be mentioned that peroxide was sometimes used as a reagent for blood detection, especially in the older literature. That blood possessed a peroxidase activity, and acting as a catalase was thus capable of evolving oxygen from peroxide, has been known since the experiments of Schönbein (1863). All the catalytic tests are based on this principle, as discussed in the preceding sections; but all have relied upon the coupling of the peroxidase activity to the oxidation of a compound which formed a colored product.

Zahn (1871) specifically noted that peroxide could be used to detect blood in stains, though it is not clear that he was aware of Schönbein's work. If peroxide is brought into contact with a bloodstain the peroxidase reaction takes place after a minute or so, and is evidenced by the formation of large numbers of tiny bubbles. Gantter (1895) suggested that the test had substantial value if negative, i.e., was a good indication of the absence of blood. Sutherland (1907) referred to the test as the Zahn-Gantter Test, and noted that many of the substances now known to give false positive catalytic tests, such as vegetable extracts, gave this test as well. Cotton (1904) studied the evolution of oxygen in the presence of H_2O_2 from the blood of a number of different species. Palleske (1905b) also conducted studies on the test with different bloods. A positive test could be obtained with a drop of blood in 1500 mℓ of water, which, if we assumed that 20 of his drops would equal 1 mℓ, would represent a sensitivity of a 1:30,000 dilution. Sutherland (1907) thought the test was useful when negative, except where the stain had been heated above 120°. Leers (1910) presented the test as a presumptive one, noting that other substances than blood gave positive results. He called the test "Die Vorprobe mit Wasserstoffsuperoxyd" or, simply, "the preliminary test with hydrogen peroxide."

6.6.2 Eosin

In 1910, Ganassini proposed the use of an eosin reagent, prepared from crystalline eosin by heating in strong base, and collection and washing of the acid precipitate. This reagent in alcoholic solution in the presence of strong alkali and H_2O_2 gave a momentary yellow to red colored product. He believed the reagent to be specific for blood. Belussi (1911) disagreed, noting that other substances gave positive tests, and that the sensitivity of Ganassini's test was far lower than that of benzidine.

6.6.3 2,7-Diaminofluorene

There is a brief report in the literature by Schmidt and Eitel (1932) on blood identification using 2,7-diaminofluorene (also called 2,7-fluorenediamine). This report has mainly to do with a problem concerning the stability of the reagent, but the implication that the reagent was in use for the detection of blood is quite clear. The structure of 2,7-diamonofluorene is indicated in Fig. 6.7. The 7th edition of the *Merck Index* indicates that the reagent is used to determine halides, nitrate, persulfate and several metals. It seems likely, therefore, that these might be expected to give false positive reactions in the test for blood using this reagent.

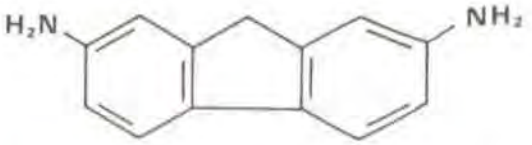


Figure 6.7 2,7 - Diaminofluorene

6.6.4 Rhodamine B

In 1917, Fuld recommended the use of a Rhodamine B reagent for blood detection. The reagent was prepared from Rhodamine B (Fig. 6.8) by reduction in base in the presence of zinc. This reagent detected blood at a dilution of $1:10^7$, according to Fuld. Alke (1922) studied the reaction in some detail, and reported that it is not given by semen, saliva, urine or a number of other biological substances. The usual inorganic oxidants will oxidize the reagents in the absence of H_2O_2. The reaction was negative with rust, and Alke reported a sensitivity limit of $1:10^5$ to $1:10^6$ dilution of blood. Ziemke (1938) notes that an investigator named Diels showed that chlorophyll gives the test. The reaction is apparently still positive with blood which is putrefied, or which has been heated above 200°.

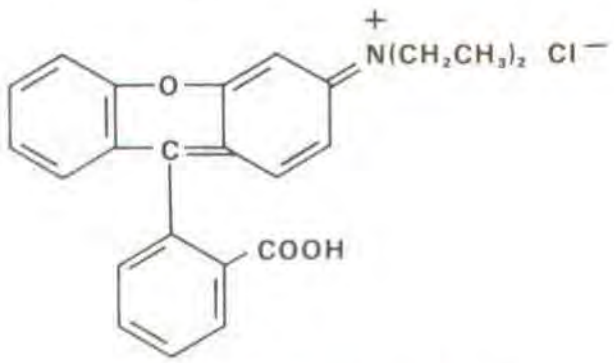


Figure 6.8 Rhodamine B

6.6.5 Para-Phenylenediamine

Boas, in 1906, recommended p-phenylenediamine (p-diaminobenzene) as a reagent for detecting occult blood in feces. Schumm and Remstedt (1906) evaluated the reaction, noting that under their conditions, the sensitivity could be as high as $1:10^5$ dilution of blood. The colors obtained varied, however, with different blood dilutions. Adler and Adler (1904) had tested this material, as well as the o- and m-isomers, in their experiments and obtained color reactions at sensitivities of 7:100,000 dilutions of blood, or about 1:14,285.

6.6.6 Ortho-Tolidine and ortho-toluidine

Ruttan and Hardisty (1912) recommended o-tolidine as a reagent for detecting occult blood. This reagent had a sensitivity limit of $1:7 \times 10^6$, an order of magnitude greater than that for benzidine, but somewhat less than that for phenolphthalin, which they reported as a $1:10^7$ dilution. The test was also recommended by Kohn and O'Kelly (1955) as a substitute for the benzidine test for occult blood, and Jacobs (1958) found the reagent to be a good substitute for benzidine for the staining of leucocyte peroxidases. In 1939, Gershenfeld wrote a paper noting that there had come to be some confusion in the literature as to the difference between o-tolidine and o-toluidine. The former is 3,3'-dimethylbenzidine (Fig. 6.9), while the latter is o-methylaniline (or 2-aminotoluene) (Fig. 6.10). Gershenfeld mentioned that some writers had erroneously credited the employment of o-toluidine for blood testing to Ruttan and Hardisty (1912), while these workers were responsible only for the employment of o-tolidine. Adler and Adler (1904) had tested p-toluidine but not o-toluidine in their studies. Either o-tolidine or o-toluidine may be used as reagents for blood testing, the former being more sensitive and giving a blue-colored oxidation product, while the latter gives a violet-colored one. The distinction is important, and should be kept in mind. Holland *et al.* (1974) confirmed Ruttan and Hardisty's earlier finding that o-tolidine is more sensitive than benzidine for blood testing, although the comparison in the more recent study was made on the basis of molar extinctions at maximum wavelength of visible absorption,

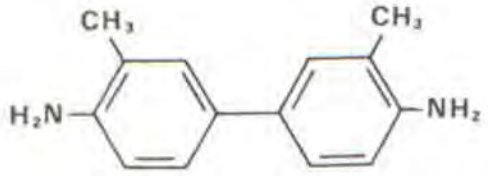


Figure 6.9 o-Tolidine

rather than in terms of maximal dilutions of blood which still gave positive reactions. Although o-tolidine has apparently not been placed on the OSHA regulations list (Cf:29 Code of Federal Regulations 1910.1000 *et seq.*), Holland *et al.* (1974) reported that it is carcinogenic in rats. Ferretti *et al.* (1977) reported that o-tolidine was mutagenic in the Salmonella/mammalian microsome test, indicating a high probability of carcinogenicity. Ortho-toluidine was not mutagenic in the test. Hunt *et al.* (1960) tested o-tolidine and found it to be quite similar to benzidine in most respects, and weak but slow positive reactions were obtained at $1:10^6$ dilutions of blood. Culliford (1971) mentioned that o-tolidine could be substituted for benzidine for routine identification testing.

6.6.7 Ortho-Dianisidine

Ortho-dianisidine is 3,3'-dimethoxybenzidine (Fig. 6.11). Owen *et al.* (1958) tested a number of compounds for the detection of the hemoglobin-haptoglobin complex in gels following zone electrophoresis. They found o-dianisidine to be the most sensitive, and to give the most stable color. The latter property led to its being adopted in clinical situations for the quantitative, colorimetric determination of heme compounds (Lupovitch and Zak, 1964; Ahlquist and Schwartz, 1975). Compton *et al.* (1976) used it to detect Hb-Hp complexes on cellulose acetate membranes following electrophoresis. Rye *et al.* (1970) said that there was no evidence that o-dianisidine was carcinogenic in humans, and that it was in fact metabolized by a different pathway than is benzidine, but Ferretti *et al.* (1977) reported that it behaved like benzidine and o-tolidine in the Salmonella/mammalian microsome test indicating that it might well be found to be carcinogenic. Culliford (1971) noted that this compound could be substituted for benzidine in identification tests for blood stains.

6.6.8 Amidopyrine

Amidopyrine, also known as aminopyrine, pyramidon, aminopyrazine, 4-dimethylaminoantipyrine, and a variety of other names, is 4-dimethylamino-2, 3-dimethyl-1-phenyl-3-pyrazolin-5-one (Fig. 6.12). Caplan and Discombe (1951) tested amidopyrine for the detection of blood in urine, and found it to be more sensitive than guaiacum, but less so than o-tolidine, benzidine or phenolphthalin. White (1977) found it to be among the least sensitive of a number of compounds he tested for the detection of Fe(III) mesoporphyrin spotted on filter paper. Owen *et al.* (1958) tested this material for

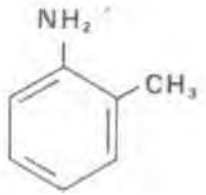


Figure 6.10 o-Toluidine

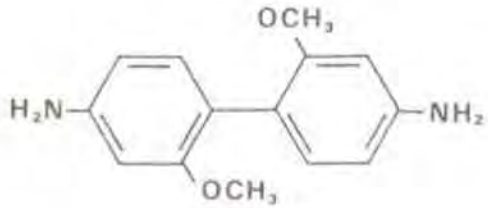


Figure 6.11 o-Dianisidine

the detection of Hb-Hp complexes following electrophoresis, but got poor results with it. As noted briefly above, however, Rentoul and Smith (1973) recommended amidopyrine as a benzidine substitute in blood identification tests. The suspect material is touched with a piece of filter paper, which is then first treated with a saturated solution of amidopyrine in 95% ethanol, and then with H_2O_2. A purple color appears in a few seconds if the test is positive, and the test was regarded as presumptive only. The test was said to work better with higher concentrations of peroxide, but these were not used because they were caustic to the skin.

6.6.9 Benzylidine dimethylaniline

In 1956, MacPhail reported that p,p′-benzylidine bis (N,N′-dimethylaniline) was an excellent reagent for the catalytic blood identification test. The compound is also known as 4,4′-tetramethyldiaminotriphenylmethane. Saline-moistened filter paper was placed in contact with the stain and then tested in a two-stage reaction. Reagent was made up 2 grains/oz 40% acetic acid. Sodium perborate (23 grains/oz 40% acetic acid) was used in the second stage. The oxidized product is momentarily green, passing quickly to a dark blue-green. Sensitivity was said to be in excess of $1:10^6$ dilution of blood, and the reaction only occurred with mammalian blood. False positives were not observed if the two-stage technique was employed. Williams (1974) recommended the reagent and said that it was superior to benzidine, there being less chance for false positive reactions.

6.6.10 3,3′,5,5′-Tetramethylbenzidine (TMB)

In an effort to find a safe substitute for benzidine, Holland *et al.* (1974) synthesized and tested 3,3′,5,5′-tetramethylbenzidine (TMB). The compound was prepared from 2,6-dimethylaniline, and its structure is shown in Fig. 6.13. There was some evidence, according to these authors, that carcinogenicity in these aromatic amines is related to the ability of the compound to undergo metabolic, or *in vivo*, o-hydroxylation. Methylation at all four o-positions renders this reaction impossible, and was expected, therefore, to decrease carcinogenicity. TMB was found to be much less carcinogenic in rats than either benzidine or o-tolidine, and more sensitive than either of them for blood testing. Garner *et al.* (1976) evaluated TMB as a reagent for the identification of blood in stains in comparison to benzidine. The sensitivity of the reagents, expressed as the weakest dilution of blood in saline that gave a positive test, was found to be identical at reagent concentrations of 0.05M, 0.1M and 0.2M, the last-mentioned being the saturating concentration of TMB in glacial acetic acid. At 0.2M strength, both reagents detected $1:10^6$ dilutions of blood. Studies on specificity were carried out by testing a number of plant extracts deposited on cotton cloth for false positive reactions. Testing was carried out on rubbings, extracts, and directly on the cloth. Positive reactions were obtained with both reagents on rubbings of horseradish stain, and with TMB but not with benzidine on rubbings of garlic stain. All other "false positive" reactions on rubbings or cloth were distinguishable from those of blood on the basis of the time of reaction or nature of the color developed. All saline extracts tested were negative with both reagents. Some types of papers gave positive reactions as well, but these too were qualitatively distinguishable from the blood reactions. Still, blood identification in stains on paper should be carried out with great care if TMB is employed. The reagents showed parallel decreases in sensitivity as a function of storage as 0.2M solutions in glacial acetic acid. The sensitivity had decreased to $1:10^5$ dilution of blood by the second day of storage, and to $1:10^4$ dilution by the eighth day. It made no difference to this decrease if the reagents were stored dark, or dark and refrigerated.

Nardelli (1976) has noted that parts of tangerine peels give positive but slow TMB reactions, a result which is not altogether surprising in view of the observation by Grodsky *et al.* (1951) that the white pulp, but not the juice, of the orange gave false positive reactions with benzidine. Nardelli also mentioned that saliva and asbestos glove material gave false positives. Garner *et al.* (1976) pointed out that as demand for and supply of TMB increases, and price decreases, the reagent may come to be used as commonly in forensic laboratories as was benzidine.

6.6.11 Chlorpromazine

Chlorpromazine is 2-chloro-10-(3-dimethylamino propyl)-phenothiazine. It can be oxidized by peroxide to a red free radical form, the reaction being catalyzed by peroxidases, including hemoglobin. The chromophore has an absorption maximum at 530 nm and Lee and Ling (1969) showed that the reagent could be used to quantitate Hb in serum. Collier (1974) confirmed these results and recommended chlorpromazine as a substitute for o-dianisidine and o-tolidine. White (1977) reported that chlorpromazine and

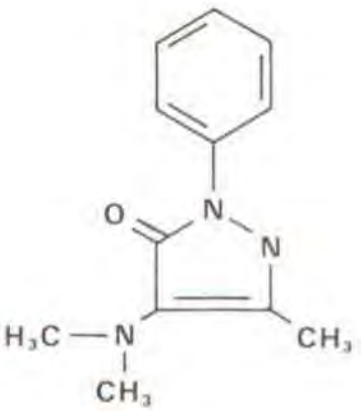


Figure 6.12 Amidopyrine

H_2O_2 detect Fe(III) mesoporphyrin IX as a microspot on filter paper.

6.6.12 Diphenylamine

In 1970, Woodman proposed the use of diphenylamine as a catalytic test reagent for blood in feces. The test was sensitive to about 1:4000 dilutions of blood in water and the oxidized chromophore is a green color. The reagent is also suitable for detecting hematin compounds on paper or cellulose acetate membranes following electrophoresis, and was recommended because it is not carcinogenic.

6.6.13 Fluorescin

Fluorescin is the reduced form of fluorescein, the latter being 9-(o-carboxyphenyl)-6-hydroxy-3H-xanthen-3-one. Fluorescein is soluble in alkali hydroxides and carbonates at room temperature, and exhibits an intense green fluorescence. In 1910, Fleig proposed using fluorescin to detect blood in the urine of patients. He prepared the reagent by reducing fluorescein in the presence of KOH, zinc dust and heat. He said that it was a more sensitive reagent than phenolphthalin, and suggested that it could be used to detect blood in dried stains as well.

We have conducted studies on the use of fluorescin as a presumptive test reagent, and have found that it is entirely satisfactory (Lee *et al.* 1979). We were unaware of Fleig's paper until quite recently. Fluorescin can be prepared from fluorescein in the same way that phenolphthalin is prepared from phenolphthalein. We find that the reagent is stable for months if kept over some zinc dust at 4°. We dilute it to about 1:60 with water for use, and the dilute solution is not as stable as the stock one. Water is preferable to ethanol as a diluent. A positive test can be obtained on blood dilutions up to $1:10^7$. It works on bloodstains on many different substrata.

6.7 Luminol Test

The luminol test is based on the fact that a number of hemoglobin derivatives greatly enhance the chemiluminescence exhibited by luminol upon its oxidation in alkaline solution. According to Proescher and Moody (1939), the compound was first synthesized by A. Schmitz in Heidelberg in 1902, under the direction of Prof. T. Curtius. Curtius and Semper (1913) then synthesized it in a different way, and referred to Schmitz's earlier work. But none of these workers noticed the chemiluminescence properties of the molecule. The intense blue chemiluminescence of the compound during its oxidation in alkaline solution was first observed by W. Lommel in Leverkusen (Germany) who brought it to the attention of H. Kautsky. Kautsky apparently interested H. O. Albrecht in looking into the properties of the phenomenon, the results of the investigation having appeared in 1928. Albrecht found that a number of oxidizing agents, which could be used in alkaline solution, brought about the luminescence, that H_2O_2 alone brought about only a feeble luminescence, and that luminescence was visible in a darkened room even at luminol concentrations of 10^{-8} M. Ferricyanide or hypochlorite greatly enhanced the luminescence obtained with H_2O_2, as did plant peroxidases and blood. Albrecht suggested a mechanism for the reaction as well, and this matter is discussed in more detail below.

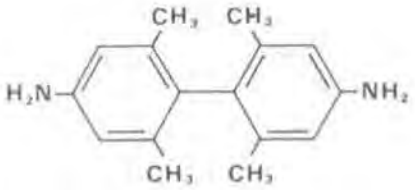


Figure 6.13 3, 3', 5, 5' - Tetramethylbenzidine

Luminol is 3-aminophthalhydrazide (Fig. 6.14). In 1934, Huntress *et al.* reported a method for the synthesis of the compound from 3-nitrophthalic acid and hydrazine sulfate, and named it "luminol". A year later, Harris and Parker, in the same laboratory, published studies on the quantum yield of the chemiluminescence. Gleu and Pfannstiel (1936) observed that crystalline hemin produced an especially intense luminescence, a fact soon confirmed by Tamamashi (1937). Specht (1937a, 1937b) did an extensive series of studies intended to design a useful medico-legal blood identification test based on luminol chemiluminescence. Old as well as recent bloodstains were examined, and able to be detected reliably using luminol reagent. Specht made two solutions, and noted that either one worked well: (1) 0.1 g luminol, 5 g $CaCO_3$, and 15 mℓ 30% H_2O_2 in 100 mℓ H_2O; (2) 0.1 g luminol in 100 mℓ 0.5% aqueous sodium peroxide. He tested a variety of substances for their ability to enhance peroxide-luminol luminescence in the same manner as did bloodstains. These included milk, coffee stains, semen, saliva, urine, feces, dyes, moldy bread, leather, fabrics, oils, varnish, wax, shoe polish, wood, grass, leaves and a number of metals, and all gave negative results. The reagent could be used in solution or sprayed onto suspected surfaces of all types using an atomizer. The spraying of luminol reagent onto bloodstain

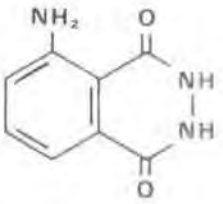


Figure 6.14 Luminol (3-aminophthalhydrazide)

material was said not to interfere with subsequent crystal or spectral tests, nor with serological tests for species or blood groups. The luminescence lasts for quite a while, at least a matter of minutes, and under proper conditions can be photographed to provide a record of the location of stains. Specht recommended the test strongly for medico-legal examinations, and believed that it was quite specific for blood. Proescher and Moody (1939) looked into the test fairly extensively, using commercial luminol at a concentration of 0.1 g in 100 mℓ 5% Na_2CO_3. This reagent solution was indefinitely stable. The test was performed by adding 15-20 mℓ 3% H_2O_2 or 1 g sodium peroxide to 100 mℓ luminol solution just prior to making the test. For the detection of fresh blood in solution, the hemoglobin was converted to hematin by the addition of concentrated HCl to the sample, which was then boiled briefly to destroy vegetable peroxidases. The test solution was then made alkaline again with sodium carbonate, and luminol reagent added. For bloodstains, the surfaces were first sprayed with 1-2% HCl, sprayed again after 10-15 min with sodium carbonate solution, and finally with luminol-peroxide reagent. Proescher and Moody regarded the test as extremely useful, but presumptive. The test could be given by hypochlorites, ferricyanide, and several other inorganic substances. Many of the substances with which Specht (1937a, 1937b) had obtained negative results were tested, and the negative results confirmed. Bloodstains were found to give more intense and longer lasting luminescence than fresh blood, and could be made luminescent many times by allowing the sprayed reagents to dry, and then re-spraying.

McGrath (1942) recommended the luminol test for use in forensic blood detection. He noted, as had others, that older stains gave more intense and longer-lived luminescence than fresher ones, because more met-Hb and hematin has formed in the older stains. He believed the test to be quite specific for blood, having obtained negative reactions with serum, bile, pus, semen, pleural fluid, earth, feces, fresh and spoiled vegetable material, various paints, metals, wood and shoe polish. He nevertheless cautioned that the test should not be used as a final, specific test for blood by itself. The main disadvantage of the test, in McGrath's mind, was that it had to be carried out in the dark. Kraul *et al.* (1941) noted that they regarded the test as a good presumptive one, but that it was not specific for blood. They also determined the wavelength of maximum chemiluminescence to be at 441 nm, with a shift to longer wavelength, 452 nm, in the presence of old blood. Schneider (1941) reported that a number of iron chlorophyll derivatives give luminescence with luminol-peroxide in sodium carbonate solution.

Grodsky *et al.* (1951) described a number of studies on the luminol test. They recommended a reagent consisting of 0.07 g sodium perborate in 10 mℓ water, to which is then added 0.01 g luminol and 0.5 g Na_2CO_3. The order of addition was important because the perborate is more soluble in water than in sodium carbonate, while the opposite is true of luminol. Reagents for the preparation of this reagent were incorporated into a field test kit recommended by the authors. The test was considered to be quite specific for blood, no false positives having been observed with a variety of materials that affected other catalytic tests. A noteworthy exception was copper salts. Grodsky *et al.* found that most brass, bronze and similar alloys which contain copper gave the reaction, a very important consideration if one is dealing with locks, door handles or other fixtures constructed of these materials. Indeed, Steigmann (1941) recommended the use of luminol for the detection of copper, as well as iron and cobalt, ions. He noted in 1942 that the reagent could be used for peroxide determinations as well.

Zweidinger *et al.* (1973) evaluated a number of types of film for the photography of bloodstains sprayed with luminol reagent. Various reagents, made with peroxide or perborate, were also tested. In aqueous solutions, the peroxide yielded a more intense, but shorter-lived chemiluminescence than the perborate. Similar results were obtained if the reagents were made up in 95% ethanol, it being necessary in this latter case to basicify the solution with 0.02 M KOH since sodium carbonate is quite insoluble in ethanol. A number of different photographic films were investigated, along with the effects of varying f/stop, exposure time and development conditions. It was possible to obtain good photographic records of bloodstains on items of evidence, and the procedure was recommended for adoption in routine practice.

One of the claims that has been made for the luminol test, particularly by those recommending sprayed reagents, is that its presence does not interfere with subsequent confirmatory blood tests or serological tests (Specht, 1937a, 1937b; Proescher and Moody, 1939; McGrath, 1942; Grodsky *et al.*, 1951). Srch (1971) reported, however, that the presence of luminol reagent on a sample may interfere with the Takayama test, the determination of ABH agglutinins by the method of Lattes, and the absorption-inhibition test for ABH agglutinogens. It did not interfere with the benzidine test, nor with species determination by the precipitin test. Schwerd and Birkenberger (1977) confirmed Srch's finding that luminol-peroxide spray can interfere with ABO grouping by inhibition technique, especially in small stains. Mixed agglutination could still be used, but did not work as well as on unsprayed controls. The precipitin test was not affected by the spray reagent.

An advantage of the luminol test is its great sensitivity. Albrecht (1928) stated that chemiluminescence was obtainable at luminol concentrations of 10^{-8} M, and Wegler (1937) reported luminescence at 10^{-10} g/mℓ luminol, or about

5.6×10^{-10} M (the MW of luminol is 177.16). The more usual way of expressing sensitivity, of course, is in terms of maximal dilutions of blood which still give positive reactions at some constant reagent concentration. Most authors have used 0.1% (w/v) luminol solutions, corresponding to about 0.056 M. Proescher and Moody (1939) said that the test was sensitive to $1:10^9$ dilutions of hematin. Grodsky *et al.* (1951) obtained luminescence lasting at least 15 minutes within 5 sec of spraying the reagent on stains made from $1:5 \times 10^6$ dilutions of blood. Weber (1966) quoted a sensitivity of $1:10^7$ dilution of blood using a photomultiplier tube detection system.

Bujan (1948) attempted to take advantage of the fact that luminol gives its intense luminescence with hematin, which is formed upon bloodstain aging. The luminescence intensity, measured photoelectrically, could be correlated with the age of the bloodstain, or with the amount of blood that was present in the stain.

The luminol reaction is somewhat more complex than those involved in the phenolphthalin, benzidine, leucomalachite green, and other catalytic tests. While it is probably not wrong to refer to the luminol test as a "catalytic test", it is not mechanistically a catalytic test in quite the same way as are the others. In dilute acid solution, luminol is relatively insoluble, and has the structure shown in Fig. 6.15(a). This compound gives a strong blue fluorescence with UV light. The tautomeric forms shown in Fig. 6.15(b) exist in alkaline solution, and it is these which produce chemiluminescence upon oxidation. Albrecht (1928) proposed a mechanism for chemiluminescence (Fig. 6.16), in which the phthalazine (I) was oxidized to form a diimide compound (II). This material would be hydrolyzed in the basic solution to yield the phthalic acid compound (III) and N_2H_2, which reacts with an additional mole of II to form nitrogen, IV and light. Compound IV, it will be noticed, is the form of luminol which exists in acid solutions (Fig. 6.15(a)), and thus presumably a partial regeneration of starting material. Tamamushi and Akiyama (1938) studied the reaction and their results were consistent with this mechanism, but Stross and Branch (1938) obtained results using fast-flow methods which could not be explained by it. Other studies were done by Sveshnikov (1938) whose results suggested a prior hydrolysis, with luminescence being due to the oxidation of a hydrolysis product. Kubal (1938) and Plotnikov and Kubal (1938) investigated the spectral changes associated with the reactions. In the presence of rhodamine or fluorescein, some of the chemiluminescent energy is apparently absorbed by the dyes, which then fluoresce at wavelengths longer than that of the luminescence. This phenomenon, they called chemifluorescence. Baur (1940) said that the decay of luminescence of luminol in the presence of hemin and peroxide followed a bimolecular rate law. Weber and co-workers carried out extensive studies on the luminescence reaction, and state, among other things, that substances which greatly increased the peroxide oxidation-dependent luminescence, such as chlorhemin, met-Hb and ferritin, do not, strictly

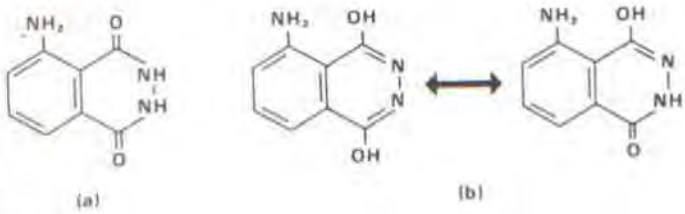


Figure 6.15 Luminol Structures in Solution

speaking, act as catalysts under all conditions (Weber, 1942; Weber *et al.*, 1942; Weber and Krajčinovič, 1942). Apparently, these compounds are best thought of as "accelerators", which may act catalytically. Weber *et al.* (1942) suggested that the products of the initial oxidation reaction included O_2 and reduced accelerator. If the reduced accelerator could be reoxidized by O_2, the compound would be acting catalytically, while if the reoxidation were not possible, the accelerator would have acted as a reactant. The subject is complex, and it may be that the mechanism is not the same with every "accelerator" or catalyst. Shevlin and Neufeld (1970) studied the mechanism of the ferricyanide-catalyzed luminescence of luminol, and proposed the scheme shown in Fig. 6.17 to explain their data. It should be noted that the ferricyanide acts catalytically in this scheme. The exact role of the catalysts or accelerators is not clear from Albrecht's scheme (Fig. 6.16). White and Roswell (1970) reviewed the chemiluminescence phenomena characteristic of organic hydrazides generally, including luminol. Isaccson and Wettermark (1974) noted that the mechanism of luminol oxidation in aqueous solution has still not been satisfactorily elucidated in spite of many studies.

It may be mentioned, finally, that Weber (1966) proposed an improved reagent for blood testing. Stock solutions were: (A) 8 g NaOH in 500 mℓ H_2O, or 0.4N; (B) 10 mℓ 30% H_2O_2 in 490 mℓ H_2O, or 0.176M; (C) 0.354 g luminol in 62.5 mℓ 0.4N NaOH to a final volume of 500 mℓ with water, or 0.004M luminol. To make up testing reagent, 10 mℓ of each of these solutions is mixed with 70 mℓ H_2O. It will be noted that the Na_2CO_3 used by others is here replaced by NaOH, and that the luminol and peroxide concentrations are very much lower. This reagent works well with both fresh and dried blood, whereas the older reagents did not readily react with fresh blood because there was too little met-Hb or hematin present. The fact that this reagent serves with fresh stains is explained by the rapid conversion of Hb to met-Hb and/or hematin in the strong base. The reagent is more sensitive than the older ones because of the lowered H_2O_2 and luminol concentrations. Higher concentrations of these compounds tend to be inhibitory.

6.8 Catalytic Tests—General Considerations

There can be no doubt that most authorities have considered the catalytic tests as presumptive when used alone. Their value is ascribed to the ease and rapidity with which

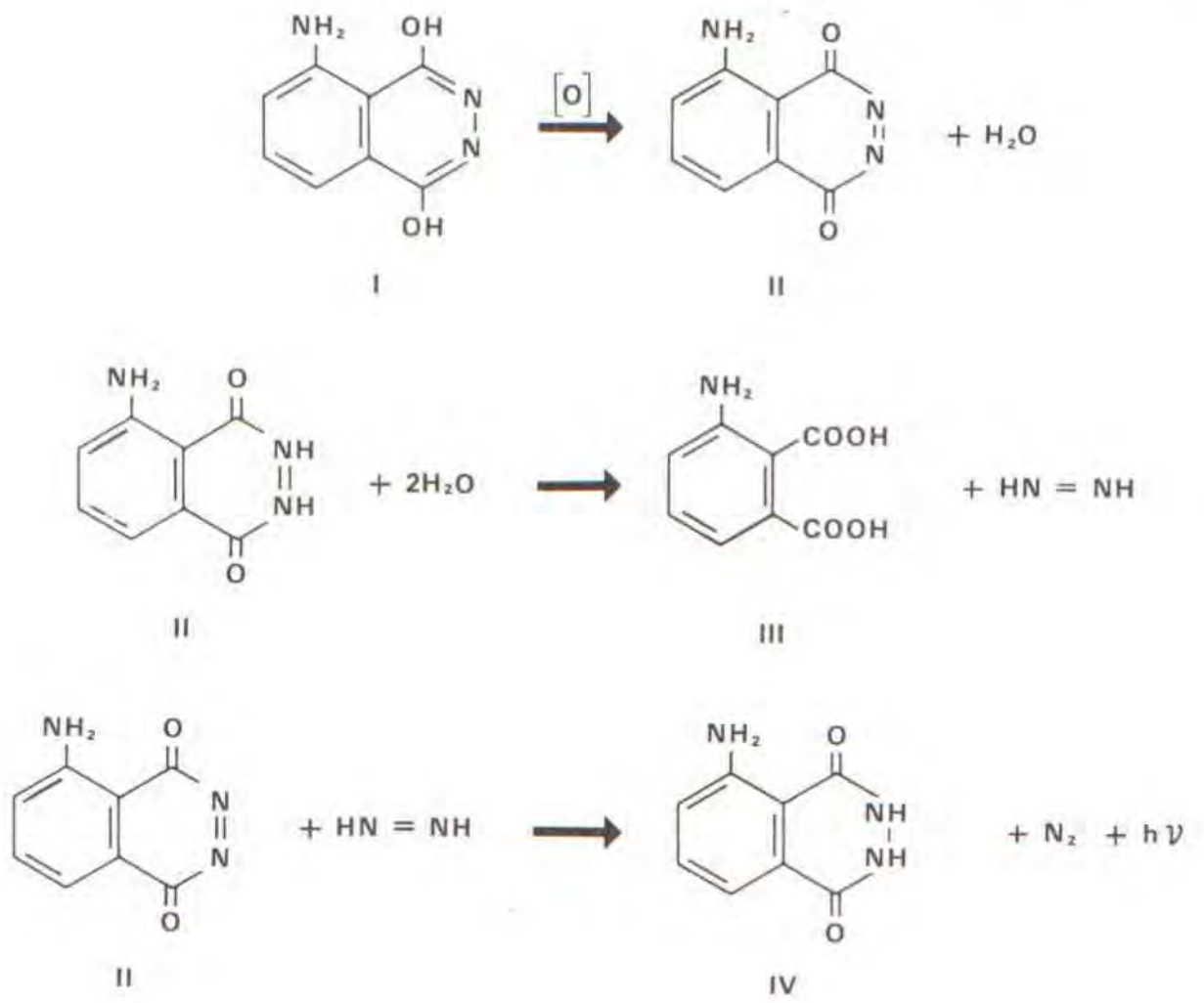


Figure 6.16 Albrecht Mechanism

they can be carried out in order to decide which exhibits should be subjected to further tests. It has occasionally been persuasively argued that in the hands of an experienced investigator, who is aware of the principles underlying the test and of the materials other than blood likely to give misleading reactions, the tests can be virtually specific. Grodsky *et al.* (1951) made something of a case for the combined use of several tests, namely benzidine, phenolphthalin, leucomalachite green and luminol, especially in cases where there was a limited amount of material to work with. These recommendations were based in part on their own studies, as well as on a set of experiments conducted by Pinker (1934). Pinker tested some 200 different biological substances and organic and inorganic chemicals for reactions with benzidine, phenophthalin and leucomalachite green reagents. A very small amount of the substance or stained material was treated directly with the reagent in a spotting plate. The reactions were carefully observed in terms of time, intensity, color produced, and other characteristic properties such as precipitate formation. Vegetable peroxidase interference could be eliminated by testing samples which had been heated, and chemical oxidants were detected by the fact that they react in the absence of peroxide. With these materials excluded, there was not found one interfering substance which would give a false positive reaction (indistinguishable from the blood reaction) with *all three* reagents. Pinker therefore argued that if positive reactions were given by all three reagents in carefully performed tests, the likelihood of error was exceedingly remote. Not all the same substances are likely to interfere with all the tests, especially with luminol as against the others. Grodsky *et al.* felt that there was no significant probability that any substance other than blood would be found which would give positive reactions in all four tests which would be indistinguishable from the blood reaction by an experienced worker. The argument was not put forth to suggest that these tests

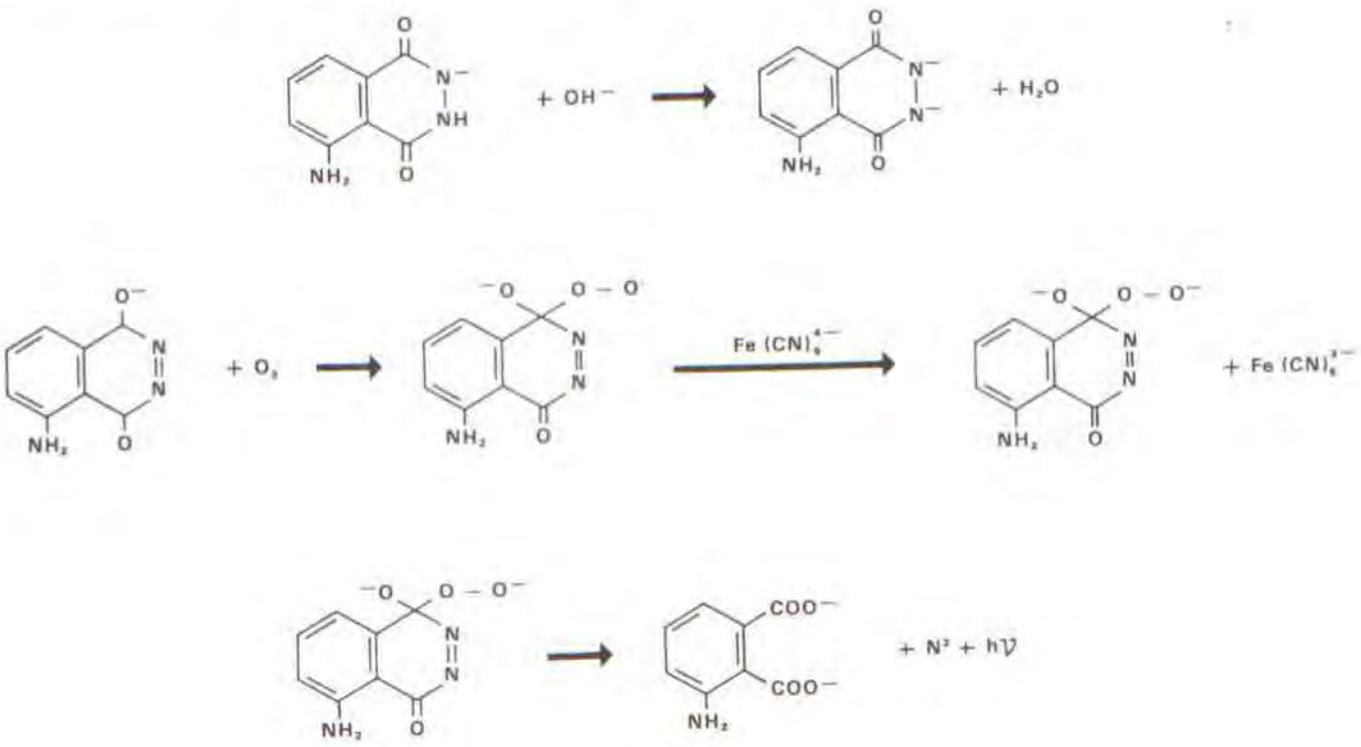


Figure 6.17 Shevlin and Neufeld Mechanism

replace crystal and spectroscopic tests; but cases do arise in which there is insufficient material for further testing. The results of Blake and Dillon (1973), discussed in Section 4.2.4, would suggest that for a number of examples of bacteria, whether in fresh culture or dried out on filter paper for 2 months, the use of several catalytic tests and/or a luminol test (Section 6.7) would not necessarily exclude false positive reactions. The ease with which suspensions of microorganisms gave the benzidine test was directly related to their catalase content, and the false positive reaction was not inhibited by exposure to high temperatures for periods of up to 30 min. Ultimately, of course, the decision concerning what proof value should be placed on the outcome of any test on a given exhibit rests with the individual expert. In the final analysis, the individual is giving his or her opinion as to what the findings mean.

A somewhat different issue, of no less concern, is raised by studies that have indicated the presence of blood on clothing and other samples that were randomly selected, and had nothing whatever to do with case material. Owen and Smalldon (1975) got positive benzidine reactions on 5 jackets of 100 tested, and on 16 pairs of trousers of 100 tested, randomly selected at a dry cleaning shop. One pair of used shoes of 100 pairs examined (50 men's and 50 women's) gave a positive reaction as well. Hunt *et al.* (1960) obtained strongly positive benzidine reactions (i.e. within 3 sec) on one of 30 used shoes randomly selected at a shoe repairer, and weakly positive reactions (i.e. within 5-10 sec) on six others. Terörde (1939) examined fingernail scrapings from 606 people in the general population for benzidine reactions. 195 samples were positive. 165 positive samples came from the 546 men in the sample, while the remaining 30 were from the 60 women. The individual's occupation or walk of life seemed to matter little, except in the case of butchers, whose scrapings were all positive. Additionally, Terörde got a positive precipitin reaction against anti-human serum with one of six of the benzidine-positive samples. The results strongly indicate that some consideration must be given to the actual evidentiary value of samples when positive results are obtained, especially in cases where little material is available for further testing.

Briggs (1978) discussed the matter of the probative value of bloodstains on clothing in connection with a case, which had required the examination of stains on a very large number of different articles. The relative occurrence of particular combinations of blood groups in the bloodstains enters into this discussion as well.

Sutherland and Mitra had earlier observed, and in another paper (1918b) suggested that it would probably be best to use rabbits for making antisera. Hektoen and McNally discussed the precipitin test in detail, including the preparation and evaluation of antisera in their review of medico-legal examination of blood stains in 1923. Fujiwara (1922a) said that heat-coagulated serum elicited antibody production as well as did fresh serum. Schmidt (1921), working with egg albumin, had shown that antibodies obtained with denatured protein could react with the denatured protein as well as with the native molecules. Blumenthal (1927) reviewed in detail the methods for making antisera, and gave what he regarded as the best protocol for immunization. Proom (1943) was able to obtain potent antisera in over 80% of the rabbits used with alum-precipitated antigen. Over the years, many investigators have looked into the preparation of antisera for the precipitin test. Many of these studies are discussed in some detail in Schleyer's (1962) review.

At present, many laboratories obtain antisera from commercial sources. Schleyer (1962) said that antisera should meet certain criteria before being employed in medico-legal work. They should be sterile, free from turbidity, of high potency and species-specific. By high potency was meant that the antiserum should have a minimum titer of $1:10^4$ to $1:2 \times 10^4$ against homologous antigen, and quite obviously the antiserum must not cross react with the sera of other animals to be exlcuded in the tests at the dilutions at which the test is performed. Otto and Somogyi (1974) said that most of the cross reactions of antihuman sera with nonhuman animal sera were accounted for by antibodies to IgG and to α-globulins.

It is worthy of mention that the use of adjuvants in immunizing animals to obtain antisera has permitted better yields of higher titer antisera in many cases. Since the work of Freund (see, for example, Freund and McDermott, 1942), many immunologists have employed adjuvants in the preparation of antisera. Herbert (1973) discussed the use of water-in-oil adjuvants, giving a review of the technique as well as some of the experimental details.

16.1.2 More recent developments—gel methods

Another important development has been the evolution of methods which use gel media for detection of the antigen-antibody reaction. Before gel media were introduced, the precipitin test was carried out in tubes. Often, antiserum, which is more dense than the usual aqueous (e.g. saline) antigen solutions, was placed into a tube and antigen solution carefully layered over the top so as to form an interface at which the precipitate could then form. Alternatively, the antigen solution could be placed in the tube first, and the antiserum solution layered underneath it by means of a capillary pipette. This procedure is sometimes called a "ring test". The earlier workers all employed this sort of method, in many cases using what would be considered by present standards excessive quantities of reagents, on the order of one to several mℓ. Material can be conserved by carrying out the test in small capillaries. The use of capillary tubes was first recommended by Hauser in 1904. Schoenherr (1952) gave a technique for the precipitin test in Pasteur pipettes. The ring test is still in use in many laboratories (see, for example, Boyd, 1946 and Hunt *et al.*, 1960). The test can be carried out on a microscope slide and the precipitate observed in the microscope under dark field illumination (Marx, 1920).

The first immunodiffusion experiment was done by Bechhold in 1905, although his primary interest was not in antigen-antibody reactions. He allowed goat serum to diffuse into a gelatin medium into which had been incorporated rabbit antiserum to the goat serum. The experiment was done to obtain a precipitate different from those obtained when inorganic chemicals were allowed to diffuse together in gels and form precipitates. One can, for example, put a drop of $Na_2Cr_2O_7$ onto a gel into which has been incorporated $AgNO_3$, and a series of concentric rings of $AgCr_2O_7$ precipitate will form. These are called "Liesegang rings", and Bechhold attributed their discovery to R. E. Liesegang in 1898. No notice appears to have been taken of Bechhold's experiments by the immunologists of the time. In 1920, Nicolle *et al.* devised a single immunodiffusion technique for the identification of bacteria. Bacteria were grown in agar medium containing specific antisera for particular bacterial species. If the bacterial cell wall antigens were homologous to the antiserum, a halo formed in the gel around the colony. None of the early workers properly appreciated the meaning and potential applicability of multiple precipitin band formations, which were occasionally observed, but thought they were similar to the Liesegang phenomenon. The possibility that they might represent more than one antigen-antibody system, present in the same test system, was overlooked.

In 1946, Oudin published his first paper on immunodiffusion. This work represented the beginning of the development of the technique as a powerful immunological tool. Antiserum containing gels were overlayed with homologous antigen solutions and the behavior of the precipitin band which formed in the gel was studied. The precipitin band appears to diffuse through the gel in proportion to the initial concentration of antigen, its diffusion coefficient, and inversely to the antibody concentration. Multiple antigens cause the formation of multiple bands, which migrate independently. In succeeding years Oudin developed the method further and devised a theoretical basis for the observations (Oudin, 1947, 1948 and 1949). A review of the principles underlying Oudin's technique and immunodiffusion generally may be found in Oudin (1971), Aladjem (1971) and Oudin and Williams (1971).

Oudin's technique is called single immunodiffusion. It is discussed in connection with the principles of immunodiffusion in section 2.2.

The late 1940's witnessed the development of techniques for double diffusion analysis of antigen-antibody reactions in gels by Elek in England and Ouchterlony in Sweden. The method was developed primarily for assessing the diphtheria bacillus toxin reaction with homologous antitoxin. Elek pub-

lished his preliminary findings in 1948, a more detailed account following in 1949. Ouchterlony published his first account in 1948 as well (Ouchterlony, 1948a and 1948b). The theoretical basis was laid down in a series of papers in the following year (Ouchterlony, 1949a, 1949b and 1949c). Elek's interest in the subject did not continue, but Ouchterlony has continued to work in the field, one of the best single references being his monograph, published in 1968. This work covers not only the agar gel method, but discusses some of the other support media such as cellulose acetate membranes. Detailed descriptions of various methods are given, as well as the underlying theoretical basis for the variations which have been described. Crowle's book also gives a very complete treatment of the subject (Crowle, 1973). Oakley and Fulthorpe (1953) developed the double diffusion technique for use in tubes, with special reference to its application to the analysis of bacterial toxins. Double diffusion is discussed in section 2.2.2.

Muller *et al.* (1950) applied Oudin's technique to medicolegal practice. Gum acacia was tried as a medium but was abandoned in favor of agar and agar-gelatin mixtures (Muller & Michaux, 1952). In 1958, Muller *et al.* noted the applicability of the double diffusion methods. Species determination could be carried out using a microdiffusion technique in agar originally devised by Hartmann and Toillez in 1957 (Muller *et al.*, 1959). Mansi (1958) devised a microimmunodiffusion technique which was used and recommended by Fiori (1963). Gajos and Brzecka (1968) said that threads from bloodstained fabrics could be incorporated directly into the gel for species determination, thus avoiding not only the time required for extraction, but the concomitant loss of material. Maresch and Wehrschütz (1963) described a precipitin test for species determination carried out on microscope slides in 2 mm thick agar gels. Feinberg (1961) devised a "microspot" double diffusion test, which could be carried out on microscope slide cover slips. Katsura (1976) utilized this method to diagnose the species of origin of very small amounts of blood using anti-human Hb antisera (see section 7.1). The precipitin lines were visualized using a phase contrast microscope, and even stains 48 years old were said to react within 3 hrs at 30°. Feinberg (1962) published a modification of his spot test for use on cellulose acetate membranes. El-Guindi (1972) reported successful results with an ordinary macroimmunodiffusion procedure in agar gels. He used several arrangements of sample and antiserum wells.

In 1959, Bussard suggested taking advantage of the electroendosmotic properties of agar in carrying out immunoelectrophoretic analysis of antigen-antibody reactions. A system could be devised in which the antigen and the antibody migrated toward one another, a precipitate forming at the point of their interaction. This technique, he called electrosyneresis. It has also been called electroprecipitation and crossed over electrophoresis or crossing over electrophoresis (see section 2.4.2). Culliford (1964 and 1967) devised a crossed over electrophoretic technique for species determination in forensic casework which allowed a large number of samples and controls to be run simultaneously in a short period of time, using only a few $\mu\ell$ of material for each test. In this method, small wells about 1.5 mm in diameter are punched in an agar gel about 5 mm apart. The stain extract (antigen) is placed in the cathodic well of a neighboring pair, and the antiserum in the anodic one. The γ-globulin antibodies migrate cathodically because of electroendosmosis, while the other serum proteins migrate anodically. The net result is a precipitin reaction occurring about midway between the paired wells. The test was performed in veronal buffers, pH 8.6, containing calcium lactate. Čarny reported a similar technique in 1971. Grunbaum (1972) noted that crossed over electrophoresis could be done on cellulose acetate membranes as well. Although it is usual practice to prepare antisera against whole human or animal sera, Tran Van Ky *et al.* (1968) recommended the use of antisera prepared specifically against the γ-globulin fraction.

Lincoln (1975) reported on a very interesting series of cases involving alleged witchcraft, illustrating how the events were unraveled by determining the species of origin of the articles involved.

16.1.3 Effects of some external influences

The age of a stain alone apparently does not prevent a positive precipitin test. Haseeb (1972) got a positive test on a 12 year old stain kept at room temperature. Linoli (1971) examined the "flesh and blood" from the eucharistic miracle that is said to have occurred at Lanciano, Italy, in the 7th Century A.D. The blood, which was about 1200 years old at the time of examination, gave a positive precipitin test with anti-human serum. Smith and Glaister (1939) reported a positive precipitin test on extracts from mummified tissue many thousands of years old. It has been known for a long time, however, that old blood stains do not easily yield serum proteins to mild, aqueous extracting solvents, like water or saline. Dorrill and Whitehead (1979) said that 5% (v/v) 0.880 ammonia was a much more effective extraction medium for older stains than water for purposes of extracting serum protein for species determination in older stains.

Muller *et al.* (1966) showed that a positive precipitin test could be obtained on stained garments which had been dry cleaned. There are circumstances, however, under which the precipitin test may fail. Since antisera are almost universally prepared against serum proteins, serum protein must be present in, and extractable from, the stain if a reaction is to be expected. There is not a necessary relationship between the amount of hemoglobin and the amount of serum protein in a stain extract. One cannot judge how much serum protein may be present on the basis of the amount of pigment present, as was pointed out by Okamoto in 1902. With fairly freshly dried stains there is a strong likelihood that considerable blood pigment will be extracted, but sometimes without sufficient serum protein to give a positive precipitin test, particularly if extraction time is short (Mueller, 1934). 24 hour extraction times are recommended in such cases. Mezger *et al.* (1933) noted that with a blood stain on wood,