Chapter 8
Identification of Blood and Body Fluids*

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Chapter Outline

*This chapter is based in part on Chapter 14, "The Identification and Characterization of Blood and Bloodstains," by Robert P. Spaulding, and Chapter 15, "Identification of Biological Fluids," by Andrew Greenfield and Monica M. Sloan in the third edition of this text.

SIDEBAR 8.1. CAREER PREPARATION AND EXPECTATIONS

Forensic serology is no longer a commonly recognized discipline due to the advent of DNA typing methods. Most of the presumptive tests described in this chapter are performed by DNA analysts or by trace evidence analysts. You will find more information about career preparation for those fields in those chapters.

8.1 Forensic Serology

Technologies such as DNA analysis have captured our attention for both the uniqueness involved and the certainty with which an individual may be identified. However, DNA is a relative newcomer to forensic science, not much more than 30 years old. What preceded DNA as a means of analysis of blood and body fluids? The discipline called forensic **serology**. A study of serology involves the examination and analysis of body fluids and, among those fluids, blood. In the medical setting, blood is analyzed to assess one's state of health. Serologists type blood into groups in systems such as ABO and Rh. For example, if your blood type is A+ (a common type), this means that your red blood cells are type A and that your Rh type is +. These notations refer to the presence or absence of substances in your blood that can react with antibodies. In the forensic realm, blood might be analyzed to determine its source at a crime scene or on an item of evidence. The difference doesn't end here. A clinical serologist typically deals with samples that are fresh, normally liquid, and usually recently acquired from a source individual. Forensic serology deals not only with a variety of body fluids (blood, saliva, semen, and urine) but also, and more frequently, with samples that are in stain form and often degraded or deteriorated, making successful analysis more difficult. The reliability and discriminatory power of DNA analysis has led to the reanalysis of a number of cases in which the innocence of a convicted defendant has been established and the individual released. The superior power of DNA in individualization has been able to greatly extend the biochemical information provided by serology. This is not to say that the original serological work was wrong or performed with any fraudulent purpose but simply that employing DNA can provide more specific information, far exceeding that of serology. In situations such as these, the evaluation of the original serological data is made easier with a reference such as this text. From the foregoing, it should be evident that knowledge of serology or at least an availability of information on the subject is important to thorough forensic study. It is the intent of this text to review and discuss the more commonly used techniques of forensic serology regarding blood, provide sources of information, and present newer information. (For a discussion of career preparation for this field, see Sidebar 8.1.)

8.2 Blood

Identification of a stain as blood is one of the most important preliminary tests performed on physical evidence and one that DNA protocols have not replaced. Because of the time, cost, and complexity of DNA typing, it is impractical to test every stain

that appears to be blood. This is where **screening** or **presumptive testing** is most important. Typically, the flow of analysis of a stain suspected to be blood moves from more general and less specific testing to DNA typing, which under the best circumstances can match a stain to an individual with reasonable scientific certainty. The analysis begins with a careful visual examination of the item of evidence to locate any stains or material visibly characteristic of blood. Any stains that look promising are then tested using a screening test; we will discuss the most common ones shortly. If the test is positive, then the species of origin of the stain may be tested but this is not always the case. We will see why in the next chapter. Finally, the blood is characterized using DNA, which will be discussed in detail in the next chapter.

8.2.1 What Is Blood?

We discussed the composition of blood briefly in Chapter 4 in the context of bloodstain patterns. To review, blood is classified as an **extracellular fluid**, meaning it is found outside of cells in the body. We discussed some of the characteristics of blood in Chapter 4, and a few points are worth highlighting. Blood has many functions and is a complex mixture of organic and inorganic materials, including electrolytes such as sodium, proteins, and several different kinds of cells. The characteristic red of blood comes from the complex formed between **hemoglobin** in red blood cells (RBCs) and oxygen. The typical adult has a blood volume of around 4.5 to 6.5 liters (~1 to 2 gallons) depending on size and gender.

By spinning a blood sample in a centrifuge, it can be divided into a cellular component (approximately 45% of the total volume) and a non-cellular component called *plasma*, which makes up the remaining 55%. Plasma can be further subdivided into serum and fibrinogen, the material that forms clots. Serum, a clear straw yellow in color, carries electrolytes, with the sodium ion (Na^+) and the chloride ion (Cl^-) being the most concentrated. The bicarbonate ion (HCO_3^-) is also found in high concentrations and this is the form in which waste carbon dioxide (CO_2) is transported in the blood. Proteins (albumins and globulins) are also carried in the serum.

The cellular portion of blood can be divided into three types of cells: red blood cells (RBCs, or erythrocytes); white blood cells (WBCs, or leukocytes); and **platelets** (**thrombocytes**). RBCs, which transport oxygen and bicarbonate, are the most numerous and are unique in that they lose their nucleus before entering into the circulatory system. The substance that transports oxygen is hemoglobin. WBCs (several types exist) are the next most numerous and are active in fighting diseases. Platelets are needed for clot formation. DNA typing targets DNA found in the nuclei of cells and therefore requires white blood cells (or a cell with a nucleus).

8.2.2 Identification of Blood

Identification of blood usually employs a presumptive test frequently followed by a **confirmatory test** to clearly establish the identification. A visual observation of the untested stain, coupled with positive chemical presumptive and confirmatory tests, then provides sound data to support the identification of blood. A presumptive test is one that, when positive, would lead the forensic examiner to strongly suspect blood is present in the tested sample. Notice this is not absolute; further testing is always required to confirm the results. When the results are negative, the test often helps to eliminate stains that need no further consideration. In the event of a positive test, when sufficient sample remains, further action to confirm the presence of blood is usually taken, since no single test is absolutely specific for blood.

Presumptive tests may be recognized as those that produce a visible color reaction or those that result in a release of light. Both types rely on the catalytic properties of blood to drive the reaction.

8.2.3 Catalytic Color Tests

Catalytic tests employ the chemical **oxidation** of a **chromogenic substance** (one capable of generating a colored species) by an oxidizing agent catalyzed by the presence of blood or, more specifically, the hemoglobin or red pigment in the blood. Those tests that produce color reactions are usually carried out by first applying a solution of the chromogen to a sample of the suspected material/stain followed by addition of the oxidizing agent (often hydrogen peroxide in a 3% solution). The catalyst is actually the peroxidase-like activity of the heme group of hemoglobin present in the RBCs. A rapidly developing color, characteristic of the chromogen used, constitutes a positive test. Some methods employ the chromogen and the oxidant in a single solution. There is a potential disadvantage in this, however, as the order of addition of the specific reagents can be important. A non-blood sample capable of producing a color reaction will normally do so without the addition of the oxidizing agent so that when the color reagent is added first there is a reaction (without the addition of the oxidant). With a single solution, a reaction due to a non-blood material might be incorrectly interpreted. Twenty or more substances have been investigated over the years as chromogens; we will focus on the ones most commonly used today.

Because these tests are presumptive in nature, inevitably there are instances of **false positive results** (a positive result from a substance other than blood for example) and **false negative results** (a negative result even when blood is present). Misleading results usually can be attributed to chemical oxidants (often producing a reaction before application of the peroxide), plant materials (vegetable peroxidases are thermolabile and can be destroyed by heat), or materials of animal origin (to include human) which are not blood but may contain contaminating traces of blood. An analyst must always be cognizant of this possibility. The proper use of positive and negative controls is an important step in minimizing false positives and false negatives.

One method of applying the presumptive test involves sampling a questioned stain with a clean, moistened cotton swab and adding a drop of the color reagent solution followed by a similar amount of hydrogen peroxide (H_2O_2). With this procedure, the immediate development of the color typical of the particular reagent used indicates the presence of blood in the test sample. Alternatively, the evidence could be sampled by removal of a thread or fragment of dried material and testing it with the above reagents in a spot plate. Color development would then be observed in the spot plate as well. Immediate (within a few seconds) reading and recording of results is an important aspect of test result interpretation. A clearly negative result may appear positive several minutes after the test is completed due to a slow oxidation that often occurs in air. These tests are not usually affected by the age of the stain.

8.2.4 Benzidine (Adler Test)

Benzidine has been used probably more extensively than any other single test for the presumptive identification of blood. The reaction (Figure 8.1), normally carried out in an ethanol/acetic acid solution, results in a characteristic blue to dark blue color. The blue, in turn, may eventually turn to a brown. Benzidine, however, is recognized as a carcinogen and is seldom used in forensic laboratories today. Other test reagents such as *o*-toluidine were largely abandoned for the same reason.

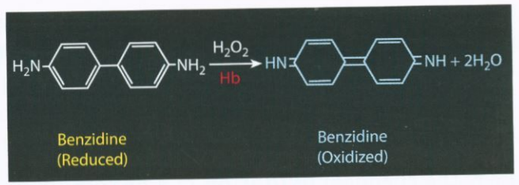

Figure 8.1 Benzidine oxidation.

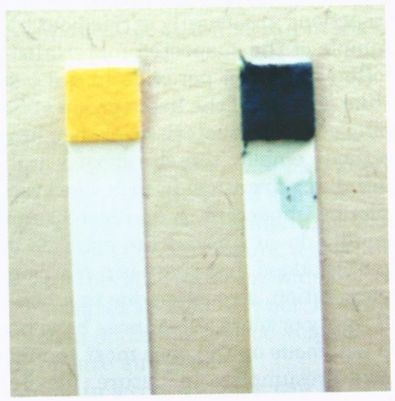

Figure 8.2 Hemastix® negative and positive reactions.

8.2.5 Tetramethylbenzidine and Hemastix®

With the recognition of benzidine as a carcinogen, the search for replacement substances was under way. One compound studied and still used is the 3,3′,5,5′-tetramethyl derivative of benzidine. **Tetramethylbenzidine (TMB)** is used in an acid medium (acetic acid) when employed as a solution, and the resultant color is green to blue-green. The **Hemastix®** test has been adopted for field use by a number of laboratories, particularly at crime scenes when containers of solutions can be hazardous or, at best, inconvenient. The test itself consists of a plastic strip with a reagent-treated filter paper tab at one end. Testing a bloodstain may be accomplished by moistening a cotton swab with distilled water, sampling the stain, and touching the swab sample to the reagent tab on the strip. The reagent tab is originally yellow, and a normally immediate color change to green or blue–green indicates the presence of blood (Figure 8.2).

8.2.6 Phenolphthalein (Kastle–Meyer Test)

A test procedure that is commonly used in many forensic laboratories today involves the simple acid–base indicator **phenolphthalein**. Phenolphthalein produces a bright pink color when used as above in testing suspected blood. The reagent consists of reduced phenolphthalein (phenolphthalin) in alkaline solution, which is oxidized by peroxide in the presence of hemoglobin in blood. The reaction shows phenolphthalin (which is colorless in alkaline solution) being oxidized to phenolphthalein (which is pink in an alkaline environment) (see Figures 8.3 and 8.4). As with any of the catalytic tests the result is read immediately, and a

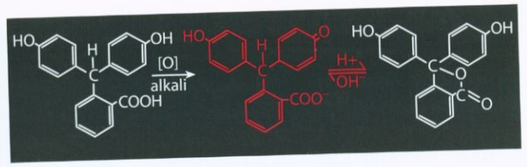

Figure 8.3 Phenolphthalin oxidation. Phenolphthalin (left, colorless) is catalyzed to phenolphthalein (pink in alkaline solution, center), which is colorless in acid (right).

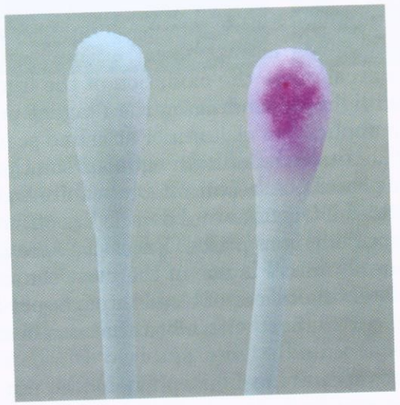

Figure 8.4 Negative and positive phenolphthalein reactions.

positive result a minute or more after the test is performed is usually not considered reliable. False positives with phenolphthalein usually are not really positives, in that the reaction is often not the characteristic pink but usually some other color change.

8.2.7 Tests Using Chemiluminescence and Fluorescence

Often, the presence of blood is suspected, based on witness information or because it would be expected in a particular location, but under normal lighting and viewing little is to be seen. A drag pattern across a floor that has been cleaned up or a washed spatter pattern on a wall might be typical examples. At this point the **luminol** and **fluorescein** tests may come into play. They involve spraying a chemical mixture on a suspected bloodstained area, usually *in situ*, and observing (sketching, photographing, etc.) the result, either in darkness or in reduced light with the aid of an **alternative light source (ALS)**. When luminol reacts with blood, light is produced that often enables the observer to determine the limits, shape, and some degree of detail in the original bloodstained area, often including an enhancement of blood patterns already present.

The presence of blood and patterns displayed by blood can provide information of value, and one should be alert to both. Further, the nature of these tests makes them potential sources of contamination of the blood. Thus, if the stain can be seen and collected, these tests probably should not be used. Because these tests do add material to the tested area, their use should be carefully considered, often as a last resort. These tests are of more value in locating and defining blood than in specifically identifying it.

Luminol and fluorescein each produce light but they do so in different ways. **Chemiluminescence** and fluorescence are two entirely different phenomena. Chemiluminescence is the process by which light is emitted as a product of a chemical reaction. No additional light is required for the reaction to take place. Luminol relies on this process. Fluorescence occurs when a chemical substance is exposed to a particular wavelength of light (usually short wavelengths, such as ultraviolet), and light energy is emitted at longer wavelengths. Light produced by fluorescein is a result of this irradiation, usually by light in the range of 425 to 485 nanometers.

Luminol reacts in a fashion similar to that of the color tests discussed above wherein luminol and an oxidizer are applied to a bloodstain. The catalytic activity of the heme group then accelerates the oxidation of the luminol, producing a blue-white to yellowish green light (depending on the reagent preparation) where blood is present. The forensic application of luminol at crime scenes involves spraying a mixture of luminol and a suitable oxidant in aqueous solution over the area thought to have traces of blood present. A resultant blue-white to yellow-green glow will indicate the presence of blood. Outlines and details are often visible for up to 30 seconds before additional spraying is required. Excessive sprayings will usually result in stain pattern diffusion. Luminol is one of the most sensitive of the presumptive tests and is capable of detecting traces of blood in parts-per-million concentrations. Interestingly, luminol can also react with blood that has been painted over, such as on walls.

Fluorescein is prepared for use much like phenolphthalein. Fluorescein is reduced in alkaline solution over zinc to fluorescin, which is then applied to the suspected bloodstained area. The catalytic activity of the heme then accelerates the oxidation by hydrogen peroxide of the fluorescin to fluorescein, which will fluoresce when treated with ultraviolet light. Fluorescein and luminol are similar in that they produce light to indicate the presence of blood; however, practical differences exist in the use of the two. Whereas an aqueous solution of the luminol reactants is simply sprayed on a surface bearing suspected bloodstain residues, a common fluorescein system includes a commercial thickener, which effectively causes the mixture to adhere to the surface and is thus more effective on vertical surfaces. Once the mixture is sprayed, visualization of fluorescence requires the use of an alternative light source, typically set at 450 nanometers.

8.2.8 *Confirmatory Tests for Blood*

Once a stain has been tentatively identified as blood, the next step is frequently a confirmation test. In some cases, the analyst may proceed directly to DNA typing, knowing that only human DNA will be detected and typed using current methods. In cases where a more definitive identification of a stain as being blood is desired, confirmatory tests such as **crystal tests** (also called **microcrystal tests**) may be used. These tests target the non-protein heme group of hemoglobin, the oxygen-carrying protein of erythrocytes that belongs to a class of compounds called *porphyrins*. The heme structure contains an iron atom that is hexavalent, meaning it can interact with six different entities. Nitrogen atoms within the ring structure bind four of the iron coordination positions and one is bound to histidine nitrogen in the globin protein. In hemoglobin, the remaining coordination position is normally bound by water or, in oxygenated hemoglobin, oxygen. In dried bloodstains, these last two positions are used in the formation of crystals that are the basis for confirming the presence of blood.

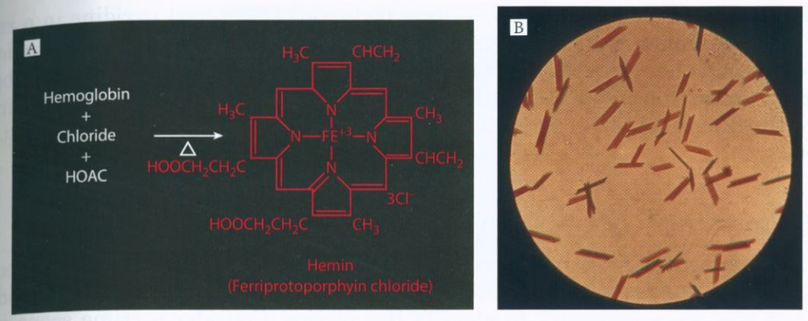

Figure 8.5 (A) Teichmann reaction. (B) Teichmann crystals.

The two common crystal tests are the **Teichmann test** and the **Takayama test**. The Teichmann test consists of heating dried blood in the presence of glacial acetic acid and a halide (usually chloride) to form the **hematin** derivative (Figure 8.5A). The crystals (Figure 8.5B) formed are observed microscopically; they are usually rhombic in shape and brownish in color. The crystals are formed by placing a sample of suspected blood on a microscope slide and adding a small amount of chloride-containing glacial acetic acid followed by heating.

If heme is gently heated with pyridine under alkaline conditions in the presence of a reducing sugar such as glucose, crystals of pyridine ferroprotoporphyrin or hemochromogen (Figure 8.6A) are formed. The reaction was studied by Takayama (1912), who examined several mixtures and found best results with a reagent containing

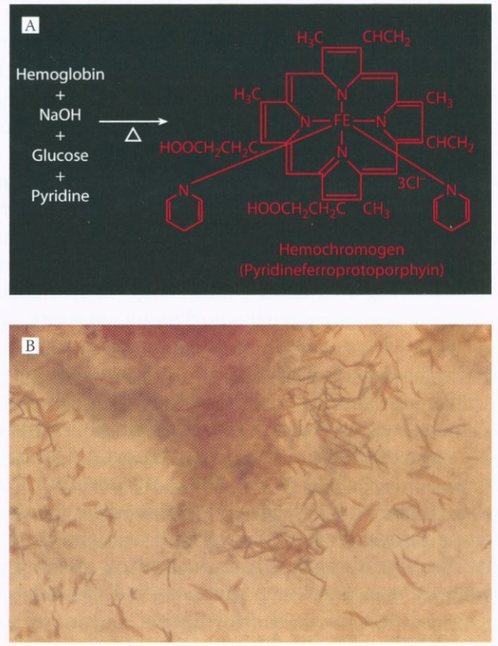

Figure 8.6 (A) Takayama reaction. (B) Takayama crystals.

water, saturated glucose solution, sodium hydroxide (10%), and pyridine in a ratio of 2:1:1:1 by volume. The normal procedure is to place a small stain sample under a cover slip and allow the reagent to flow under and saturate the sample. After a brief heating period, the crystals are viewed microscopically (Figure 8.6B). A very small cover slip (2-mm square or smaller) allows the test to be carried out on a small stain quantity.

8.2.9 Species Origin Determination in Bloodstains

Current methods of DNA typing target human DNA, so there is usually no need to identify what species a blood sample came from. Before DNA methods were widely used, it was often necessary to identify the species. Consider a crime scene in a kitchen where meat is prepared. Blood in a sink could come from a human victim but it also might have come from fresh steak. Serologists had to be sure that the blood was human before doing more testing, as the testing itself was not species specific. Most of the tests used are based on **immunological reactions** in which an **antigen (Ag)** reacts with an **antibody (Ab)** to produce a visible solid or precipitate. In this context, antibodies are proteins found in the serum portion of blood. A wide variety of tests is available to determine the species origin of an identified bloodstain, and most employ immunoprecipitation.

If a host animal (e.g., a rabbit) is inoculated with a serum protein (e.g., human), the immune system of the animal will normally recognize the protein as foreign and produce antibodies against it. Harvesting the antibodies provides an antiserum to the protein (antigen), and when a sample of the antiserum and the antigen are brought into contact a precipitin reaction normally occurs. The precipitate may form in a gel or in a solution, but its formation signals a reaction has occurred. This visible result is the basis of several species tests.

The ring **precipitin test** employs simple diffusion between two liquids in contact with one another in a test tube (see the photograph at the start of the chapter). The two liquids are the antiserum and an extract of the bloodstain in question. If the antiserum (antihuman) is placed in a small tube, and a portion of the bloodstain extract (human) is carefully layered over the denser antiserum, dissolved antigens and antibodies from the respective layers will begin to diffuse into the other layer. The result will be a fine line of precipitate at the interface of the two solutions. In the case where the bloodstain extract is not human, there should be no reaction. Occasionally, there will be more than one precipitin band, indicating different reacting components with different diffusion coefficients. A standard operating procedure would be to include necessary positive and negative controls with a questioned sample.

The Ouchterlony double diffusion test is carried out in a gel on a glass plate. A hot agar solution is plated on small glass plates or a Petri dish and allowed to cool, producing a gel layer usually 2 to 3 millimeters thick. Holes (wells) are punched in the gel layer at specified locations and the gel is removed. Commonly used patterns are a square of four wells, a rosette of six wells surrounding a center well, or two rows of parallel wells. When the antiserum and stain extracts (antigen) are prepared, the antiserum is placed in the central well and the different stain extracts in the surrounding wells. The system is then incubated at constant temperature (4, 18, or 37°C are commonly used). After a given period of time, the diffusion of the reactants through the gel results in the formation of immunoprecipitate lines in the gel between the wells (Figure 8.7). This precipitate is a stable antigen–antibody complex that has grown beyond the limits of its solubility.

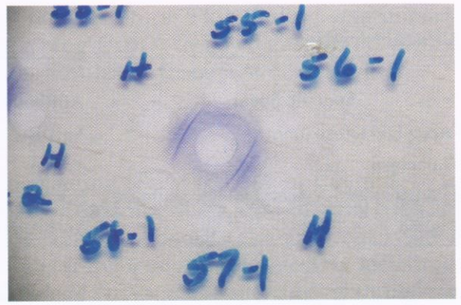

Figure 8.7 The Ouchterlony double diffusion test.

Antihuman serum from the center well forming an immunoprecipitate with a sample from one or more of the surrounding wells indicates the presence of human protein in the respective outer wells. The precipitate may be stained with a suitable protein stain. Although the point at which the immunoprecipitate forms is controlled by the diffusion coefficients of the reactants, its formation is dependent on the concentration of reactants and the identities of the reactants. When antigen and antibody are present in relatively equivalent amounts, the line grows in an arc between the two wells. Incubation at cooler temperatures is a slower process but has the advantage of producing sharper lines and sometimes additional precipitin lines, due to reduced solubility at lower temperatures.

8.2.10 Prelude to DNA: Genetic Markers

Prior to DNA typing, blood was characterized by other tests, also broadly referred to as typing. Like DNA, these tests targeted inherited characteristics under hereditary control and are referred to generically as **genetic marker systems**. Some, such as the **ABO blood group** system (see Sidebar 8.2), are amenable to forensic

SIDEBAR 8.2. HISTORICAL NOTE: BLOOD TYPES AND KARL LANDSTEINER

Karl Landsteiner (1868–1943) was an Austrian physician who was the first to study and identify the ABO blood group at the turn of the 20th century. His research was not focused on forensic science, but on fatal reactions being observed with blood transfusions that were tried in the 1800s. These early attempts frequently caused the red blood cells of the recipient to clump together (agglutinate). Landsteiner noted that this reaction was not universal in that the blood of some individuals was compatible. These observations coupled with his research led to the identification of the ABO system, which was the first blood group system identified. Landsteiner also recognized that a person's blood group was inherited and thus would be useful in paternity cases. Landsteiner's discovery led to systematic typing for blood transfusions and saved untold thousands of lives. As a result, he was awarded the Nobel Prize in Medicine in 1930. Landsteiner eventually moved to New York and continued to work in the field of immunology, participating in the discovery of several more blood group systems, including the Rh system discussed briefly in this chapter.

TABLE 8.1
ABO Blood Group Characteristics

Blood Group	Antigen Present	Specific Sugar	Antibody Present	Population Frequency
A	A	*N*-Acetylgalactose amine	Anti-B	40%
B	B	D-Galactose	Anti-A	10%
AB	A, B	D-Galactose and *N*-acetylgalactose amine	None	5%
O	H	L-Fucose	Anti-A and anti-B	45%

settings, and types can be retrieved from stains, even old and degraded ones. Other systems that are easy to type in whole blood are not typable in stains. We will examine a few of the genetic marker systems used in forensic serology.

The first and best-known blood grouping system is the ABO system, discovered by Karl Landsteiner in 1900. The types A, B, O, and AB refer to the antigens (**agglutinogens**) on the surface of the red blood cells, which are glycoprotein substances and an integral part of the cell membrane. The corresponding antibodies (**agglutinins**) of the system—anti-A (α-A) and anti-B (α-B)—are present in the plasma. These characteristics are summarized in Table 8.1. A person of blood group A will have anti-B (α-B) antibody in his or her plasma, and if that plasma is mixed with group B cells the two are said to be homologous, with agglutination being the result. The characteristics of the person who is group O present a little different picture. There are no routinely occurring antibodies in humans for the red cell H antigens possessed by the group O person. However, certain seed extracts called *lectins* are capable of achieving agglutination with blood cells. One of the reasons for knowing blood types is for transfusions; if a person with type B blood is given type A blood, a fatal reaction can occur.

Forensic testing for the ABO system in dried bloodstains centers on identifying both the antigen and antibodies present. A variety of methods have been devised to accomplish both tasks. The **absorption elution** test involves the exposure of a portion (a cutting or thread) of the stain bearing the blood (and antigen) to absorb the homologous antibody. Unreacted antibody is then washed away and the absorbed antibody is eluted by warming and then mixed with a cell suspension to be identified. As an example, a group A stain exposed to anti-A (α-A), anti-B (α-B), and anti-H lectin (α-H lectin) antibodies in separate containers (tubes, wells, etc.) would absorb the anti-A and not the anti-B or anti-H antibodies. After allowing sufficient time for absorption, the unreacted antibodies (and lectin) would be washed away and gentle heating would be applied to release (elute) the absorbed anti-A. This anti-A antibody would be detected by the addition of group A cells, which would then agglutinate and be viewed microscopically. The other two containers would exhibit no reaction as no antibody or lectin was absorbed and then eluted to react with the B and O cells added.

The **Lattes crust test** focuses on the antibodies found in the stain and involves exposing three separate stain samples to dilute suspensions of A, B, and O cells (usually on glass microscope slides), allowing a suitable period of time for elution of the antibodies from the stain and agglutination of the cells. A positive result or agglutination on the slide with the B cells would indicate anti-B antibody in the stain and thus confirm the stain as blood group A. It is well known that some individuals secrete their ABO biochemical (antigenic) characteristics into body fluids such as saliva, semen, and vaginal fluid. Such individuals are called **secretors**, which represent approximately 80% of the general population. The remaining 20%

of the population are non-secretors. This finding was important forensically as it allowed blood group typing (ABO) to be conducted on evidence such as cigarette butts, using techniques similar to those just described.

8.2.11 Enzyme Markers

Between 1971 and the coming of age of DNA in the mid- to late 1980s, interest in polymorphic **enzyme systems** and their analysis was high. The result was a flourishing of technology designed to identify the different types of a host of enzymes, centering mainly on electrophoresis and isoelectric focusing (IEF) methods. This advancement in technology led to a greater ability to discriminate between individuals and evidentiary material and paved the way for the more advanced technologies of DNA analysis. Methods have been developed for numerous enzymes with regard to their use in individualizing bloodstains (Table 8.2), but space precludes a complete discussion of them. **Phosphoglucomutase (PGM)** has been chosen as a representative enzyme for discussion (see Case Study 8.1).

Although the development of techniques enabled a greater discriminatory capability, there was still the basic nature of the macromolecules to contend with. The basic structure of proteins and enzymes is complex, both in physical structure and chemical nature. While many of the systems mentioned above are sensitive to unfavorable environmental conditions, proteins and enzymes are much more susceptible to such antagonism. A change in the molecule's physical structure or the ability to perform its chemical task will often result in an inability to detect any phenotype in evidentiary stains. Thus, the results of heat and humidity become even more significant when dealing with these markers. A discussion of unfavorable environmental conditions often brings up the question of altered phenotypes being observed in the analysis of improperly stored evidentiary stains. In other words, would a PGM 1+1B become a PGM 2+2B? In fact, it may be shown that the result of subjecting evidence to unfavorable conditions is likely to be the loss of any detectable phenotypes rather than the introduction of new ones. A term one will find mentioned in connection with forensic enzyme assays is **polymorphism**. Polymorphism may be described as the occurrence in a population of two or more genetically determined alternative

TABLE 8.2
Forensically Important Enzymes

Enzyme	Abbreviation	Common Phenotypes	Remarks
Adenosine deaminase	ADA	1, 2, 2–1	Rare variants exist (3–1, 4–1, 5–1, 6–1, 7–1)
Adenylate kinase	AK	1, 2, 2–1	Rare variants exist (3–1, 4–1, 5–1)
Carbonic anhydrase	CA II	1, 2, 2–1	Takes 4 to 6 years to reach adult levels
Erythrocyte acid phosphatase	EAP, ACP	A, B, BA, C, CA, CB	Stability of bands C > B > A
Esterase D	ESD	1, 2, 2–1, 1–5, 2–5, 5	Five allelic products observable with IEF
Glucose-6-phosphate dehydrogenase	G6PD	A, B, AB	Mainly polymorphic in blacks; AB normally
Glyoxalase	GLO I	1, 2, 2–1	In sperm and plasma
Peptidase A	PEP A	1, 2, 2–1	In plasma more than sperm

CASE STUDY 8.1: APPLICATION OF ABO

During the early morning hours of a June day in a northeastern community, the teenaged daughter of the headmaster of a prestigious girl's school was awakened in her own bed and carried downstairs and into the back yard, where she was sexually assaulted and forced to commit sodomy. She had limited vision without her glasses and was later unable to identify her attacker except to provide a general description. When released, she fled to wake her parents who immediately reported the incident to the police. The girl's injuries included a vaginal laceration, which bled profusely.

Shortly after the police were notified, an individual who fit the general description given by the girl was located walking on the street about five blocks from the scene. The individual explained that the blood-like stains around the fly area on his blue jeans came from a nosebleed suffered by his girlfriend's daughter earlier that day.

The blue jeans, the girl's nightgown, and liquid blood samples from all three individuals were subjected to forensic examination. Human blood was, indeed, identified on the nightgown and the blue jeans. ABO analysis of the blood samples disclosed that the girl, the suspect, and the blood on the pants and the nightgown were all of blood group A, while the girlfriend's daughter was group O. Additional analysis of the known samples disclosed the girl to be PGM 2–1, EAP BA, Hp 1; the suspect, PGM 1, EAP A, Hp 1; and the girlfriend's daughter, PGM 1, EAP B, Hp 2–1. The blood on the pants and nightgown was PGM 2–1, EAP BA, and Hp 1, indicating the young girl as a possible source but eliminating the others. All additional testing was inconclusive. No semen was identified on the evidence. The blood on the jeans could have come from the victim but not the suspect or his girlfriend's daughter.

The significance of these results becomes clearer when we look at the relative frequency of the victim's set of types in the population. The population frequencies for the victim's characteristics are ABO A, 40%; PGM 1, 35.9%; EAP A, 41.4%; and Hp 1, 16.5%. The combination of these frequencies ($0.40 \times 0.359 \times 0.414 \times 0.165$) gives us 0.0098; in other words, 0.98% of the population can be expected to have all the characteristics of the victim. Stated differently, there is roughly 1 chance in 100 that we can randomly select a person from the population of that town with that combination of phenotypes. Had this case been examined when PGM subtyping was available and the victim had been the most common subtype of PGM 1, the figure would have been 0.006 or 0.6%, or 1 chance in 167. The result for the least common PGM subtype would have been 0.0004 or 0.04%, or 1 chance in 2500. Although this may sound impressive, DNA typing methods, described in the next chapter, can yield numbers in

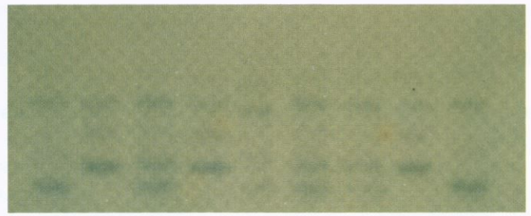

Figure 8.8 PGM phenotypes (electrophoresis). From left to right: PGM 1, PGM 2, PGM 2-1, PGM 2, PGM 1, PGM 2-1, PGM 2-1, PGM 2, PGM 1 (anode at top).

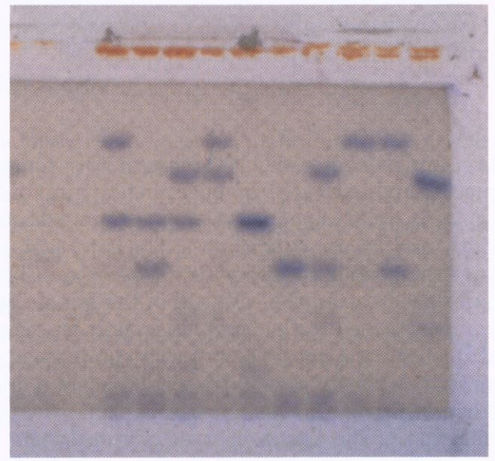

Figure 8.9 PGM phenotypes, (isoelectric focusing). From left to right: PGM 1+2+, PGM 1+1–, PGM 1+2–, PGM 2+2–, PGM 1+, PGM 1–, PGM 1–2–, PGM 2+, PGM 1–2+, PGM 2– (anode at top).

animals, and microorganisms. In humans the enzyme exists in significant concentrations in blood and semen and in small amounts in vaginal secretions and cervical mucus. It is known to be inhibited by heavy metals and fluoride, a significant point as blood samples taken by medical personnel may contain fluoride. When stored under cool dry conditions the enzyme survives well. It should always be the concern of the forensic investigator, however, to avoid exposing biological evidence to prolonged heat and humidity.

There are actually three genetic loci that control PGM polymorphism. Locus 1 is on chromosome 1, locus 2 is on chromosome 6, and locus 3 is on chromosome 4. Only

8.3 Seminal Fluid

Next to blood, the body fluid most often seen in forensic cases is semen or seminal fluid. Semen is produced by post-pubescent males and ejaculated following sexual stimulation. It is a semifluid mixture of cells, amino acids, sugars, salts, ions, and other organic and inorganic materials elaborated as a heterogeneous gelatinous mass contributed by the seminal vesicles, the prostate gland, and Cowper's glands. Ejaculate volumes of human males range from 2 to 6 milliliters and typically contain between 100 and 150 million sperm cells per milliliter. Certain disease states, genetic conditions, excessive abuse of alcohol or drugs, prolonged exposure to certain chemicals, and elective surgery procedures may result in a drastically reduced sperm count or complete absence of sperm cells from semen.

8.3.1 Sperm Cells

The principal cellular component of semen is the spermatozoa or sperm cell, a specialized, flagellated structure approximately 55 micrometers (μm) in length. The human sperm cell head is typically ovoid in shape with approximate dimensions of 4.5 μm in length, 2.5 μm in width, and 1.5 μm in thickness. The head contains the cell nucleus. The anterior portion of the head is capped with the acrosome. This structure is rich in enzymes to assist in penetrating the cell wall of the female egg during fertilization. A flagellated tail is attached to the head via a short midpiece and accounts for about 90% of the total length of a sperm cell. A common method of detecting semen is to stain the sperm cells to allow for visual detection using a microscope.

The condition of **azoospermia** (semen lacking spermatozoa) requires that other tests besides identification of the sperm be used. Crystal tests in which chemical components of semen such as spermine and choline could be detected were developed to assist with the identification of such samples. Characterization of enzymes in animal body fluids and tissues began in the 1920s, and by the mid-1930s a test was developed to test for **seminal acid phosphatase (SAP)**. Modifications of the test remain in widespread use today. By the 1970s, analysis of stains for protein components of seminal fluid was commonplace.

8.3.2 Acid Phosphatase

Acid phosphatases (APs) are a class of enzymes that can catalyze the hydrolysis of certain organic phosphates. These enzymes are ubiquitous in nature and may be found in materials as diverse as mammalian liver and cauliflower stem juice. Seminal acid phosphatase (SAP) is a phosphatase found in human semen at uniquely high levels compared with other body fluids and plant tissues. In males, puberty stimulates the large-scale synthesis of SAP by secretory epithelial cells that line the prostate gland. SAP levels remain high until the age of about 40, after which they gradually decline. No correlation exists between the level of SAP and the number of sperm cells present in an ejaculate, and no variation has been found between males with normal sperm counts and those who are clinically infertile or who underwent vasectomies.

Over the years, many methods for the presumptive identification of semen have been devised. Only one, the test for SAP activity, has withstood the test of time and is now used for this purpose. It involves a reaction that generates a color by combining a substrate (here, something that will react with the phosphatase enzyme) with

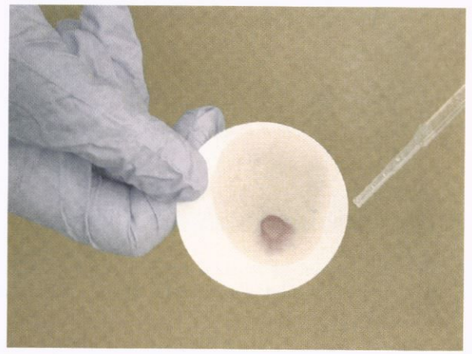

Figure 8.10 Test for SAP activity.

a color developer. In forensic laboratories, alpha-naphthyl phosphate is the preferred substrate and Brentamine Fast Blue B is used as the color developer. These two components are prepared separately in anhydrous sodium acetate and mixed to create a working solution or reagent that is sensitive enough to produce a positive result with semen diluted 500 times. This test is especially practical because it does not require that a suspected semen stain first be localized visually before applying it.

Application of the test is a very simple two-step procedure. Because SAP is readily soluble in aqueous media, a piece of absorbent paper (filter paper is ideal since it comes in a variety of sizes) or cotton swab is moistened with sterile water and applied to the questioned stain. The reagent is added to the paper or swab and development of an intense purple color noted (Figure 8.10). If an obvious purple coloration does not develop within 2 minutes, the test is recorded as negative. The intensity of the purple color of a positive reaction and the time taken for the color to develop should be recorded. The one-step process of adding reagent directly to a stain or swab *in situ* conveys no advantage if the method described above is correctly applied.

A strong reaction within 30 seconds is diagnostic for semen presence, notwithstanding that identification of spermatozoa or **prostate-specific antigen (PSA)** is the generally accepted standard (see the section below on confirmatory tests for semen). A positive reaction observed in less than 2 minutes in a stain located in an area with little likelihood of containing a contaminant (for example, the knee area of a pair of pants) should also merit confirmation. The forensic biologist must consider the presence of other body fluids, the time elapsed since alleged intercourse, and the fabric type when weak to moderate positive reactions are observed after a minute or so. Vaginal secretions, perspiration, feces, urine, or any combination of these fluids, particularly when repeatedly deposited, will typically exhibit such a reaction, especially in the crotch areas and seams of undergarments. Laboratory policy and individual discretion following thorough review of these issues will dictate whether a confirmatory test is undertaken in these situations.

8.3.3 Confirmatory Tests for Semen

Microscopic identification of sperm cells provides unambiguous proof that a stain under scrutiny contains semen. It is unusual for a forensic scientist to examine semen in which sperm cells are motile since motility is lost within 3 to 6 hours of ejaculation; however, established staining techniques greatly assist the trained eye

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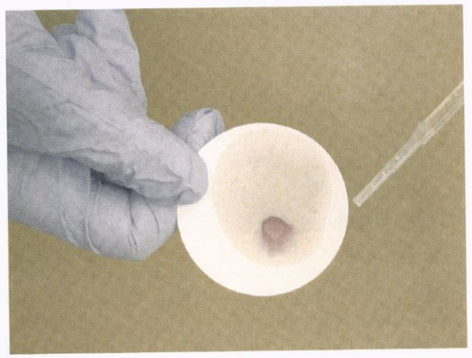

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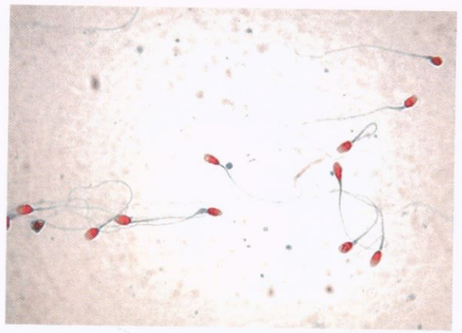

Figure 8.11 Human spermatozoa stained with Christmas tree stain (Nuclear Fast Red and picroindigocarmine). (Original magnification 1000×.)

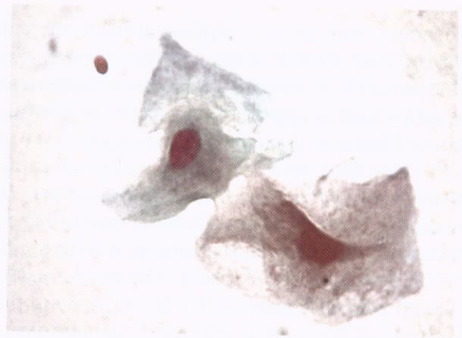

Figure 8.12 Human spermatozoon and nucleated epithelial cells stained with Christmas tree stain (Nuclear Fast Red and picroindigocarmine). (Original magnification 1000×.)

to easily distinguish sperm cells from extraneous material such as epithelial cells. The most commonly encountered staining technique uses **picroindigocarmine (PIC)** and nuclear fast red dyes and is colloquially referred to as the *Christmas tree stain*. It was developed specifically for sperm cell visualization. Different parts of the sperm structures are singularly colored and contrast well with the colors taken up by epithelial cells. Stained sperm cells can be identified using microscopy. Sperm cell heads are ovoid and exhibit characteristic differential staining. The anterior portion is pink and the posterior is dark red or purple and often appears shiny (Figure 8.11). Sperm cell tails stain yellow–green and the midpiece stains blue. Epithelial cells also take up the stain and appear blue–green with red nuclei (Figure 8.12).

8.3.3.1 Prostate-Specific Antigen

You may have heard of the prostate-specific antigen (PSA, p30) test which is used to detect prostate cancer in men. This antigen has also proven to be useful for identifying a stain as semen. In the absence of spermatozoa in an acid phosphatase-positive specimen, the detection of PSA is proof positive for semen. PSA is secreted into seminal plasma by the prostate gland and varies in concentration from 300 to 4000 nanograms per milliliter. Because the prostate gland lies distal to the customary point of interruption in a vasectomy, this procedure has no effect on the elaboration

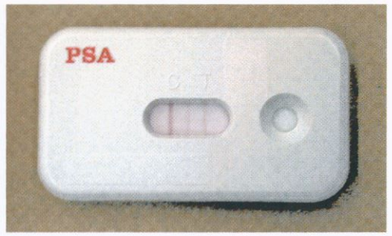

Figure 8.13 Seratec™ PSA test kit. The line closest to the sample well indicates a positive result. The middle line is an internal standard. The line on the left is the positive control.

of PSA into the semen. The urine and serum of males, breast milk, and sweat glands contain levels of PSA that are usually below the limits of forensic detection. PSA is found in elevated levels in the serum of males with prostate cancer, but because a positive AP reaction is usually required in tandem with PSA detection for semen to be unambiguously identified, this, too, is of little consequence forensically. Current tests for PSA are based on the appearance of a blue color as shown in Figure 8.13.

8.4 Saliva

Saliva is a slightly alkaline secretion comprised of water, mucus, proteins, salts, and enzymes found in the mouth. Humans produce 1 to 1.5 liters of saliva per day. Its primary purpose is to aid in the initial stages of digestion by lubricating food masses for easier swallowing and initiating the digestion of starches using the enzyme amylase. No test is specific for saliva. Traditionally, forensic tests for saliva relied primarily on the detection of **alpha-amylase**, an enzyme sometimes referred to as *ptyalin* in older references. **Amylases** are ubiquitous enzymes found in both animals and plants. They are responsible for catalysis of the components of starch, amylose, and amylopectin into smaller, less complex sugars. Amylases can be subdivided into alpha and beta categories. beta-Amylases are found in plants and alpha-amylases in animals, including humans. beta-Amylase attacks only the bonds at the ends of polyglucan chains, unlike alpha-amylase, which catalyzes bonds within the chains. Primates, pigs, elephants, and certain rodents have high levels of amylase.

In humans, two DNA loci, AMY1 and AMY2 found on chromosome 1, code for amylase. The AMY1 locus codes for the amylase found in saliva, breast milk, and perspiration. The AMY2 locus codes for the amylase found in the pancreas, semen, and vaginal secretions. In the mouth, alpha-amylase is secreted primarily from the parotid glands as a component of saliva. Although alpha-amylase is found in many body fluids and tissues, it still serves as a good marker because it is found at levels 50 times higher in saliva than in most other body fluids and is relatively stable. Some activity can be detected for up to 28 months.

8.4.1 Starch–Iodine Test

In the presence of iodine, starch appears blue. As amylase acts on starch to break it down, the color changes and subsides. This test has several drawbacks; for example, the presence of proteins, particularly albumin and gamma-globulin originating in other body fluids (e.g., blood, semen), compete with starch for iodine and produce

Figure 8.14 Phadebas™ press test. The position of the test paper is marked on the item for accurate relocation.

false positive results. This test is also difficult to use as a locator test for stains on items. One version of this method is still in use as the radial diffusion test. An agar gel containing a known concentration of starch is prepared, and iodine is poured over the gel. An extract of the questioned sample is added to the wells in the gel. As the starch diffuses, it breaks down, leaving a circular void area proportional to the amount of amylase present.

8.4.2 Phadebas® Reagent

More common is the use of commercial products in which starch is linked to a dye molecule to form an insoluble complex. When starch is cleaved from the dye by amylase, the dye molecule becomes soluble, producing a colored product that can be measured with a spectrophotometer. The degree of coloration is proportional to the amount of amylase in the sample. This procedure is commonly referred to as a *tube test*. Alternatively, the reagent can be dissolved and applied to large sheets of filter paper, which can then be placed on an item to map the location of amylase-containing stains for further analysis. This procedure is referred to as a **press test**. Procion red amylopectin (PRA) or Phadebas® reagents are the most commonly used. The Phadebas® reagent is manufactured by Pharmacia and is produced in tablet form. It is used clinically to detect alpha-amylase in urine, serum, and plasma, which may denote certain medical conditions, such as pancreatitis. An example application of the Phadebas® test is shown in Figures 8.14 and 8.15.

8.5 Other Body Fluids

8.5.1 Urine

Although the chemical detection of urine may play a role in sexual assault, harassment, and mischief cases, it is performed less frequently by forensic laboratories because of the insensitivity of the tests and the low success rate with DNA profiling. Like many other analyses, detecting urine relies on visual examination for stains with characteristic appearances. Stains may fluoresce or luminesce under alternative light sources, but diluted stains are more difficult to detect. Odor is another

Figure 8.15 Phadebas™ press test. A positive reaction appears as a smooth blue region. These areas are circled on the paper and on the item.

characteristic of urine, but it generally permeates an entire item and is not localized to the urine stain itself. Historically, methods for the identification of urine have relied on identifying inorganic ions or organic compounds that concentrate in urine. It is advisable in the forensic context to attempt to identify more than one component for confirmation due to the ubiquity of the substances in question. Urine detection relies on identifying two organic compounds: **urea** and **creatinine**. Both components are found in other body fluids, such as perspiration, blood, saliva, and semen. Urea is present in high levels, approximately 1400 to 3500 milligrams per 100 milliliters. Creatinine is present at about one tenth of these values, with average concentrations of 105 to 210 milligrams per 100 milliliters. While urea and creatinine are found at relatively high levels in liquid urine, they may be difficult to detect in stains, the most commonly encountered forensic samples. As liquid urine is absorbed into fabric surfaces, it spreads across the surface, effectively diluting the test components. Testing for urea relies on the use of urease, an enzyme that breaks down urea and releases ammonia and carbon dioxide. The ammonia is then detected using an indicator chemical, such as Nessler's reagent (mercuric iodide in potassium iodide) or ***p*-DMAC** (*p*-dimethylaminocin-namaldehyde). **Azostix®**, a commercial test strip used for the clinical detection of urea in blood, relies on the same principle, except it measures a shift in pH caused by the formation of ammonia hydroxide.

8.5.2 Vaginal Secretions

The identification of vaginal secretions is especially important when a case involves an allegation of a foreign object inserted into the vagina as part of a sexual assault. Vaginal secretions are usually identified on the basis of detecting glycogenated epithelial cells. A **periodic acid–Schiff (PAS) reagent** serves to stain the glycogen in the cellular cytoplasm a bright magenta color. The cells can then be rated based on staining intensity on a scale of 1 to 4, with 4 being the most intense.

The test is not conclusive as the amount of glycogenation varies depending on the stage in the menstrual cycle; glycogenation levels are highest around the time of ovulation. Glycogenated cells are absent from pre-pubescent females and are uncommon in postmenopausal women, although estrogen replacement therapy will affect the measurement. Glycogenated epithelial cells are also not unique to the vaginal tract and can be found in smaller numbers in the mouth and in the urethral tracts of males. Thus, the finding of only a few cells is problematic for interpretation.

The test may also consume a large amount of the questioned material and reduce the amount available for DNA testing. It may be prudent to forego PAS testing in favor of retaining material for DNA testing. However, if the origin of the cells is particularly important (e.g., whether a DNA profile from a bottle can be attributed to someone drinking from it or from vaginal insertion), this testing may have to be done.

Chapter Summary

The field of forensic serology has experienced many changes in technology, ranging from simple improvements in technique to the addition of newer methods of analysis and the recognition of additional biochemical sources of information. Twenty years ago the possibility of establishing the degree of identity possible today with DNA and doing it with the minute sample quantity currently used seemed impossible. These kinds of changes are ongoing and do not show any indication of stopping; however, by knowing the past, we can better understand the future. Because the material presented here is a basic primer and not by any means all inclusive, the reader is heartily encouraged to consult additional references for more in-depth information.

8.6 Review Material: Key Concepts and Questions

8.6.1 Key Terms and Concepts

ABO blood group system
Absorption elution
Acid phosphatases
Agglutinins
Agglutinogens
alpha-Amylase
Alternative light source (ALS)
Amylases
Antibody (Ab)
Antigen (Ag)
Azoospermia
Azostix®
Benzidine
Chemiluminescence
Chromogenic substance
Confirmatory test
Creatinine
Crystal tests
Enzyme systems
Extracellular fluid
False negative
False positive
Fluorescein
Genetic marker systems
Hemastix®
Hematin
Hemoglobin
Immunological reactions
Lattes crust test
Luminol
Microcrystal tests
Oxidation
p-DMAC
Periodic acid–Schiff (PAS) reagent
Phadebas® reagent
Phenolphthalein test (Kastle–Meyer test)
Phosphoglucomutase (PGM)
Picroindigocarmine (PIC)
Platelets
Polymorphism
Precipitin test
Press test
Presumptive testing
Prostate-specific antigen (PSA)
Screening
Secretors
Seminal acid phosphatase (SAP)
Serology
Starch–iodine test
Takayama test
Teichmann test
Tetramethylbenzidine (TMB)
Thrombocytes
Urea

8.6.2 Review Questions

1. What two environmental factors are most important to consider in preserving blood evidence?
2. List three possible causes of false positive reactions with presumptive screening tests for blood.
3. Describe the principle behind a presumptive test for blood (what is done and what it means).
4. An analyst conducted a phenolphthalein test on a stain he thought looked like blood. He observed a positive test immediately after adding the phenolphthalein reagent. He concluded that blood was, in fact, present. Was he correct?
5. True or false: Luminol is so sensitive that a positive reaction can be taken to prove that blood is present.
6. A person having the A antigen on his or her red cells will have serum antibodies that will agglutinate cells from:
   _____ a group "A" person
   _____ a group "B" person
   _____ a group "O" person
   _____ a group "AB" person
7. When called to testify regarding evidence he had examined, an analyst was questioned about the possibility of errant enzyme results being introduced into the evidence as a result of inadequate preservation. How should he have responded?
12. Define polymorphism.
13. What are the five classes of serum proteins?
14. Where in the male reproductive tract is acid phosphatase produced?
15. Name two tests considered confirmatory for the presence of semen.
16. Give reasons for the absence of tails on spermatozoa on a microscope slide preparation.
17. How long might seminal fluid constituents be detectable after deposition (a) inside the vagina, (b) in dried form, and (c) on fabric after laundering or dry cleaning?
18. beta-Amylase is found in plants. Will plant extracts or stains react with the Phadebas test? Why or why not?
19. Assume that you detected amylase using a Phadebas press test on a pair of underpants worn after a sexual assault in which cunnilingus is alleged. Discuss the factors that would determine your choice of area for DNA analysis.
20. Why is it preferable to identify both urea and creatinine in suspected urine stains?

8.7 References and Further Reading

8.7.1 Books

Bell, S. C. *Crime and Circumstance: Investigating the History of Forensic Science*. Westport, CT: Praeger Publishers, 2008.

Gaensslen, R. E., and H. C. Lee. *Forensic Science: An Introduction to Criminalistics*, 2nd ed. New York: McGraw-Hill, 2007.