I

General Principles of Laboratory Testing and Diagnosis

1 Laboratory Technology for Veterinary Medicine

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This chapter presents an overview of the laboratory technology used to generate data for hematology and clinical biochemistry. For the procedures and technologies likely to be employed within veterinary hospitals, general instructions and descriptions provide a review of the principles previously learned in laboratory courses. This, in conjunction with the instructions accompanying different devices and consumables, should enable users to reproduce the procedures to a satisfactory performance standard. For technologies more likely to be used only in large commercial or research laboratories, the overview provides familiarity with the basic principles.

Hematologic techniques

Basic techniques applicable for any veterinary hospital

The procedures outlined here are most appropriate for the in-house veterinary laboratory in most practice settings. These procedures, with the exception of a cell counting hematology system, require minimal investment in instrumentation and technical training. These basic hematologic procedures include:

- Blood mixing—for all hematologic measurements
- Packed cell volume or hematocrit by centrifugation
- Plasma protein estimation by refractometry
- Cell counting instrumentation
- Preparation of blood films
- Differential leukocyte count and blood film examination

Blood mixing

The blood sample is assumed to have been freshly and properly collected into an ethylenediaminetetraacetic acid (EDTA) tube (as described in Chapter 2). When performing any hematologic procedure, it is important that the blood is thoroughly mixed. Cellular components may settle rapidly while the tube sits on a counter or in a tube rack (Fig. 1.1). As a result, failure to mix the sample before removing an aliquot for hematologic measurement may result in a serious error. Mixing can be performed by manually tipping the tube back and forth a minimum of 10–15 times (Fig. 1.1). Alternatively, the tube may be placed on a rotating wheel or tilting rack designed specifically to mix blood (Fig. 1.2).

Packed cell volume

The packed cell volume value is the percentage of whole blood composed of erythrocytes. It is measured in a column of blood after centrifugation that results in maximal packing of the erythrocytes. Tools for performing the packed cell volume include 75×1.5-mm tubes (i.e., microhematocrit tubes), tube sealant, a microhematocrit centrifuge, and a tube-reading device.

The procedure is performed using the following steps. First, the microhematocrit tube is filled via capillary action by holding it horizontally or slightly downward and then touching the upper end to the blood of the opened EDTA tube (Fig. 1.3).

Next, allow the tube to fill to approximately 70–90% of its length. Hold the tube horizontally to prevent blood from dripping out of the tube, and seal one end by pressing the tube into the tube sealant once or twice (Fig. 1.4). Note that air may be present between the sealant and the blood (Fig. 1.4). This is not a problem, however, because the trapped air is removed during centrifugation.

The tube is then loaded into the microhematocrit centrifuge according to the manufacturer's instructions (Figs. 1.5 and 1.6). The microhematocrit centrifuge is designed to spin the lightweight tube at very high speeds to generate sufficient centrifugal force to completely pack the red cells within 2–3 minutes. With such centrifugal force, most (or all) of the plasma is removed from the layers of packed cells.

Veterinary Hematology and Clinical Chemistry, Second Edition. Edited by Mary Anna Thrall, Glade Weiser, Robin W. Allison, and Terry W. Campbell.

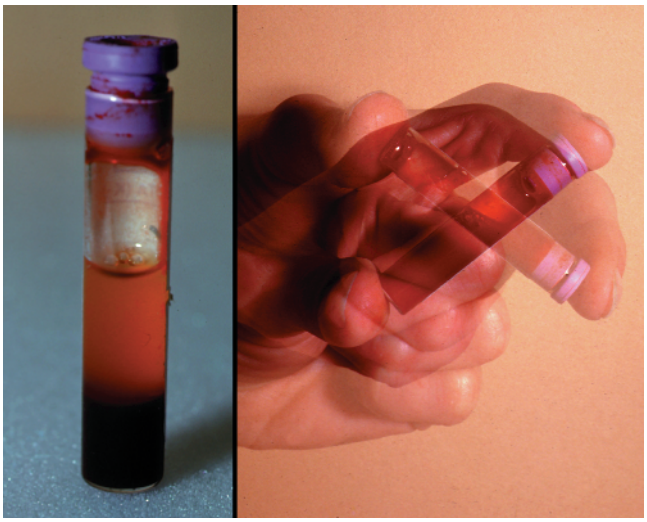


Figure 1.1 Left. Gravity sedimentation of whole blood. Right. A gentle, repetitive, back-and-forth tube inversion technique used to manually mix blood before removing aliquots for hematologic procedures.

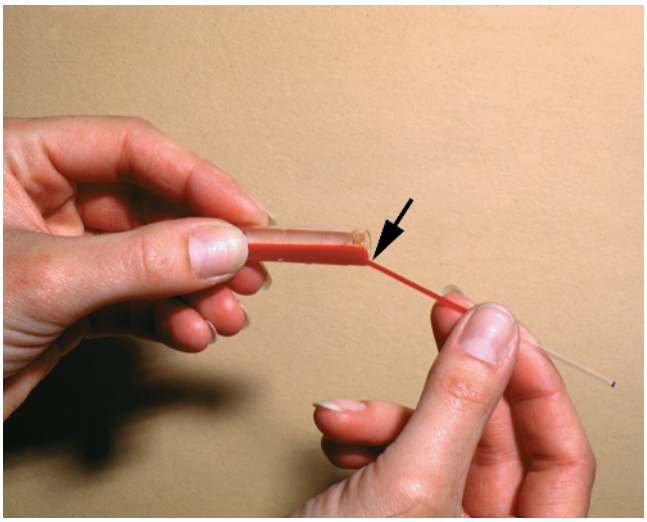


Figure 1.3 Proper technique for filling a microhematocrit tube. The tube should be positioned horizontally or tilted slightly downward to facilitate filling by capillary action. Capillary action is established by touching the upper end of the tube to the blood (arrow).

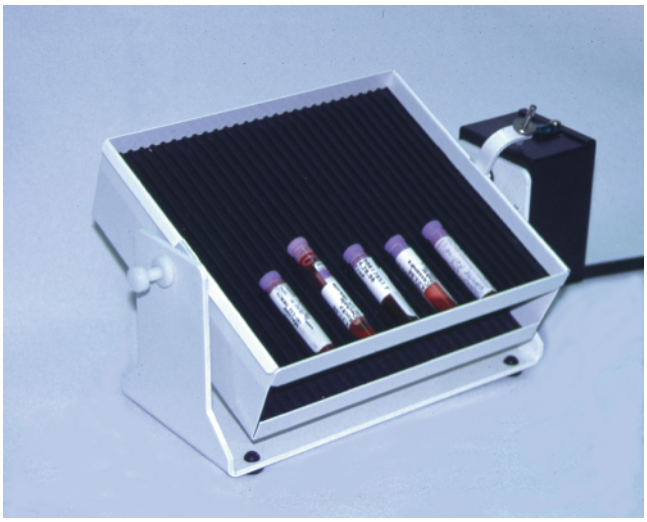


Figure 1.2 Representative mechanical blood-mixing table. The surface holds several tubes on a ribbed rubber surface and tilts back and forth at the rate of 20–30 oscillations per minute.

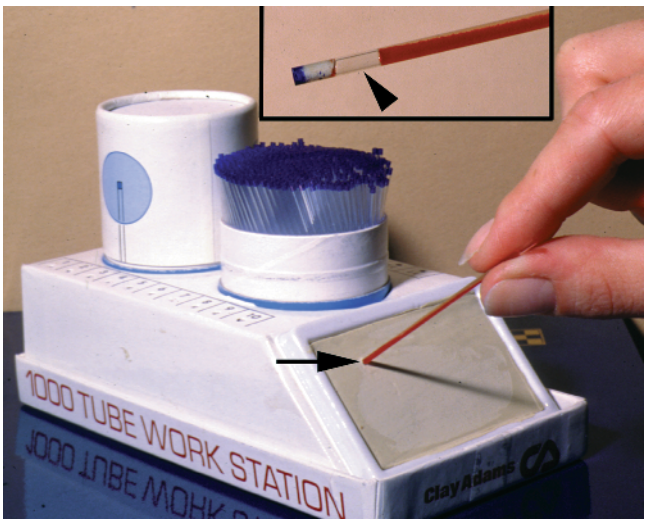


Figure 1.4 A microhematocrit tube is sealed by pressing two to three times into the clay sealant (arrow). Note that a small amount of air trapped between the blood and white clay is not a problem (arrowhead in the inset).

Three distinct layers may be observed in the tube after removal from the centrifuge: the plasma column at the top, the packed erythrocytes at the bottom, and a small, middle white band known as the buffy coat (Fig. 1.7). The buffy coat consists of nucleated cells (predominantly leukocytes) and platelets, and it may be discolored red when the nucleated erythrocyte concentration is prominently increased. Observations of any abnormalities in the plasma column above the red cells should be recorded. Common abnormalities such as icterus, lipemia, and hemolysis are shown in Figure 1.7. Icterus is excessively yellow pigmentation of the plasma column that suggests hyperbilirubinemia; the magnitude of this hyperbilirubinemia should be confirmed by a biochemical determination of serum bilirubin concentration (see Chapter 26). The observation of an icteric coloration to the plasma is diagnostically useful in small animals. It is not reliable in large animal species, however, because their serum usually has a yellow coloration from the normal carotene pigments associated with their herbivorous diet. Lipemia is a white, opaque coloration of the plasma column

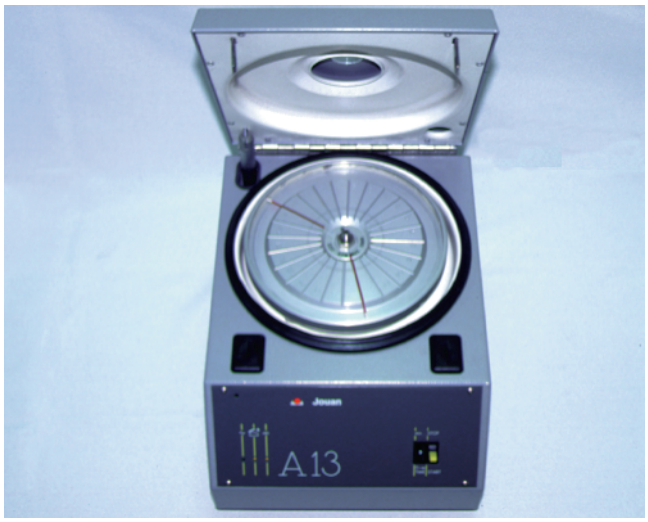


Figure 1.5 Representative microhematocrit centrifuge. The head and motor are designed to spin the tubes at very high speeds to achieve maximal erythrocyte packing.

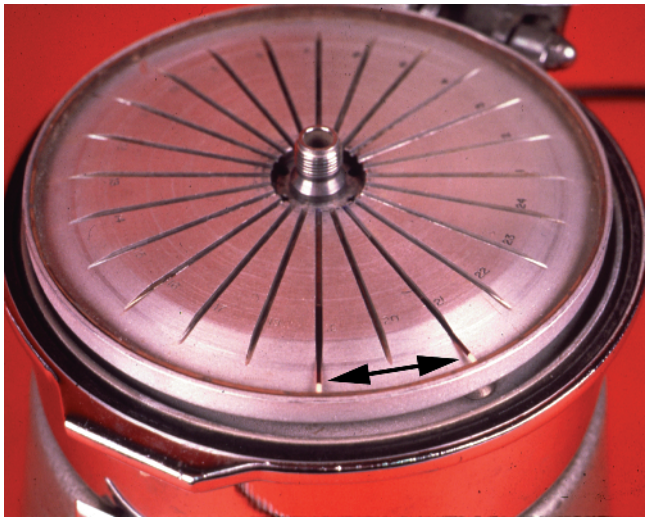


Figure 1.6 Placement of microhematocrit tubes on a microhematocrit centrifuge head. Note the proper orientation of two microhematocrit tubes, with the clay-sealed end positioned at the outer ring of the centrifuge head (double arrow).

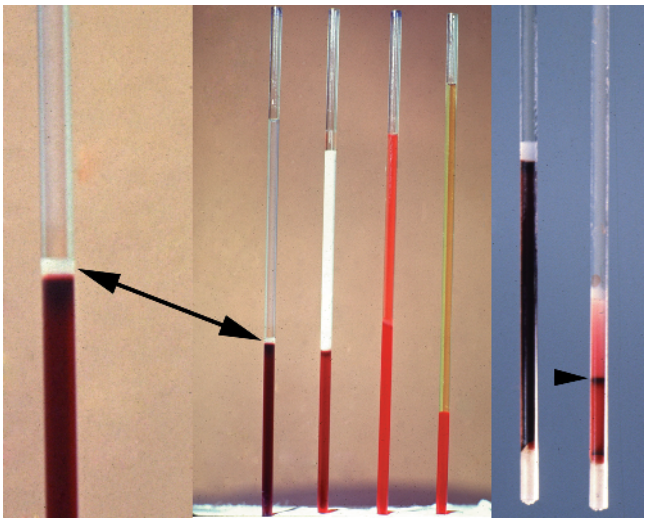


Figure 1.7 Normal and abnormal spun microhematocrit tubes (4 tubes in middle panel). The tube on the left is normal. Note the packed erythrocytes at the bottom, plasma layer at the top, and buffy coat in the middle (arrow; enlarged at left). The second tube illustrates lipemia, the third hemolysis, and the fourth icterus. Note also that the hematocrit is considerably decreased in the fourth tube. Two additional tubes illustrate buffy-coat abnormalities (enlarged at right). The first of these tubes has an increased buffy coat that correlates with an increased leukocyte concentration. The second (right) is from a sheep with leukemia and has a dramatically increased buffy coat. The leukocyte concentration was greater than 400,000 cells/μL. There is also severe anemia. With such major abnormalities in cell concentration, separation of erythrocytes and leukocytes is not complete, and division may be blurred. What is interpreted as being the "top" of the erythrocyte column is indicated by the arrowhead. The red discoloration of the buffy coat may be caused by a prominent increase in nucleated erythrocytes.

because of the presence of chylomicrons. Lipemia most commonly is associated with the postprandial collection of blood, but it also may be associated with disorders involving lipid metabolism (see Chapter 31). Hemolysis is a red discoloration of the plasma column, which usually results from artifactual lysis of red cells induced during the collection of blood. A small quantity of lysed erythrocytes is sufficient to impart visual hemolysis. Therefore, if the hematocrit is normal, one may assume it is an artifact. Less commonly, causes of anemia that result in intravascular hemolysis give rise to observable hemolysis in the plasma fraction, which also is known as hemoglobinemia (see Chapter 8). This will typically also be associated with hemoglobinuria.

The packed cell volume is measured on a reading device, such as a microhematocrit card reader (Fig. 1.8). The procedure is performed by positioning the erythrocyte–clay interface on the 0 line and the top of the plasma column on the 100 line. The position of the top of the erythrocyte column is then read on the scale as the packed cell volume.

Plasma proteins by refractometry

After measurement and observation of the microhematocrit tube, the plasma column may be used to estimate the plasma protein concentration on the refractometer (Fig. 1.9). This instrument may be used to estimate the concentration of any solute in fluid according to the principle that the solute refracts (or bends) light passing through the fluid to a degree that is proportional to the solute concentration. The principle or property being measured is the refractive index relative to distilled water. The scale for a particular solute can

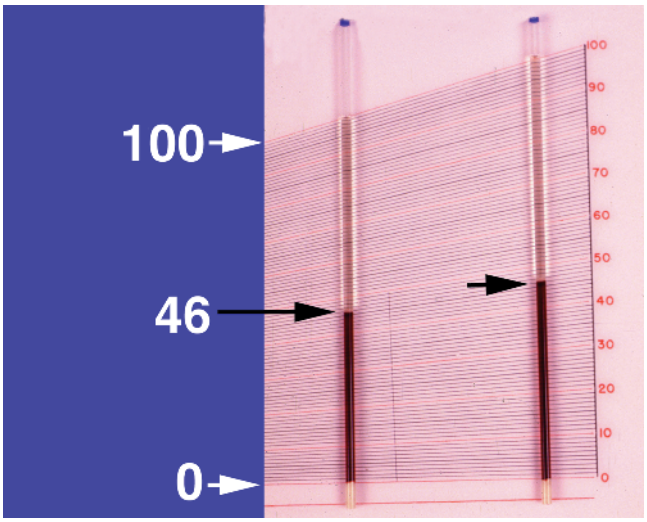


Figure 1.8 Determination of packed cell volume on a microhematocrit tube card reader using two tubes of blood from the same patient sample. Note that the scale allows the tube to be read over a considerable range of filling levels. The steps are to line up the erythrocyte–clay interface with the 0 line, line up the top of the plasma column with the 100 line, and then read the top of the erythrocyte column on the scale. The positions of these steps are indicated by the arrows. Note in this example that the packed cell volume is 46%.

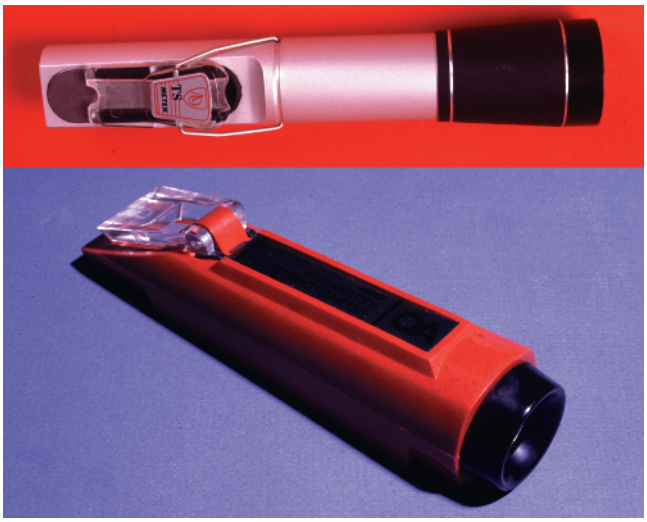


Figure 1.9 Refractometers. The lower refractometer is more rugged, because it is encased in rubber. It is known as a veterinary refractometer, and it has a canine and feline urine specific gravity scale that calibrates for minor differences between species during this determination.

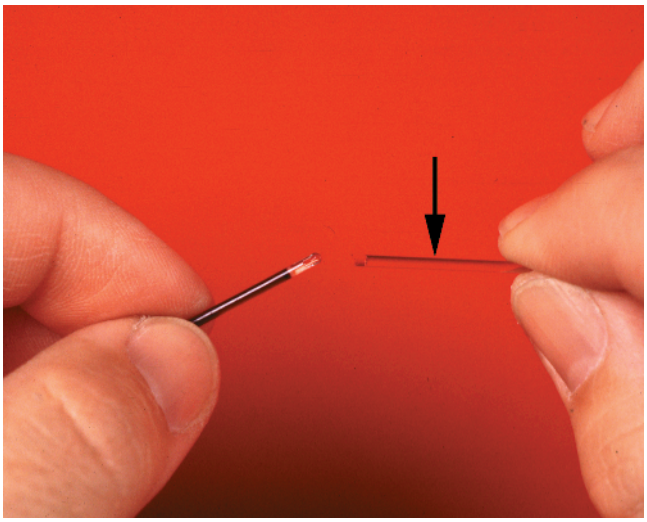


Figure 1.10 Preparation of the microhematocrit tube for measuring plasma protein concentration. The tube is broken just above the buffy coat to yield a column of plasma (arrow).

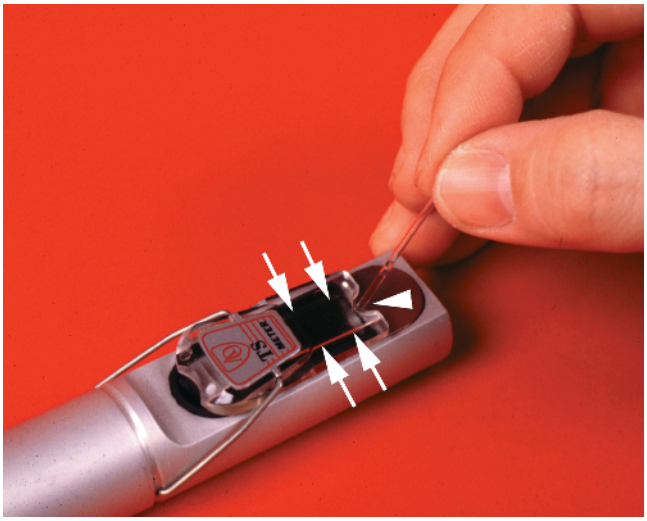


Figure 1.11 Loading plasma from the microhematocrit tube to the refractometer. To wick plasma onto the refractometer, capillary action is established by touching the end of the plasma tube at the notch of the prism cover (arrowhead). Flow should establish a thin layer of plasma under the plastic cover to fill the area delineated by arrows. After reading, the plastic cover is flipped back and wiped clean with a laboratory tissue.

be developed from refractive index measurements calibrated to solutions with known solute concentrations. In clinical diagnostics, refractometry is used to estimate the plasma protein concentration and urine specific gravity.

Plasma protein is measured using the plasma column in the microhematocrit tube. The tube is broken above the buffy coat layer (Fig. 1.10), and the portion of the tube containing the plasma is used to load the refractometer (Fig. 1.11). The instrument then is held so that an ambient light source can pass through the prism wetted with plasma, and the light refraction is read on a scale through an eyepiece (Fig. 1.12).

The protein measurement is regarded as being an estimate based on calibration, assuming that other solutes in the serum are present in normal concentrations. The measure-

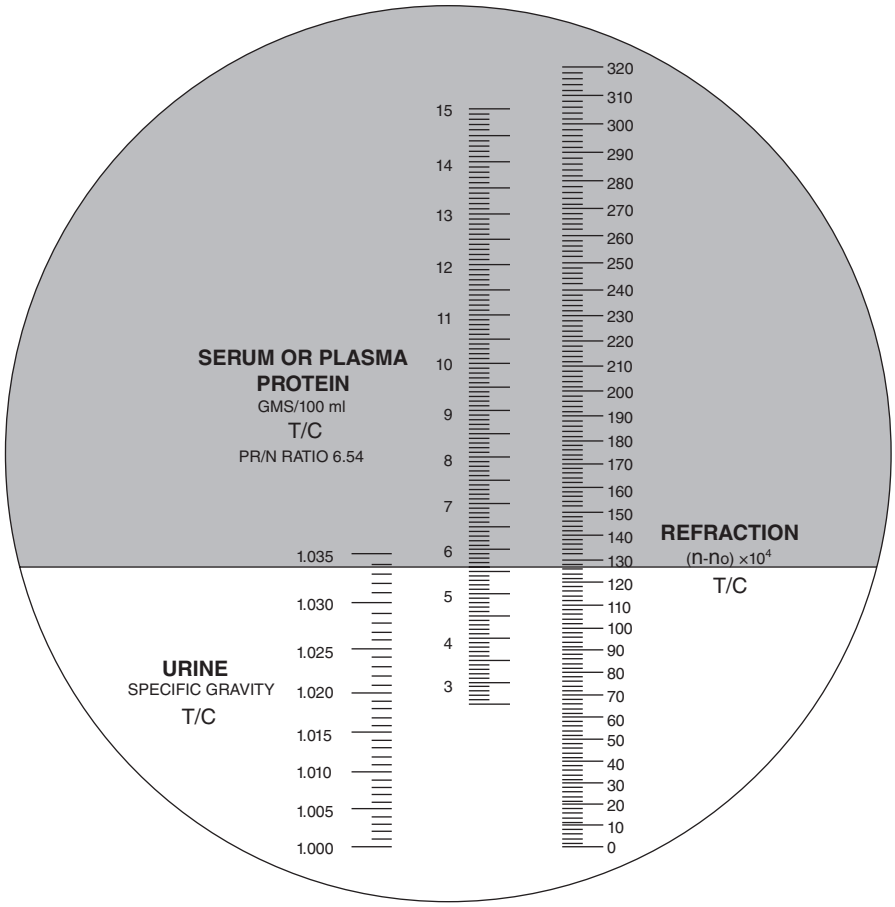


Figure 1.12 Representative refractometer scale as seen through the eyepiece. Light refraction creates a shadow–bright area interface that is read on the appropriate scale.

ment may be influenced by alterations in other solutes. Most notably, lipemia may artificially increase the protein estimate by as much as 2 g/dL. Other alterations of solutes such as urea and glucose influence the protein estimate to a much lesser, and usually negligible, degree.

Determination of total leukocyte concentration

Two general approaches are available to determine the leukocyte concentration. Historically, cell concentrations were measured manually using a blood dilution placed onto a hemocytometer and counted while observing by microscopy. This procedure, and associated consumables, is regarded as obsolete for the veterinary practice setting. Over the past 30 years this procedure has been progressively replaced by automated cell counting hematology systems or alternatively expanded buffy coat analysis technology in which cellular estimates are made from layers in a specialized hematocrit tube. The total leukocyte count is the concentration of nucleated cells, because the techniques detect all the nuclei in solutions from which erythrocytes have been removed by lysis or centrifugation. Therefore, nucleated erythrocytes typically are included in this count. In most cases the concentration of NRBC is negligible, but on rare occasion they may make up an appreciable fraction of the total nucleated cell concentration.

A variety of electronic cell counters operate by enumerating nuclear particles in an isotonic dilution in which a detergent is used to lyse the erythrocytes. These systems must be engineered for animal blood, however, to generate accurate measurements of cell concentrations. There are also continued advances in these hematology systems for performing leukocyte differentiation. Three-, four-, and five-part differential systems exist. The differential capability works best with normal blood, but there are individual exceptions. All systems may produce questionable results when there is leukocyte pathology and none properly detects abnormalities such as left shift, toxic change, and cell types outside the routine five normal cell types (see Chapters 10 and 12). (For principles of hematology system operation, see the discussion of advanced hematologic procedures later in this chapter.) The quantitative buffy coat analysis system (QBC, Becton Dickinson) estimates the leukocyte concentration by

measurement of the buffy coat layer in a specialized microhematocrit tube, in which a float is present to expand the buffy coat region for optical scanning.

In isolation, the total leukocyte count is not particularly useful for interpretive purposes; this measurement is used to determine the concentration of various leukocyte types that make up the differential count. The concentration of individual leukocytes is the most useful value for the interpretation of disease processes. This information is determined by evaluating the stained blood film (discussed below). Because of the limitations in automated leukocyte differentiation described above, it is important to utilize blood film examination in conjunction with automated hematology systems. This is essential not only for leukocyte characterization, but also for evaluation of erythrocytes in cases of anemia and platelets when the instrument produces a decreased platelet concentration value.

Preparation of blood films

The stained blood film is an essential tool for determining the concentrations of individual leukocyte types (i.e., differential count) and for evaluating important pathologic abnormalities involving leukocytes, erythrocytes, and platelets. Successful derivation of information from the blood film requires a proper technique, which both creates a monolayer of individually dispersed cells and a minimal disturbance of relative cell distributions that reflect the cell concentrations in mixed blood. A poorly prepared film presents confusing artifacts and may result in cell distributions on the slide that lead to serious errors in the differential count.

Preparation of a good-quality blood film requires mastery of a specific technique (Figs. 1.13–1.15). The most common procedure is known as the wedge or push technique and uses two glass microscope slides. A drop of blood is placed near one end of the first slide supported on the counter. The second slide is placed on the first in a way that forms a "wedge" consisting of a 30–45° angle in front of the drop of blood. The second slide, which is known as the pusher slide, then is backed into the drop of blood and advanced forward to the end. This should be accomplished in one rapid motion that involves a flip of the wrist holding the pusher slide. Downward pressure on the pusher slide should be minimal.

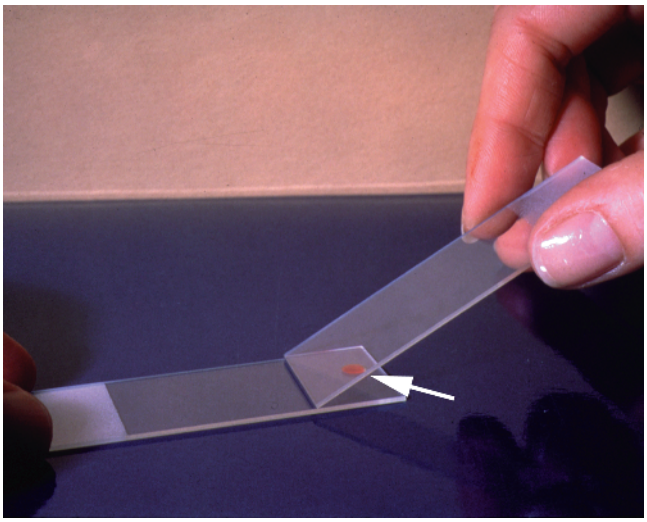


Figure 1.13 Blood film preparation. The blood slide is held on a firm surface, and a drop of blood is placed near the end (arrow). The pusher slide then is placed on the blood slide in front of the drop of blood to form an angle of approximately 30°.

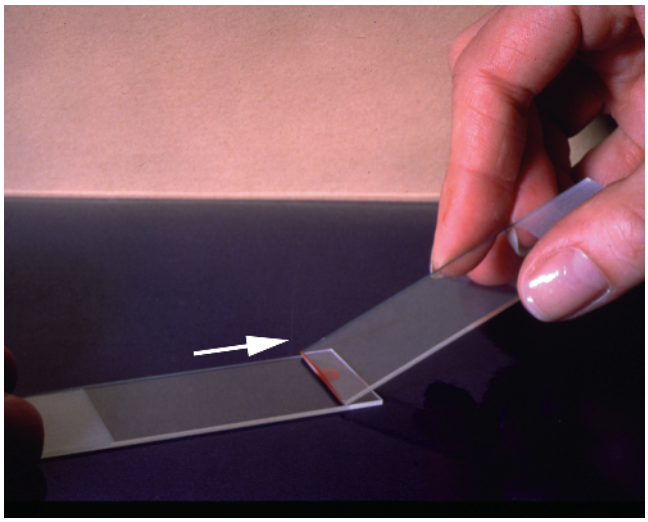


Figure 1.14 Blood film preparation. The pusher slide is backed into the drop of blood with a directional movement (arrow).

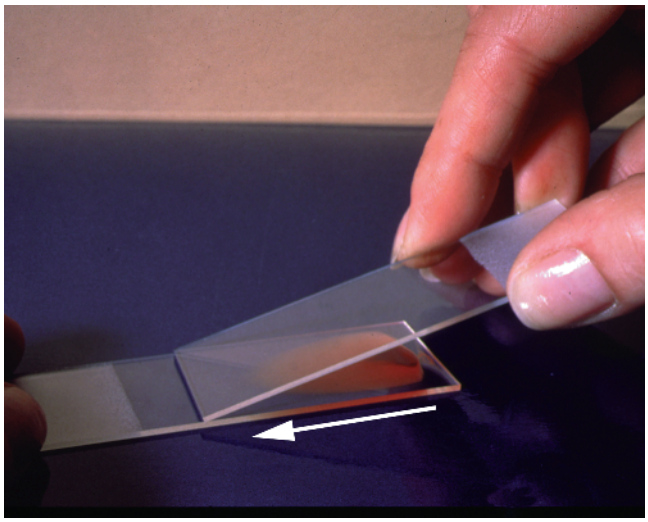


Figure 1.15 Blood film preparation. The pusher slide is pushed forward with a rapid directional movement (arrow). It is important that the movements shown in Figures 1.13 through 1.15 are a single, rapid procedure involving a flip of the wrist. Considerable practice is required to develop this skill. The result should be a uniform film of blood that gets progressively thinner (see Fig. 1.17).

Learning this technique in the presence of someone experienced with making good films is helpful, and considerable practice is advised. A common poor technique is to push the pusher slide too slowly, thereby creating a film that is too thin. This results in very poor distribution of leukocytes at the end of the film and artifacts in the evaluation of erythrocytes. In blood with reduced viscosity, such as that from patients with severe anemia, increasing the angle to avoid a slide that is too thin is useful.

Staining

After preparation, the blood film is usually stained within minutes. However, it may be stained within hours to days if it is being sent to a diagnostic laboratory. The staining system used for microscopic evaluation of cellular elements is the Wright stain, or a Wright stain modified by the addition of Giemsa. This is a relatively complex procedure that requires care and maintenance, thus often being limited to larger laboratory facilities. Quick-stain procedures that mimic the classical Wright stain are available, however, and for convenience, these are the most commonly used stains in the veterinary practice setting. The best-known stain kit is Diff-Quick (Dade Behring Inc., Newark, DE). Quick stains may result in nuclear overstaining and blurring of chromatin detail, but they provide sufficient quality for differential leukocyte counting and screening for morphologic abnormalities. Examples of manual to automated staining systems are shown in Figure 1.16.

Figure 1.16 Blood film and cytology staining apparatus. Top. Manual staining jars containing Diff-Quick stain. Slides are manually moved from one jar to the next according to the manufacturer's instructions. Bottom. An automated stainer used for higher-throughput situations. Note the mechanical arm that moves a rack of slides (not shown) through the sequence of staining procedure baths (arrow). The stainer may be programmed to control the timing in each bath. Most such machines provide the ability to stain as many as 20–25 slides per cycle.

Expertise for examination of blood films

Once stained, the anatomy of a blood film must be known to properly orient the slide for microscopic viewing (Fig. 1.17). The largest part of the film is the thick area or body, in which cells are superimposed and leukocytes are rounded up, thereby making microscopic evaluation of all components difficult. The feathered edge occurs at the end of the film. Artifacts in this area include broken leukocytes and the inability to evaluate the erythrocyte central pallor. The counting area is a small area between the thick portion and the feathered edge, and it consists of a monolayer of cells in which microscopy is optimal. Leukocytes are flattened out so that the internal detail is most evident.

The amount of interpretive disease relevance that can be gained from examination of the blood film is proportional to the expertise available for the examination. Success in dealing with all components of such examination depends on the quality of film making, stain maintenance, ability to look in the correct place, ability to differentiate preparation artifacts from morphologic abnormalities, and experience with interpretive blood film pathology. To the extent that the user cannot make these distinctions, abnormal blood films should be referred to a specialist for examination and/or second opinion.

It is important to examine the gross appearance of blood films as a correlate to artifact recognition. Improper preparation can be recognized, thereby alerting the observer to

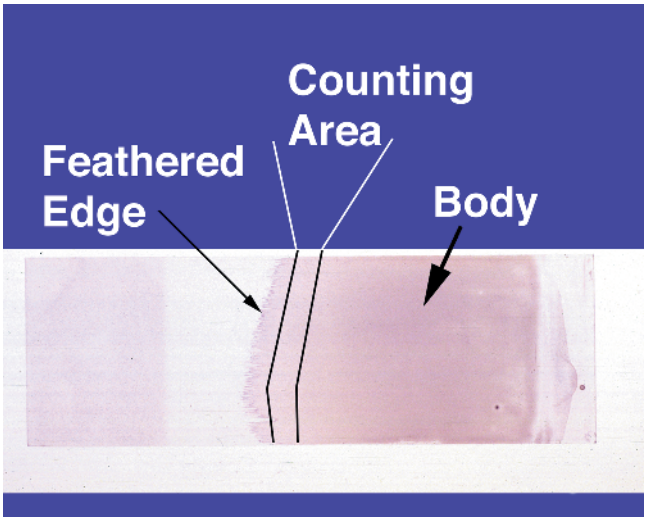


Figure 1.17 Anatomy of a stained blood film. Note the feathered edge (thin arrow) and the thick area or body of the slide (thick arrow). The counting area containing a monolayer of cells is present in a relatively small area, which is delineated approximately by the lines across the slide. This gross examination of the slide is very helpful in orienting the observer before placing the slide on the microscope stage. This facilitates alignment of the optics over the proper area of the slide, making it easier and faster to perform low-magnification observations and to find the counting area.

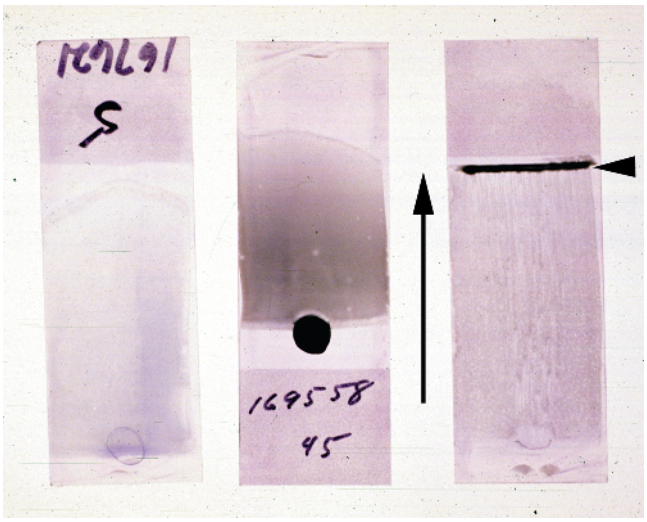


Figure 1.18 Gross appearance of blood films. All three of these films are oriented the same way. The drop of blood was placed near the bottom of the picture, and the film was made by pushing in the direction of the arrow. The middle film has a normal appearance and intensity of color. The appearance is homogeneous but gets progressively thinner as one approaches the feathered edge. The film on the left is very pale; this is the appearance when severe anemia is present. With severe anemia, blood viscosity is reduced, resulting in a much thinner film. The film on the right is made improperly and does not yield accurate information. The pusher slide was pushed too slowly, making a thin film with streaks. Note the streaking and irregularity over most of the slide. Blood was still present at the end of the slide as well, resulting in a line of densely concentrated cells (arrowhead). It is not possible to find a good monolayer for evaluation of erythrocyte morphology on this slide. In addition, the leukocytes are disproportionately concentrated at the end of the slide, which ordinarily has a feathered edge. Performing a differential count will be difficult in this case—and likely not accurate. A thin slide as a result of pushing too slowly is the most common problem in technique found at veterinary facilities.

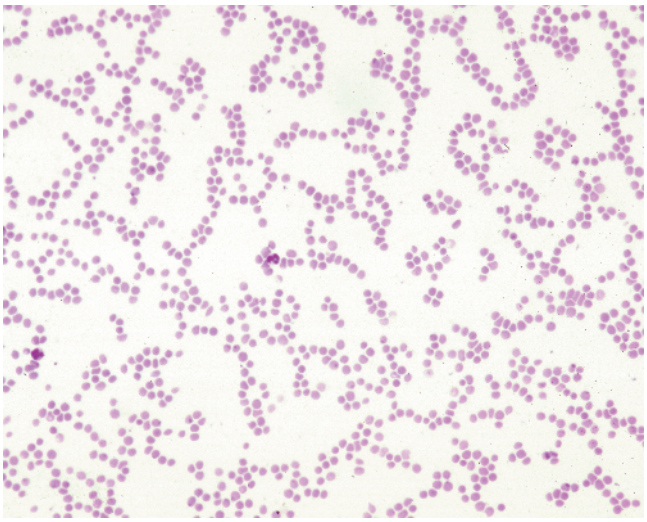


Figure 1.19 Low-magnification appearance of the feathered edge. Note the reticulated pattern of erythrocyte distribution. Artifactual loss of central pallor makes evaluation of erythrocyte morphology difficult, and false interpretation of pathologic abnormalities is likely to occur in this area.

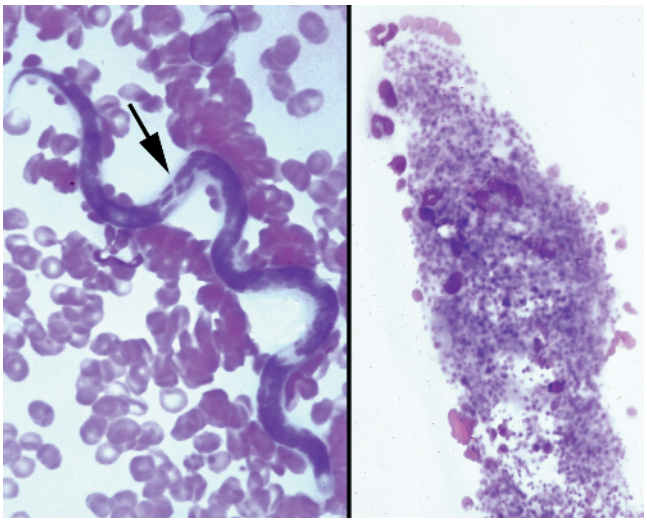


Figure 1.20 Large items pushed to the feathered edge. Left. Microfilaria (arrow) in an animal with heartworm disease. Right. A large clump of platelets with trapped leukocytes. Several hundred platelets are contained in this microclot.

artifacts that can be avoided and preventing any associated, errant interpretations. Common abnormalities that may be recognized grossly are presented in Figure 1.18. The most common and important abnormality is a slide that is too thin, which can be recognized by streaks progressing toward the feathered edge. This results in a leukocyte distribution that presents major errors in the differential count. In addition, there is not an area adequate for the evaluation of erythrocyte abnormalities.

The observer should locate the counting area using the 10× objective. The feathered edge is recognized by a loss of erythrocyte central pallor and a reticulated pattern of erythrocyte distribution on the film (Fig. 1.19). Quick, low-power examination of the feathered edge is useful for the detection and identification of abnormalities such as microfilaria, platelet clumps, and unusual, large cells that are preferentially deposited here (Fig. 1.20). The thick area is recognized by a progressive superimposition of erythrocytes as the observer moves further into the thick area of the slide. In very thick areas, the evaluation of cells is severely compromised (Fig. 1.21). The counting area is recognized by a monolayer of evenly dispersed cells (Figs 1.22 and 1.23).

Once the counting area is located, the experienced observer can estimate the leukocyte concentration on a well-prepared blood film. This is useful as a gross quality-control measure, and it is recommended that the observer

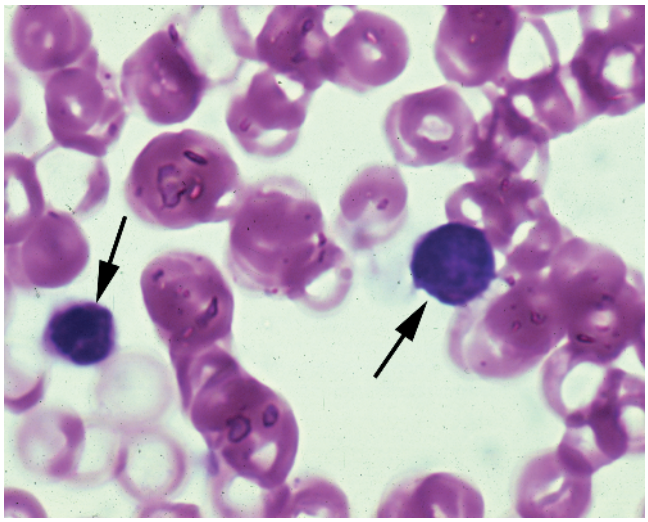


Figure 1.21 High-magnification appearance of cells in the thick area or body of slide. Note the superimposition of erythrocytes, thus making evaluation of erythrocyte morphology difficult. In addition, specifically identifying leukocytes (arrows) is difficult to impossible. In this area, leukocytes are spherical or rounded-up rather than flattened. It is not possible to see intracellular detail or even the delineation between the cytoplasm and the nucleus.

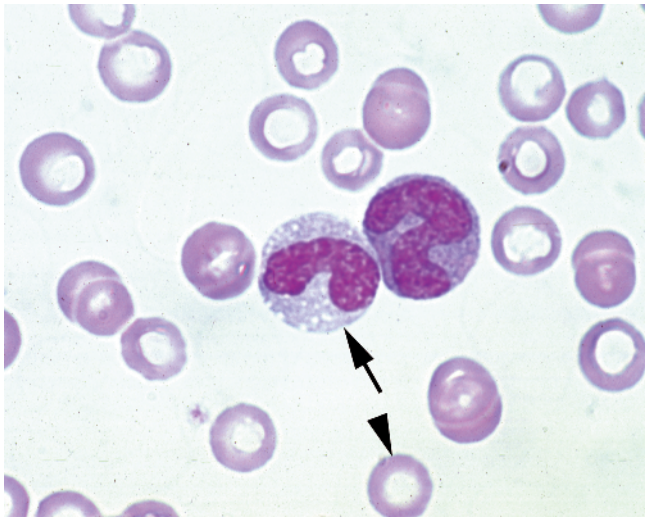


Figure 1.22 High-magnification appearance of cells in the counting area or monolayer. Note the minimal superimposition of erythrocytes, which facilitates evaluation of erythrocyte morphology (arrowhead). Leukocytes (arrow) are flattened on the slide, which makes it possible to see details of the cytoplasm and nucleus. Note that the nuclear borders are sharply delineated from the surrounding cytoplasm.

gain experience at this by repetitive comparison of leukocyte density on well-prepared blood films with total leukocyte counts from a cell counter. The low-power appearances of a leukocyte count in the normal range, marked leukopenia, and marked leukocytosis are shown in Figures 1.23, 1.24, and 1.25, respectively.

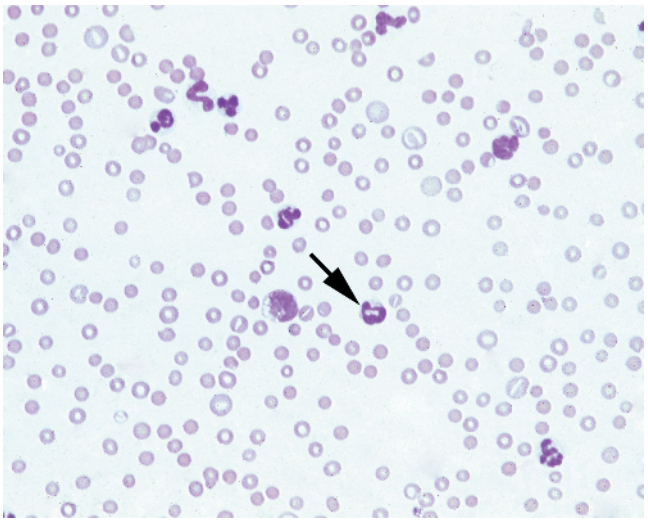


Figure 1.23 Low-magnification appearance of the counting area. Note the evenly dispersed cells and the ability to visualize the erythrocyte central pallor. The density of leukocytes (arrow) is that expected with a leukocyte concentration in the normal range.

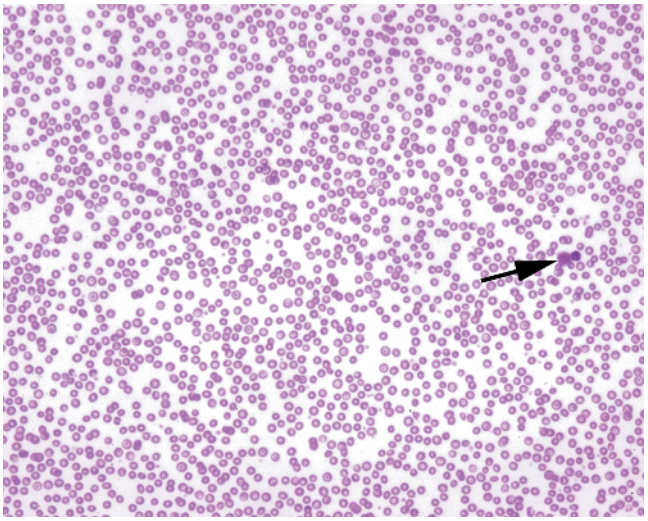


Figure 1.24 Low-magnification appearance of the counting area with a marked decrease in the leukocyte concentration. A rare leukocyte per field is present (arrow).

Procedures using the 100×, oil-immersion objective

Once the counting area is located and these assessments are completed the microscope should be adjusted for oil immersion, high magnification observation. The observer will then perform a systematic evaluation of the three major cell lines. This includes a differential count for leukocytes with notation about any abnormal cells, evaluation of erythrocyte morphology, and evaluation of platelets.

Within the counting area, the observer will move across fields and obtain the differential leukocyte count by classifying a

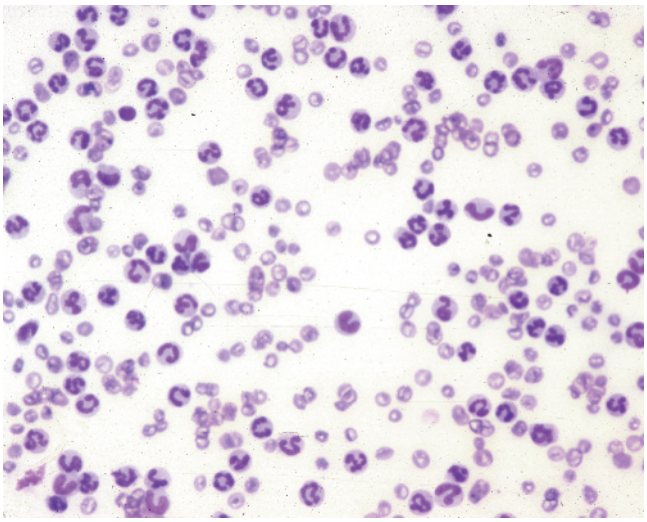


Figure 1.25 Low-magnification appearance of the counting area with a marked increase in leukocyte concentration. The density of leukocytes is considerably greater than that seen in Figure 1.23.

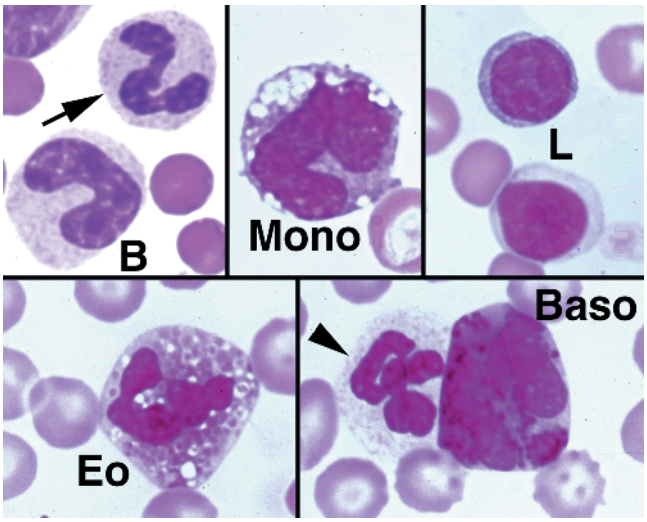


Figure 1.26 Basic leukocytes encountered in the differential count. Upper left. Neutrophils. Note the segmented neutrophil (arrow) and the constrictions in the nuclear contour. The band neutrophil (B) has smooth, parallel nuclear contours. Upper middle. Monocyte (Mono). The nucleus may have any shape, from round to bean-shaped to ameboid and band-shaped, as in this example. The cytoplasm is blue-gray and may variably contain vacuoles. Upper right. Two lymphocytes (L). Lower left. An eosinophil (Eo). Note that granules stain similar to the surrounding erythrocytes. Occasionally, granules may wash out in the staining procedure, leaving vacuoles. Lower right. Basophil (B) with dark granules that stain similar to nuclear chromatin. Note the adjacent neutrophil (arrowhead) and that neutrophils may have small, poorly staining granules that are much smaller than those of eosinophils or basophils.

minimum of 100 consecutively encountered cells. Cells are classified into a minimum of five to six categories, with the presence of abnormal cells being recorded into a category of "other," in which a specification is made for the individual sample. The common six categories of normal cells—neutrophil, band neutrophil, lymphocyte, monocyte, eosinophil, and basophil—are shown in Figure 1.26. (See Chapter 10 for additional visual details regarding leukocyte identification that may be helpful in differential counts.)

The result of counting 100 cells is that the number of each leukocyte type is a fraction of 100, or a percentage of the leukocyte population. Once cells are categorized into percentages, they must be converted to absolute numbers for interpretation purposes. This is done by multiplying the total leukocyte concentration by the percentage of each leukocyte type, which yields the absolute number or concentration of each leukocyte in the blood sample. The following example illustrates the conversion of percentages to absolute numbers:

Example 1.1. Conversion of Percentage Counts to Absolute Concentrations

Total white-blood-cell count = 10,000/µL
Differential white-blood-cell count:

	Percentages	Absolute Numbers/µL
Neutrophils	60%	(6000)
Lymphocytes	30%	(3000)
Monocytes	5%	(500)
Eosinophils	5%	(500)

Any abnormalities in leukocyte morphology also should be noted. Important morphologic abnormalities are detailed in Chapter 12.

Erythrocyte morphology then is systematically evaluated. The observer should note any important erythrocyte shape or color abnormalities; this is particularly important for evaluating anemias. (See Chapter 5 for a review of morphologic erythrocyte abnormalities.)

The presence of platelet adequacy may be interpreted from a properly prepared blood film. A minimum of 8–12 platelets per oil immersion high-power (1000×) field may be interpreted as adequate. The number seen may be considerably greater than described, however, because of the wide range of normal platelet concentrations. This number is only a guideline for most microscopes with a wide field of view. It should be adjusted downward when using a microscope with a narrow field of view and upward if using one with a superwide field of view. If the platelets appear to be decreased, a search for platelet clumps on a low-power setting at the feathered edge should be performed. The ability to look for platelet clumps is also important when a cell counter produces a decreased platelet concentration value; this is a frequent problem in cats. Morphology of platelets also may be noted. Platelets that approach the

diameter of erythrocytes or larger are referred to as macroplatelets or giant platelets. In dogs, these suggest accelerated platelet regeneration, but this interpretation usually is not applied to macroplatelets in cats.

Advanced hematologic techniques

Historically, these capabilities were limited to central laboratories. Over the past 20 years there has been rapid technological evolution resulting in reduced cost and complexity such that these capabilities are now available to the common veterinary facility. Currently, the predominant differences of the larger, more expensive systems used by commercial laboratories are higher throughput rate, automated tube handling, and more sophisticated differential counting technology. (See Chapter 2 for additional discussion of equipment and laboratories.) Hemograms performed on modern hematologic instrumentation provide the following additional measurements.

Items determined by spectrophotometry or calculation:

- Hemoglobin concentration of blood, g/dL
- Mean cell hemoglobin content, pg
- Mean cell hemoglobin concentration (MCHC), g/dL

Items determined by cell (particle) counting and sizing:

- Erythrocyte concentration of blood, $\times 10^6$ cells/μL
- Mean cell volume (the average size of erythrocytes; MCV), fL
- Hematocrit (equivalent to the packed cell volume), %
- Platelet concentration of blood, $\times 10^3$ cells/μL
- Mean platelet volume (MPV), fL
- Total and differential leukocyte concentrations, $\times 10^3$ cells/μL
- Reticulocyte concentration, $\times 10^3$ cells/μL

The method and applicability for each of these measurements are now described.

Items determined by spectrophotometry or calculation

Hemoglobin concentration

This measurement of the quantity of hemoglobin per unit volume, expressed as g/dL, is performed in conjunction with the total leukocyte count. Briefly, a blood sample is diluted, and a chemical agent is added to rapidly lyse cells, thereby liberating hemoglobin into the fluid phase. Nucleated cells remain present in the form of a nucleus with organelles collapsed around it. The absorbance of light at a specific wavelength then may be measured by spectrophotometry in a small flow cell known as a hemoglobinometer. The absorbance of light is proportional to the concentration of hemoglobin. The system is calibrated with material of known hemoglobin concentration using reference techniques.

Interpretation of the hemoglobin concentration is the same as that of the packed cell volume, or hematocrit. It is an index of the red cell mass per unit volume of blood in the patient. Because it is roughly equivalent to the packed cell volume, however, it is not particularly useful for clinical interpretations. Most clinicians are more familiar or experienced with interpreting packed cell volumes. The hemoglobin value is always proportional to hematocrit and is a separate, independent measurement. Therefore, the hemoglobin value may serve as a quality-control adjunct for laboratory personnel when used to calculate the MCHC.

Mean cell hemoglobin

The mean cell hemoglobin is calculated from the hemoglobin concentration and erythrocyte concentration. It is regarded as being redundant to other measurements and, therefore, is not useful.

Mean cell hemoglobin concentration

The MCHC is calculated from the hemoglobin concentration and the hematocrit. It provides an index for the quantity of hemoglobin (HGB) relative to the volume of packed erythrocytes (expressed as g/dL):

$$\frac{\text{HGB (g/dL)}}{\text{PCV (\%)}} \times 100 = \text{MCHC (g/dL)}$$

where PCV is the packed cell volume. An example calculation is

$$\frac{10\ \text{g/dL}}{30\%} \times 100 = 33.3\ \text{g/dL}$$

A universal relationship among mammalian species, other than the camel family, is that the hemoglobin value normally is approximately one-third of the hematocrit value. Thus, from the relationship described, the MCHC for all mammalian species ranges from approximately 33 to 38 g/dL. Because members of the camel family (camel, llama, alpaca, vicuna) have relatively more hemoglobin within their cells, their MCHCs are expected to range from 41 to 45 g/dL.

The MCHC is not particularly useful for clinical interpretations; however, it is useful to laboratorians for monitoring instrument performance. The rationale is that the hematocrit and hemoglobin are determined on different blood aliquots, which are diluted in two different subsystems of the instrument. A malfunction in either of these subsystems may result in a mismatch between the hemoglobin and the packed cell volume, which is reflected by a deviation from the reference interval. In addition, some abnormalities of blood can result in an artifactually increased MCHC, and these can include any factor that causes a false increase in the spectrophotometric determination of hemoglobin relative to the hematocrit. Severe hemolysis in the sample is a common cause of an increased MCHC. Alternatively, common examples of increased turbidity that interfere with

light transmittance are lipemia and a very large number of Heinz bodies (see Chapter 8) in cats. Erythrocyte agglutination, as may occur in immune-mediated hemolytic anemia, may result in a false high MCHC. In this situation, the hemoglobin measurement is accurate, but the hematocrit is falsely low because the agglutinated erythrocytes are out of the system's measuring range and are therefore not counted or sized in derivation of hematocrit.

Two erythrocyte responses related to anemia may be associated with a slightly decreased MCHC. The first is marked regenerative anemia. Reticulocytes or polychromatophilic cells are still synthesizing hemoglobin and, therefore, have not yet attained the cellular hemoglobin concentration of a mature erythrocyte. A very high fraction of reticulocytes is required, however, such as greater than 20%, to develop a detectable decrease in MCHC. The second is severe iron deficiency, in which cells have a reduction in hemoglobin content because they are smaller (i.e., microcytic) but also may have a minor reduction in cellular hemoglobin concentration. There are no causes of a dramatically decreased MCHC (<28 g/dL) other than an analytic instrument error.

Items determined by cell (particle) counting and sizing

Cell counting and sizing technologies

A brief overview of cell counting and sizing technology common to all of these measurements is appropriate. One of two technologies is used by most hematology instrument systems.

The first is light-scatter measurement of cells passing through a light source. Cells are passed through a flow cell that is intersected by a focused laser beam. The physical properties of the cell scatter light to different degrees and at different angles relative to the light source. Cell passages eliciting scatter events may be counted to derive the cell concentration. The degree of scatter in the direction of the light beam, which is known as forward-angle scatter, is proportional to the size of the cell. In addition, measurement of light scattered to different angles may be correlated with cellular properties, which leads to the ability to differentiate nucleated cell types.

The second is more common and incorporated into a wider range of instrument designs and may also be used as a second measuring principle in light-scatter systems. This is electronic cell counting, which is also known as impedance technology or Coulter technology (after the original inventor). It is based on the principle that cells are suspended in an electrolyte medium, such as saline, that is a good conductor of electricity. The suspended cells, however, are relatively poor conductors of electricity. Thus, these cells impede the ability of the medium to conduct current in a sensing zone known as an aperture. By simultaneously passing current and cells through a small space or aperture, deflections in current can be measured (Fig. 1.27). In addition, the size of the cell is proportional to the resultant deflection in current. This volumetric size discrimination may be used to measure the size distribution of erythrocytes, to discriminate platelets from erythrocytes, and to partially differentiate leukocytes. Cells within a given population are counted and assigned to a size distribution by particle-size analyzer circuitry (Fig. 1.28). The particle-size analyzer assigns each cell to a size scale that is divided into a large number of discrete size "bins" of equal size. The size scale is calibrated with particles of known size. By rapidly accumulating several thousand cells, a frequency distribution of the sizes of the cell population may be constructed (Fig. 1.29).

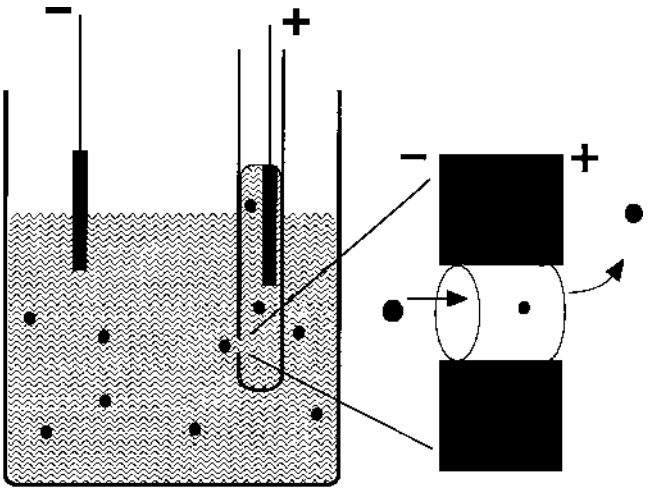


Figure 1.27 Principle of electronic impedance cell counting. Left. Overview of the fluidic chamber. Cells (dots) are diluted in an isotonic fluid (wavy lines). Two electrodes (+ and –) are separated by a glass tube containing a small opening or aperture. Electric current is conducted by the isotonic fluid across the electrodes via the aperture. Vacuum is applied to move the fluid and cells through the aperture. Right. Magnified, diagrammatic view of the aperture. Cells flow through the aperture (arrows). The aperture is a cylindric shape with a volume called the sensing zone. While occupying space within the aperture, cells transiently impede the flow of current. Cell passages are counted as deflections in the current voltage. In addition, the magnitude of voltage deflection is proportional to the volume of the cell.

The size distribution curve is most useful for the evaluation of erythrocytes in the laboratory. It also may be used to derive leukocyte differential and platelet information.

The following measurements derive from the described cell counting and sizing technology. Because of the considerable differences in erythrocyte and platelet sizes between species, instrument systems require careful design and/or adjustment to accurately obtain the various measurements. For example, instruments manufactured for the analysis of human blood do not perform accurately for most animal species without modification.

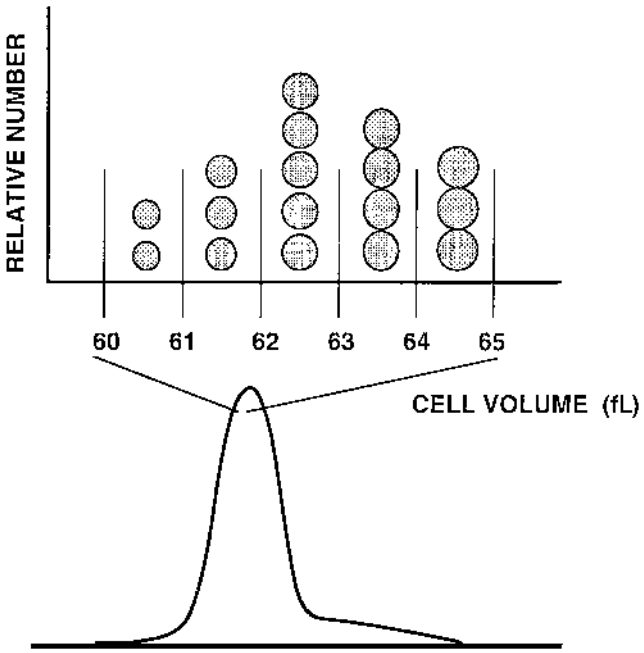


Figure 1.28 Cell volumes assigned to size bins. In the case of erythrocytes, a cell volume scale of approximately 30 to 250 fL is divided into a large number of discrete size bins (e.g., 60–61 fL, 61–62 fL). As the cells are counted, they are assigned to size bins (circles). Accumulation of many cells allows the construction of a size distribution histogram on the cell-volume scale (curve tracing at bottom). The drawing of bins at the top would represent a small area of the total curve.

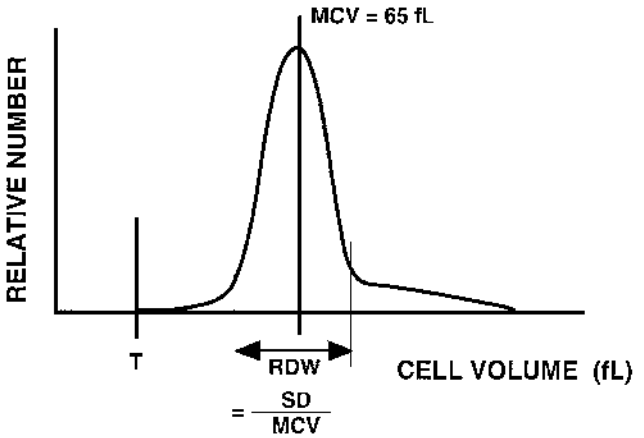


Figure 1.29 Histogram of erythrocyte size distribution. The x-axis is the cell volume, and the y-axis is the relative number of cells at each volume. Only cells above a specified volume or threshold are included in the analysis; this is indicated by the vertical bar (T). The mean cell volume (MCV) is indicated by the large vertical bar. The RDW (red-cell distribution width) value, an index of volume heterogeneity, is the standard deviation (SD) divided by the MCV, with the SD being that of the volumes of erythrocytes within the region indicated by the fine lines marked by the double arrow.

Erythrocyte concentration

The erythrocyte concentration is measured directly by counting the erythrocyte particles in an isotonic dilution of blood. This value is not useful for purposes of clinical interpretation. It generally parallels the packed cell volume and hemoglobin concentration, but the packed cell volume is the preferred value for the interpretation of erythrocyte mass. The erythrocyte concentration is used by the instrument to calculate the packed cell volume (described later).

Mean cell volume, erythrocyte histogram, and red cell distribution width

As the erythrocytes are counted, their size distribution is simultaneously constructed (Fig. 1.29), and from this size distribution, the MCV is easily calculated. The red cell distribution width (RDW) is a mathematic index describing the relative width of the size distribution curve. It is the standard deviation of most the erythrocytes divided by the MCV. The tails of the erythrocyte distribution usually are excluded from this mathematic treatment.

These values are useful for the evaluation of anemia. Iron deficiency results in the production of microcytic erythrocytes, and accelerated erythrocyte regeneration results in the production of macrocytic erythrocytes. Early in these responses, a widening of the erythrocyte size distribution and RDW value may be observed (Fig. 1.29). As a larger proportion of these cells accumulate during the response, the curve shifts in the respective direction, and eventually, the MCV may fall out of the reference interval. The RDW is more useful in the laboratory, in conjunction with the examination of blood films, whereas the laboratorian and the clinician both may interpret the MCV. Examples of interspecies variation and representative reference intervals for MCV are

Humans	80–100 fL
Dogs	60–72 fL
Cats, horses, and cows	39–50 fL
Sheep	25–35 fL
Llama	21–29 fL
Goat	15–25 fL

For additional detail on microcytic and macrocytic anemias and other breed-specific information regarding erythrocyte size, see Chapter 6.

Hematocrit

One of the advantages of hematology instrumentation is that the hematocrit may be determined by calculation, thereby avoiding the need for microhematocrit centrifugation. The instrument calculates hematocrit (HCT) using the erythrocyte concentration (RBC) and the MCV:

$$(\text{MCV} \times 10^{-15}\ \text{L}) \times (\text{RBC} \times 10^{12}\ \text{L}) = \text{HCT}$$

Or, simplified:

$$\frac{\text{MCV} \times \text{RBC}}{10} = \text{HCT}$$

Thus, for example:

$$\frac{\text{MCV 70 fL} \times 7.00\ \text{RBC}}{10} = \text{HCT 49\%}$$

Platelet concentration

Platelets may be counted simultaneously with erythrocytes. Because platelets are considerably smaller than erythrocytes, however, they are analyzed in a separate area of the particle-size analyzer scale. Most species have little or no overlap between platelet and erythrocyte volume, thereby making such analysis both simple and accurate. Cats are an exception, in that their platelets are approximately twice the volume of those in other domestic species. In addition, macroplatelet production is a frequent response during most hematologic disturbances in cats. This response is not specific for any specific disease pattern, but it results in considerable overlap between erythrocyte and platelet size distributions, thus making determination of accurate counts difficult. Therefore, feline platelet counts should be regarded as being estimates only. Because large platelets tend to get counted as erythrocytes, the platelet concentration frequently may be artifactually low. In general, if the platelet concentration falls in the reference interval, it may be regarded as being adequate. If the platelet concentration is decreased, however, the blood film should be examined by a laboratorian to confirm this finding.

White blood cell and differential leukocyte concentrations

To analyze leukocytes, a lytic agent is first added to a dilution of blood. This agent rapidly lyses or dissolves cytoplasmic membranes, thereby making the erythrocytes and platelets "invisible" to the detection technologies. Only nuclear particles of nucleated cells remain, around which is found a "collapse" or condensation of cytoskeletal elements and any attached organelles. These particles are measured by one of the detection technologies previously described to obtain the total leukocyte concentration. Using specially formulated lytic reagents, the degree of collapse may be controlled to different degrees in different leukocyte types. The result is a differential size that can be measured by a particle-size analyzer or light-scatter technology. Automated differential leukocyte counting is not as perfected in domestic animals as in humans; however, the procedure is reasonably accurate for normal blood and, therefore, is very useful in situations such as safety assessment trials, in which most (or all) of the blood samples to be analyzed are normal. When blood is abnormal, however, the frequency of analytic error in the differential count increases considerably. Analytic errors are handled by using the blood film for comparison and the visual differential count whenever an instrument analytic error is either present or suspected. It is essential to monitor instrument performance by visual inspection of the histogram or cytogram display for each sample to know when analytic failure occurs. It is very difficult, if not impossible, to determine this simply by monitoring numeric data from the instrument. Therefore, use of this technology requires considerable training and expertise by the operator to monitor the instrument performance and appropriately intervene with visual inspection of the blood film.

Summary of blood analysis by automated or semiautomated instrumentation

The flow of dilutions, analysis, and calculations within an automated hematology instrument is summarized in Figure 1.30. This flow has two main pathways. In one, an isotonic dilution of blood is made for erythrocyte and platelet analysis. In the other, a dilution is made, into which a lytic agent is added; in this pathway, leukocytes and hemoglobin are measured.

Reticulocyte concentration

Reticulocyte enumeration

The reticulocyte concentration is very useful in the evaluation of anemias. The rate of release of reticulocytes from the bone marrow is the best assessment regarding the function of the erythroid component of bone marrow. (See Chapters 6–8 for a more detailed discussion of the anemias.)

The basis for the reticulocyte count involves the events in the maturation of erythroid cells. The developing erythroid cell is heavily involved in aerobic metabolism and protein (i.e., hemoglobin) synthesis. As it nears the final stages of

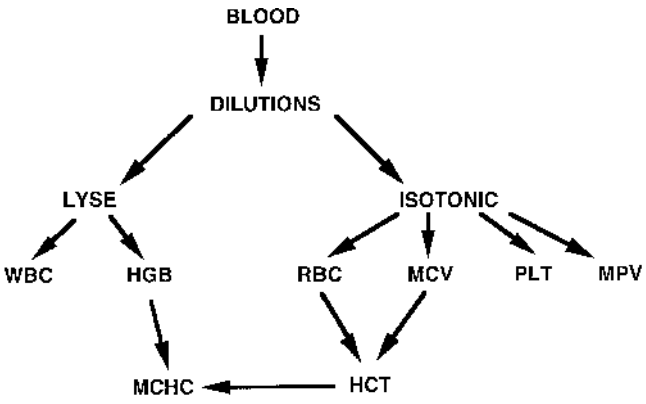


Figure 1.30 Summary of blood analysis pathways in an automated instrument. Two major dilutions are made (see text). In the left pathway, a lytic agent is added, and leukocytes are counted and the hemoglobin concentration measured. In the right pathway, erythrocytes and platelets are counted and sized. From the direct measurements, the hematocrit is calculated. A cross-check between the two pathways is provided by calculation of the mean cell hemoglobin concentration (MCHC).

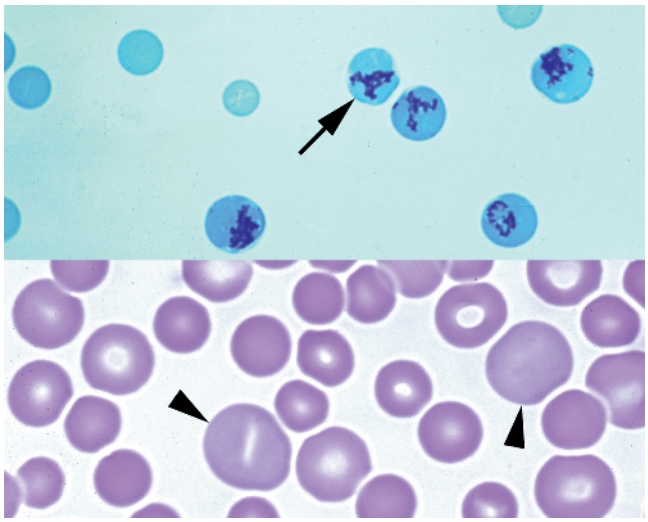


Figure 1.31 Reticulocytes. Top. Representative reticulocyte (arrow) using new methylene blue stain. Note the dark-staining, aggregated organelles in several reticulocytes. Bottom. Blood film stained with Wright-Giemsa stain. Polychromatophilic cells (arrowheads) are roughly equivalent to reticulocytes on the counterpart stain.

Figure 1.32 Examples of reticulocyte stains. Left. New methylene blue in a liquid dropper bottle. Right. Commercial preparation of brilliant cresyl blue. The stain is coated on the bottom of disposable tubes.

maturity, the nucleus undergoes degeneration and is extruded from the cell, and the organelles supporting the synthetic and metabolic events are removed. After denucleation of the metarubricyte, the remaining erythrocyte undergoes its final maturation, which involves the loss of ribosomes and mitochondria during a period of 1–2 days. To enumerate reticulocytes, a stain is applied to erythrocytes, thereby causing aggregation of these residual organelles. This results in visible, clumped granular material that can be seen microscopically (Fig. 1.31). The aggregation is referred to as reticulum, hence the name reticulocyte. Reticulocytes are equivalent to the polychromatophilic cells observed on the Wright-stained blood films (Fig. 1.31). Evaluation of polychromatophilic cells on the Wright-stained blood film can provide an assessment of the bone marrow response to anemia. The appearance of these cells, however, is more subjective, and they are more difficult to quantitate than counting the corresponding cells on the reticulocyte stain.

Stains that can be used are new methylene blue (liquid) and brilliant cresyl blue, which is available in disposable tubes that facilitate the procedure (Fig. 1.32). First, several drops of blood are added to the stain in a tube. The tube then is mixed and incubated for 10 minutes. From this mixture, a conventional blood film is made and air-dried. A total of 1000 erythrocytes are counted and categorized as either reticulocytes or normal cells. From this, the percentage of reticulocytes is derived. Interpretation of the percentage reticulocytes is somewhat misleading, however, because it does not account for the degree of anemia. Thus, for purposes of interpretation, the absolute reticulocyte concentration should be calculated by multiplying the erythrocyte concentration (RBC) by the percentage of erythrocytes that are reticulocytes:

$$\text{RBC}/\mu\text{L} \times \%\ \text{Reticulocyte} = \text{Reticulocytes}/\mu\text{L}$$

Some instrument systems are also capable of reticulocyte enumeration. The method involves staining erythrocytes with a fluorescent dye that binds to residual RNA in the reticulocyte that is not present in the mature erythrocyte. RNA content, proportional to fluorochrome per cell, is measured and gated to differentiate reticulocytes from mature erythrocytes and other nonerythroid cell types. The percent and absolute values are presented as described above.

Interpretation of the reticulocyte concentration

The reticulocyte concentration is most useful in dogs and cats, and it also has some application in cows. It is not used in horses, however. Reticulocyte maturation is confined to the marrow space in the horse, and reticulocytes almost never are released into their circulation. Reticulocyte concentration guidelines for domestic mammals are the concentrations to be expected when the hematocrit is normal:

Dogs and cats	0–60,000 cells/μL
Cows	0 cells/μL
Horses	Do not release reticulocytes

When anemia is present, a greater degree of release from the marrow is to be expected if the marrow can respond to the anemia. This gives rise to the following guidelines for the interpretation of reticulocyte concentrations with respect to the type of anemia present:

Nonregenerative anemia to very poor regeneration	0–10,000 cells/μL
Nonregenerative to poorly regenerative anemia	10,000–60,000 cells/μL
Regenerative anemia with mild to moderate output	60,000–200,000 cells/μL
Maximal regeneration	200,000–500,000 cells/μL

Reticulocyte maturation

In dogs, reticulocyte maturation occurs in 24–48 hours. Maturation involves a continuum of progressive loss of the visible organelles (Fig. 1.33).

Cats are unique in that more than one kind of reticulocyte may be present. These reticulocytes are of the aggregate and the punctate forms (Fig. 1.34). The aggregate reticulocyte has a clumped reticulum that appears to be identical to that of other species. In the punctate reticulocyte, discrete dots are seen without any clumping; other species do not have this reticulocyte counterpart. Only aggregate reticulocytes appear to be polychromatophilic with Wright stain. Punctate reticulocytes are indistinguishable from normal, mature erythrocytes with Wright stain.

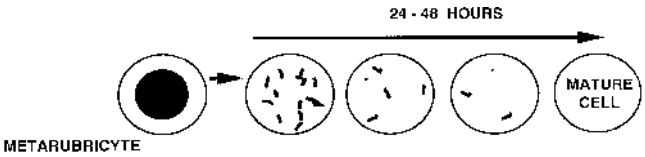


Figure 1.33 Sequential erythroid maturation as related to the reticulocyte stain and interpreted in dogs. The metarubricyte denucleates on leaving the reticulocyte. Reticulum is progressively lost during a 24–48-hour period, resulting in a mature erythrocyte.

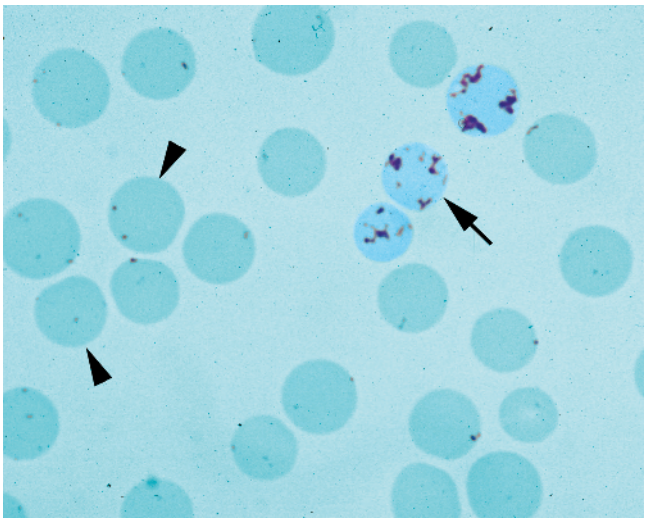


Figure 1.34 Feline reticulocyte morphology with new methylene blue stain. Three aggregate reticulocytes are in the field; note the representative one (arrow). The remainder of the cells are punctate reticulocytes; note the representative cells (arrowheads).

Reticulocyte maturation in cats also may be viewed as a continuum (Fig. 1.35). Aggregate reticulocytes mature to the punctate form in approximately 12 hours; the punctate cells may continue to mature for another 10–12 days. Because of the short maturation time of aggregate reticulocytes, these cells are the best indicator of active marrow release. Therefore, only aggregate cells are counted in cats, and interpretive guidelines apply to this cell type only. Experience is required to exclude punctate cells when performing the reticulocyte count.

Organization of the complete blood count (hemogram)

It is useful to summarize the described basic and advanced determinations in a way that shows the organization of how they are performed and interpreted. This provides a mental framework for simplifying the complexity of this information into an everyday, intuitive tool: the hemogram. The techniques for generating data may be organized conceptually as direct measurements, microscopic procedures, and calculations. The complete blood count may include:

Direct Measurements

- Packed cell volume (by microhematocrit centrifugation)
- Hemoglobin concentration
- Red cell concentration (RBC)
- Mean cell volume (MCV)
- White cell concentration
- Plasma proteins (by refractometer)

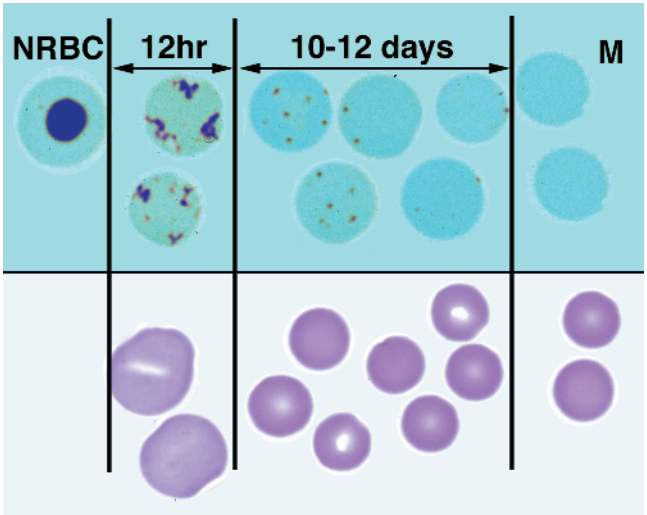


Figure 1.35 Feline reticulocyte maturation, progressing from left to right. Top. Cells stained with new methylene blue. After denucleation of the metarubricyte (NRBC), an aggregate reticulocyte is formed. This cell matures to the punctate form in approximately 12 hours. The punctate forms continue to mature by slow loss of punctate granules during a 10–12-day period. Mature cells (M) on the right have no granularity. Bottom. Corresponding cells stained with Wright-Giemsa stain. Note that polychromatophilic cells correspond to aggregate reticulocytes. Punctate and mature cells are indistinguishable with Wright-Giemsa stain.

- Platelet concentration
- Mean platelet volume (MPV)

Microscopic Procedures

- Differential white cell count
- Red cell morphology
- Platelet morphology and assessment of adequacy
- Microscopic reticulocyte enumeration in patients with anemia

Calculations

- Hematocrit, when instrument derived
- Erythrocyte indices (e.g., MCHC, MCH, and RDW)
- Absolute white blood cell differential values
- Absolute reticulocyte count

These determinations are organized into a report form that aids the clinician in efficiently interpreting the information. The best way for this information to be organized is into banks of data that relate to the three major cell lines (i.e., erythrocytes, leukocytes, and platelets). For each cell line, all pieces of relevant information are organized in one place on the form.

Laboratory tests useful in the diagnosis of immune-mediated hemolytic anemia

Coombs or antiglobulin test

The Coombs or antiglobulin test is used as an aid in establishing the diagnosis of immune-mediated hemolytic anemia by detecting species-specific immunoglobulin that is adsorbed or attached to the surface of erythrocytes. The test uses the Coombs reagent, which is a polyclonal serum (usually prepared in rabbits) to the immunoglobulins of the species of interest. Some reagent manufacturers claim their reagent also detects complement. The procedure involves washing the erythrocytes in saline to remove plasma proteins and immunoglobulin that may be nonspecifically associated with erythrocytes. An aliquot of washed cells then is incubated with the Coombs serum. If appreciable patient immunoglobulin is attached to the erythrocytes, the Coombs serum induces erythrocyte agglutination. By means of two binding sites per molecule, the Coombs reagent immunoglobulin binds the patient immunoglobulin attached to the erythrocytes. The two binding sites result in progressive bridging of erythrocytes, which is visualized as agglutination. The absence of agglutination is interpreted as being a negative result, whereas the presence of agglutination is interpreted as being a positive result. Appropriate controls are performed as well.

False-negative reactions are a common problem with the Coombs test, likely because of the elution of pathologically adsorbed immunoglobulin or immune complexes during washing of the erythrocytes in preparation for the test. The best evidence for this is that prominent autoagglutination may disappear with washing. Autoagglutination, if confirmed microscopically, may be interpreted as being equivalent to a positive Coombs test. False-positive reactions also may occur, but are less well documented because the test is typically only performed when one suspects the disease.

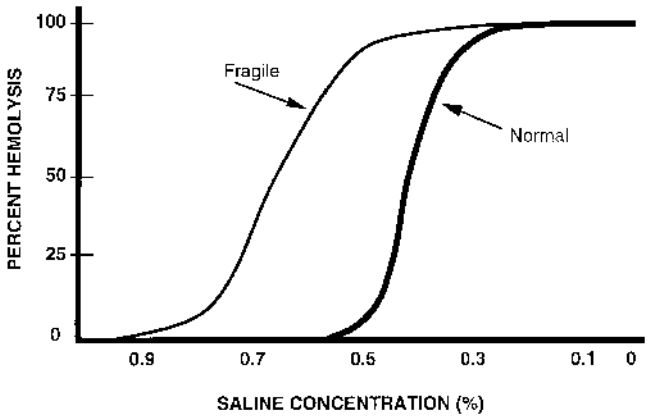


Figure 1.36 Erythrocyte fragility curve. Percentage hemolysis is plotted against decreasing saline concentration. Note the normal curve (arrow marked Normal). Increased erythrocyte fragility is recognized by a shift of the curve to the left (arrow marked Fragile).

Saline fragility test

Resistance of patient erythrocytes to hemolysis is measured in decreasing concentrations of saline. This test is not commonly used because of its complexity and labor intensity. It remains a useful diagnostic aid, however, in occasional cases of immune-mediated hemolytic anemia in which other hallmark pieces of information are not clearly interpretable. An equal aliquot of erythrocytes is added to a series of tubes containing decreasing concentrations of saline. After incubation, the tubes undergo centrifugation, and the hemoglobin concentration then is measured on the supernatant. A tube with distilled water serves as an index for 100% hemolysis. A plot of the percentage hemolysis and the concentration of saline facilitates interpretation, as shown in Figure 1.36.

These tests must not be used or interpreted in isolation. They are to be used in conjunction with analysis of other hematologic data and morphologic evaluation of the blood film by the laboratorian. Because of the frequency of false-negative and -positive results with the Coombs test, interpreting the results of this test in the light of the other available hematologic information is important. (See Chapter 8 for a detailed discussion of the strategy for diagnosing immune-mediated hemolytic anemia.)

Chemistry techniques

A wide variety of techniques, which have been incorporated into many different instrument designs, are used in veterinary clinical chemistry. No attempt is made here to discuss all of these techniques and instruments, but the basic information

on a variety of chemistry techniques used in analyzing samples from animals is provided. A complete understanding of these techniques is not necessary for veterinarians who send clinical chemistry samples to a reference laboratory; however, an increasing number of chemistry instruments are being marketed to veterinarians for in-practice use. Therefore, an understanding of how these instruments work is important for understanding the advantages and disadvantages of the various instruments, the laboratory techniques necessary for their use, the problems that might arise during their use, and the basic principles underlying their variations in design.

The chemistry techniques discussed in this chapter and the substances that may be measured with them are listed in Table 1.1. Absorbance or reflectance photometry is used to measure most of the substances in clinical chemistry profiles. Fluorometry also is used to measure certain analytes in some clinical chemistry analyzers. Blood pH, partial pressures of carbon dioxide and oxygen, and concentrations of electrolytes such as sodium, potassium, and chloride most commonly are measured by electrochemical methods. Atomic absorption spectrophotometers are not commonly used in clinical chemistry laboratories; rather, they are more common in laboratories testing for elements considered nutrients and/or toxicants. Osmometers are common in clinical chemistry laboratories and are used to measure blood and urine osmolality or osmolarity. Protein electrophoresis is used to measure concentrations of the various protein fractions comprising the total serum protein, especially in samples with either decreased or increased protein concentrations. Light-scatter techniques that quantitate turbidity are used less commonly to measure the concentrations of substances such as large protein molecules.

Photometry

Photometry is a general term used to describe an analytical chemistry technique in which the concentrations of substances and the activities of enzymes are determined by measuring the intensity of light passing through or emitted from a test chamber. This test chamber contains the substance to be detected and, in most cases, reagents intended to react with that substance to produce a color reaction. Strictly speaking, the term spectrophotometry should be applied when the instrument being used has the ability to produce light of a variety of wavelengths through some type of light-fractionating device, such as filters, prisms, or diffraction gratings.

Absorbance spectrophotometry

Absorbance spectrophotometry is an analytic technique in which concentrations of substances are determined by directing a beam of light through a solution containing the

Table 1.1 Techniques in veterinary clinical chemistry and substances measured with those techniques.

Technique	Substances Measured
Photometry	
Absorbance photometry	Glucose, BUN, creatine, calcium, phosphorus, magnesium, protein, albumin, bilirubin, bile acids, ammonia, cholesterol, bicarbonate, total CO_2, enzymes
Reflectance photometry	Similar to those measured by absorbance photometry
Atomic absorption spectrophotometry	Many elements including nutrients and toxicants (e.g., calcium,[a] magnesium,[a] lead, arsenic)
Fluorometry	Glucose, bilirubin, bile acids, calcium, magnesium, enzymes, antithrombin III, heparin, plasminogen, hormones, drugs
Light-scatter techniques	
Turbidimetry	Immunoglobulins, antigen–antibody complexes, other large proteins, drugs
Nephalometry	Immunoglobulins, antigen–antibody complexes, other large proteins, drugs
Electrochemical methods	
Potentiometry	Blood pH, PCO_2, sodium,[b] potassium,[b] chloride[b]
Amperometry	PO_2
Coulometry and conductometry[c]	BUN
Osmometry	Osmolality or osmolarity
Protein electrophoresis	Albumin, α-globulin, β-globulin, γ-globulin

BUN, blood urea nitrogen.

[a] May be used to measure the concentration of these substances in solid tissues that have been ashed or digested. Absorbance photometry is more commonly used to measure concentrations of these substances in serum or plasma.

[b] Electrodes used to measure concentrations of these electrolytes are called ion-selective electrodes.

[c] Conductometry also is used to perform cell counts in some hematology analyzers.

substance to be detected (or a product of that substance) and then measuring the amount of light that either of these absorb. The principles described here are incorporated into automated and semiautomated processes on today's chemistry analyzers. Automation, from sample and reagent addition management to calculation of test results to generation of a patient diagnostic report, is made possible by computer control and information processing integral to these systems.

To understand absorbance spectrophotometry, some basic knowledge regarding light is necessary. Typically, light is classified by its wavelength, which is measured in nanometers (nm). Light with the shortest wavelengths (<380 nm) is termed ultraviolet (UV) light (Table 1.2). Light in the visible spectrum has wavelengths of 380–750 nm. Light with the longest wavelengths (>750–2000 nm) is termed infrared (IR) light. The energy of light is inversely proportional to its wavelength; therefore, UV light has the highest energy and IR light the lowest.

The visible spectrum includes a variety of wavelengths that represent the colors with which we are familiar. It is important to remember that color results from the transmittance or reflectance of light. In other words, a green object is that color because it reflects the green area of the visible spectrum and has absorbed the other wavelengths of light in that spectrum. Likewise, a green solution is green because it allows light in the green area of the visible spectrum to be transmitted through it and has absorbed the visible light of other wavelengths. These same principles also apply to light outside the visible spectrum. Different substances absorb and reflect different wavelengths in a pattern that is typical for that substance. The pattern in which a substance absorbs light at various wavelengths is known as its absorption spectrum, and each substance has its own unique absorption spectrum.

Table 1.2 Wavelengths resulting in ultraviolet light, various colors of visible light, and infrared light.

Wavelength (nm)	Color
<380	None (ultraviolet)
380–440	Violet
440–500	Blue
500–580	Green
580–600	Yellow
600–620	Orange
620–750	Red
750–2000	None (infrared)

A basic absorbance spectrophotometer is diagrammed in Figure 1.37. Various sources of light can be used, with the choice being based on the portion of the spectrum desired plus issues such as longevity of the bulb and the basic instrument design. In the application of absorbance spectrophotometry for measuring the concentration of a substance, a wavelength of light that is absorbed by that substance (or by a product of that substance) is used. This wavelength is determined by examining the absorption spectrum of the substance of interest. Usually, the wavelength chosen is the one at which the maximum absorbance occurs. Occasionally, however, some other wavelength may be chosen to avoid interference with substances such as hemoglobin and bilirubin, which may be present in serum samples secondary to hemolysis (in vitro or in vivo) or disease leading to high bilirubin concentration. Hemoglobin and bilirubin have their own absorption spectrums, and methods attempt to avoid using the wavelengths that these substances strongly absorb.

A monochromator is an optical device between the light source and the measuring cuvette. It will narrow the spectrum of light that passes to and through the cuvette. Monochromators can be filters, prisms, or diffraction gratings. When attempting to produce light of a specific wavelength, the actual range of wavelengths produced by a monochromator is called the spectral bandwidth. Each type of monochromator can produce rays of light at certain spectral

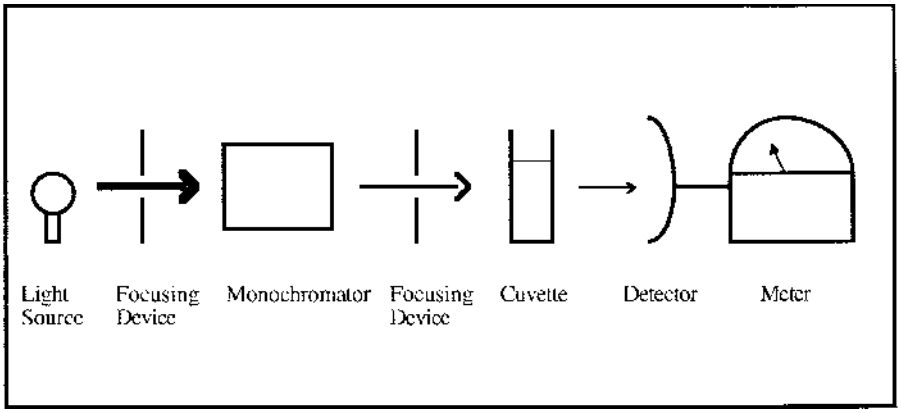


Figure 1.37 Components of a simple absorbance spectrophotometer. Arrows represent light.

bandwidths. Monochromators capable of producing light of a narrow spectral bandwidth have more spectral purity. The importance of spectral purity varies with the type of spectrophotometry, however, and with the substance being analyzed. Filters may be a thin layer of colored glass that transmits light at wavelengths corresponding to the filter's color, or they may be more complex structures, with a layer of dielectric material sandwiched between two pieces of glass coated with a thin layer of silver. The latter type of filter transmits light at wavelengths equal to or at multiples of the thickness of the dielectric layer. In some cases, multiple filters may be placed in series to produce light of greater spectral purity. Prisms separate the wavelengths of white light by refracting this light. As light passes through a prism, shorter wavelengths are bent more than longer wavelengths, thus separating them. The desired wavelength then can be selected from this spectrum for transmission. Diffraction gratings are a metal or glass plate covered with a layer of metal alloy into which multiple parallel grooves have been etched. When the grating is illuminated, each groove separates the light into a spectrum, and light of specific wavelengths is produced as wavelengths that are in phase are reinforced and those that are not in phase are cancelled.

The focusing devices usually are lenses or slits that are inserted before and/or after the monochromator. This placement varies with the instrument. Focusing devices are used to narrow the light beam, to produce parallel light rays, and/or to regulate the intensity of the light reaching the photodetector. In some modern instruments, application of fiber optics has eliminated some of the lens and slits used for narrowing and directing the light beams.

Cuvettes are also known as absorption cells. They have constant dimensions for a given instrument, and they can be made of various materials (e.g., glass, quartz, or plastic) and be of various shapes (e.g., round, square, or rectangular). The materials or shapes used depend on the instrument design and on the portion of the light spectrum being used. During analysis, a solution containing the absorbing substance is placed in the cuvette, and the light rays that have been produced pass through the cuvette walls and the solution. If the correct wavelength has been chosen, the substance absorbs this light in direct proportion to its concentration. In addition to the absorbing substance, the cuvette walls and the solution in which the substance is suspended also absorb small amounts of light. It is, therefore, necessary to "zero" spectrophotometers in order to eliminate the effect of these other factors, and this typically is accomplished by taking an absorbance reading on a cuvette containing only the solution in which the substance is suspended (i.e., the solution contains none of the absorbing substance). The absorbance reading of the instrument typically is set to zero while reading the absorbance of this "blank." Some spectrophotometers are designed to read the absorbance of the test solution and the blank solution simultaneously, which requires splitting the light beam and then shining each beam through either the test or the blank cuvette.

Photodetectors collect the light that has passed through the cuvette (i.e., the light that has not been absorbed). Several different technologies can be used in photodetectors. Factors such as cost, sensitivity, speed of response to changes in light intensity, propensity to fatigue (i.e., decreased response over time despite constant light intensity), and heat sensitivity help to determine which technology is used in a given application. Regardless of the type of photodetector, the underlying mechanism involves the production of electrons and, therefore, an electrical current in response to light striking the detector. This electrical current then is transmitted to a readout device or meter.

Readout devices or meters measure the electrical current produced by the photodetector. This current can be read out directly, but more commonly, this information is converted to a readout that gives either the absorbance or the actual concentration of the substance being measured. This conversion usually requires some type of microprocessor, which can store and use calibration information (discussed later) and also automatically adjust for the reading of the blank sample. The actual readout might be presented as some type of digital display, but it more commonly is printed.

Modern readout devices also incorporate recorders for obtaining multiple absorbance readings on the same sample over time. This is most useful in kinetic assays. In such assays, a reaction is allowed to occur over a period of time, and the production or disappearance of the absorbing substance is evaluated at multiple time points by measuring the absorbance of light normally absorbed by that substance. The change in absorbance over the time period is proportional to the activity of an enzyme or to the concentration of a substance, depending on which is being assayed. Such an assay obviously requires a device that can record and use data produced over time.

In addition to the basic instrumentation of absorbance spectrophotometry, the basic physical chemistry principles used in obtaining measurements via this technology also should be understood. When a light beam of a certain wavelength is projected through a solution containing a substance that absorbs light at that wavelength, the light is absorbed in direct proportion to the concentration of that substance. The intensity of the light leaving the solution, therefore, is less than the intensity of the light entering the solution. If these two intensities are known, the percentage transmittance of light (%T) can be calculated. For instance, if the intensity of light entering the cuvette is designated as I_1 and the intensity of light leaving the cuvette as I_2, then %T is calculated as

$$\%T = \frac{I_2}{I_1}$$

The intensity of light entering the cuvette is measured by projecting light of the appropriate wavelength through a

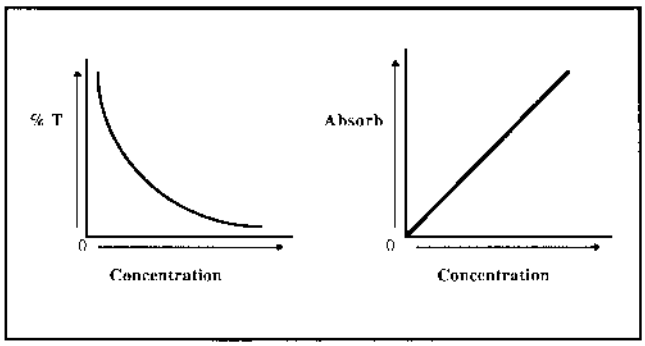


Figure 1.38 The relationships between percentage transmittance (%T), absorbance (Absorb), and concentration of a substance being measured. Note that as the concentration increases, %T decreases logarithmically or nonlinearly and absorbance increases linearly.

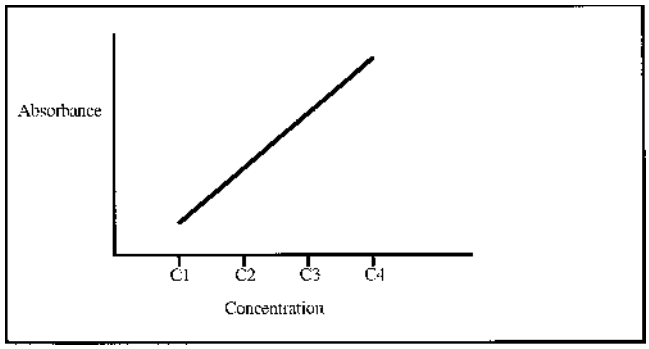


Figure 1.39 Use of calibrators to establish a calibration curve. In this case, four calibrators (C1, C2, C3, C4) were used. Note the linear relationship between concentration of the substance being measured and resulting absorbance.

cuvette containing the solution in which the substance to be measured is suspended. In this case, however, the solution contains none of the substance. Therefore, $\%T$ is set at 100% for this "blank" solution. The solution containing the substance to be measured is then placed in a similar cuvette, and the light is intensity measured, after which the $\%T$ can be assessed.

In the described situation, transmittance varies inversely and logarithmically with the concentration of the substance being measured. If $\%T$ versus the concentration of such a substance is plotted, a curved line results (Fig. 1.38). Light that is not transmitted is absorbed; therefore, transmittance and absorbance are inversely related, as described by the formula:

$$\text{Absorbance} = 2 - \log \%T$$

Because of this relationship, absorbance of light increases linearly with increasing concentration of the substance being measured (Fig. 1.38). This linear relationship between absorbance and concentration makes it more convenient to deal with absorbance than with transmittance during spectrophotometric analysis. Modern spectrophotometers measure transmittance, but then convert transmittance to absorbance. In addition, microprocessors in most spectrophotometers convert absorbance results to concentrations or activities and then report these in a final diagnostic test result format.

The concentration of a substance can be calculated from the absorbance by use of Beer's law:

$$A = abc$$

where A is the absorbance measured, a is the molar absorptivity (also known as the proportionality constant), b is the light path in centimeters (the diameter or width of the cuvette through which the light passes), and c is the concentration of the substance in question. The concentration (c) then can be calculated as

$$\text{Concentration} = \frac{A}{ab}$$

For Beer's law to apply, a linear relationship must exist between concentration and absorbance. In some cases, this might be true only up to certain concentrations or absorbance levels. To assure that Beer's law applies to a given assay, calibration solutions (also known as calibrators), which contain known concentrations of the substance to be measured, are used. The ranges of concentrations used as calibrators should include those that might be measured in samples from patients. Absorbance results for each calibrator are plotted against the concentrations of these calibrators to establish a calibration curve. Ideally, this curve is a straight line rather than an actual curve, showing that a linear relationship exists between absorbance and concentration (Fig. 1.39). In most applications, one or more calibrators are included with each series of sample measurements. It is best, however, to reestablish the calibration curve at frequent intervals (at least daily), because many slight day-to-day changes in the conditions of the test can affect this curve. These changes (e.g., light intensity, temperature, condition of reagents) can occur even in situations when instruments and reagents have been designed to minimize such variation. If a linear relationship does exist between the concentrations of the calibrators and the resulting absorbances, the solutions are said to obey Beer's law, and the calibrators can be used to establish a calibration constant (K):

$$K = \frac{\text{Concentration of the calibration solution}}{\text{Absorbance of the calibration solution}}$$

If K is known, then the concentration of an unknown solution can be calculated as:

Concentration of unknown = (Absorbance of unknown) × K

Microprocessors in instruments can plot absorbance results from calibrators, assure that a linear relationship exists, and calculate the calibration constant. These results are stored, and the concentrations of unknowns are calculated by measuring their absorbances and calibration constant.

A linear relationship between concentration and absorbance over the possible range of unknown concentrations is highly desirable, but a nonlinear calibration curve also can be used to derive unknown concentrations. In such a case, enough calibrators must be used to define the shape of the calibration curve, and as with a linear calibration curve, the range of calibrator concentrations should include the possible range of concentrations that might be found in samples from patients.

In absorbance spectrophotometry, two types of assay methods—endpoint or kinetic—may be used. In both types, the same principles of spectrophotometry described earlier apply. Endpoint assays usually are applied when measuring the concentration of some preexisting substance in serum or plasma. In such an assay, reagent(s) is added to a quantity of serum, and a chemical reaction occurs. The product resulting from this reaction then is measured by spectrophotometry. In other words, the solution in which the reaction has occurred is placed in a cuvette (or the reaction itself might have occurred in the cuvette), a light beam of a wavelength absorbed by the product is projected through a cuvette, and the absorbance is measured. By using a calibration curve and/or a calibration constant, the concentration of the substance being measured then is calculated. An example of an endpoint assay is a method for measuring the concentration of serum calcium:

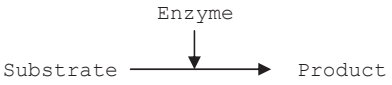
calcium + o-cresolphthalein complexone
→ calcium–cresolphthalein complexone

In this assay, the substance of interest (i.e., calcium) is complexed with cresolphthalein complexone, which has a purple color and absorbs light at a wavelength of 570 nm. This reaction is allowed to occur long enough to allow nearly all of the calcium in the sample to be complexed. More calcium–cresolphthalein complexone results in more light being absorbed, and a higher concentration of calcium reported by the instrument. After the absorbance is determined, it is compared with the absorbance of a calibration solution, and the absorbance of the unknown then is calculated as:

$$\text{Concentration of the unknown} = \text{Absorbance of the unknown} \times \frac{\text{Concentration of the calibration solution}}{\text{Absorbance of the calibration solution}}$$

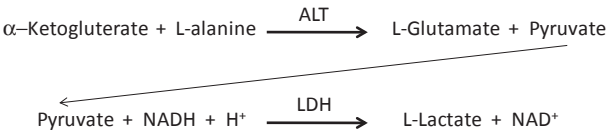
Note that the second portion of this formula is the calibration constant (K).

Kinetic assays typically have been used to measure enzyme activities, but also have been adapted to measure the concentrations of many analytes in blood. Typically, enzyme concentrations are not measured directly in clinical chemistry. Rather, the amount of enzyme in the serum usually is gauged indirectly, by the activity of that enzyme. Enzymes are proteins that catalyze (i.e., speed-up) chemical reactions, with the result that substrate is converted to product more quickly:



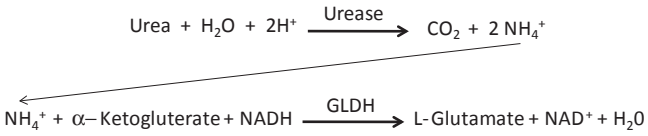
To measure an enzyme's activity, the rate at which it converts a substrate to a product must be assessed. The more quickly conversion occurs, the higher the enzyme activity is assumed to be. To measure the rate of conversion from substrate to product, the rate at which the product is being produced must be assessed, and this requires multiple measurements of the product concentration over time. Because this type of assay is a dynamic process, it is termed a kinetic assay. In a kinetic assay of enzyme activity, a solution containing the substrate of the enzyme of interest is added to the sample serum in a cuvette that already is in a spectrophotometer. When enzyme in this serum begins to convert substrate to product, absorbance is measured periodically by the same methods and using the same principles of spectrophotometry described previously (i.e., using a light beam of a wavelength absorbed by the product). In this process, the conversion rate of substrate to product is monitored. This rate can be converted to enzyme activity by using a formula involving the rate of absorbance change and several constants related to the absorptivity of the product as well as to test characteristics such as sample volume, total sample volume, and light path.

An example of a kinetic enzyme assay is an assay of alanine aminotransferase (ALT) activity:



where LDH is lactate dehydrogenase. In this assay, NADH is converted to NAD^+ at a rate proportional to the activity of ALT in the sample. The NADH absorbs light at 340 nm, and its rate of disappearance is measured by periodically assessing the absorbance of the reaction mixture. The rate of absorbance change in this mixture can be converted to units of ALT activity.

As previously noted, kinetic assays also are used for measuring the concentrations of preexisting substances in the blood. In these assays, the rate of appearance or disappearance of an absorbing substance is monitored by periodically measuring the absorbance of the reaction mixture. An example of a kinetic assay for measuring the concentration of a preexisting substance is an assay of the blood urea nitrogen (BUN) concentration, which uses the chemical reaction



where GLDH is glutamate dehydrogenase. In this reaction, the disappearance rate of NADH is monitored by periodically assessing the absorbance of the reaction mixture at a wavelength of 340 nm. The disappearance rate is proportional to the urea nitrogen concentration in the serum being tested. The BUN concentration is calculated by relating the rate of change in the absorbance of the sample with that of a calibrator.

Enzyme activity also can be measured by endpoint methods, which involve mixing serum with reagent containing substrate for the enzyme and then allowing the conversion of substrate to product to proceed for a specific period of time. At the end of that period, the concentration of substrate or product is measured. The more substrate used or product produced during the time period, the higher the enzyme activity is assumed to be.

Reflectance photometry

The principle of reflectance photometry is used in a few large, automated clinical chemistry analyzers and in several of the smaller clinical chemistry analyzers designed for in-practice use. Most of these instruments use "dry chemistry" systems, in which the fluid to be analyzed is placed on a carrier that contains the reagents for the assay. This carrier can take different forms, including a dry fiber pad or a multilayer of film. After the sample is applied, the chemical reaction occurs in this carrier, and a product is formed in a concentration proportional to that of the substance being measured. The carrier then is illuminated with diffused light, and the intensity of the light reflected from the carrier is measured and compared with that of either the original illuminating light or the intensity of light reflected off a reference surface. Reflectance photometry, therefore, is analogous to absorbance photometry in that the chemical reaction occurring in the carrier results in a product that absorbs a portion of the illuminating light. The remaining light is reflected, analogous to transmittance in absorbance spectrophotometry, to a photodetector that measures its intensity. The intensity of the reflected light is not related linearly to the concentration of the substance being produced. As a result, formulas are required to convert the reflectance results to concentrations. These formulas vary with the type of instrument being used.

Atomic absorption spectrophotometry

Atomic absorption spectrophotometry (AA) is used for measuring the concentrations of many elements. Advantages of AA include its superior sensitivity (i.e., it can detect smaller concentrations) and its ability to measure the concentrations of various elements. AA is typically limited to toxicology laboratories for clinical purposes. Applications include measurement of concentrations of elements such as lead, copper, and selenium in fluids or tissues. As the name implies, AA involves measuring absorption of energy by atoms. This technique involves heating a sample in a flame that is hot enough to cause the element in question to dissociate from its chemical bonds and form neutral atoms—but not hot enough to cause large numbers of electrons to jump to the excited state. These atoms then are in a low-energy (i.e., ground) state and can absorb light of a narrow wavelength that is specific for that element. If a light of this wavelength is projected through the flame, the amount of light absorbed is proportional to the concentration of the element in the sample. Measurement of the amount of light absorbed, therefore, allows the concentration of that element in the sample to be calculated. Focusing devices, photodetectors, meters, and readout devices serve the same purposes in AA as in other types of spectrophotometry.

Fluorometry

Fluorometric techniques can be used in a wide variety of applications, ranging from measurement of the concentrations of substances to assessment of the numbers and other characteristics of larger particles, including cells. This section discusses use of these techniques in measuring concentrations of various substances in body fluids.

Among the substances that can be measured by these techniques are some that commonly are measured in clinical chemistry analysis (e.g., bilirubin, bile acids, glucose, calcium, magnesium, and various enzymes), substances related to coagulation (e.g., antithrombin III, heparin, and plasminogen), drugs, and hormones. Some of these substances are fluorescent; in other cases, measurement of these substances is possible by linking other fluorescent substances to the analyte of interest, either directly or indirectly, as the result of a series of chemical reactions.

The basic principle underlying use of fluorometry is that certain substances, when exposed to light of the proper wavelength, will fluoresce. Fluorescence results when a substance absorbs light at one wavelength and then emits light at a longer (i.e., lower energy) wavelength. The ability to fluoresce varies with a compound's chemical structure; therefore, not all compounds can be readily measured by fluorometry.

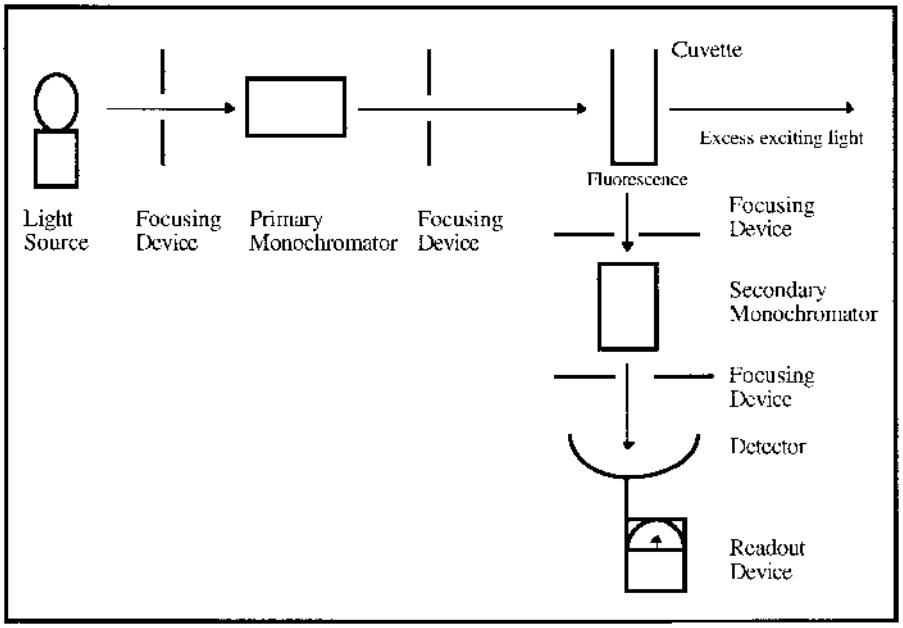


Figure 1.40 The basic design of a fluorometer. Arrows represent light.

The basic design of a fluorometer is shown in Figure 1.40. A variety of light sources, including various types of bulbs and lasers, can be used. Most fluorescent compounds absorb light at 300–550 nm; therefore, light sources must produce light at these wavelengths. The primary monochromator isolates light at the proper wavelength to produce fluorescence in the substance being analyzed. Each compound can best be caused to fluoresce at specific wavelengths, and these wavelengths are known as the apparent excitation spectrum of the compound. Of these wavelengths, a narrow band at which peak fluorescence is caused usually is chosen to be isolated by the primary monochromator and, from there, transmitted to the cuvette. When light strikes the solution in the cuvette, it produces fluorescence in the substance being measured. The detector of this fluorescent energy usually is placed at a 90° angle from the projected (i.e., the exciting) light beam. This placement means that light from the exciting light beam continues straight through the cuvette and does not need to be dealt with by the secondary monochromator or the detector. Because fluorescent energy is projected in all directions, this energy can be measured at 90° without measuring the energy from the exciting light beam. Some fluorometers incorporated into absorbance spectrophotometers measure fluorescence directly in the path of exciting light (i.e., an end-on design), because this is the typical light path for absorbance spectrophotometers. In such cases, mechanisms must be incorporated to exclude excitation light that has passed through the cuvette.

The secondary monochromator excludes light from sources other than the fluorescence itself and allows only a narrow band of wavelengths to pass to the photodetector. Just as each fluorescent compound has an apparent excitation spectrum of light in which optimum fluorescence occurs, each compound also has an emission spectrum, which is the spectrum of wavelengths in which most of the emitted fluorescent energy from that compound is found. To develop a fluorescent assay, the emission spectrum of the compound of interest must be determined. Then, the narrow band of wavelengths in which maximum emission occurs is isolated by the secondary monochromator. Light passing from the monochromator is collected by a photodetector, measured, and processed in a manner similar to that described for spectrophotometry. Various lenses, slits and in some cases, polarizing devices are included in fluorometers to help direct and/or polarize light as well as to reduce stray light in the system.

A wide variety of fluorometer designs are available. Strictly speaking, fluorometers are instruments that can produce light at only a few wavelengths, because their primary monochromator is a filter. Many instruments that use fluorometry have primary monochromators that are diffraction gratings or prisms. These instruments can produce a spectrum of excitation wavelengths and are known as spectrofluorometers. Some fluorometers are designed to compensate for variations in the intensity of the light source and, therefore, decrease the frequency with which calibration is required. Fluorometers also might use a pulsed light source and measure fluorescence only during those periods of time when the source is off. This technique, which is known as time-resolved fluorometry, eliminates the effects of light scatter.

Interference by other molecules is a potential problem when biologic fluids are being analyzed by fluorometry. Some

of these molecules fluoresce (e.g., bilirubin and some proteins), whereas others scatter light (e.g., proteins and lipids). When developing assays on biologic fluids, adjustments must be made to minimize the effects of these molecules.

Although the mechanism of measuring concentrations is different, the basic procedure for performing fluorometry is similar to that for absorbance spectrophotometry. Calibrators are used to establish a calibration curve, and blanks are used to negate any effects other than those attributable to the substance of interest. At low concentrations of fluorescing substances (e.g., resulting in an absorbance of <2% of the exciting light), a direct, linear relationship usually exists between fluorescence and concentration. If the concentration of the fluorescing substance is high (e.g., >2% of the exciting light is absorbed), the relationship between fluorescence and concentration might be nonlinear.

Light-scatter techniques

Light-scatter techniques can be used to measure the concentrations of larger molecules in fluids. When light is projected through solutions containing large molecules such as immunoglobulins and other large proteins, antigen–antibody complexes, and some drugs, these molecules cause light to scatter in all directions. These techniques, therefore, are potentially useful in measuring the concentrations of these substances. With light scattering, the wavelength of the light being scattered is the same as that of the light being projected into the solution. By assessing the degree of light scattering, the concentration of the substance of interest can be measured. Two techniques, turbidimetry and nephelometry, use the principles of light scattering to make such measurements.

In turbidimetry, the decreased intensity of a light beam passing through a turbid solution is measured. The intensity of light decreases, because a portion of it has been scattered by the large molecules of interest. A basic turbidimeter is diagrammed in Figure 1.41. In a turbidimeter, light rays are projected through a cuvette containing the analyte in solution, and the intensity of light leaving the solution (i.e., the transmitted light) is measured in a straight line from the transmitted light. The decrease in transmitted light intensity is proportional to the concentration of the analyte. A turbidimeter, therefore, is similar in principle to an absorbance spectrophotometer.

In nephelometry, a beam of light also is projected through a solution containing the analyte, but the photodetector is placed at a 90° angle to the cuvette (Fig. 1.42). In addition, scattered rather than transmitted light is measured. The intensity of the scattered light is proportional to the concentration of the analyte. Nephelometry, therefore, is analogous to fluorometry in terms of configuration of the light path. If a solution is not visibly turbid, nephelometry is a somewhat better technique than turbidimetry.

A direct relationship exists between the concentrations of light-scattering molecules and the degree of light scattering. A direct relationship also exists between the sizes of the light-scattering molecules and the degree of light scattering. When developing light-scatter techniques, the size of the particles being measured must be considered, because larger particles (e.g., immunoglobulin M, chylomicrons, and antigen–antibody complexes) cause an asymmetric distribution of scattered light. In some cases, the position of the photodetector must be altered to adjust for this. Large molecules or particles other than those of interest can interfere with light-scatter techniques as well.

With light-scatter techniques, the analytic procedures are similar to those of absorbance spectrophotometry. Calibrators are used to establish a calibration curve, and blanks are used to negate the effects of reagents and other light-scattering molecules.

Electrochemical techniques

A variety of electrochemical techniques are used in clinical chemistry and most often are applied in measurements of electrolytes and acid-base status. This includes electrolytes such as sodium (Na^+), potassium (K^+), chloride, (Cl^-), ionized

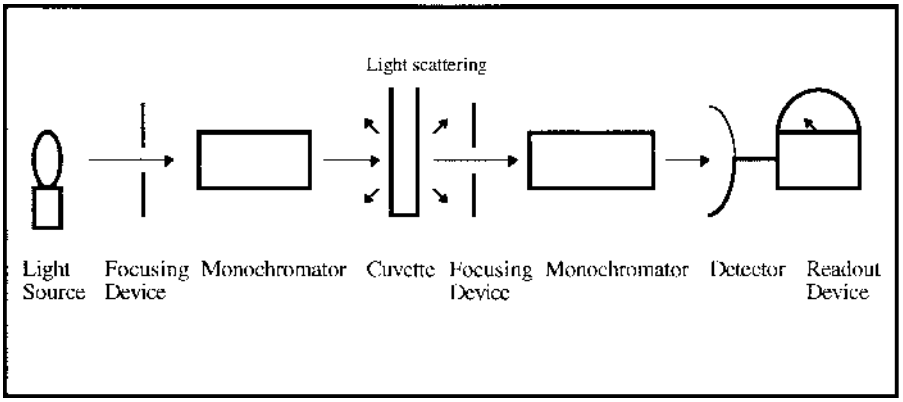


Figure 1.41 A basic turbidimeter. Arrows represent light.

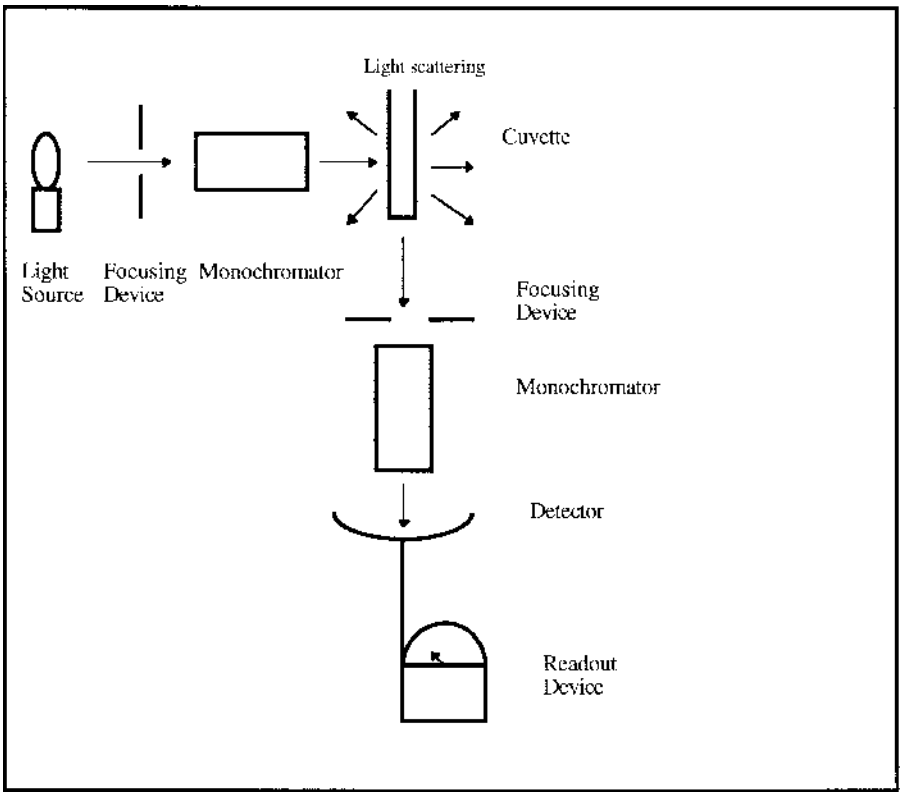


Figure 1.42 A basic nephalometer. Arrows represent light.

calcium (Ca^{+2}), pH (H^{+}), and partial pressures of oxygen (pO_2) and carbon dioxide (pCO_2) in whole blood. These techniques also can be used to measure other substances if the chemical reactions used in the assay system result in production or consumption of an ion. For example, such reactions exist for determination of glucose, urea, and creatinine concentrations. Basic electrochemical techniques and examples of some of their applications are described in this section. Electrochemical methods are applied through a wide variety of electrode and instrument configurations. In recent years several electrochemical systems have had complexity, cost, and applications reduced to practice in point-of-care formats. These systems have rendered blood gas, electrolyte, and selected chemistry capability both affordable and practical in the typical veterinary facility. Some of these devices utilize microfabricated disposable cartridges in which these measurements are made on whole blood. Other systems use small volumes of blood injected into a port leading to sample flow-through fluidics within the analyzer. Regardless of design, these instrument systems typically combine potentiometry, amperometry, and conductometry to provide acid-base and electrolyte panels, as described below.

Potentiometry

Potentiometry is commonly used for measurement of pH (i.e., hydrogen ion concentration), partial pressures of carbon dioxide and oxygen, and concentrations of electrolytes in whole blood or serum. In potentiometry, the electrical potential between two electrodes is measured thereby giving a value that can be used to calculate the concentrations of various electrolytes.

Potentiometry involves the development and measurement of the potential difference between two electrodes. This technique is used to measure electrolyte concentrations using ion-selective membrane electrodes, also known as ion-specific electrodes (ISE). The technique is used to measure ion concentrations in whole blood, plasma, serum, and occasionally other body fluids. The ISE is the variable electrode sensor immersed in the sample of measurement interest; see Figure 1.43. The ISE has a barrier or membrane that isolates the internal electrode from the body fluid. Only the specific ion being measured is allowed to cross or interact with the barrier, leading to accumulation of charge on the internal electrode. At equilibrium, the potential in the ISE will vary depending on the concentration of ionic interaction with the sample. The second electrode is a reference electrode that has constant, fixed potential. The basic principle is that contact of the ion-selective membrane with the body fluid results in ion selective passage or interaction with the ISE membrane leading to development of a potential difference from a reference electrode. A sensitive voltmeter is used to measure the potential difference when the ISE has

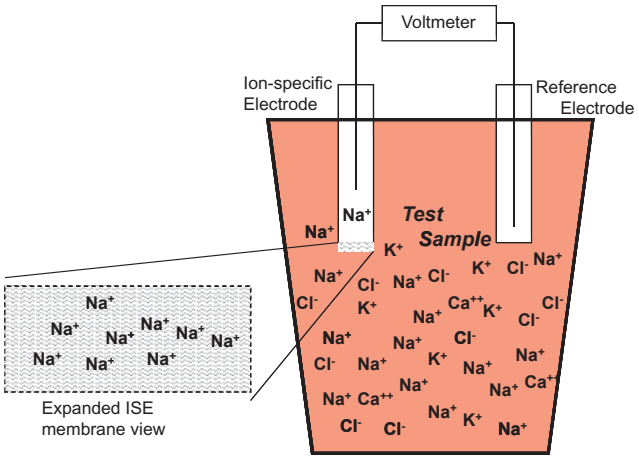


Figure 1.43 Schematic drawing of an ion-selective electrode (ISE) for potentiometric measurement; see text for further explanation. There is a reference electrode, chemically saturated to have fixed potential. The test sample contains differing concentrations of various ions. The ISE selectively allows movement of the ion of interest (e.g. Na^+) into or across the membrane resulting in a potential difference between the two electrodes (expanded view). The potential difference is proportional to the concentration of specific analyte in being measured.

come to equilibrium with the sample. The potential difference that develops is due to the activity of the ion being measured. The potential difference is used to calculate the concentration of ion in the sample. The ISE system is calibrated with solution containing known concentration of the ion of interest.

ISEs are the core technology in most or all modern blood gas and electrolyte analyzers, including those recommended for in-clinic applications. The design and materials used to manufacture these electrodes vary considerably. An important component of each electrode is a membrane that is selective for the ion that the electrode measures. The membrane may be composed of thin glass specially formulated to allow diffusion of a specific ion; glass is used in ISEs for pH and Na^+ measurement. A second type of membrane involves a water insoluble ion exchange chemistry coupled with a barrier membrane matrix. This type of electrode may be used to measure K^+, NH_4^+, and Ca^{2+}. There are also solid-state electrodes consisting of a single crystal of some ion-selective material or salt imbedded in an inert matrix membrane. This type of electrode is typically used to measure chloride (Cl^-).

The partial pressure of carbon dioxide (PCO_2) in the blood also is measured by potentiometry. This method is used in blood-gas analyzers. Whereas CO_2 is not an ion, the CO_2 electrode is designed to produce an ion in proportion to the PCO_2 in the blood. The design of such an electrode is shown in Figure 1.44 as a modified pH electrode. In this electrode, a chamber containing sodium bicarbonate solution is separated from the blood sample by a thin membrane. The CO_2 diffuses through the membrane into the sodium bicarbonate solution, and the following chemical reaction occurs:

$$CO_2 + H_2O \rightarrow H_2CO_3$$
$$H_2CO_3 \rightarrow H^+ + HCO_3^-$$

The amount of CO_2 that diffuses through the membrane affects the H^+ concentration in the sodium bicarbonate solution in direct proportion to the PCO_2. The remainder of this electrode is a pH electrode that senses the change in H^+ concentration of the sodium bicarbonate solution. These changes alter the electrical potential of this electrode, and the instrument then calculates the PCO_2 from these changes.

Amperometry

Amperometry is a technique that measures the electrical current passing between two electrodes in a chemical cell while a constant voltage is applied. This differentiates the technique from potentiometry, in which no electrical current flows and no voltage is applied. The most common application of amperometry in clinical chemistry is electrochemical measurement of the partial pressure of oxygen (PO_2) in blood.

The technique is most easily understood by considering how this electrochemical cell operates. A typical PO_2 electrode is diagrammed in Figure 1.45. An electrical potential of –0.65 V is applied to this electrode, and almost no current passes through this electrode if no oxygen is present. When this electrode is submersed in blood, O_2 from the blood diffuses through the O_2-permeable membrane and comes into contact with the tip of the platinum electrode. The O_2 then is reduced by the reaction:

$$O_2 + 2H_2O + 4 \text{ electrons} \rightarrow 4\ OH^-$$

This process consumes electrons and, therefore, produces an electrical current under these conditions. An ammeter is used to measure this current as amperage. The amount of current produced is proportional to the PO_2 of the blood. Calibration solutions are used to relate the amperage to the PO_2 of the unknown.

Coulometry and conductometry

Coulometry and conductometry are two other electrochemical methods that occasionally are used to measure the concentrations of substances. Coulometry involves measurement of the amount of electrical energy passing between two electrodes in an electrochemical cell. This electrical current is produced by chemical reactions occurring at the surfaces of each of two electrodes, resulting in the loss or gain of electrons by these electrodes. The amount of electrical current produced is directly proportional to the concentration of the substance being measured. This substance is

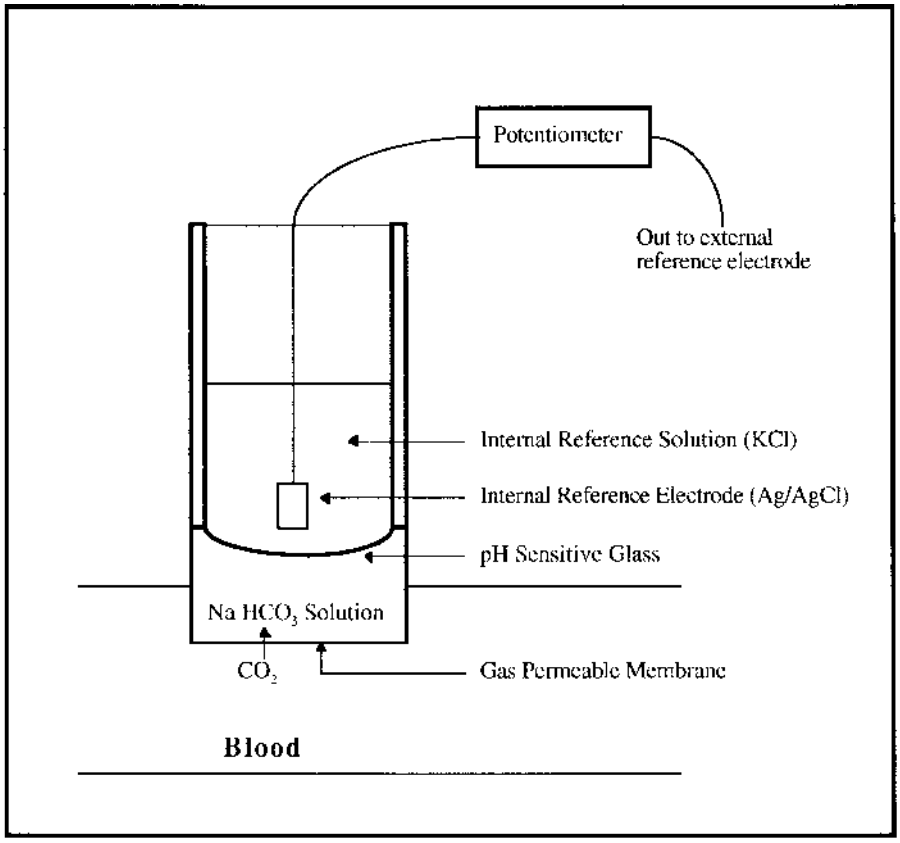


Figure 1.44 An electrode designed to measure the partial pressure of carbon dioxide in the blood.

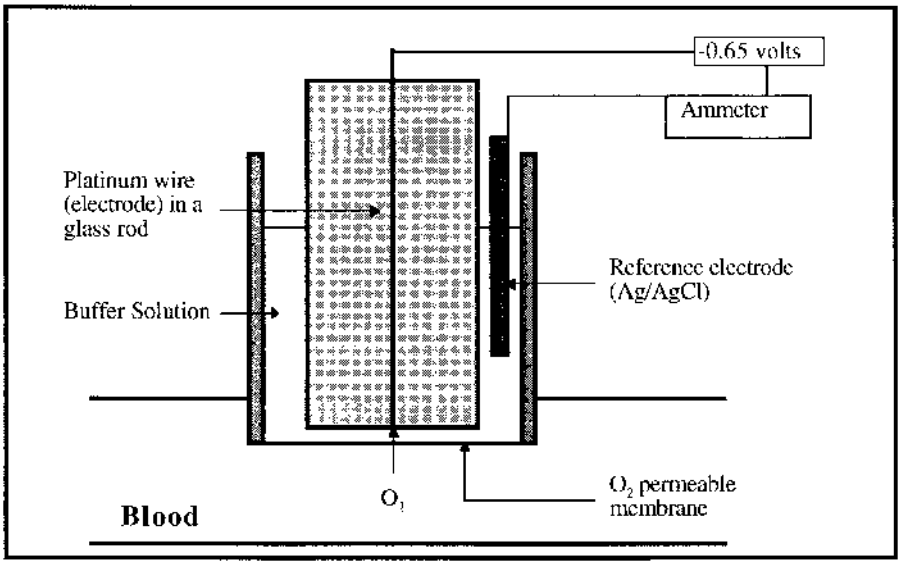


Figure 1.45 An electrochemical cell designed to measure the partial pressure of oxygen in the blood.

consumed in an electron-using or electron-producing process. Unlike potentiometry, the actual current rather than the potential between two electrodes is measured, and unlike amperometry, no outside voltage is applied to the system. This method has been applied to the measurement of serum chloride concentrations.

Conductometry involves measurement of a fluid's ability to conduct an electrical current between two electrodes when a voltage is applied to the sample in the system. This property, which is known as electrolytic conductance, occurs via movement of ions in the fluid. The conductivity of an aqueous fluid depends on the concentration and ionic strength of the electrolytes in that fluid: the higher the electrolyte concentration, the higher the conductivity. Conductometry can be used to measure the production of ions by chemical reactions. Therefore, it is possible to measure the concentration of a substance in a fluid if it is used in a chemical reaction producing ions in numbers proportional to the substance of interest. The increased conductivity resulting from the production of these ions would then be proportional to the original concentration of the substance being measured. It is also possible to measure hematocrit by conductometry on some clinical systems. The plasma fraction readily conducts current while cellular mass acts as an insulator, impeding current. As the hematocrit increases, the ability of the sample to conduct current decreases. This measurement can be calibrated. The calculation factors in electrolyte concentrations simultaneously measured in the same sample.

Osmometry

Osmometry involves measurement of the concentrations of particles in a fluid. The clinical significance of these concentrations, which are reported as osmolality (particles per kilogram of solvent (osmol/kg)) or osmolarity (particles per liter of solvent (osmol/L)), is discussed in Chapter 24. To understand osmometry, the changes that occur in a solution when concentrations of particles (i.e., solute) dissolved in a fluid (i.e., solvent) increase must be understood. These changes, which are known as colligative properties, are increased osmotic pressure, decreased vapor pressure, increased boiling point (because of decreased vapor pressure), and decreased freezing point. Any of these colligative properties could be used to measure osmolality or osmolarity. Among those properties that actually are used to make these measurements are freezing point depression and decreased vapor pressure.

The freezing-point depression technique is the most commonly used. As the name implies, this type of osmometer measures the freezing point of a solution through a number of steps involving freezing, thawing, and freezing again. This process is monitored by a thermistor, which measures temperature, and it determines the freezing point by determining the temperature at equilibrium between freezing and thawing. The osmolality or osmolarity of the fluid then is determined by comparing this temperature with those of various calibration fluids with known osmolality or osmolarity.

Vapor pressure osmometers are less commonly used. These instruments measure the osmolality or osmolarity of a fluid by determining the dew point (i.e., the temperature at the point of equilibrium between vaporization and condensation) of that fluid. The dew point is a gauge of vapor pressure: the higher the osmolality or osmolarity of a fluid, the lower its dew point. In general, vapor pressure osmometers are not considered to be as precise as freezing-point osmometers. In addition, volatile substances such as ethanol are not detected by vapor pressure osmometers, whereas they are detected by the freezing-point depression technique.

Protein electrophoresis

Electrophoresis is an analytic technique based on the movement of charged particles through a solution under the influence of an electrical field. In clinical chemistry, electrophoretic techniques most commonly are used to separate and analyze serum proteins. When serum is placed on or in a supporting substance that allows migration of these proteins and can carry an electrical charge, these proteins move through this material just as other charged particles do. The movement of proteins through such a substance depends on the net charge on the protein molecule, the size and shape of the protein molecule, the strength of the electrical field applied, the type of supporting medium, and the temperature. In a given electrophoresis application, the latter three items are held constant. Therefore, the migration of protein molecules depends on the net charge and on the size and shape of the molecules. As a result, different serum proteins migrate at different rates and, possibly, in different directions in the supporting substance.

A simple electrophoresis chamber is demonstrated in Figure 1.46. Small amounts of serum are placed in specific areas on the surface of the supporting substance or in small depressions cut at one end. Supporting substances commonly used include agarose gel and cellulose acetate. Starch gel is less commonly used in clinical applications. Polyacrylamide gel also can be used for protein electrophoresis and separates more serum protein fractions than the other supporting substances. Polyacrylamide electrophoresis does produce interesting information, but the clinical applications of this information in veterinary medicine are not understood. The common supporting substances usually are in the form of a sheet, and they either have buffer incorporated into them when they are produced or are soaked in buffer before use. The buffer determines the pH at which the process occurs, and the pH determines the type of charge as well as the net charge on each type of protein molecule. Both ends of the supporting substance are in contact with

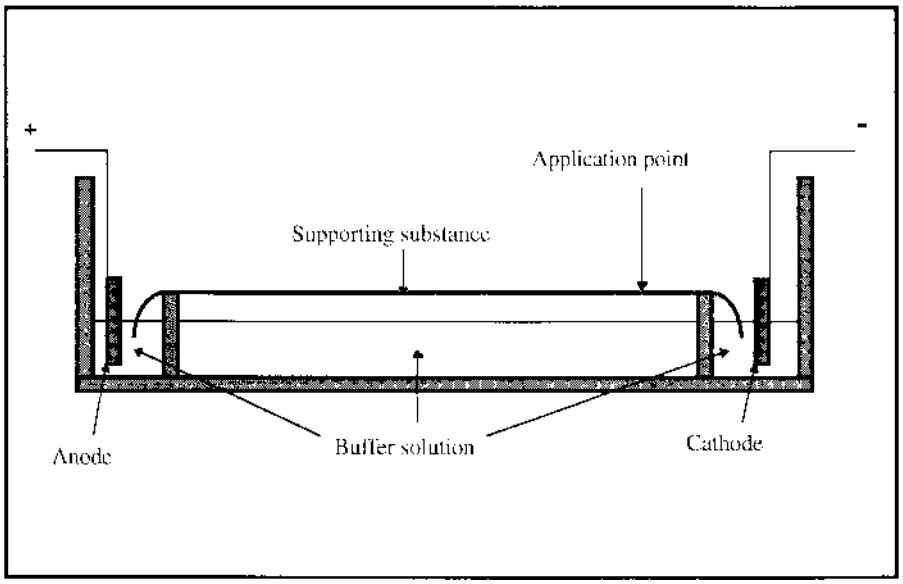


Figure 1.46 A simple electrophoresis chamber.

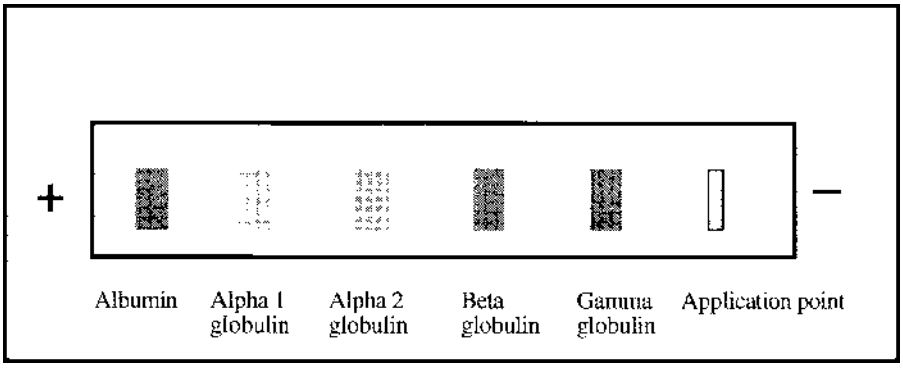


Figure 1.47 Typical electrophoretic separation of serum proteins in a sheet of supporting substance. The type and number of fractions actually separated depends on the type of electrophoresis application and on the species from which the serum was sampled.

buffer solution in an adjacent well. These buffer solutions are not in contact with each other, however, or with the buffer solution in the center well. The electrical current is applied to the system by electrodes placed into each of these wells. A negatively charged cathode is placed in the well at one end, and a positively charged anode is placed in the well at the other end. The serum sample typically is applied at the end near the cathode, because most proteins are negatively charged and migrate toward the anode. When an electrical current is applied to this system, proteins migrate toward either the anode or the cathode, depending on whether they are negatively charged (i.e., toward the anode) or positively charged (i.e., toward the cathode). As noted, the rate of this migration depends on both the net charge of the molecule and its size and shape, and because these vary with the different types of proteins, different proteins migrate at different rates. If this migration is allowed to occur for a fixed period of time, various protein fractions are isolated along a straight line in the supporting substance.

A typical distribution of serum protein fractions in a sheet of supporting substance after electrophoretic separation is shown in Figure 1.47. Albumin is the smallest of the serum proteins and has the highest net negative charge relative to its size. Albumin, therefore, migrates faster than the other proteins, and it advances further toward the anode during the time allowed for separation. The globulins are larger than albumin and therefore do not migrate as far toward the anode. The relative migration distances of the globulins depend on the relationship of their size to their net negative charge. The gamma globulins have the smallest net negative charge relative to their size and, therefore, migrate the shortest distance toward the anode. In some techniques, the application point actually might lie in the gamma-globulin region, with some gamma globulins migrating to the cathode side of this point. The number of fractions separated depends on the electrophoretic technique used and the species being analyzed. (These separations are discussed in more detail in Chapter 29.)

Once electrophoretic separation is completed, the protein fractions usually are identified and quantified. Staining these fractions aids in this process. Various types of dye that stain protein can be used, including amido black, bromphenol blue, Coomassie brilliant blue, nigrosin, and ponceau s. After staining, it is possible, with experience, to visually identify the various proteins fractions based on their order of migration. Visual examination also sometimes reveals apparently increased quantities of some protein fractions. This quantitation is more easily accomplished using a densitometer to scan the protein pattern and calculate the percentages and absolute quantities of protein in each fraction. A densitometer measures the amount of protein in each fraction by projecting light through these fractions as these are mechanically passed over the light source. A photodetector determines the width and density of each fraction. Results are reported as a densitometer scan, which more commonly is known as an electrophoretic pattern or electrophoretogram, as shown in Figure 1.48 and as both a percentage and an absolute value for each protein fraction. The absolute value for each fraction is calculated by the microprocessor in the instrument using the total protein concentration, which is entered by the operator, and the percentage of each fraction as determined by the densitometer:

$$\text{Absolute quantity of each fraction} = \frac{\text{Percentage of each fraction} \times \text{Total serum protein}}{100}$$

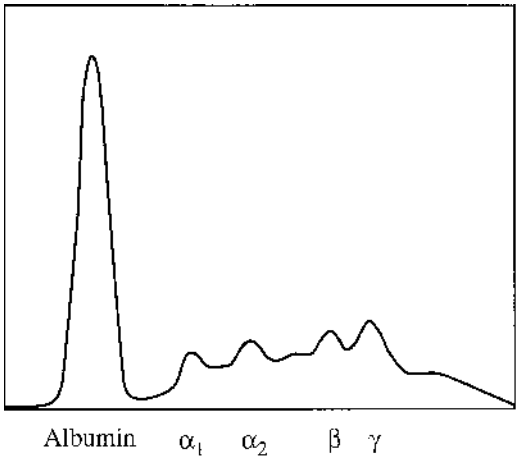


Figure 1.48 A densitometer scan (electrophoretic scan) of a serum protein electrophoresis separation.

Most densitometers automatically identify each fraction as well as the boundaries between these fractions. The operator can and should change these in some cases.

Once the absolute quantities in the various fractions are determined, they can be compared with known reference intervals for that species, and any abnormalities can be identified. Use of such data in clinical chemistry of proteins is discussed in Chapter 29.

Suggested Reading

Hematology

Weiss DJ, Wardrop KJ (eds.) (2010) *Schalm's Veterinary Hematology*, 6th ed. Ames, IA: Blackwell Publishing Ltd.

Chemistry

Burtis CA, Ashwood ER, Bruns D (eds.) (2006) *Tietz Textbook of Clinical Chemistry and Molecular Diagnostics*, 4th ed. St. Louis, MO: Elsevier Health Sciences.