DIAGNOSTIC LABORATORY HEMATOLOGY

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Preface

The purpose of this manual is to present in concise form the essential details for the performance and critical evaluation of procedures used commonly in student laboratories and doctors' offices for the diagnosis of hematologic disorders.

For each determination a multiplicity of technics is available. As a general rule, the more numerous the technics available, the less satisfactory is the determination. The student, house staff officer, and practicing physician for whom this manual is written have neither the time nor the experience to evaluate all of the modifications suggested in the great mass of literature. Therefore, in each case, a single method for each determination has been selected on the basis of the author's experience. Simplicity, accuracy, reliability, time of performance, cost and availability of equipment are all factors which have been taken into consideration in making the selection. When a variety of technics of equal merit and demerit have been available to the author for selection, the simplest method to perform has always been selected. However, only those methods have been selected which can be performed in student laboratories and doctors' offices with a minimum of low cost equipment and a minimum of skill and experience. In many cases this has not resulted in the selection of the most specific, accurate, or best available method, but the most practical method under the circumstances. For more specific and elaborate methods, *A Syllabus of Laboratory Examinations in Clinical Diagnosis*, edited by T. H. Ham, Harvard University Press, Cambridge, Massachusetts, should be consulted.

For corollary reading concerning the clinical aspects of hematology, *Clinical Hematology* by M. M. Wintrobe, Lea and Febiger, Philadelphia, Pa., is recommended.

An appendix concerning the preparation of reagents has been included because when former students have found themselves responsible for their own office laboratory, they frequently have found it necessary to inquire concerning these rather elementary and yet important matters.

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TABLE OF CONTENTS

Statistical Considerations

In clinical hematology, as in medicine, the clinician's primary interest is in significant deviations from the normal, that is, disease. Unfortunately, because of the technical as well as the biologic variation, it is not possible to deal in absolute or true values. The best that can be obtained is a series of approximations to the correct value. That is to say, the probable limits within which the true value lies are defined, rather than the actual value itself. Therefore, to determine if a given value is abnormal, it is imperative to know the limits of the "normal" variation and the possible error of the value which is to be compared to the "normal". For this it is necessary to understand a few simple statistical concepts.

Average or Mean. The average or mean ($\bar{X}$) is defined as the sum of all of the observations (SX) divided by the number of observations (N).

$$\overline{X} = \frac{SX}{N}$$

$\overline{X}$ = the mean
X = the various individual measurements
S = means take the sum of _____.
N = the number of determinations.

Standard Deviation. The standard deviation (S.D.) is a measure of the variability about the mean. One standard deviation on each side of the mean (±1 S.D.) is defined as that value which takes in approximately 68 per cent of the determinations. Two standard deviations on each side of the mean (±2 S.D.) are defined as that value which takes in 95 per cent of the determinations. The standard deviation is expressed in the same units as the quantity measured.

$$\text{S.D.} = \sqrt{\frac{S(X - \overline{X})^2}{(N - 1)}}$$

Coefficient of Variation. The coefficient of variation (C.V.) is the standard deviation (S.D.) expressed as per cent of the mean. It is

TABLE 1.—*Calculation of the Mean, Standard Deviation and Coefficient of Variation*

Determination N	Red Cell Count 10^6/mm.^{3} X	$X - \bar{X}$	$(X - \bar{X})^2$
1	5.20	0.20	.04
2	4.80	0.20	.04
3	5.55	0.55	.30
4	5.10	0.10	.01
5	5.60	0.60	.36
6	4.40	0.60	.36
7	5.30	0.30	.09
8	4.45	0.55	.30
9	4.70	0.30	.09
10	4.90	0.10	.01
SX	50.00		1.60

$N = 10$

$$\bar{X} = \frac{SX}{N} = \frac{50}{10} = 5.00$$

$$\text{S. D.} = \sqrt{\frac{S(X - \bar{X})^2}{(N - 1)}} = \sqrt{\frac{1.60}{9}} = \sqrt{0.178} = 0.42$$

$$\text{C. V.} = \frac{0.42}{5.00} \times 100 = \pm 8.4 \text{ per cent}$$

useful in comparing the standard deviations of quantities which are expressed in different units.

$$\text{C.V.} = \frac{\text{S.D.}}{\bar{X}} \times 100$$

An example of the above calculations is given in table 1.

THE EQUIPMENT AND ITS CARE

Hemocytometer. The hemocytometer consists of two chambers separated from one another by a transverse trench and bordered bilaterally by a longitudinal trench. The "improved Neubauer"

ruling is used. Each chamber is 3 x 3 x 0.1 mm. and has a volume of 0.9 mm.^{3} The ruled area consists of 9 large squares, each 1 x 1 x 0.1 mm. with a volume of 0.1 mm.^{3} and bordered by a triple line. The center line of the three is the boundary line of the square. Each of the 4 corner squares is subdivided into 16 smaller squares measuring 0.25 x 0.25 x 0.1 mm. with a volume of 0.00625 mm.^{3} The center square of the 9 large squares is divided in 25 smaller squares each measuring 0.2 x 0.2 x 0.1 mm. with a volume of 0.0040 mm.^{3} (fig. 1).

The cover glass used over the chambers is made with precision. According to U. S. Bureau of Standards requirements the cover glass must be free of visible defects and must be optically plane on both

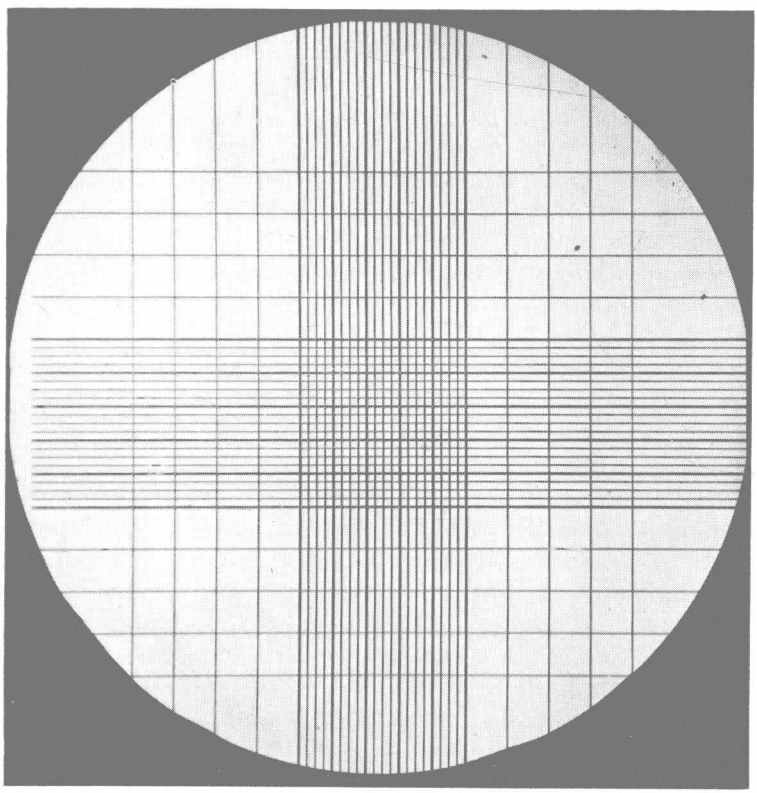

FIG. 1.—The improved Neubauer ruling of the counting chamber

sides within ±0.002 mm. From this it is evident that the cover glass must be handled carefully like any other piece of calibrated microchemical equipment. Obviously, ordinary cover glasses cannot be substituted.

To clean the hemocytometer and cover glass, rinse immediately after use in cold or lukewarm water. Never use hot water. Never allow the diluted blood to dry on the chamber. If the diluted blood has been allowed to dry on the chamber, it may be washed off with soap and water (lukewarm), gently using the ball of the thumb to accelerate its action. Then thoroughly wash and rinse in running water. A soft cloth may be used to wipe off the water. The chamber and cover glass are allowed to dry in air. Before using, the chamber and cover glass must be absolutely clean and free from lint and water marks. Never touch the chamber or cover glass except at the edges after it has been cleaned. A scratch across the chamber or cover glass renders them useless.

Pipets. Under U. S. Bureau of Standard specifications, the allowable error of the red cell pipet is ±5 per cent and of the white cell pipet ±3.5 per cent. However, at little or no extra cost, certain manufacturers are now marketing both red and white cell pipets which are guaranteed to be accurate within ±1 per cent. Obviously, such pipets are to be preferred. Because pipets are carefully calibrated precision instruments they must be properly cared for. The marks on the pipets are arbitrary in order to accomplish a certain dilution of the sample when the pipet is filled. The volume of the red cell pipet is made up of 1 part in the capillary portion and 100 parts in bulb. When blood is drawn to the 0.5 mark and subsequently diluted to the 101 mark, all of the red cells are washed into the bulb thus making a dilution of 0.5 parts in 100 parts of the bulb (1/200), the cell free contents in the capillary portion not participating in the dilution. In the white cell pipet, when blood is drawn to the 0.5 mark and subsequently diluted to the 11 mark, the dilution is 1/20.

Cleaning of the pipets may be performed as follows:

1. Fill the bulb 3 times with distilled water, each time filling through the capillary end, shaking, and emptying through the large bore end.

2. Repeat the above procedure by filling 3 times with 95 per cent alcohol.

3. Then fill the bulb 3 times with ether.

4. Thoroughly dry the interior of the pipet with a current of dry air. Air as discharged from the lungs is unsatisfactory since it contains much moisture. If a suction apparatus is not available, the pipet may be dried by removing the rubber tubing and shaking in the air. The bead must roll freely and the pipet must be absolutely dry and free from solvents. If any solvent remains in the pipet, protein precipitation will occur when blood is introduced and this may be extremely difficult to remove. The interior of the pipet should always be dry before storing. If moisture remains in the bulb, the bead may adhere to the wall and then be removed only with difficulty.

In the above procedure, acetone may be substituted for alcohol and ether.

If albuminous material has been allowed to remain in the bulb and cannot be removed by the above method, the pipet should be filled with cleaning solution and allowed to stand overnight.

Take all precautions against breaking the point of the pipet. The slightest nick which enters the bore renders the pipet useless because of the resulting inaccuracy in dilution.

Hemoglobinometer. A multitude of hemoglobinometers have been developed and are available. In no single instrument are the 4 essential qualities, accuracy, speed, simplicity, and low cost, incorporated. Although the Sahli hemoglobinometer leaves much to be desired from the standpoint of accuracy, it is the most suitable low priced instrument available at present. The Spencer hemoglobinometer is more satisfactory from the standpoint of accuracy, speed and simplicity but is approximately twice as costly. A properly standarized photoelectric colorimeter is the most accurate practical method for the routine determination of hemoglobin and should be used whenever possible. However, even the most expensive photoelectric colorimeter if not standardized might be less accurate than a properly standardized Sahli hemoglobinometer. High cost is not necessarily a substitute for careful standardization, skill, and experience.

Many types of Sahli hemoglobinometers are on the market. The square type of Sahli tube with the prismatic-shaped glass standards, although slightly more expensive, is the most satisfactory. It is important that the tubes used should be made by the same manu-

facturer as that of the standards. The hemoglobin pipets are manufactured to contain 0.02 ml. of blood. It is important to purchase a pipet guaranteed to be accurate within ±1 per cent. Pipets accurate within ±5 per cent (U. S. Bureau of Standards) are available but cost as much as the more accurate pipets. Uncalibrated and less expensive pipets may vary as much as ±30 per cent of the true volume.

The calibrated tube is easily cleaned by brushing with soap and water. The pipet is cleaned as noted above for the red and white cell pipets.

Hematocrit. The hematocrit is a tube of uniform bore (3 mm.) and flat bottom which is calibrated longitudinally into centimeters and millimeters. The numbers on the left of the scale read down from 0 to 10 cm. The numbers on the right read up from 0 to 10 cm. Since no absolute volumetric measurement is required, the volume contained within the tube is of no consequence.

The hematocrit tube is most easily cleaned by inserting a pipet (preferably stainless steel rather than glass) into the bottom of the tube, applying suction (either by a water suction pump or by a syringe fitted to the steel needle) and simultaneously holding the hematocrit tube under a stream of water. The hematocrit is then removed from the current of water and water from within is withdrawn by suction. If it is then allowed to stand in an inverted position, drying will be completed. When it is necessary to use the tube immediately, rinsing with alcohol and then ether will hasten drying. From time to time the hematocrit should be cleaned with cleaning solution to remove the thin film of coagulated protein which may accumulate on the glass.

Solution Bottles. For students working individually and moving from bedside to bedside and ward to ward, it is convenient to tape 9 one-ounce bottles together. These bottles can then be filled with buffer, Wright's stain, distilled water, n/10 HCl, reticulocyte stain, alcohol, ether, red cell fluid, and white cell fluid. The first 2 named should each contain a screw cap with a built in dropper. Corks should be used in the remaining bottles. A Bard-Parker blade may be inserted into the cork stopper of the alcohol bottle. All bottles and stoppers should be carefully labeled.

Obtaining the Specimen of Blood

Capillary Blood. For small samples such as are needed for the enumeration of red cells, white cells, reticulocytes and platelets and the determination of hemoglobin, puncture of the finger, or, in the case of infants, the plantar surface of the heel provides an adequate specimen with a minimum of difficulty, effort and inconvenience to the patient. For these purposes venous blood has no advantages over capillary blood. Blood smears should always be made from capillary blood in preference to venous blood to which an anticoagulant has been added. Blood from an earlobe is less satisfactory than blood from the finger or heel.

The part to be punctured should not be cyanotic, edematous or cold. If it is cyanotic it should be immersed in warm water for 5 minutes. The side of the finger is less sensitive than the ball of the finger. The skin is cleaned with alcohol and then wiped dry.

The best instrument for capillary puncture is a Bard-Parker № 11 scalpel blade. The blade can be mounted in a cork stopper that serves as a grip for the operator and as a guard for the depth of the puncture. The cork with the blade may be kept in a bottle containing alcohol and is, therefore, always sterile and ready for use after the alcohol is wiped off. If the blade becomes dull or rusty it can be readily replaced. Many types of automatic blood lancets are available but are more expensive, require sharpening, are more difficult to sterilize, and offer no advantage over the simple, inexpensive, easily replaceable Bard-Parker blade.

A puncture, approximately 3 mm. deep is made by a simple quick stroke. The first drop or two of the freely flowing blood is discarded by allowing it to flow off to the side. Slight pressure may be made along the sides of the finger some distance from the wound. Undue pressure in securing drops of blood should be avoided as this will cause dilution of the blood with tissue fluid. If free flowing blood is not obtained, it is better to repeat the puncture. All equipment must be readily available. Speed, dexterity and freely flowing blood are essential for good results.

Venous Blood. The advantage of venous blood is that a number of determinations may be performed and repeated if necessary, at the

leisure of the physician, from a single specimen of blood. The blood counts, with the exception of the platelet count, may be made at any time up to 24 hours after the blood has been drawn provided there is no hemolysis evident in the plasma and the bottle has been kept tightly corked and refrigerated at 5 C. when not in use. The sedimentation rate should be performed within 2 hours and the platelet count within an hour. Venous blood may be used for erythrocyte, reticulocyte, platelet and leukocyte counts, hemoglobin, sedimentation rate, volume of packed red cells, and icterus index tests.

A 20 or 18 gauge needle with a short bevel should be employed. The needle must fit tightly to the syringe. If the tip of the syringe is chipped, it must be discarded. All air should be expelled from the syringe before it is used. The needle and syringe must be clean, dry, and sterile.

With the tourniquet in place, a vein is selected, if possible one that is visible, palpable, and well fixed to the surrounding tissue. If a suitable vein cannot be located easily the patient should be instructed to open and close his fist several times, or the site may be gently rubbed. If this fails, it may be necessary to remove the tourniquet and warm the extremity by wrapping in a warm towel or by soaking it in warm water. It is frequently advantageous in place of a tourniquet, to apply a blood pressure cuff equidistant between systolic and diastolic pressure.

A suitable vein having been found and the equipment checked and ready, the area to be punctured is cleansed with 70 per cent alcohol and a tourniquet applied. The skin over the vein is drawn tense by the thumb of the left hand. The syringe is held in the right hand with the bevel of the needle turned up. If the vein is large and well fixed to the surrounding tissues, the skin and the vein may be pierced by one short thrust. If the vein is small or easily movable, it is better to puncture the skin over the vein first and then enter the vein. Only gentle pressure is applied to the plunger of the syringe. Vigorous pull on the plunger of the syringe may cause the vein to collapse, air to enter the syringe, and hemolysis of the blood sample to occur The tourniquet is removed *before* the needle is withdrawn. The tourniquet should not be applied for more than two minutes since the blood may then be altered by concentration. Slow removal of the needle is less painful than sudden removal. Gentle pressure is applied over the vein

by a pad of clean dry gauze or cotton ball and the patient is instructed to maintain this pressure for several minutes to prevent bleeding and the development of a subcutaneous hematoma.

Before emptying the syringe into the bottle containing the proper anticoagulant, the needle is removed. The contents of the syringe are then gently delivered into the bottle, the plunger of the syringe pulled back so as to prevent "freezing", and the bottle corked. Thorough mixing of the anticoagulant with the blood must be ensured by gently inverting and shaking the sample for about one minute. The blood must be thoroughly mixed each time before a sample is withdrawn.

It is absolutely essential to use the *proper type and amount of anticoagulant*. Heparin is satisfactory but expensive. Potassium oxalate is unsatisfactory since it causes shrinkage of the red cells. Ammonium oxalate causes the red cells to swell. The most satisfactory anticoagulant is a mixture of dry ammonium and potassium oxalate. When 6 mg. of ammonium oxalate and 4 mg. of potassium oxalate are used for 5 ml. of blood, no alteration in the volume of packed red cells takes place and the other corpuscular constituents remain unchanged. The volume of blood added to this amount of anticoagulant may be varied from 3.5 to 6.0 ml. without appreciable influence on any of the values except the sedimentation rate of the red cells. The anticoagulant is used dry to prevent dilution of the blood. This mixture is referred to as the "double oxalate mixture" and its preparation is described under "Preparation of Solutions".

Enumeration of Erythrocytes

Technic

1. Blood is drawn into the red cell pipet to point 0.5 with great accuracy.

This must be done meticulously since any error is magnified 200 times by the subsequent dilution. If the pipet is held in a nearly horizontal position better control of the column of blood can be obtained. If there is a slight excess (2 mm.) of blood above the 0.5

mark it may be drawn down by touching the point of the pipet lightly with a finger or cloth. A great excess of blood should not be drawn into the pipet since even though it is withdrawn, enough blood will adhere to the side of the pipet to invalidate the results. If this happens the pipet must be cleaned and the procedure begun again.

2. Wipe off the blood adhering to the outside of the pipet.

3. Diluting fluid (Gower's or Hayem's Solution) is drawn to the 101 mark.

The pipet should be gently rotated between the finger and the thumb while filling the bulb. The bead contained in the bulb should move freely. It is important to get good mixing while the pipet is being filled. When the bulb is almost full the pipet should be raised to the vertical position and the level of the fluid is drawn slowly to exactly the 101 mark. The pipet is then shaken immediately for about 30 seconds to facilitate the initial mixing. For this purpose the pipet may be held loosely in one hand while the attached rubber tubing is revolved between the thumb and forefinger of the other hand. If blood is being obtained from the finger, the manipulations up to this point must be carried out rapidly, otherwise coagulation is likely to begin before the blood is mixed with the diluting fluid and the cells will be clumped in the preparation, thus introducing a gross error.

4. Repeat the above procedures with a second pipet.

5. Shake the pipets for approximately 3 minutes.

The pipets can conveniently be held between the thumb and second finger. Shaking should be done in several directions rather than in one direction only. When the shaking has been completed, proceed immediately to the next step. If the pipet is allowed to stand for any period of time (even one minute), the contents of the pipet must be remixed for 3 minutes.

6. Expel and discard the first 4 drops from each pipet. This is done in order to remove the fluid in the capillary portion of the pipet which has not come in contact with the blood.

7. Load the counting chambers of the hemocytometer.

Fill one chamber from each pipet. With the cover glass on the chambers over the ruled platforms allow a drop of the diluted blood to run by capillary attraction under the cover glass without any forcing. If this does not occur, the counting chamber or cover glass is dirty and both should be washed in water, dipped in alcohol and dried free of lint. In placing the diluted blood in the counting cham-

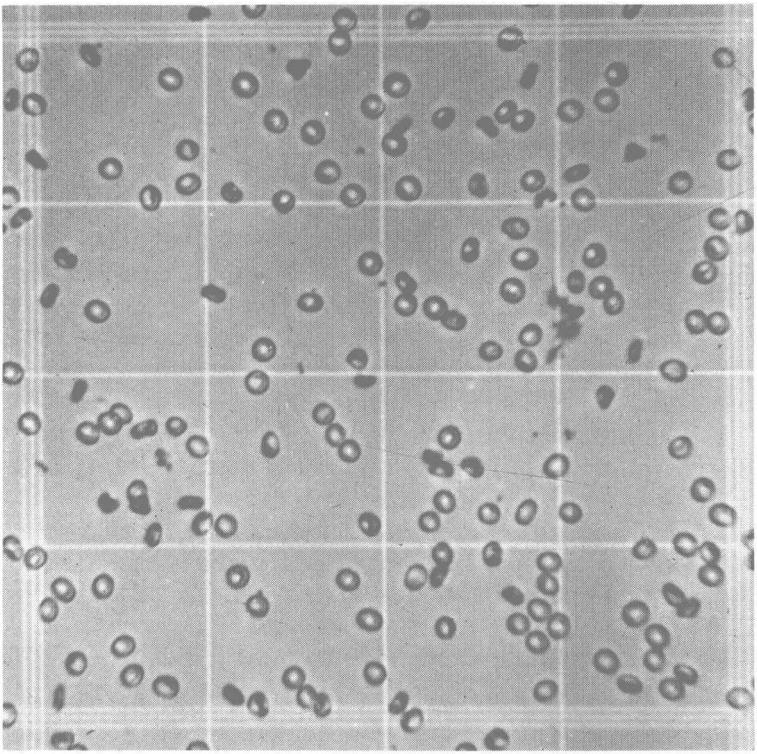

FIG. 2.—Red blood cell count. The group of 16 squares shown is one of the 25 squares in the central large square. The count by each small square is as follows:

8 → 7 → 14 → 6
↓
6 ← 8 ← 12 ← 8
↓
11 → 10 → 8 → 6
↓
9 ← 7 ← 13 → 13

bers, the tubing should be kept in the mouth so that the flow can be controlled. The pipet should be held as one holds a pencil, with the tip at the edge of the cover glass. The size of the drop must be gauged by practice. It must be sufficiently large to cover the whole platform