AN EFFICIENT AND PRACTICAL METHOD FOR THE COUNTING OF RED BLOOD CELLS*

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Dreyer has quite recently described a method for the enumeration of blood cells and bacteria without the use of a counting chamber. His method simulates that first elaborated by Wright for the counting of bacteria in that it is dependent for its determination on a comparison of the relative number of corpuscles, which are of known titer per unit volume, and the elements to be counted. It differs principally, however, and has a much broader field of usefulness in that, instead of using normal erythrocytes and making smears for examination, a suspension of the red corpuscles of the hen carefully titrated is used as a standard and this suspension mixed in known proportion with the elements, i.e. patient's erythrocytes, leukocytes, etc., to be counted allows for examination and comparative enumeration under the microscope. He points out the following distinct advantages: (1) it can be used with equal facility for counting erythrocytes, leukocytes and bacteria; (2) it does away with the use of a costly counting chamber; (3) it is quicker and less fatiguing to the eyes.

The method is to be commended for its universality and accuracy; however, it possesses certain disadvantages which greatly interfere with its practical clinical employment in enumerating erythrocytes. These are: (1) although the blood of the hen is obtainable it is not a common laboratory animal or readily secured in many hospitals; (2) the differentiation of the human and avian erythrocytes, while easy, is not instantaneous; (3) the method recommended for diluting the blood and then adding an equal volume of the suspension is an extra step over the hemocytometer method; (4) the use of a suspension standardized to contain about 20,000 corpuscles per cmm. makes calculation of the patient's erythrocytes mathematically cumbersome. These disadvantages, though they in no way interfere with the accuracy of the method, do tend to limit it to a special laboratory technic and remove it from the sphere of clinical usefulness.

To overcome these disadvantages a procedure has been devised which has been found to be practical and efficient. Instead of avian erythrocytes, a suspension of crenated, fixed and stained human red blood cells is employed. This suspension is made up to contain 25,000 cells per cmm.

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Technic.—The technic for enumeration requires: (1) glass stoppered bottle containing the standard suspension; (2) pipets graded to deliver 1 c.c. and 0.005 c.c.¹; (3) test tube, plain glass slide, and cover slip.

Preparation of the Standard Suspension.—Fifty c.c. sterile 2 per cent. sodium citrate in 0.85 per cent. saline is placed in a sterile 300 c.c. Erlenmeyer flask. To this is added approximately 5 c.c. blood freshly drawn from a vein and free from clot. The blood should come from a person free from disease which alters the red blood cells, as anemia, etc., and may readily be obtained at the time of taking specimens for routine Wassermann examination. As the blood is added the mixture should be agitated slowly and the cotton wool plug then replaced in the mouth of the flask. The flask is kept at room temperature with gentle shaking up of the settled corpuscles daily or oftener to prevent their becoming too firmly precipitated and adherent at the bottom. At the end of three or four days, depending on the rate of evaporation, it will be noted that the color has changed from a bright to a slightly darker red, and if a drop is examined under the microscope, the cells will be found darker, smaller than when fresh and slightly crenated. Some show the alteration to a greater degree than others. There should be no clumping.

To the suspension, when the cells have attained this appearance, is added 200 c.c. Hayem's solution made up according to the following formula: Sodium chlorid, 1 gm.; sodium sulphate, 5 gm.; mercuric chlorid, 0.5 gm.; distilled water, 200 c.c. This must be filtered and perfectly clear. It should be borne in mind that all glassware employed throughout the process of preparing the suspension must be perfectly clean and rinsed in distilled water and all solutions free from insoluble dust particles, etc.

It is convenient if the suspension be now transferred to a tall, rather narrow glass stoppered bottle which will hold at least 500 c.c. or, better still, a 1,000 c.c. graduated glass stoppered cylinder. During the day the suspension is agitated and on the following day it will be found to have become light brown in color. The supernatant fluid is then siphoned off and replaced by fresh Hayem's solution. The cells are allowed to settle, the supernatant fluid is withdrawn and fresh solution is again added. The suspension is thus practically freed of the sodium citrate and the erythrocytes have become fixed. Under the microscope they appear small, crenated, brownish and quite highly refractile.

1. Capillary pipets to measure 0.005 c.c. may be obtained from the Will Corporation, Rochester, N. Y., or the Topley Company, Ottawa, Ont.

The cells are now stained by adding 100 c.c. filtered eosin solution such as is employed for routine tissue staining or made up as follows: Saturated solution of water soluble eosin, 10 c.c.; distilled water, 85 c.c.; saturated solution of picric acid, 5 c.c., filter. The mixture is shaken up well from time to time and the stain is allowed to act for twenty-four hours at the end of which time the supernatant fluid is siphoned off and replaced by Hayem's solution. The excess of stain is thus removed and the process may be repeated if it is found after settling that the solution still contains much eosin.

Titering the Suspension.—Depending on the amount of blood originally obtained, the suspension is now diluted with Hayem's solution so as to contain approximately 25,000 cells per cmm. Before counting it is best to transfer definite amounts, e. g., 250 or 300 c.c., to smaller glass stoppered bottles. These are then brought to exact titer of 25,000 by making several careful counts with an accurate hemocytometer, fluid being added or withdrawn after settling as required.

The suspension thus prepared is, so far as we have been able to judge, absolutely stable. There should be no clumping which is inclined to occur if centrifugalization is employed. The cells stand out clearly under the dry high power of the microscope as pink or red irregularly spherical masses about 5 microns in diameter.

Technic for Adding Patient's Blood.—The blood of which the erythrocyte count is to be determined is added to the standard suspension in the proportion of 1 to 200. This may be brought about in several ways with satisfactory results. That procedure which is most convenient under the circumstances can be employed. Where, as usually, it is desired not to withdraw a large amount of blood, the following procedure has been found very efficient.

After thoroughly shaking the suspension, exactly 1 c. c. is measured into a test tube. The tube should be perfectly clean but rinsing in distilled water without drying will not alter the result. Dwarf test tubes measuring 10x1 cm. are a convenient size. The blood is obtained by finger or ear prick in the usual way and is allowed to run by capillarity or is sucked by means of rubber tubing and mouthpiece into a pipet graded to measure 0.005 c. c. The end is then wiped off on a piece of gauze and the contents discharged directly into the 1 c. c. of suspension in the test tube. The pipet is washed clear by drawing up and blowing out the suspension several times. The mixture is then shaken thoroughly and may be counted at once or the tube may be corked to prevent evaporation and consequent crenation of the blood disks and set aside to be examined any time when convenient within twenty-four hours.

Another method consists in the use of the regular erythrocyte pipet provided with hemocytometers. One with a relatively large bore

so that the contents may be expelled readily is preferable. The pipet is filled with patient's blood to the 0.5 mark and then diluted with suspension to 101. It is best to remove a small quantity of the suspension from the large bottle for this purpose. The pipet is then shaken, and after discharging the first three or four drops, the balance is expelled into a clean test tube and either counted or corked and set aside, as stated above. It is obvious that there is a slight fallacy here as the mixture is minus a quantity of suspension equal to the amount of blood employed, namely $\frac{1}{200}$ part. For practical purposes, however, this can be disregarded as it alters the count of corpuscles per cubic millimeter by only a similar proportion and this is of no clinical consequence.

When a large quantity of blood is withdrawn by venous puncture, as in connection with chemical metabolic blood calculations, and the fluid preserved free from clot by the use of a few crystals of potassium oxalate, a larger quantity of blood may be conveniently employed. It is only necessary to keep in mind to use a relatively larger amount of suspension, so that the proportion of 1:200 may be preserved, as for example 0.05 c. c. blood to 10 c. c. suspension.

Counting the Erythrocytes.—The erythrocytes may be counted any time within twenty-four hours, provided the suspension has been properly freed of a large amount of uncombined eosin and care is taken by corking to avoid crenation of the cells. This possibility is of considerable practical importance, as, for example, in ward work where several specimens may be taken at one time and removed to the laboratory, or in private practice when examining blood of persons outside the hospital or office.

When ready to proceed, the mixture should be shaken thoroughly. This is quickly accomplished by removing the cork and placing the thumb over the orifice of the tube. A medium sized drop of the mixture is now transferred to a clean glass slide and covered by a cover slip. Bubbles should be avoided. It will then be found that the cells settle almost instantaneously into quiet position. The cells are now examined under the high dry power of the microscope. Reduced light facilitates differentiation, and the pale biconcave circular disks of the patient stand out in distinct contrast to the red crenated corpuscles of the standard suspension.

Considering the technic employed, it is obvious that if the blood which is being examined has a normal erythrocyte count, that is between 4,500,000 and 5,000,000 cells per c. mm. the corpuscles and standard suspension cells in the mixture will be in the proportion of approximately 1 to 1 and will appear in practically equal numbers in the microscopic field. Thus, almost at a glance and without counting one may obtain this information and draw immediate conclusion as regards a normality or deficiency in red blood cells.

For enumeration it is necessary to count and record the exact number of each type of cell in several fields. The total number of cells in a field may be altered by the size of the drop which has been placed on the slide and the size of the cover slip employed. Furthermore counting may be facilitated, as Dreyer suggests,² by inserting on the metal diaphragm within the eyepiece a small circle cut from light cardboard in the center of which a square aperture has been made. After a little practice 100 cells per field may be counted rapidly and accurately.

The number of fields examined and the total number of cells counted can be modified by the degree of accuracy which it is desired to attain. If the cells have been mixed properly by shaking, the proportion will be found to vary but slightly in different fields, especially if the total number of cells per field is high. For accurate enumeration it is advisable that a total of 1,000 cells be counted from each of two drops examined.

Calculation of the Patient's Erythrocyte Count.—The total number of each type of cell is now computed and by multiplying the sum of the fresh corpuscles by 5,000,000 and dividing by the sum of the standard suspension cells, the number of patient's red blood corpuscles per cubic millimeter is quickly determined. As a matter of fact, the six ciphers may be disregarded and simply "5" used, care being exercised to preserve the decimal.

For example: Suppose the sum of standard suspension cells in several fields to be 625, and the sum of the patient's corpuscles in the same fields 456. Then

$$625 : 456 :: 5 : X$$

$$X = \frac{456 \times 5}{625} \text{ or } 3.648$$

$$\text{Erythrocyte count} = 3,648,000.$$

Unless a very large number of cells is counted, it of course, adds nothing to the accuracy to carry the decimal beyond three figures.

CONCLUSIONS

Employing the same principle of comparing the elements to be counted with those of a titered standard suspension, as in Dreyer's method, a procedure is devised which has distinct advantages for the enumeration of erythrocytes and offers a method which is rapid, accurate and clinically practicable.

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2. Dreyer, Georges: A Simple Procedure for the Accurate Enumeration of Blood Cells and Bacteria Without the Use of a Counting Chamber, *Lancet* **1**: 219 (Jan. 29) 1921. The Counting of Blood Cells and Bacteria, *J. A. M. A.* **77**: 1166 (Oct. 8) 1921.