

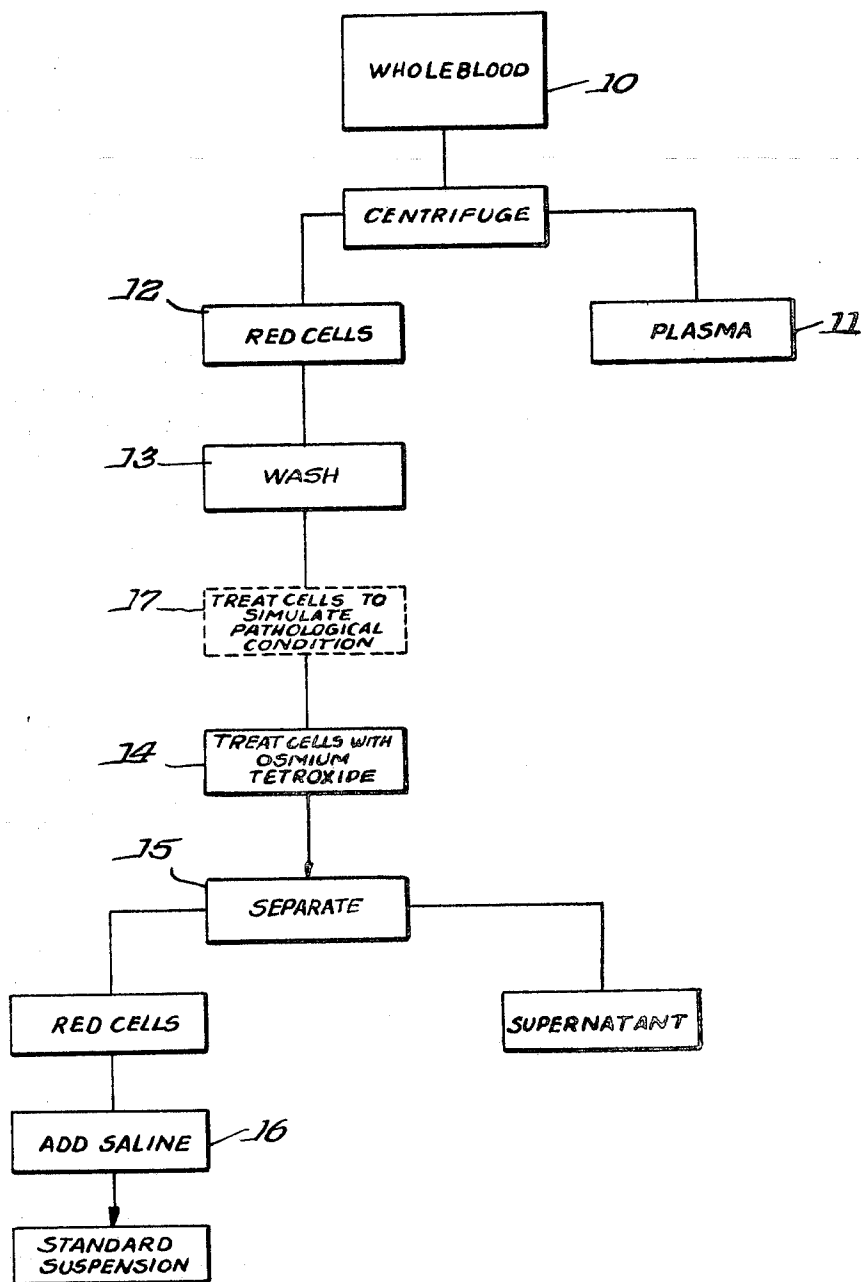
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BLOOD CELL COUNTING STANDARD AND METHOD OF PREPARING THE SAME

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BLOOD CELL COUNTING STANDARD AND METHOD OF PREPARING THE SAME

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ABSTRACT OF THE DISCLOSURE

A blood cell counting standard, and its method of preparation, in which a quantity of red blood cells are suspended in a stabilizing medium consisting essentially of physiologic saline solution containing a small percentage of osmium tetroxide, the stabilizing medium acting to stabilize the size, shape, and appearance of the red cells.

This invention relates to the preservation of red blood cells, and more specifically, to a red blood cell counting standard and its method of preparation.

A main object of the present invention lies in providing a standardized suspension of red cells which is particularly suitable for use in checking the accuracy of automatic or manual cell counting procedures. In the use of automatic methods for particle counting, there is always the possibility of malfunctioning of the instrument which could result in counting errors; by providing a standardized particle suspension in which the number of particles in a given volume of fluid is predetermined, the accuracy of the instrument may be readily checked. The various manual techniques of particle counting may be similarly checked. By giving a technician a "blind" sample containing a known concentration of particles on which he is to obtain a particle count, the accuracy of the technician's work may be easily determined.

Another object is to provide a cell counting standard, and its method of preparation, in which no appreciable change occurs in the size, shape, and suspension stability of the red cells over extended periods of time. A still further object is to provide a standardized suspension of red cells, and its method of preparation, in which the cells are altered to simulate pathological conditions and are fixed or preserved in such an altered state without any significant changes occurring in the size or shape of the cells for periods of several months or more.

Other objects will appear from the specification and drawings. The drawing illustrates in diagrammatic fashion the steps of treating whole blood to obtain a standardized suspension of red cells embodying the present invention.

A main aspect of the invention lies in the discovery that when red blood cells are suspended in an isotonic saline solution containing small amounts of osmium tetroxide, such cells are stabilized against changes in size and shape which would otherwise occur in a relatively short period of time. While osmium tetroxide has been previously used in biological fixatives to demonstrate the presence of fatty material in tissue specimens, because of the formation of a black pigment when osmium tetroxide contacts fatty material, its effectiveness as a stabilizing agent for red cells in a counting standard, to maintain the appearance, size and shape of such cells, is believed surprising and unexpected.

The counting standard of the present invention comprises a quantity of red blood cells in a physiologic or isotonic saline solution (approximately 0.9 percent sodium chloride in water) containing a small percentage of osmium tetroxide. The concentration of osmium tetroxide may vary from 0.1 percent to 1.5 percent, depending largely upon the degree of fixation required. Generally, concentrations between 0.5 percent to 1.0 percent have

been found most satisfactory for the preparation of counting standards.

The preparation of such a counting standard, in which the red cells are stabilized against changes in size, shape, and appearance, is illustrated diagrammatically in the accompanying drawing. As the first step of the procedure, a quantity of whole blood 10 collected from a donor is mixed with an anticoagulant and the plasma 11 and red cells 12 are then separated by centrifugation or settling. The anticoagulant may be sodium citrate, ammonium oxalate, potassium oxalate, or any other conventional anticoagulant which will not effect a change in the size or shape of the red cells, and may be used in conventional quantities. Separation is preferably achieved by centrifugation; however, the same result may be achieved by permitting the blood to settle for a period of time.

The separated red cells are then repeatedly washed, as indicated by 13, in isotonic saline solution. Each time, the saline wash solution is separated from the red cells by centrifugation or by settling. It has been found desirable to repeat the washing step at least three times, the purpose being to separate from the cells all traces of plasma and to obtain a quantity of red cells free of all other agents or materials.

The final wash solution is removed from the packed red cells as completely as possible. The red cells are then resuspended in an equal volume of isotonic saline solution (50 percent suspension) and added with constant agitation (as indicated at 14) to a volume of osmium tetroxide solution equal to the combined volume of red cells and saline. The use of osmium tetroxide in the concentration ranges previously indicated, stabilizes the cells against changes in size, shape, or appearance, thereby producing a stable counting standard for use in the laboratory.

To suppress the growth of microorganisms which might accidentally contaminate the suspension and which might contribute to a cell count as an artifact, it is desirable to refrigerate the suspension. The addition of a growth-suppressing agent such as mixtures of conventional antibiotics or actual antiseptics (i.e., phenol in a concentration of 0.1 percent) is preferable, especially if extended storage periods are contemplated. Since the suspension contains no heat labile substances, refrigeration is recommended as a precautionary measure but is not essential if complete asepsis can be maintained during preparation and storage.

It has been found that a counting standard prepared as described above reveals no appreciable change in the size, shape, or appearance of the red cells over a storage period of six months, and it is believed that substantially longer periods of storage will produce no significant changes in the physical appearance of the cells.

When the suspension of red cells in the osmium tetroxide solution has been left standing for several days or more, the red cells slowly settle out of suspension leaving a clear supernatant layer of osmium tetroxide solution. It has been found that after approximately two hours of suspension of the cells in the osmium tetroxide solution, the stabilizing effects of the osmium tetroxide on the red cells are fully achieved and, therefore, if the presence of free osmium tetroxide is for any reason considered undesirable in subsequent analytical procedures, the supernatant layer may be aspirated and replaced by physiologic saline, as indicated in the diagram at 15 and 16. Here again, it has been found that the stabilized red cells in the resulting counting standard show no appreciable changes in size, shape, or appearance over a storage period of at least six months.

The preparation resulting from the above steps is a suspension of stabilized red cells in a normal or physiologic condition and, as such, is highly suitable for use as a par-

ticle counting standard. In some cases, however, it may be desirable to preserve or fix the cells to simulate pathological conditions. This may be achieved, as indicated at 17, by introducing certain substances into the blood prior to contact with the osmium tetroxide solution, or by briefly subjecting the cells to an environment which causes them to swell or shrink in size prior to mixing with the stabilizing osmium tetroxide solution, the osmium tetroxide then acting to fix the cells in their pseudo-pathological state.

The method of the present invention may be more fully understood by reference to the following illustrative examples:

Example I

Whole blood is collected from a normal healthy donor by anyone of the standardized blood collecting procedures. The blood-receiving container of about 600 cc. capacity may be of the evacuated type (either a bottle or a collapsed blood bag) or of the non-evacuated type having an airway. Container, connecting tube, needle, and other equipment used must be sterile, and the container must contain a suitable anticoagulant.

An effective anticoagulant consists of 12 grams ammonium oxalate and 8 grams of potassium oxalate in 1000 ml. of distilled water, with 50 ml. of the anticoagulant solution being used for 500 ml. of collected whole blood.

The contents of the container (500 ml. of whole blood and 50 ml. anticoagulant) are then centrifuged at 1500 to 2000 G for approximately 30 minutes. The supernatant plasma is aspirated and is replaced by a similar volume of sterile physiological saline. The collected red cells are thoroughly mixed with the saline, centrifugation is repeated, and the supernatant saline is removed by aspiration. The washing process is repeated at least three times in order to remove entirely any soluble proteinaceous material.

The concentrated red cells remaining after the final wash are then mixed with an equal volume of sterile physiologic saline and the mixture is added, with constant agitation, to an equal volume (i.e., equal to the combined volumes of red cells and saline) of a 1.0 percent solution of osmium tetroxide in physiologic saline. The final concentration of osmium tetroxide is therefore approximately 0.75 percent. Constant agitation is achieved by securing the flask of osmium tetroxide solution to a standard oscillating table pre-set to oscillate at about 100 cycles per minute. The addition of cells in the osmium tetroxide solution should be achieved within an interval of three minutes or less. The flask is then stoppered and transferred to a refrigerator for a period of two hours or more during which the cell stabilization reaction takes place.

The contents of the flask are then transferred to a suitable settling vessel (a 500 ml. graduated cylinder) and are allowed to settle for 24 hours. Thereafter, the osmium tetroxide solution is aspirated and replaced by physiologic saline. The cells are thoroughly mixed with the saline and are allowed to resediment. Three repetitions of this process are effective in removing the free osmium tetroxide and the amount of physiologic saline added to the cell suspension to make the final volume is sufficient to achieve a total volume for the suspension approximating the original volume of the collected whole blood (500 ml.). Liquid phenol is added to the final suspension to achieve a concentration of 0.1 percent.

For use as a counting standard, the suspension is thoroughly mixed to insure a uniform distribution of red cells throughout, and is then dispensed into 0.5 ml. vials. Dilutions of the contents of a random selection of vials are assayed in a conventional automatic particle counter (Coulter Electronic Blood Cell Counter) to determine the average particle count. The total deviation of counts should in no case exceed 2 percent of the mean whether comparison is being made among replicate counts of a single vial or from different vials.

A second assay should be made to determine the size

distribution of the cells. The technic of using the automatic particle counter for deriving such values is known, as set forth in the manufacturer's literature.

Example II

Samples of a suspension prepared in accordance with the teachings of Example I have been observed at weekly intervals for a period of six months, such samples being stored both at refrigeration temperatures and at room temperature. No change in cell concentration or size distribution has been observed over that period.

Such a suspension has also been found to withstand repeated freezing and thawing (which would destroy normal cells), and boiling and autoclaving, without any observed changes in cell concentration or size distribution. Prolonged exposure to weak acids or alkalies, or brief exposure to strong acids or alkalies, also fails to produce any such changes. The ability of the suspension to withstand extreme chemical and physical abuse over the six month test period suggests that an unabused suspension would have a life span of years without dissolution or alteration of the red cells.

Example III

To simulate pathological conditions in which red cell sizes are below normal, or to check the ability of an automatic blood cell counting machine to count in the lower size ranges, it is desirable to provide a suspension in which the cells are of lesser mean size than normal. This is accomplished by the procedure set forth in Example I with the following modifications:

The saline-washed normal red cells are exposed, at the final wash stage, to a concentration of saline (2.0 percent) which is greater than normal. The red cells in the solution of increased osmotic strength lose water and undergo shrinkage. Periodic microscopic examination (at 10-15 minute intervals) is used to determine the extent of shrinkage and, when the degree of shrinkage is deemed appropriate, the shrinking is arrested by stirring the red cell suspension into sufficiently concentrated osmium tetroxide to achieve a final concentration of 1.0 percent in the liquid phase.

Example IV

The procedure of Example III may be used to obtain a suspension of cells having a greater mean size than normal by exposing the normal cells at the stage of the final saline wash to a concentration of saline lower than normal, and by controlling and arresting the size alteration of the cells as already set forth.

While in the foregoing I have disclosed the counting standard and its method of preparation in considerable detail for purposes of illustration, it will be understood by those skilled in the art that many of these details may be varied without departing from the spirit and scope of the invention.

I claim:

1. A stable suspension of red blood cells for use as a counting standard comprising a known quantity of red cells in a predetermined volume of an aqueous stabilizing medium, said stabilizing medium consisting essentially of a physiologic saline solution containing osmium tetroxide in a concentration falling within the range of approximately 0.1 to 1.5 percent weight per volume.

2. The suspension of claim 1 in which the concentration of osmium tetroxide falls within the range of 0.5 to 1.0 percent weight per volume.

3. A method of preparing a red blood cell counting standard in which the size and shape of the red cells is stabilized, comprising the steps of separating red blood cells from a quantity of whole blood containing a standard anticoagulant, washing the red cells in a physiologic saline solution and removing such solution therefrom, and mixing with the washed red cells a stabilizing medium consisting essentially of an aqueous physiologic saline

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solution containing osmium tetroxide in the concentration range of approximately 0.1 to 1.5 percent weight per volume.

4. The method of claim 3 in which the concentration of osmium tetroxide falls within the range of 0.5 to 1.0 percent weight per volume.

5. The method of claim 3 in which there are the further steps of separating supernatant osmium tetroxide solution from the stabilized red cells, and substituting a substantially equal volume of physiologic saline for the removed osmium tetroxide solution.

6. A method of preparing a red blood cell counting standard in which the size and shape of the red cells is stabilized, comprising the steps of separating red blood cells from a quantity of whole blood containing a standard anticoagulant, washing the separated red cells in a physiologic saline solution and removing such solution therefrom, subjecting said washed cells to an environment capable of producing a simulated pathological condition in such cells, and thereafter mixing said washed cells with a stabilizing medium consisting essentially of a physiologic saline solution containing osmium tetroxide in a concentration falling within the range of approximately 0.1 to 1.5 percent weight per volume.

7. The method of claim 6 in which the concentration of osmium tetroxide is 0.5 to 1.0 percent weight per volume.

8. The method of claim 6 in which said washed cells are allowed to remain in said osmium tetroxide solution for an interval of at least two hours, and thereafter separating said cells from said osmium tetroxide solution and

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suspending said cells in a physiologic saline solution to provide a total suspension volume substantially equal to the original volume of whole blood.

9. A method of preparing a red cell counting standard in which the size and shape of the red cells are stabilized, comprising the steps of centrifuging a quantity of whole blood containing a standard anticoagulant to separate the red blood cells from plasma, repeatedly washing the red cells in physiologic saline solution, removing the physiologic saline solution of the final wash from the red blood cells, then suspending said washed red cells in a stabilizing solution consisting essentially of physiologic saline containing osmium tetroxide in a concentration falling within the range of approximately 0.1 to 1.5 percent weight per volume, and thereafter separating said cells from said stabilizing solution and resuspending said cells in a physiologic saline solution to provide a total suspension volume substantially equal to the original volume of whole blood.

10. The method of claim 9 in which the concentration of osmium tetroxide in said stabilizing solution falls within the range of 0.5 to 1.0 percent weight per volume.

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