June 26, 1973 H. R. ANSLEY ET AL 3,741,875

PROCESS AND APPARATUS FOR OBTAINING A DIFFERENTIAL WHITE BLOOD CELL COUNT

Filed Oct. 30, 1970 2 Sheets-Sheet 1

FIG. 1

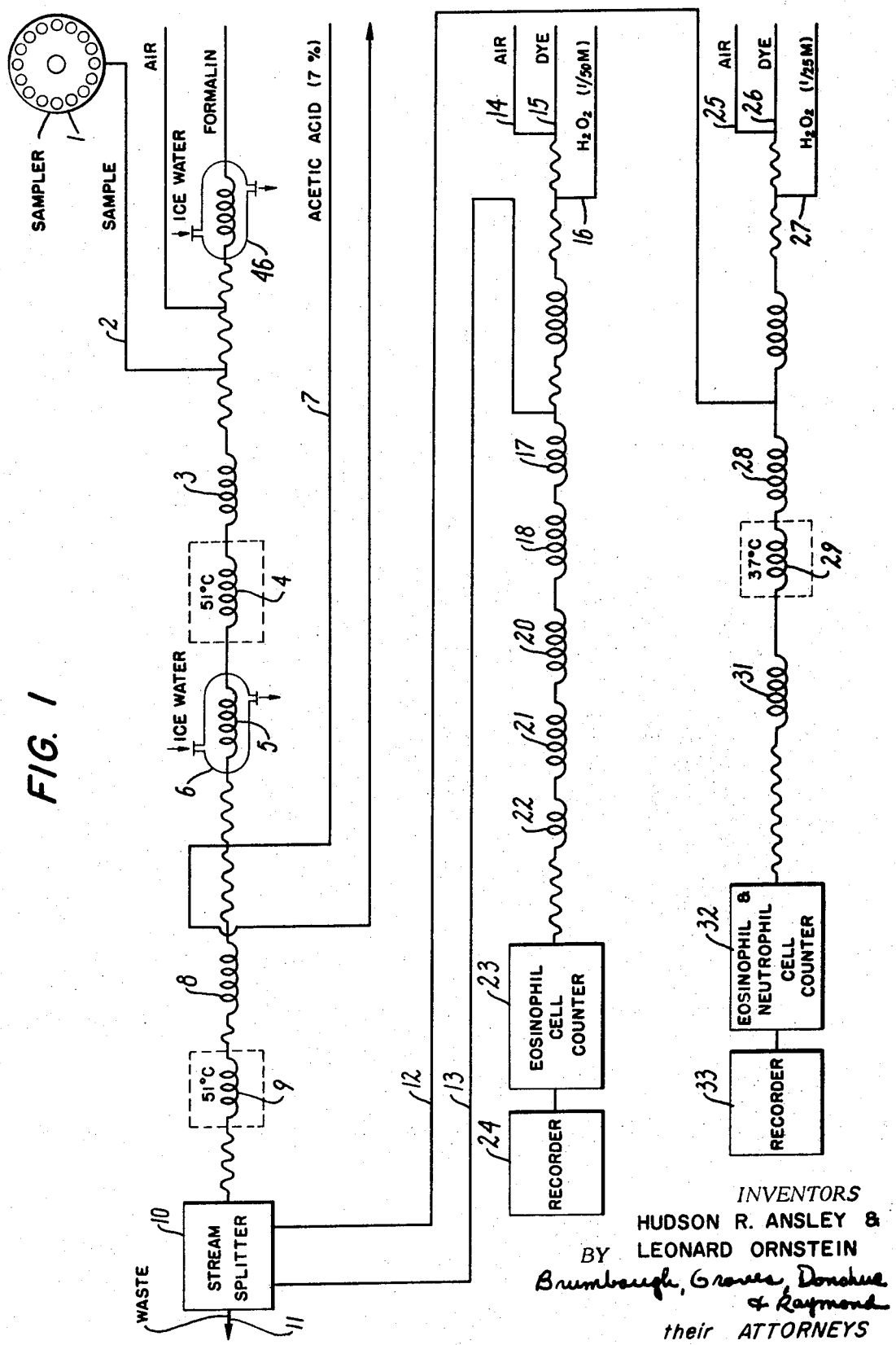

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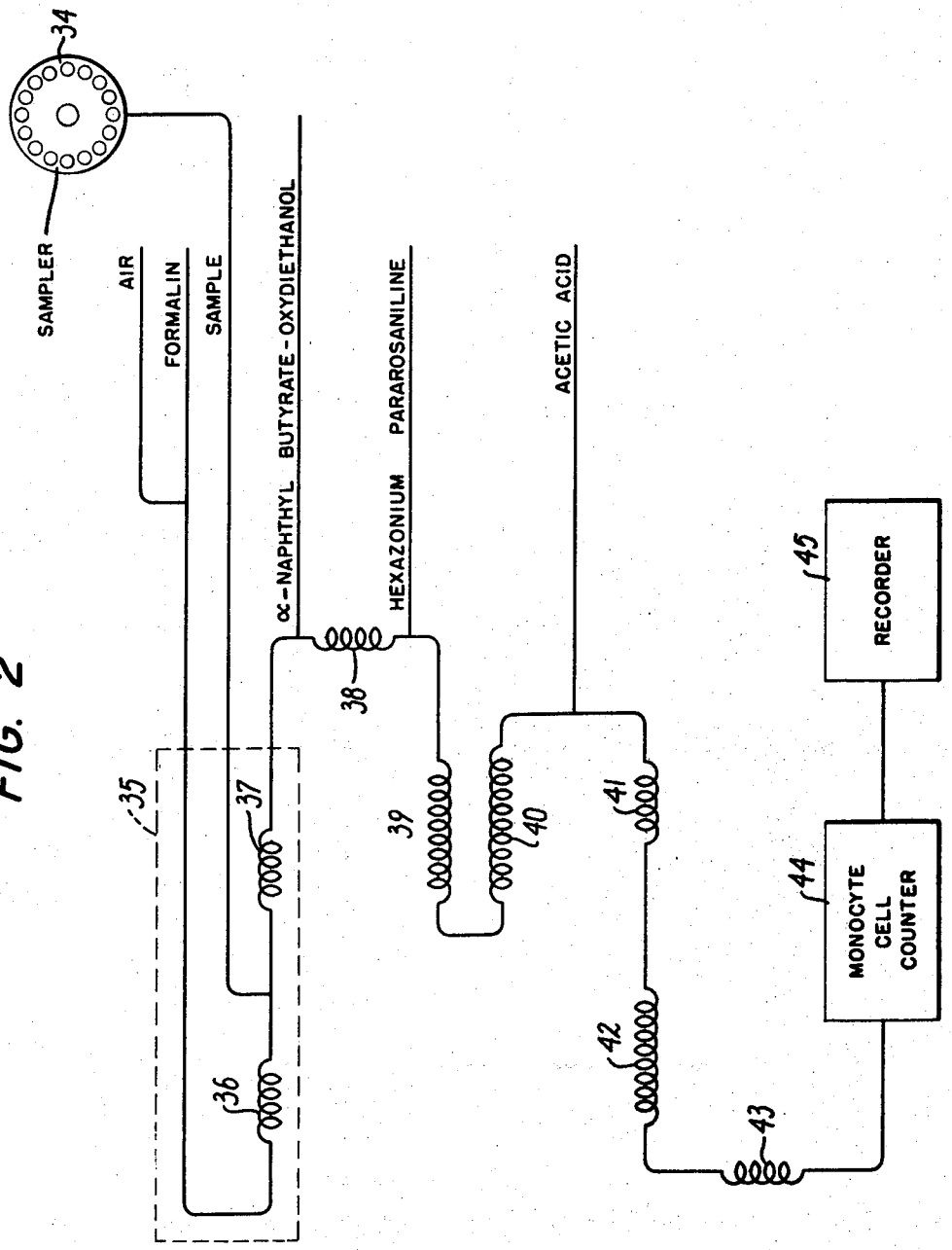
FIG. 2



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PROCESS AND APPARATUS FOR OBTAINING A DIFFERENTIAL WHITE BLOOD CELL COUNT

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ABSTRACT OF THE DISCLOSURE

A process and apparatus for obtaining a differential white blood cell count is disclosed. A cytological fixing agent is added to a sample of body fluid, such as blood, containing white blood cells to kill the blood cells contained in the solution and to immobilize the catalytic enzymes in the cells. The activity of the enzymes is not seriously impaired, and soluble components in the extracellular solution are not precipitated. When the body fluid also contains red blood cells a hemolyzing reagent is added after the fixation step to cause the red blood cells to release their hemoglobin content into solution. Addition of a specific cytochemical substrate, chromogenic precipitating coupling reagent, and pH buffer causes deposition of an insoluble dye in a specific type of cell containing the immobilized enzyme. The solution containing the dyed blood cells is then passed through a photometric counter which rapidly and accurately gives a count of the dyed cells. Using different specific substrates for different enzymes contained in specific kinds of cells, absolute and relative counts of the different cells can be obtained.

The present invention is a process and apparatus for providing a count of white blood cells.

There are five classes of normal white blood cells or leukocytes: neutrophils, lymphocytes, monocytes, eosinophils and basophils. It is a common medical diagnostic procedure to examine a dried, stained smear of human blood on a microscopic slide to determine the percentages of these five normal classes of white blood cells as well as the percentages of any abnormal cells. This is classically referred to as a differential white blood cell count and is described in Laboratory Medicine—Hematology (3rd ed.) by J. B. Miale (The C. V. Mosby Company, St. Louis, Mo., 1967), pages 822–830, 1126, 1127 and 1130. This type of blood count is performed by preparing a stained blood smear and examining from about 100 to 200 white blood cells in the smear under the microscope. From this examination a crude percentage breakdown of the various types of cells present is obtained. This method is notoriously tedious and time-consuming and is also inaccurate since only a relatively small number of cells are examined for each reported. There is a clear need for an automated process which will provide the equivalent of a differential white blood cell count more rapidly on a larger number of cells. Such an automated process could count approximately 10,000 white blood cells within one or two minutes for each patient. This process would be more accurate and would also be cheaper than the conventional process.

Classical differential white blood cell counts rely upon the different staining properties of the nucleic acids and proteins of the different parts of the various cell types and upon the abiltiy of the trained human eye and brain to recognize the distinct coloration and structural patterns that are characteristic of each kind of cell when viewed through a microscope. Each of the various kinds of white blood cells contains different proportions of various kinds of catalytic enzymes which permit them to carry out their unique functions. The general class of circumstances

under which enzyme-containing cells can be dyed is the subject of enzyme histochemical or enzyme cytochemical methodology. The following relate generally to this area of scientific investigation: Histochemistry: Theoretical and Applied by A. G. E. Pearse (Little & Brown, Boston, 1968); Enzyme Cytology by D. B. Roodyn (Academic Press, London/New York, 1967); and Laboratory Medicine—Hematology by John B. Miale, at pages 184–185 and 212–217.

The present invention in contradistinction to the conventional differential white cell count procedures is an automated operation. The invention includes addition of a cytological fixing agent to a solution containing white blood cells (e.g. whole blood) to kill the blood cells contained therein and to immobilize the catalytic enzymes contained within the cells. Samples of body fluid, such as whole blood and spinal fluid, are preferred. A suspension of bone marrow may also be analyzed by the present invention. The fixing agent does not seriously damage the catalytic properties of the enzyme, does not cause the precipitation of soluble components normally contained in the solution surrounding the cells, and preserves the morphology of the cells so that the catalytic enzymes remain within the cell with the classical identifying properties of cells essentially undistorted. After the addition of the fixing agent the solution is treated with a particular cytochemical substrate, a chromogenic precipitating coupling reagent, and pH buffer. The enzyme in the presence of the coupling reagent acts upon the substrate to cause a heavy insoluble dye deposit within the particular white blood cell to be counted. When whole blood is analyzed the procedure also includes a hemolysis step to cause the red cells to rupture and release their hemoglobin content into solution. The hemolysis step may either precede or follow the substrate, coupling reagent, and pH buffer addition step as described in greater detail hereinafter. After the particular type of white blood cell has been dyed, the solution containing it is passed through a photometric counting station which automatically records the number of dyed cells present. The formation of a heavy dye deposit in one type of cell enables the photometric counter to discriminate these cells from others in solution as will be described below.

The process of the present invention has a number of specific requirements which must be met. Since many cell enzymes are easily damaged or released from their cells by the mildest manipulation, it is necessary to simultaneously stop the metabolic processes (i.e. kill the cells) and to immobilize the enzyme of interest within the cells without significantly damaging the catalytic activity of the enzyme. These are the same requirements for cytological fixation in classical enzyme cytochemistry which are described in a number of publications including, A. Wachtel et al., J. Histochem. and Cytochem., vol. 7, p. 291 (1959) and B. J. Davis et al., J Histochem. and Cytochem., vol. 7, pp. 291–292 (1959).

It is also necessary that the fixing solution not precipitate any of the previously soluble components in the extra-cellular solution. The cells in the present process must remain suspended in a solution that is free of other particulate matter which could be mistaken at the photometric counting station for a cell. Any precipitate may also clog the channel through which the suspension of fixed, specifically dyed cells flow on their way to the counting station. These added requirements in the present process are converse to the requirements of classical histological fixation procedures which aim at insolubilizing a maximum amount of intra- and extra-cellular materials. A number of cytological fixing agents used by the prior art, such as acetone, cannot therefore be used in the present invention.

A cytological fixing solution is preferably a 0.2%–40% aqueous solution of a monoaldehyde, such as formaldehyde, butyraldehyde, propionaldehyde and acetaldehyde. Dialdehydes are unsuitable since they cross-link and produce extra-cellular precipitates. Acetone, acids and most alcohols are also unsuitable for similar reasons. The concentration of the monoaldehyde must be strong enough to kill the cells without, however, destroying the activity of the enzymes. Low concentrations at low temperatures need additional time to perform this function as compared with higher concentrations at higher temperatures. For example, at 2% formaldehyde concentration in the solution at 4° C. requires twelve hours, whereas a 4% concentration at 50° C. requires two minutes. Since equal volumes of body fluid and monoaldehyde solution are usually mixed, the strength of the aldehyde solution is double the concentration at the time of fixation of the aldehyde in solution. The following formalin solutions are satisfactory:

SOLUTION I

(20% Formalin) (pH=7.2)

Na_2HPO_4 ---------- g--	6.5
NaH_2PO_4 ---------- g--	4.0
Formalin stock solution ---------- ml--	200

Water is added to bring the final volume to 1,000 ml. This reagent is used in determining the eosinophil and neutrophil content by peroxidase dyeing.

SOLUTION II

(20% Formalin) (pH=7.2)

KCl, 1.1 g.

	Ml.
1/15 M KH_2PO_4 ----------	5.0
1/15 M K_2HPO_4 ----------	32.0
Formalin stock solution ----------	20.0
H_2O ----------	43.0
	100.0

The following stock solutions are used to prepare the above formalin working solutions:

1/15 M KH_2PO_4 (monobasic): Approximately 9.07 g. of KH_2PO_4 is dissolved in 1 liter of distilled water.

1/15 M K_2HPO_4 (dibasic): Approximately 11.61 g. of K_2HPO_4 is dissolved in 1 liter of distilled water.

Formalin stock solution: A 37% commercial solution of formaldehyde.

When the body fluid containing the white blood cells is whole blood it is also necessary to hemolyze the red blood cells since there is a danger that coincident passage of two or more red cells through a photometric counting station could be mistaken for dyed white blood cells or abnormal cells. The danger of such coincident passage could be reduced by diluting the solution but this leads to lower counting rates. A preferred way to solve the problem is to hemolyze the red blood cells by addition of a reagent to the suspension of cells to cause the red cells only to rupture and release their contents (e.g. hemoglobin) into the solution.

The hemolyzing reagent must not clot suspended cells and must not interfere with any subsequent histochemical reaction as, for example, by reacting with any of the compounds used to dye the cells in later steps. It must not cause the loss of white blood cell enzymes of interest into solution and must not cause precipitation of soluble components in the extra-cellular medium.

The hemolysis step is performed either before or after the staining reaction. If it is performed after staining it must not substantially modify the dye or the dyed or undyed white cells.

The removal of red blood cells by hemolysis in the present process is preferred over mechanical removal since the use of a hemolyzing reagent insures that the entire process remains simple. When such a reagent is used, the process requires only the sequential addition of volumes of solution to the original volume of blood in either a continuous flow or a batch process.

The hemolysis step may be carried out by adding to the solution containing the red and white blood cells an aqueous solution of an aliphatic acid having a pK_a of about 3.0 to 5.5. Acetic, propionic, butyric and lactic acids in a concentration of about 1%–10% are satisfactory. An acetic acid solution having a concentration of about 7% is preferred. The solution may be heated at temperatures of from about 40° C. for eight minutes to 55° C. for one minute, respectively, to inactivate catalase enzyme, to reduce pseudoperoxidase activity of hemoglobin, and to hasten hemolysis. Lower temperatures require more time for the reaction. Use of still higher temperatures inactivates peroxidase enzymes and promotes clotting after addition of the hemolyzing reagent.

The staining steps used in the present invention are enzymatic stainings in which the morphologically intact cell is directly responsible for production of the stain. The substrate and coupling reagent are turned into an insoluble dye within the cell by one of the enzymes in the cell. Such staining requires a rapid coupling to the cleaved substrate and also requires an insoluble product within the cell. These criteria are mentioned by Lehrer, G. M., et al., J. Biophysic. and Biochem. Cytol., vol. 6, No. 3, pp. 399–404 (1959); Davis, B. J., et al., J. Histochem. and Cytochem., vol. 7, pp. 297 ff. (1959); and Davis, B. J. Proc. Soc. Exp. Biol. & Med., vol. 101, pp. 90 ff. (1959).

The staining step of the present invention requires a careful choice of reagents. For example, two classical histochemical substrates for peroxidase enzymes are benzidine and paraphenylene diamine as described by Pearse in Histochemistry: Theoretical and Applied, and L. Ornstein in J. Histochem. and Cytochem., vol. 16, p. 504 (1968). Such reagents in contrast to 4-chloro-1-naphthol are unsuitable in the present invention since they react rapidly with the monoaldehyde fixing agent and produce compounds which precipitate and which cannot serve as peroxidase substrates for dye production.

The present invention utilizes a number of known cytochemical substrates but does so in a unique manner. For example, a mixture of an inorganic or organic peroxide and 4-chloro-1-naphthol may be used to dye a peroxidase-containing cell, such as an eosinophil or neutrophil. The peroxide and 4-chloro-1-naphthol serve as enzyme substrates and the 4-chloro-1-naphthol also serves as the coupling reagent. The 4-chloro-1-naphthol produces a blue-black dye deposit within the peroxidase-containing cell without the addition of a separate chromogenic precipitating coupling reagent. A mixture of o-tolidine and 4-chloro-1-naphthol may also be used to produce a different purple-red dye if the formaldehyde used in the fixing step is inactivated.

In order that a truly selective dyeing procedure may be attained, the pH of the solution is controlled. It has been found that when the pH of the solution is under 3.0, heavy deposits of dye are formed only in eosinophils. When the pH in the solution ranges between 3.0 and 5.0, heavy dye deposits are formed only in eosinophils and moderate deposits only in neutrophils. If the pH is buffered in the range between 5.0 and 7.0, heavy dye deposits are formed only in eosinophils and neutrophils.

When lipase-containing cells, i.e. monocytes, are dyed, the present invention preferably uses a combination of a naphthol ester, such as 1-naphthyl acetate or 1-naphthyl butyrate and a diazonium salt, such as hexazonium pararosaniline, which is a chromogenic precipitating coupling reagent. The optimum pH for this staining reaction is about 5.5–6.5, preferably about 6.0. At such a pH heavy dye deposits are formed in monocytes only.

The substrates normally used in classical esterase-lipase histochemistry are somewhat insoluble. In order that an adequate color may be developed in monocytes in a short time, e.g. five minutes, the concentration of the substrate must exceed about 0.5 mg./ml. in the final incubation

solution. Naphthol-AS esters and their derivatives and 1-naphthyl butyrate are less soluble than 0.5 mg./ml. This problem has been overcome by adding up to one-quarter of the final volume of either 2,2'-oxydiethanol (diethylene glycol) or other water-like alcohols or ethers (e.g. ethylene glycol, propylene glycol, dimethyl ether of diethylene glycol and diethyl carbitol). When this is done, 1-naphthyl butyrate and 1-naphthyl acetate can be dissolved in concentrations of up to nearly 1 mg./ml. Other substrates, such as α-naphthyl chloroacetate, indoxyl esters, such as indoxylacetate, indoxylpropionate, and indoxylbutyrate, 8-hydroquinoline esters, such as 8-hydroquinoline acetate, 8-hydroquinoline propionate and 8-hydroquinoline butyrate can also be dissolved. Only about 0.1 mg./ml. of naphthol-AS acetate will dissolve under these same conditions thus leading to a much slower color development. The other esters of naphthol-AS derivatives dissolve to an even lesser extent.

The following are typical preferred reagents which may be used to stain monocytes, eosinophils and neutrophils:

EOSINOPHIL-NEUTROPHIL REAGENTS

Stock solutions

0.003% and 0.006% part by weight of H_2O_2 in water
0.5 g. of 4-chloro-1-naphthol in 25 ml. of 2,2'-oxydiethanol
0.1 N acetic acid

Working solution

Eosinophil dyeing solution
(pH = ≤2.5)

	Ml.
0.1 N acetic acid	40
2,2'-oxydiethanol (100%)	20
4-chloro-1-naphthol stock solution	8
Concentrated acetic acid	3.4

Eosinophil-neutrophil dyeing solution
(pH = >3.3)

	Ml.
0.1 N acetic acid	40
2,2'-oxydiethanol (100%)	20
4-chloro-1-naphthol stock solution	13

Hydrogen peroxide solutions are used in conjunction with the 4-chloro-1-naphthol solutions mentioned above. A number of other organic and inorganic peroxides may be used as well. Examples are sodium perborate tetrahydrate, sodium carbonate peroxide, sodium pyrophosphate peroxide, ethyl hydroperoxide, tertiary butyl hydroperoxide and urea peroxide. All may be used in final concentrations of 0.1% to 0.0001% by weight final concentration.

The 4-chloro-1-naphthol is used in final concentrations of 0.001% to 0.06% by weight.

MONOCYTE REAGENTS

Stock solutions

(1) α-naphthyl butyrate—one percent by weight in 2,2'-oxydiethanol
(2) 1/15 M KH_2PO_4 (dibasic)—about 9.07 g./l.
(3) 1/15 M K_2HPO_4 (dibasic)—About 11.61 g./l.
(4) Basic Fuchsin (e.g. Matheson, Coleman & Bell)—1 g. in 25 ml. of 2 N HCl
(5) 2,2'-oxydiethanol
(6) 0.1 M K_2HPO_4
(7) 1 g. of sodium nitrite in 25 ml. of H_2O

Working reagents

α-naphthyl butyrate:	Ml.
α-Naphthyl butyrate (stock)	2.0
2,2'-oxydiethanol	6.0
1/15 M KH_2PO_4 (monobasic)	8.0
Total	16.0

The above solution should be prepared daily.

Hexazonium pararosaniline coupler

	Ml.
Basic Fuchsin (stock)	0.8
Sodium nitrite (stock)	0.8

The above are mixed for about one minute and are added to—

0.1 M K_2HPO_4 (stock—to adjust the pH to 5.8 to 6.2)	17.0
Total	18.6

The amount of basic Fuchsin is used in a final concentration of 0.001% to 0.01%.

For the monocyte staining step, a preferred flow embodiment calls for addition of about 0.32 ml./min. of formalin-fixed sample, about 0.42 ml./min. of α-naphthyl butyrate, and about 0.42 ml./min. of hexazonium pararosaniline. The eosinophil dyeing step adds about 0.60 ml./min. of eosinophil dyeing solution and about 0.10 ml./min. of 0.06% H_2O_2 to about 0.42 ml./min. of the formalin-fixed, hemolyzed sample. The eosinophil and neutrophil dyeing step combines about 0.32 ml./min. of eosinophil-neutrophil dye, about 0.10 ml./min. of 0.03% hydrogen peroxide, and about 0.23 ml./min. of formalin-fixed, hemolyzed sample.

A number of components in whole blood interfere with both the peroxidase and monocyte staining procedures.

When the peroxidase reaction is performed serum catalase can overwhelm the granulocytic peroxidase and thereby exhaust the substrate. Catalase is heat-labile at about 50° C. whereas peroxidase is stable up to about 80° C. Heating at a temperature of 40° C. for eight minutes to 55° C. for one minute, or appropriate intermediate times and temperatures, during or after the formalin fixation step, inactivates the catalase. A preferred heating time is 50° C. for three minutes. Dilution of the blood further enhances the ratio of intra-cellular peroxidase activity to residual catalase concentration. When the catalase concentration is reduced to the point where it competes successfully with the neutrophil peroxidase, the more active eosinophil peroxide is still intensely stained.

Heating of the blood solution also destroys the pseudoperoxidase activity of hemoglobin.

The interfering component during the monocyte staining is C'1-esterase inhibitor, a well-known component of serum which keeps C'1-esterase inactive. The action of C'1-esterase inhibitor can be blocked in a number of ways. One way is to add a mixture of heparin (an anticoagulant) and C'1-esterase to the sample to tie up the inhibitor. This is not a preferred step since C'1-esterase is very expensive. A combination of heparin and streptokinase can be added instead. An inexpensive way is to dilute the sample with potassium saline. A 50-fold dilution is normally required. This, however, greatly reduces the counting rate. A preferred method is to add 2,2'-oxydiethanol to the fixed blood sample in concentration of from 10% to 30%. This results in much greater damage to the inhibitor than to the monocyte lipase.

FIGS. 1 and 2 show apparatus which may be used to carry out the addition of the various reagents to the sample. The apparatus in FIG. 1 is designed to be used in the determination of the eosinophil and neutrophil count, whereas the one in FIG. 2 is designed to determine the monocyte content in a sample. These apparatus are similar to that described by Skeggs, L. T., Am. J. Clinical Path., vol. 28, p. 311 (1957) which was previously utilized for clinical chemical analysis.

With further reference to FIG. 1, it can be seen that a sample is fed from a sampler **1** through a conduit or tubing **2** and is mixed with a Formalin solution which was cooled in cooler **46**. Air bubbles are added to divide the liquid stream into segments to prevent mixing of successive samples. The mixture passes through a mixing

coil 3, a heated mixing coil 4, and a third mixing coil 5 which is cooled in cooler 6 by ice water. Acetic acid for the hemolysis step is added by conduit 7 to the fixed sample, and this mixture is passed through mixing coil 8 and heated mixing coil 9 to a stream splitter 10 where excess sample and bubbles are removed through line 11 and where the sample stream is split and sent through two conduits, 12 and 13. The stream in line 13 is added to a mixture of eosinophil dye and hydrogen peroxide added by conduits 15 and 16, respectively, and is again segmented by air introduced by conduit 14. The mixture is fed through mixing coils 17 and 18. The total mixture is fed through mixing coils 20, 21 and 22 and is placed in an eosinophil cell counter 23 which is attached to a recorder 24.

Meanwhile, the stream in conduit 12 is combined with dye for eosinophils and neutrophils, and hydrogen peroxide supplied by conduits 26 and 27, respectively, and is again segmented by air introduced by conduit 25. This mixture is fed through mixing coil 28, is heated to 37° C. while passing through coil 29. The sample containing the dyed cells is passed through coil 31 and is fed to counter 32 which counts the combined number of eosinophils and neutrophils. A recorder 33 is attached to this counter.

The above apparatus enables a technician to obtain a count of the eosinophils and neutrophils present in a sample.

FIG. 2 shows an apparatus which can be used to count the number of monocytes in a sample. The sample is fed from sampler 34 and is combined in a jacketed ice bath 35 with air-segmented Formalin which has been cooled in coil 36. The mixture is combined with α-naphthyl butyrate, oxydiethanol, and hexazonium pararosaniline and acetic acid. Complete mixing is obtained by sending the solution through mixing coils 37 through 43. The solution containing the dyed white blood cells is passed through monocyte cell counter 44 attached to a recorder 45.

The prior art staining methods caused only subtle differences in the dye deposits formed in varions types of cells. This, of course, precludes use of simple counters such as used herein since such are not capable of discriminating subtle differences in dye distribution within the cell. Consequently, more sophisticated apparatus which usually had to recognize the pattern of dyed or undyed structures usually had to be used.

Examples of such apparatus are described in Ingram et al., Annals N.Y. Acad. Sci., vol. 157, Article I, pages 275 ff. (1969); Prewitt et al., Annals N.Y. Acad. Sci., vol. 128, pages 1035 ff. (1966); Young, I. T., Quantitative Cytochemistry, vol. 2, Wied et al., eds. (Academic Press, N.Y., 1970); Wied et al., Cytology Automation, D. M. D. Evans, ed. (E. & S. Livingstone, London, 1970) and U.S. Pats. Nos. 3,327,117; 3,413,464 and 3,497,690. The present invention, however, enables a technician to impart a dye deposit to a particular type or types of cells in such a manner that a relatively simple counter is able to discriminate between heavily dyed, moderately dyed, and undyed cells. Examples of such apparatus are described in Kamentsky, L. A., et al., Annals N.Y. Acad. Sci., vol. 157, Article I, pages 310 ff. (1969) and Kamentsky, L. A., et al., Proc. I.E.E.E., vol. 57, page 2007 (1969).

The counter used in the present invention illuminates the solution of suspended cells with a beam of light. Dyed cells remove a certain amount of light from the beam. This amount is measurably different from the amount removed by undyed cells or less heavily dyed cells. This difference leads to discrimination between two or more types of cells and enables them to be counted accurately.

Cells may be counted in the sample counter described above at high rates of from about 1,000 to 30,000 per minute per channel. Blood samples from different patients follow each other sequentially separated by intervening short washes which prevent contamination of a succeeding sample by a preceding sample.

We claim:

1. A process for detecting a type of white blood cell comprising adding a cytological fixing agent to a solution containing white blood cells to kill the white blood cells contained in the solution and to immobilize catalytic enzymes within the cells without seriously damaging their catalytic properties, thereafter adding a cytochemical substrate to enzymatically stain a type of white blood cell, the fixing agent and the cytochemical substrate both being chosen to avoid precipitation of soluble components present in the extra-cellular solution, and detecting the stained cell.

2. A process as claimed in claim 1 which further comprises addition of a separate chromogenic precipitating coupling reagent with the cytochemical substrate, said reagent being chosen to avoid the precipitation of soluble components in the extra-cellular solution.

3. A process as claimed in claim 2 which further comprises addition of a pH buffer to maintain the pH in the solution during reaction of the chromogenic precipitating coupling reagent with a product of the enzymatic modification of the cytochemical substrate.

4. A process as claimed in claim 2 in which the coupling reagent comprises hexazonium pararosaniline.

5. A process as claimed in claim 2 in which the cytochemical substrate is selected from the group consisting of naphthol esters, indoxyl esters and 8-hydroquinoline esters, and the cell to be counted is a monocyte.

6. A process as claimed in claim 5 in which the pH is controlled in the range of 5.5 to 6.5.

7. A process as claimed in claim 5 in which the substrate is 1-naphthyl acetate.

8. A process as claimed in claim 5 in which the substrate is 1-naphthyl butyrate.

9. A process as claimed in claim 5 which further comprises addition of an inactivator of soluble heat-labile plasma esterase inhibitor.

10. A process as claimed in claim 5 which further comprises addition of a mixture selected from the group consisting of heparin and streptokinase, and heparin and compliment C'1-esterase.

11. A process as claimed in claim 5 which further comprises addition of formaldehyde and 2,2'-oxydiethanol.

12. A process as claimed in claim 2 in which a hemolyzing reagent is added to the solution after addition of the fixing agent and prior to addition of the cytochemical substrate, coupling reagent, and pH buffer.

13. A process as claimed in claim 2 in which a hemolyzing reagent is added to the solution after addition of the cytochemical substrate, coupling reagent, and pH buffer.

14. A process as claimed in claim 2 in which a hemolyzing reagent is added to the solution to cause red blood cells to release their contents into the solution, the hemolyzing reagent being selected so that it does not dissolve the stain formed in the type of white blood cell and does not cause precipitation of soluble components present in the extra-cellular solution.

15. A process as claimed in claim 2 in which the fixing agent comprises a monoaldehyde, the monoaldehyde being thereafter inactivated prior to addition of the cytochemical substrate, the cytochemical substrate being a mixture of a peroxide and o-tolidine and the chromogenic precipitating coupling reagent being 4-chloro-1-naphthol.

16. A process as claimed in claim 1 in which the solution also contains red blood cells and in which a hemolyzing reagent is added to the solution to cause the red blood cells to release their contents into the solution, the hemolyzing reagent being chosen so that it does not dissolve the stain formed in the type of white blood cell and also does not cause precipitation of soluble components present in the extra-cellular solution.

17. A process as claimed in claim 16 in which the solution is heated to inactivate catalase without precipitating soluble components in the extra-cellular solution.

18. A process as claimed in claim 17 in which the solution is heated to a temperature of from about 37° C. for ten minutes to about 55° C. for one minute.

19. A process as claimed in claim 16 in which the hemolyzing reagent is a 1%–50% solution of an aliphatic acid having a pK_a of 3.0 to 5.5.

20. A process as claimed in claim 19 in which the hemolyzing reagent is a 7% solution of acetic acid.

21. A process as claimed in claim 1 in which the cytochemical substrate is a mixture of a peroxide and 4-chloro-1-naphthol.

22. A process as claimed in claim 21 in which a weak acid is added to produce a pH in the solution of under 3.0 and in which a heavy dye deposit is caused in eosinophils only.

23. A process as claimed in claim 2 in which the pH buffer produces a pH between about 3.0 and 5.0 and in which a heavy dye is imparted to eosinophils only and a moderate dye is imparted to neutrophils only.

24. A process as claimed in claim 21 in which the pH buffer produces a pH in the solution of about 5.0 to 7.0 and in which a heavy dye is imparted to eosinophils and neutrophils only.

25. A process as claimed in claim 1 in which the fixing agent comprises a monoaldehyde.

26. A process as claimed in claim 25 in which the monoaldehyde is selected from the group consisting of formaldehyde, butyraldehyde, propionaldehyde and acetaldehyde.

27. A process as claimed in claim 26 in which the monoaldehyde is a 0.2% to 40% aqueous solution of formaldehyde.

28. A process as claimed in claim 1 in which a hemolyzing reagent is added to the solution after addition of the fixing agent and prior to addition of the cytochemical substrate and pH buffer.

29. A process as claimed in claim 1 in which a hemolyzing reagent is added to the solution after addition of the cytochemical substrate and pH buffer.

30. A process for detecting a type of white blood cell comprising adding a cytological fixing agent selected from the group consisting of formaldehyde, butyraldehyde, propionaldehyde and acetaldehyde to a solution containing white blood cells to kill the white blood cells contained in the solution and to immobilize catalytic enzymes within the cells without seriously damaging their catalytic properties, thereafter adding a cytochemical substrate to enzymatically stain a type of white blood cell, the cytochemical substrate being a mixture of a compound selected from the group consisting of o-tolidine and a peroxide with 4-chloro-1-naphthol when the cell to be counted contains peroxidase and the cytochemical substrate being a mixture of a naphthol ester and a diazonium salt when the cell to be counted contains lipase, the fixing agent and cytochemical substrate not causing precipitation of soluble components present in the extra-cellular solution, and detecting a stained type of white blood cell.

31. A process as claimed in claim 30 wherein the pH in the solution is controlled below 7.0 when peroxidase-containing cells are present and where the pH is controlled between 5.5 and 6.5 when lipase-containing cells are present.

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A. LOUIS MONACELL, Primary Examiner

R. J. WARDEN, Assistant Examiner

U.S. Cl. X.R.

23—230 B