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# Separation Of Leucocyte Types From Peripheral Blood

Peter Beverley Noble

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SEPARATION OF LEUCOCYTE TYPES  
FROM PERIPHERAL BLOOD

by

Peter Beverley Noble

Department of Medical Research

Submitted in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy

Faculty of Graduate Studies  
The University of Western Ontario  
London, Canada  
August, 1967

-iii-

To  
Sue, Andy  
&  
Gill

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Abstract

A study of the haematological response produced by the chloroform soluble extract (MPA) of *Listeria monocytogenes* and also with animals infected with the live organism, demonstrated the inability of conventional morphological techniques to distinguish between the leucocyte types so produced. It was thought that an immunological approach might provide a means of identifying leucocyte types by their antigenic components. However no method was available for the separation of the individual leucocyte types required for an immunological study of specific cell antigens. Experiments were performed with the aim of providing a separation of the individual leucocyte types. Chicken blood was used initially because of its high monocyte count.

The method of Rabinowitz, developed for human blood, could not be applied to the separation of the various leucocyte types from chicken blood; dextran does not encourage rouleaux formation of chicken erythrocytes and EDTA, in calcium and magnesium free buffered saline, failed to elute monocytes from glass beads.

Monocytes were recovered using 2 mm. waxed bead columns (eicosane m.p. 35.5 to 37°C) and melting the wax after

the other cell types had been eluted from the column with Hank's balanced salt solution and EDTA in calcium and magnesium free buffered saline. In all these experiments the gross contamination in the leucocyte fractions by erythrocytes made it necessary to remove the erythrocytes prior to attempting a separation of the individual leucocyte types.

A separation of leucocytes from erythrocytes was achieved by the floatation technique using Ficoll, a polysucrose material. The types of leucocytes separated from erythrocytes are dependent upon the balanced salt solution and anticoagulant used. Seligmann's balanced salt solution with EDTA as the anticoagulant result in the separation of lymphocytes, monocytes and thrombocytes from the erythrocytes. The granulocytes pass through the interface and remain intimately mixed with the erythrocytes. A separation of all leucocyte types from erythrocytes was accomplished using Hank's balanced salt solution with heparin as the anticoagulant, which promoted clumping of all leucocyte types so that the granulocytes remained with the other leucocyte types after the Ficoll floatation procedure. The clumped cells could be individually resuspended by Seligmann's balanced salt solution.

A separation of the various leucocyte types was accomplished on the 2mm. wax bead column. After incubation for one hour at 32°C the lymphocytes were eluted with Hank's balanced salt solution and the granulocytes by EDTA

in calcium and magnesium free buffered saline. The monocytes were obtained by raising the column temperature to 40°C and the cells collected in the molten wax. Unfortunately it was not possible to completely remove the wax from the monocytes, making it very difficult to work further with these cells.

Individual leucocyte types were separated using Ficoll density gradients. The problems associated with the floatation technique and with viewing and recovering the bands of isolated cells are discussed. Solutions to these problems are presented.

The method was also applied to human leucocyte separation from erythrocytes and to the separation of individual leucocyte types. Recovery data and viability studies are presented.

## Introduction

Initially the intention was to use the chloroform soluble extract of *Listeria monocytogenes* (MPA), which is credited with producing a monocytosis, as a tool for the study of the origin of monocytes (104). After having experienced the effect produced by both MPA and live *Listeria monocytogenes* infection in three animal species, rabbit, rat and mouse, it became apparent that conventional morphological techniques are limiting in their ability to distinguish cell types other than the mature cell of each cell line.

In response to MPA or *Listeria monocytogenes* infection numerous leucocyte types arise which cannot be classified as either lymphocytes or monocytes but appear to have morphological characteristics somewhat in between these two cell types. It was therefore impracticable to study the origin of monocytes under these circumstances. Realizing that this problem was due mainly to the difficulties inherent in the morphological approach used to classify leucocyte types; it became of practical importance to attempt to classify leucocytes by means other than those of conventional morphology.

An immunological approach to this problem might provide

the answer. Can leucocyte types be distinguished by their antigenic components? If there are leucocyte type specific antigens, and the literature certainly suggests that this is so, then fluorescent antibody studies using the cell specific antigen-antibody reaction might be a useful aid in the identification and classification of leucocyte types.

However, before this approach can be undertaken it was essential to be able to separate out the individual leucocyte types from the peripheral blood of normal animals.

The following study presents methods for the separation of leucocyte types of both chicken and human peripheral blood with an assesment of the viability of these isolated leucocyte types.



A Review of the Limitations of Conventional Morphological  
Techniques in Distinguishing Leucocyte Types

The development of the Romanowsky type stains has led to a system for the classification of leucocytes (121). This classification is based on morphological and tinctorial characteristics of the cell and as such is adequate for distinguishing the mature leucocyte of each cell line. It is, however, far less suited to characterizing immature cells or cells whose morphological characteristics overlap.

The terminology itself suffers from being developed to a large extent on only one species, man. The monocytes of rat, mouse and chicken, with whose blood the author is familiar, do not comply with the morphological description given to human monocytes. There is an even greater morphological difference between granulocytes of these species, making the terminology based on this morphological approach completely misleading. Lucas discusses this point in further detail (74).

The lymphocytic and monocytic series have to a certain extent an overlapping of morphological characteristics. Maximow and Bloom (81) in their "Textbook of Histology" comment:-

"When preparations of blood are examined and viewed objectively, it is seen that

the nongranular leucocytes consist of a series of transitional forms which begins with the smaller lymphocyte and ends with larger cells of quite different appearance, the monocytes ... but in the mid-portion of this series of transition is a group of cells which cannot be classified as either typical lymphocytes or typical monocytes."

Suffice it to say that thirty-four years later Bloom and Fawcett (10) make an identical statement. It is apparent that the conventional morphological approach to leucocyte identity and relationships many years ago reached the limit of its usefulness, particularly as a research tool. The diversity of opinions regarding the interrelationship of leucocytes is adequate proof of this state of affairs.

The appearance of cells is often modified by the techniques employed, further limiting the value of a classification based purely on morphology. Kindred (64), using the rat, showed striking differences in appearances of the nuclei of cells when prepared by smear and section methods. A second demonstration of this point is provided by Kirschbaum and Downey (65) when they compared some of Maximow's (80) drawings made from colloidin sections with cells prepared by air drying. Such differences merely add to the confusion about leucocyte interrelationships.

The following controversial topics are the direct result of the limitations of conventional morphological techniques.

*Listeria monocytogenes* can in certain species provoke an increase in large mononuclear cells. Using animals infected with *L. monocytogenes*, Witts and Webb (122) propounded that the monocyte was a distinct cell line with its own stem cell,

whilst Bloom (8) and later Conway (22, 23) favoured transition from lymphocytes.

The identity of the leucocyte which gives rise to the macrophage is still a matter for debate. Rebeck and Crowley (92) favour the view that they are derived from small lymphocytes. Volkman and Gowans (115) have shown that lymphocytes from lymph and lymphoid tissue do not give rise to macrophages but that cells from the bone marrow, which cannot be distinguished from lymphocytes by conventional morphological means, seem to play a roll in macrophage production. Osmond and Everett (89) have reported the production in the bone marrow of cells resembling small lymphocytes.

Conventional morphology cannot tell us if there are different types of small lymphocytes. If there is more than one type, then the apparent contradictions of the above paragraph are no longer present. Yoffey (124) in giving his reasons why the lymphocyte problem is so controversial states:-

"Lymphocytes which look alike may in fact be quite different in their subsequent development."

There are many recent reports which suggest that there are at least two types of small lymphocytes. It is now well documented that two types of small lymphocyte exist in the chicken; lymphocytes of the thymus and the Bursa of Fabricius have different immunological properties (24, 25). There is suggestive evidence that this is the case for man also (15, 27, 48).

The electron microscope has revealed subtle differences

in lymphocytes. Braunsteiner and Pakesch (14) have observed lymphocytes in the thoracic duct with features characteristic of plasma cells. More recently in their studies of antibody producing lymph node cells Harris, Hummeler and Harris (54) raise the same question. Are there in fact two cell lines, lymphocytic and plasmacytic producing antibody or are the forms that Harris et al observed representative of different stages in the development of a single cell line? Conventional morphology can supply no answer. Johnson, Schnappauf, Chanana and Cronkite (61) have also shown differences in lymphocyte ribosomal aggregation in lymph depleted calves.

Perhaps one should mention the so-called "specific methods" of cell identification which have been used to help distinguish lymphocytes from monocytes.

Sabin's (95) supravital stain has been used by many workers who have used the "rosette" formation in the centrosome as a specific property of monocytes. However it is still uncertain as to how specific this "rosette" really is. Bloom (9) maintains that the "rosette" is not specific for monocytes as he has seen such "rosettes" in 50% of the circulating lymphocytes in the rat. It is interesting to recall that Hall (9a) also found "rosette" formation in lymphocytes from the monkey.

Leucocytes have also been credited with the ability to move in specific, defined ways characteristic of the cell type in question. Bloom (9) again has cast doubt upon this as a specific criterion for distinguishing cells. His studies with time lapse photography led him to conclude that the environment

through which the cell was moving was of greater importance in determining the type of motion exhibited than the cell type itself.

In the granulocytic series, the process of identification is greatly aided by the specific inclusions that these cells contain. However, the problem of identification remains when one considers the immature cells of this series. The greater the degree of immaturity of the cell, the greater the difficulty in placing it in any series, be it monocytic, lymphocytic or granulocytic. Such difficulty has resulted in the unitarian, neo-unitarian, dualistic and trialistic theories of blood cell interrelationship.

In summary, conventional morphology is neither specific nor sensitive enough to reveal subtle important differences in cells. Nor does it reveal much about the function of the cell which should form the basis of any classification. Secondly it is too prone to human interference in that, the final conclusion drawn from a morphological study depends to too large an extent on the personal whims of the individual. As Lucas (75) so adequately states:-

".... but the diversity of opinions on hematologic principles of cell relationships is evidence enough that such an approach (morphological\*) falls short of carrying the weight often assigned to it."

Recently the extent to which the more modern techniques can help in answering the questions raised by morphological

\* Authors insertion

studies, has been criticized as follows:-

"Unfortunately (as Carter wisely makes clear) modern techniques such as cytochemistry, electron microscopy, autoradiography and cell-culture have so far failed to resolve the basic uncertainties which morphological studies have left. In short, we still do not know what the atypical mononuclear cells are, what kind of cell they are derived from, what they are doing, why they circulate in the blood, or what becomes of them." (69)

A Review of the Methods for the Separation  
of Leucocytes from Whole Blood

The difficulty of isolating leucocytes from whole blood and separating them into the various cell types has been a major stumbling block in leucocyte research. The many existing methods are evidence that no one method in itself is entirely satisfactory. The following is a review of the basic techniques used in the separation of leucocytes.

Sedimenting Agents

Early workers prepared leucocyte suspensions by allowing leucemic blood to settle, thus obtaining a leucocyte rich plasma. This made use of the tendency of erythrocytes to settle more rapidly than leucocytes. This differential settling is greatly enhanced by plasma components which, when mixed with the blood, encourage the erythrocytes to form rouleaux (35). Gray and Mitchell (51) studied the effect of four purified plasma protein preparations; albumin,  $\gamma$  globulin, fibrinogen and a fraction containing  $\alpha$  and  $\beta$  globulins, in the sedimentation rate of erythrocytes. Fibrinogen was found to be the most effective whereas albumin apparently inhibited the sedimentation of erythrocytes.

In 1948, Minor and Burnett (84) employed fibrinogen to separate leucocytes from whole human blood. Buckley, Powell and Gibson (16) defined the conditions for optimum separation using fibrinogen. Phytohaemagglutinin has been used since 1916 (32) for the separation of leucocytes from erythrocytes and has been used more recently for this purpose by Li and Osgood (72). However the recent demonstration that phytohaemagglutinin is able to induce proliferation and transformation of leucocytes rather limits its use for the separation of leucocytes (59, 87, 125).

Numerous substances are now used to enhance erythrocytes sedimentation with dextran (17, 70) and polyvinylpyrrolidone (P.V.P.) (13) being used most frequently. A comparative study on the use of several agents has been presented by Skoog and Beck (102). Gold and Cole (47) separated leucocytes from erythrocytes by layering whole blood over a 28.5% P.V.P. solution in 0.9% saline. Erythrocytes at the blood / P.V.P. interface formed rouleaux and sank into the P.V.P. solution until finally only a layer of leucocytes remained at the interface. Between 70 and 80% of the original leucocytes were obtained 50 minutes after layering the blood on top of the P.V.P. solution. Boyum (11) used essentially the same principle with a mixture of methylcellulose and N-methyl-3, 5-diacetamido, 2-4-6-trijodbenzoas (Isopaque). Different leucocyte yields could be obtained by varying the proportions of methylcellulose and Ispaque.



The sedimentation procedure as a whole has several disadvantages. The yield of leucocytes varies from 30 to 80% (102) of the total leucocytes in the whole blood. The agents are difficult to wash off the surface of the cells and so may interfere with subsequent experiments, e.g. dextran could interfere with carbohydrate studies. There is always some degree of erythrocyte contamination. Lastly, the formation of rouleaux and the subsequent sedimentation occurs best in the temperature range of 12°C - 25°C. Lower temperatures result in slower rouleaux formation, probably due to reduced molecular movement and increased viscosity. Thus the optimum temperature for rouleaux formation conflicts with the optimum temperature (4°C) for the maintenance of leucocyte metabolism (111).

#### Selective Destruction of Erythrocytes

The use of sedimenting agents in the separation of leucocytes from erythrocytes results in appreciable erythrocyte contamination. These erythrocytes can be selectively lysed by one of the following substances; saponin (97), gramicidin, lysolecithin (101), anti A or anti B haemolytic sera (29), alcohol - water mixture (39), distilled water followed by concentrated buffer solution to produce osmolarity (117), and acetic acid - tartaric acid mixture (108).

These substances, however, also have an injurious effect on leucocytes e.g., saponin will eventually cause lysis (118), anti A or anti B sera causes clumping and a risk of

erythrophagocytosis (17) and acetic acid - tartaric acid affects respiration of the cells (57). On the whole, selective lysis is rather a crude procedure which damages the leucocytes to varying degrees.

#### Methods Requiring Centrifugation

Various types of equipment have been designed to separate leucocytes from erythrocytes. Maupin and Chary (79) centrifuged whole blood until a buffy coat formed. A high density solution was then added via a side arm so that the contents of the bottle were forced upward through an inverted glass cone. The buffy layer elongated in the narrow part of the cone and so could be removed without disturbing the erythrocytes. Aeberhardt (1) produced a similar apparatus for handling small quantities of blood. Use of a plastic tube instead of the glass cone enabled Juhlin and Shelley (62) to slice the elongated buffy layer and so obtain sections with cells of predominantly one type.

By centrifugation, in an oil bottle, of the leucocyte rich supernatant obtained by dextran sedimentation, Green and Soloman (52) were able to prepare a platelet layer, lymphocyte-monocyte layer and polymorphonuclear leucocyte-red cell layer. Recently an instrument has been devised by Freireich, Judson and Levine (44) for the separation and collection of leucocytes on a large scale. This instrument is still in the experimental stage but has achieved a degree of success in that fractions consisting of predominantly one cell type can be obtained.

However, contamination by other cell types is rather high and the equipment is complex and requires a long time for cleaning and assembly. As there is great need for a simple method for large scale production of separated leucocyte types, this approach has much to commend it but it remains to be seen whether it can be developed further. There is some evidence that the high centrifugal force and the long period of centrifugation required by the above methods result in deterioration of leucocyte function (77).

If one is prepared to sacrifice yield for purity these techniques can produce relatively pure samples of leucocytes. The yield of granulocytes and monocytes is poor however due to their proximity to the erythrocyte interface so that high yields of these cell types are accompanied by gross erythrocyte contamination.

Gum acacia (103) and bovine albumin (36, 112) have been used to prepare fluids of different specific gravities for use in floatation techniques. A solution of the desired specific gravity is made and the whole blood layered onto this solution. After centrifugation the erythrocytes are found at the bottom of the tube whereas the leucocytes remain at the albumin / plasma interface because of their lower specific gravity. Both albumin and gum acacia require extensive reworking to bring them within range of the physiological conditions required by the cells. Albumin gives an incomplete separation from erythrocytes and produces severe clumping at the plasma / albumin interface (16, 36). Some

doubt as to the viability of the leucocytes so produced has been expressed (16). Weiss and Fawcett (119), on the other hand, used albumin for the preparation of leucocytes and cultured the cells for a histochemical study on macrophages. This shows that at least some cells were viable after this procedure. Szenberg and Shortman (107) have used albumin to fractionate lymphocytes from chicken peripheral blood. At pH 7 pronounced clumping occurred and they therefore used pH 5.5 at which clumping was inhibited. The large range in the specific gravity of a particular cell type found by these workers is, in our experience, suggestive of overloading the gradient. The use of pH 5.5 is also open to question. The greatest disadvantage in the use of albumin or gum acacia is the time required for their preparation. Albumin has the added disadvantage of being very expensive.

Biggers and McFeely (7) used a polysucrose material (Ficoll) as a floatation medium for the separation of leucocytes from erythrocytes of ox blood. The authors state however that there was incomplete separation of leucocytes from erythrocytes.

#### The Separation of Individual Leucocyte Types

A frequently used method of obtaining a relatively pure sample of a leucocyte types has been the production of exudates by injection of irritant substances into either the peritoneal or pleural cavity. Walford (116) gives details of the irritants used and the differential counts of the cellular exudates

produced.

There are two disadvantages to this method of producing leucocytes; firstly there is always a fair degree of erythrocyte contamination and secondly, due to the lack of knowledge concerning the interrelationships of leucocytes, particularly lymphocytes, monocytes and macrophages, one cannot be sure of the origin of the cells found in exudates. An example of this is in "monocyte-macrophage" exudates where the cells not only have different properties regarding dye uptake when compared with blood monocytes, but also show different morphological characteristics i.e. cells having monocytic and lymphocytic characteristics are found (21, 34, 43, 92, 114, 122).

Fairly pure lymphocyte preparations have been obtained by several methods. Green and Soloman's work using an oil bottle has already been referred to (52). The separation of lymphocytes from granulocytes was achieved by Kline (66) who used a swimmer. The lymphocyte fraction was about 90% pure but granulocytes and erythrocytes were mixed together. Ficteluis (38) obtained lymphocytes from rabbit blood by passing it through absorbent cotton and allowing a one hour incubation, during which the granulocytes adhered to the cotton fibres. Lymphocyte suspensions in the order of 100% pure were apparently obtained. Gentle centrifugation of heparinized blood has also produced pure suspensions of lymphocytes (60). Using chicken blood, Terasaki (109) produced lymphocytes 100% pure by allowing the granulocytes

and monocytes to adhere to the sides of a glass tube before carrying out gentle centrifugation.

Thierfelder (110) used iron particles suspended in dextran to prepare pure suspensions of lymphocytes for use in agglutination tests. Phagocytes, after ingesting the particles, settled with the erythrocytes leaving the non-phagocytic, lighter lymphocytes. The purity of the suspensions varied from 95 to 99.8% but the final yield was only in the order of 30 to 50%.

Coulson and Chalmers (26) used defibrinated blood with a 3% w/v solution of gelatin in physiological saline and after standing for thirty minutes at 37°C the gelatin-blood mixture settled into distinct layers. The lower layer contained erythrocytes, monocytes and granulocytes, whereas the upper layer contained 90 to 99% lymphocytes with eosinophils forming a significant proportion of the contaminating cells. Cassen, Hilt and Hays (18) modified the procedure of Levine (71) and by centrifugation removed the heavier iron-containing neutrophils to obtain a pure suspension of lymphocytes.

Granulocyte suspensions have been prepared mainly from exudates (90), although they have also been prepared from peripheral blood (85). Granulocyte suspensions of 90% purity, have been obtained by using two iso-osmotic albumin solutions of different specific gravity (2). Levine (71) obtained neutrophils by allowing them to ingest starch iron particles and then transferred them to a dish mounted over

an electromagnet. The neutrophils stuck to the bottom of the dish while the other cell types were removed by gentle washing. Dioguardi, Agostoni, Fiorelli and Lomanto, using repeated centrifugation of blood with 0.83% ammonium chloride to remove the erythrocytes, and recentrifugation in Tyrode's solution to remove lymphocytes and platelets, obtained granulocytes with reduced lymphocyte numbers but with normal monocyte numbers as contaminants (30).

Morphology as judged by electron microscopy was excellent.

"Monocyte" suspensions have been prepared by the exudate technique and objections to this method have already been discussed. Recently Bennett and Cohn (5) presented a method for the separation of monocytes from horse blood. The erythrocytes were allowed to settle and the leucocyte rich supernatant was mixed with an albumin solution previously diluted with phosphate saline to produce a final albumin concentration of 27%. After centrifugation at 2,400g for 36 minutes at 12°C, the less dense monocytes and lymphocytes were concentrated in a pellicle above the pellet containing erythrocytes and granulocytes. Use was then made of the adhesive property of monocytes to separate them from the lymphocytes. The only criticism of this method is the high centrifugal force required for the formation of the pellicle.

Durrin, Keppie and Smith (33) devised a large scale method for the separation of phagocytes and lymphocytes. Blood was centrifuged in polythene bottles and the buffy

layer floated off by "dense plasma." A separation of phagocytes and lymphocytes was obtained by layering the buffy coat on top of a layer of dense plasma; the phagocytes tended to sink while the lymphocytes remained suspended. The process was repeated for further purification. The lymphocytes obtained were reasonably pure but the granulocytes contained numerous lymphocytes as contaminants.

Wildy and Ridley (120) have presented a procedure for the preparation of granulocytes and monocytes. Sedimentation was first performed and the leucocyte rich supernatant was placed in a Roux bottle coated with "Vaspar" (equal parts of vaseline and paraffin wax). After one hour incubation at 28°C, the granulocytes and monocytes adhered to the wax whereas the erythrocytes and lymphocytes were removed by washing with buffered saline. Buffered EDTA solution was then used to remove the granulocytes and monocytes from the wax. The yield of leucocytes was quite low, in the region of 20%.

The separation of lymphocytes, granulocytes and monocytes from the same sample of blood is difficult. It was not until 1964 that a method was presented by Rabinowitz (91), who based his work on both Wildy and Ridley's use of a chelating agent to remove adhered cells, and on Garvin's (46) excellent study of the factors influencing leucocyte adherence to glass beads. Basically the method consists of the preparation of a leucocyte-rich supernatant by using dextran as a sedimenting agent. The suspension of leucocytes



is then incubated for 30 minutes at 37°C on a glass bead column. Lymphocytes and erythrocytes, platelets, granulocytes and monocytes are eluted from the column by fresh plasma, 20% fresh plasma in BSS and 0.02% EDTA solution respectively. With EDTA there is an overlap between the elution peaks of granulocytes and monocytes but a further separation is obtained by replacing the cells on a smaller column, allowing the cells to adhere and eluting with EDTA solution again. Fairly pure samples of individual cell types are obtained by this method but the yields are very low.

The methods of cell separation presented make use of one or more of the properties of the individual cells. There is however a considerable overlap of these properties, which makes the separation of individual cell types very difficult. There are numerous variations based on the above methods but these have not solved the problem of obtaining pure samples of the various leucocyte types, in high yield, from the peripheral blood.

A Review of the Literature on the Specificity  
of Leucocyte Antigens

The recent interest in the study of leucocyte antigens is due to their possible identity with transplantation antigens. Many leucocyte antigen groups are now known, but our knowledge of specific antigens to the various types of leucocytes is very limited. Of particular interest to this study are those cell components which are antigenic, and which confer on the individual leucocyte type such characteristics and properties pertinent to its specialized function.

This review therefore will be concerned only with work which suggests that there might be antigens specific to individual leucocyte types.

One of the earliest studies of anti-leucocyte-antibody was that of Metchnikoff (82, 83), in which, guinea pigs were used to prepare an antiserum against rabbit lymph nodes. The resulting antibodies proved to be active against granulocytes as well as lymphocytes, and both leucolysis and leucoagglutination were observed. Funk (45) used granulocytes obtained from peritoneal exudates produced by broth, and mononuclear cells from exudates produced by pilocarpine. The anti-

mononuclear serum had the same effect on both mononuclear and polynuclear leucocytes. However, using rabbit bone marrow, a serum was obtained which proved to be somewhat more specific, the polynuclear cells being destroyed more rapidly than mononuclear cells.

These studies, all carried out around the year 1900 were the first attempts to examine and compare the antigenicity of leucocytes. Almost 30 years passed before this aspect of haematology was studied again. For convenience, further discussion of leucocyte antibodies will be on the basis of the type of antigen-antibody reaction encountered.

### Agglutinins

Agglutination has been the method most widely used for detecting and comparing leucocyte antibodies. False positive results due to non-specific clumping are an ever present possibility. The difficulties involved in this test and the reasons for these difficulties have been discussed in detail by Walford (116).

Steinberg and Martin in 1944 (105) were the first to successfully titrate an antileucocyte serum by agglutination. In 1945 (106) these workers produced evidence that leucocyte types could be distinguished antigenically. They used leucocytes from individuals with acute and chronic myelocytic leucemia, acute and chronic lymphocytic leucemia, normal blood and normal lymph nodes. Leucocytes of acute myelocytic leucemia were agglutinated only by their own antiserum, which

also agglutinated cells from chronic myelocytic and lymphocytic leucemias. Leucocytes of chronic myelocyte leucemia were not agglutinated by antisera to normal lymph node and chronic lymphocytic leucemia, which had been absorbed with granulocytes. Varying titers were observed with other leucocyte types when compared with the antisera. The authors concluded from this study that mature lymphocytes, mature granulocytes, immature lymphocytes and immature granulocytes possess specific cell antigens. No differences between chronic leucemia and normal leucocytes were detected.

Finch, Ross and Ebaugh (41) produced an antisera in rabbits against human granulocytes and lymphocytes. The rabbit anti-human-granulocyte-antisera produced marked agglutination of human leucemia granulocytes, but not of lymphocytes. On the other hand, the anti-lymphocyte sera produced slight agglutination both of leucemic granulocytes and of lymphocytes. Andre, Dreyfus and Bessis (4) also were able to distinguish between granulocytes and lymphocytes antigenically. A non-lytic, thermostable, neutrophil agglutinin and a lytic, thermolabile, lymphocyte agglutinin were obtained. After two absorptions with lymphocytes, the lymphocyte agglutinin could be removed without effecting the granulocyte agglutinin, although repeated lymphocyte absorption weakened the latter.

Lille-Szyszkowicz (73), in 1956, compared the antigens of several cell types. The thermolabile, alcohol-soluble

antigens of both normal and exudate leucocytes were similar. A distinction could be made, however, between immature and mature neutrophil antigens, the former being alcohol-insoluble. Thermolabile, alcohol-insoluble antigens of normal and leucemic lymphocytes were similar, but were quite distinct from immature granulocyte antigens. These results are similar to those of Steinberg and Martin which have already been mentioned.

In a study of neonatal neutropenia, Lalizari and Bernard (68) detected a new antigen specific for neutrophils which was responsible for maternal immunization and the subsequent development of neonatal neutropenia.

### Precipitins

As early as 1922, Heptoen and Menne (55) demonstrated in three animal species, dogs, guinea pigs and humans, that there existed in leucocytes precipitins which were not found in red cells, platelets or blood serum. Leucocytes, therefore, could be distinguished antigenically from other cells.

A considerable amount of data on leucocyte precipitins has been collected by Seligmann and co-workers at the Pasteur Institute. In 1954 (98) this group applied a gel diffusion technique to the analysis of leucocyte antigens in cells obtained from various blood leucemias. Five sera rich in leucocyte precipitins reacted with lysates of leucocytes from normal and chronic myeloid leucemic blood, but not with a lysate of leucocytes from chronic lymphocytic leucemia.

These results are similar to the agglutination studies in that lymphocytes and granulocytes could be differentiated antigenically.

In the same year (49) they showed that washed lysates of leucocytes did not contain any detectable serum proteins. Using Ouchterlony gel diffusion techniques, they found that normal leucocytes contained three or four antigens which were not detected in leucemic leucocytes. No difference was obtained in the number of precipitin bands formed when whole cells or lysates were used to raise the antiserum. However, the route of injection appeared to be of importance as intravenous and subcutaneous injections produced marked differences in the number and distribution of the precipitin lines (50, 99).

Using immunoelectrophoresis as well as simple gel diffusion, Seligmann, Grabar and Bernard (100) have detected up to ten different leucocyte antigens. In the discussion of this paper, Seligmann makes this pertinent statement:-

"Il est très difficile de comparer les antigènes d'un granulocyte normal à ceux d'un lymphocyte ou d'un monocyte normal, car on ne peut pas isoler du sang chacune de ces variétés de cellules à l'état pur."

Walford, Peterson and Doyle (117) prepared leucocyte antibody in rabbits and demonstrated only two antigens to human leucocytes. This poor response as compared with Seligmann's work reflects on the method of preparing the lysates. The freeze-thaw method used by Walford et al is

inferior to that of Seligmann, who used ultrasonication, for the production of lysates with a high protein content.

Korngold, van Leeuwen and Miller (67) have given details of their method for the preparation of human leucocyte antigens. Normal leucocytes and leucocytes from patients with chronic and acute leucemia were used. The antisera to these leucocytes were compared with a variety of tissue cell antigens also. Analysis of the antisera after the appropriate absorptions revealed an antigen specific for granulocytes, but no antigen specific for lymphocytes was detected. However, it is interesting that lymphocytes from different patients varied in their content of several antigens.

#### Miscellaneous (Cytotoxic)

Grouped together under this heading are the few experiments in which the antigen-antibody reaction has been assessed by techniques other than either agglutination or precipitation.

Several experiments have been attempted to demonstrate specific antibody by injecting an immune serum into an animal, and noting the decrease in the circulating level of a particular cell type. It is quite possible that this decrease could be brought about by a mechanism which is not of an immunological nature. An experiment which suggests that such an alternative mechanism exists has been done by Woodruff and Forman (123). These authors used an

anti-lymphocytic serum known to be potent *in vivo*, yet failed to demonstrate any cell lysis, agglutination or increase in dye uptake when lymphocytes were exposed to the serum *in vitro*.

Extracts of the adrenal cortex are known to effect levels of circulating leucocytes. In this respect it is of interest that Robertson (93) found that adrenal cortical extracts which were anti-lymphocytic *in vivo*, had no apparent effect on lymphocytes *in vitro*.

Chew, Stephens and Lawrence (20) produced granulocyte exudates in the peritoneal cavity of guinea pigs and used rabbits to produce the antisera. Upon injection of the antiserum into guinea pigs, the neutrophil granulocytes disappeared almost completely from the circulating blood. Little or no effect on the level of lymphocytes, monocytes or basophils was seen. The lack of large numbers of neutrophils in the many tissues they examined, led them to conclude that neutrophils were rapidly disintegrated upon contact with the antibody. They showed also that neutrophils were not available for local tissue reaction to subcutaneous injection of virulent staphylococci, or to the intra-abdominal injection of a beef broth-gum acacia mixture in animals made neutropenic by the injection of an anti-neutrophilic serum.

In a similar experiment using lymphocytes from teased lymph nodes, injection of the anti-lymphocytic serum produced a marked lymphopenia which lasted for about 40 hours after



a single injection. The levels of the other leucocyte types were similar to those obtained when control sera were given (19).

Doan and Sabin in 1930 attempted to prepare specific anti-sera to monocytes, using the lungs of tuberculous rabbits as antigen and raising the anti-sera in Jersey Giant roosters. A serum was obtained which depressed the level of circulating monocytes to a greater degree than that of any other cell type (31).

Finch (40), using cytoplasmic fractions of exudate granulocytes for the production of an immune sera, obtained persistent granulocytopenia in all the animals injected. Mitochondrial and microsomal fractions appeared to be the most potent.

The results of all the experiments carried out on leucocyte antigens suggest that different leucocyte types do possess different antigens. The inability to produce relatively pure samples of the various leucocyte types has been the major limiting factor to the study of leucocyte antigens. Various leucemic bloods have been used because they offer leucocytes of a predominant cell type. Unfortunately, leucemic blood is a poor starting point for a comparative study of specific leucocyte antigens, for not only are the cells in various stages of development but they also have different morphological characteristics compared to normal cells. Exudate techniques produce cells of reasonable purity with regard to cell type but unfortunately, these cells

have morphological differences when compared to the corresponding cell type of normal peripheral blood.

Finally, the work of Lundkvist, Goeringer and Perlmann (76) demonstrates that immunological characterization of cell types is possible, providing relatively pure samples of the cells can be obtained for immunization and adsorption. Rat liver parenchymal and reticuloendothelial cells were separated on a sucrose density gradient and the anti-sera to these cell types revealed quite distinct differences in antigenic composition. Fluorescent antibody staining of tissue sections substantiated the specificity of the antisera.

## Experimental

### The Separation of Chicken Leucocyte Types

#### Introduction

Chicken blood was used initially because of its high monocyte count - up to 5,000 per cu. mm. - as compared with mammals. Chickens are easily bled by heart puncture, and up to 50ml. of blood can be withdrawn from an adult bird, or 20ml. from a 4 month old bird, with no apparent effects on the health of the chicken. White leghorns of a lymphomatosis resistant strain were supplied by Dr. Jerome, Department of Poultry Science, University of Guelph, or were hatched from eggs obtained from the same source and raised in this laboratory. Only cocks were used in this study as the circulating blood of the laying hen contains a large amount of fat. On the whole, chickens provided blood with a high leucocyte count per cu.mm. with abundant numbers of all cell types and a low erythrocyte to leucocyte ratio. The leucocyte types found in the peripheral blood of chicken and human are shown in plates 1 and 2.

#### Development of the Method

In order to compare the antigens of the various types of leucocytes, it was essential to be able to separate these cell types from the peripheral blood.

30  
Plate 1

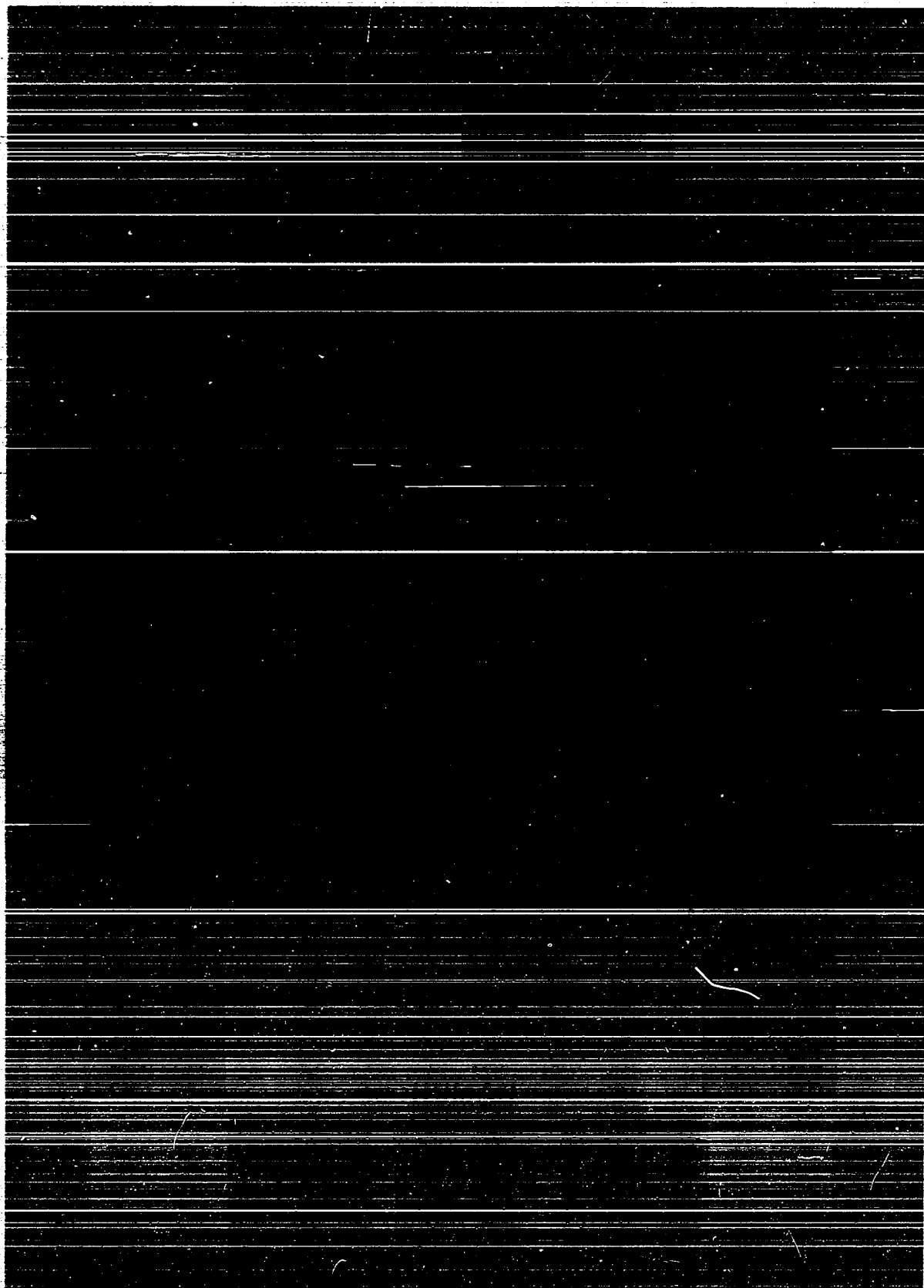
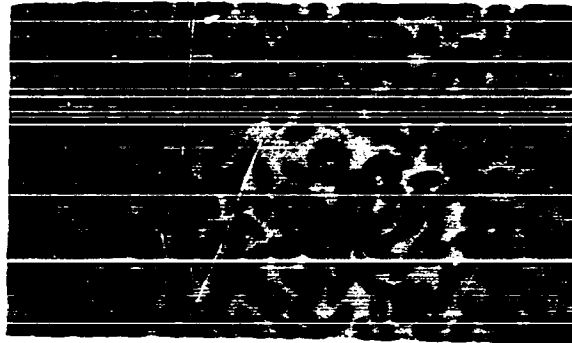
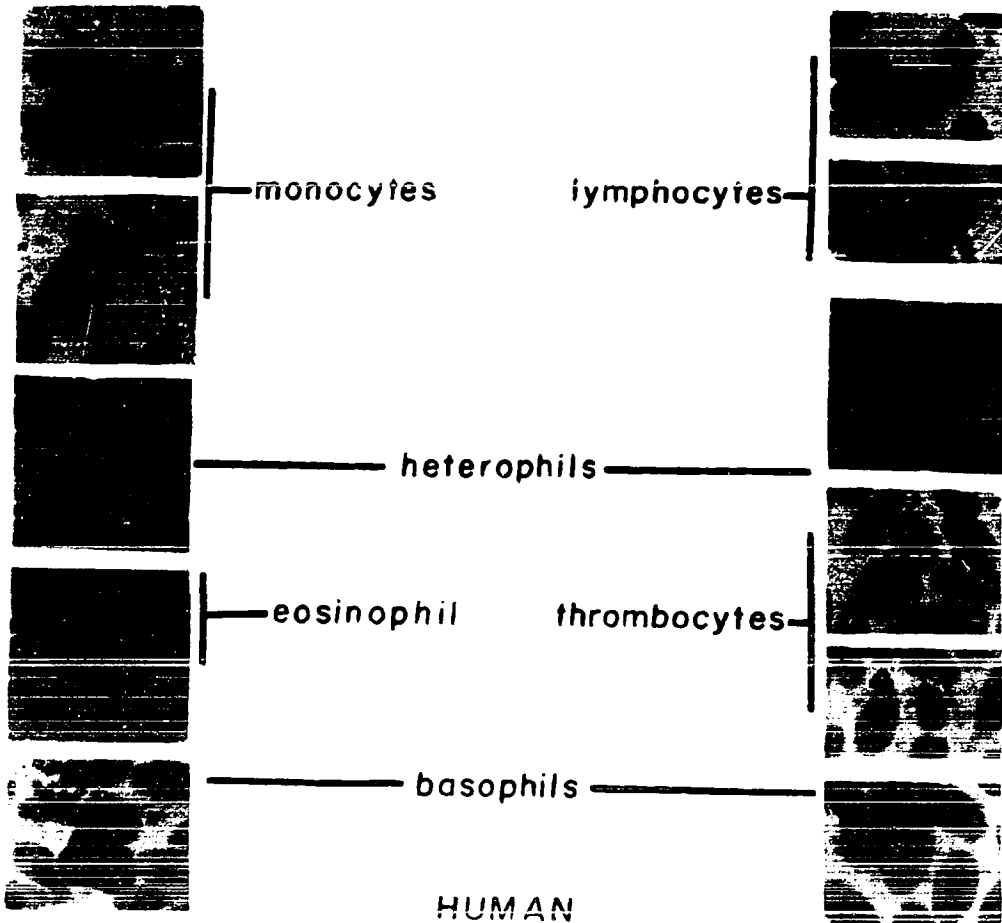


Plate 1

CHICKEN



Peripheral Blood



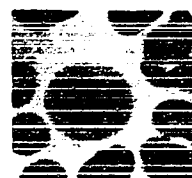
HUMAN



monocyte



lymphocyte



granulocyte

**Legend Plate 2**

**Rbc = Erythrocyte**

**H = Heterophil**

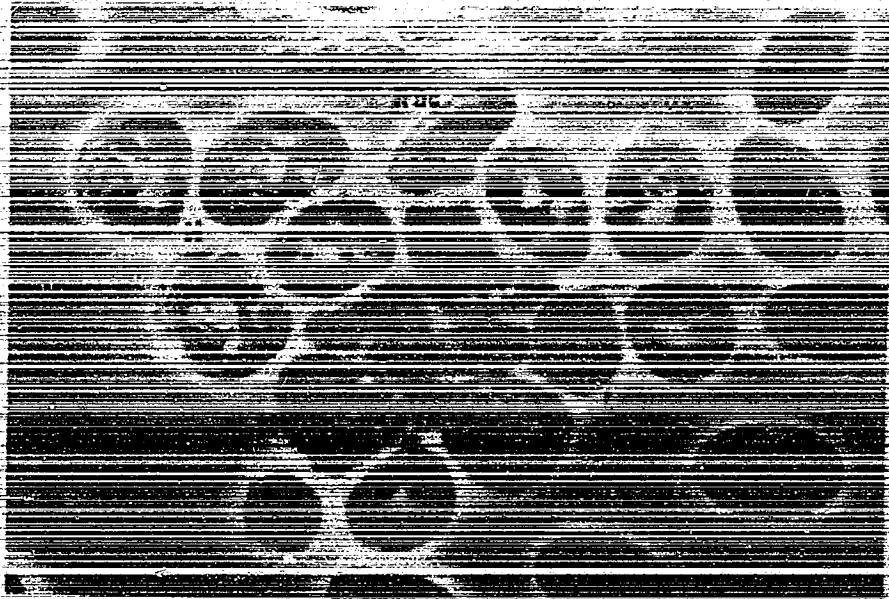
**Th = Thrombocyte**

**M = Monocyte**

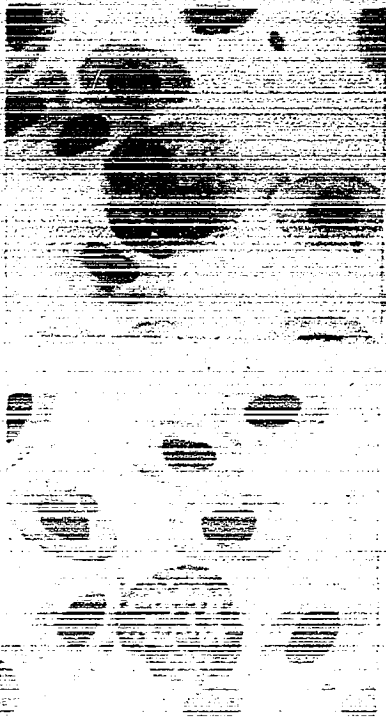
**L = Lymphocyte**

**E = Eosinophil**

# CHICKEN BLOOD LEUCOCYTES



WRIGHTS STAIN



PHASE CONTRAST



MONOCYTE

NEUTROPHIL

LYMPHOCYTE

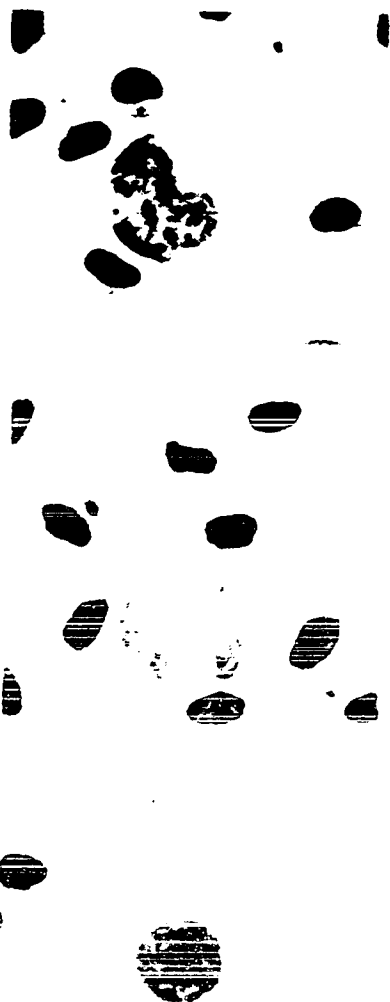
3

CHICKEN BLOOD LEUCOCYTES



WRIGHTS STAIN

PHASE CONTRAST



MONOCYTE

HETEROPHIL

LYMPHOCYTE





Prior to 1964 most leucocyte preparations were obtained from cell exudates, and these stimulated cells differed in many respects from those found in the circulating blood of the normal animal. For this reason leucocytes from normal peripheral blood would be the most suitable for a comparative study of leucocyte antigens.

In 1964, Rabinowitz (91) presented a method for the separation of the various types of leucocytes from human peripheral blood, using dextran to increase the sedimentation of erythrocytes, and a column of glass beads to separate the various types of leucocytes. Difficulties were encountered in applying this method to the separation of chicken leucocytes. In smears, or in phase contrast preparations of some species of mammalian blood, it is not uncommon to see erythrocytes in rouleaux formation. The initial step in the method of Rabinowitz takes advantage of the ability of dextran to encourage the formation of rouleaux by erythrocytes and thus increase their sedimentation, leaving a leucocyte rich supernatant. Unfortunately, the chicken erythrocytes, in common with red cells of other species, (96) showed nothing comparable to rouleaux formation, and the first stage of this method could not be used.

Separation of blood leucocytes was attempted by incubating chicken whole blood on 200 $\mu$  glass beads\* packed into a column 1cm. diam. x 20cm. length. The column was

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\* 3M; Siliconised with Siliclad, Clay Adams.

incubated at 37°C for one hour, and then eluted with plasma/Hank's BSS\* and EDTA\* in the same manner as in the method of Rabinowitz. Erythrocytes and lymphocytes were eluted from the column by plasma/HBSS and gentle centrifugation (5 minutes at 50g.) of the elute produced a relatively pure lymphocyte suspension in the upper portion of the supernatant. Elution with EDTA solution resulted in the appearance of granulocytes heavily contaminated with erythrocytes and thrombocytes. Monocytes were not recovered. The gross contamination of the granulocyte fraction was thought to be the result of erythrocytes sedimenting onto the glass bead surface and so becoming enmeshed by granulocytes, monocytes and thrombocytes, which readily adhere to the glass.

In an attempt to overcome this effect, whole blood was recirculated through a 200 $\mu$  glass bead column at various flow rates. The slow movement of the blood over the glass beads prevented the erythrocytes from becoming trapped, but allowed the granulocytes and monocytes to become attached to the glass beads. Differential counts on the effluent blood from the column showed that most of the granulocytes and monocytes adhered to the glass bead column after one hour incubation at 37°C. Elution in the same manner as that previously described resulted in lymphocyte and granulocyte fractions. Although contamination by erythrocytes was much reduced, thrombocyte contamination was still appreciable.

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\* Appendix II

Chicken granulocytes that were eluted from the column with EDTA solution, showed marked deterioration, in the form of severe swelling of their granules if left for more than 10 minutes in the EDTA solution at room temperature. No monocytes appeared despite continued elution with EDTA.

The failure of EDTA to remove chicken monocytes that were adherent to a glass surface has been noted also by Terasaki (109). Cohn and Benson (21) similarly could not remove mouse macrophages from glass with EDTA and they finally resorted to alternate freezing and thawing to remove the cells.

In our experience, alternative freezing and thawing of chicken leucocytes, obtained from the buffy coat produced by centrifugation, readily lysed all cell types. Cooling the column to 4°C did not release chicken monocytes from the glass beads, and incubation with trypsin\*, neuraminidase (800 units)\*\* and lysozyme (800 $\mu$ g)\*\* were equally unsuccessful in dislodging the adhered monocytes. Leucocytes obtained from buffy coat preparations, and resuspended in HBSS, were incubated with these substances at the same concentration as was used for the column incubation; morphologically no adverse effect was seen on the cells within three hours. Incubation of one hour would not therefore be likely to lyse the monocytes on the column.

Thus, the second difficulty encountered in the application of Rabinowitz's method was the failure of EDTA to remove monocytes from siliconised 200 $\mu$  glass beads. This presented

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\* 4 ml. of 0.5% 1:250 Difco Trypsin in HBSS. Balance to 16ml. column volume produced by HBSS.

\*\* In 1 column volume 16ml. HBSS.

a greater problem than the inability of sedimenting agents to increase the sedimentation rate of chicken erythrocytes.

Wildy and Ridley (120) used wax-coated surfaces as a medium for the separation of leucocytes from erythrocytes, and it seemed possible that wax might provide a medium suitable for the separation of chicken monocytes.

The mixture of vaseline and paraffin wax (vaspar) used by Wildy and Ridley, was found to have too high a melting point, but by mixing varying proportions of paraffin wax, (mp. 56°C) and liquid paraffin, mixtures could be obtained which had any desired melting point. Unfortunately, the liquid paraffin component came out of the mixture and floated on top of the balanced salt solution whenever the two came into contact.

From the homologous series of paraffins, eicosane and hen-eicosane\* were chosen as having suitable melting points of 35.5 to 37°C and 41°C respectively. Although the temperature of chicken blood is 41°C, eicosane was chosen because a temperature could be used in excess of the melting point of the wax and yet below the normal temperature of chicken blood. Glass beads were used as a support for the wax instead of waxed petri dishes, for the former allow control over the flow rate in the elution process. Two m.m. glass beads were used as 200 $\mu$  beads would not penetrate the wax-water interface in the waxing process.

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\* Eastman, Organic Chemicals, Rochester 3, N.Y.

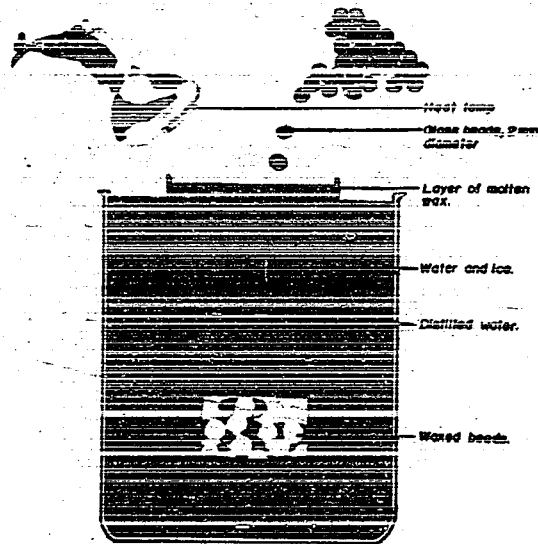
### Waxing of Glass Beads

Fig. 1 (top) illustrates the method used to produce wax coated glass beads. A beaker containing distilled water and melted wax was placed in an ice bath. The level of the water in the bath was kept about 0.5cm. below the level of the water-wax interface within the beaker. The wax was kept in the molten state by an infra-red heat lamp.

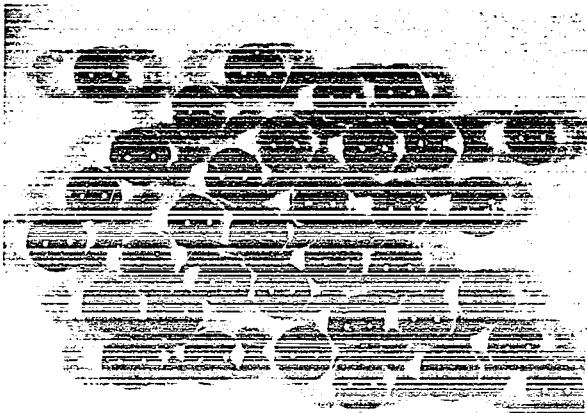
Glass beads were dropped into the wax from a height just sufficient to enable them to penetrate the wax-distilled water interface. The glass bead, on passing through the wax, obtained a wax coating which immediately solidified on contact with the cold distilled water beneath. Fig. 1 (bottom). Two factors were of importance in obtaining reasonably spherical waxed beads. If the glass bead was dropped from too low a height it failed to penetrate the wax-water interface; if dropped from too great a height the bead penetrated the wax-water interface with too great a velocity so that the wax formed a prominent "tail" on the bead, while the leading edge of the bead was not waxed at all. The thickness of the wax layer through which the bead passed was also of importance. The repeated passage of beads through the wax layer resulted in its becoming progressively thinner until only partial waxing of the bead resulted. A 3 - 4mm. layer of wax was used and replenished when partial waxing of the beads occurred. Glass beads at room temperature were used for waxing, as cold beads ( $4^{\circ}\text{C}$ ) accumulated very thick wax coats which reduced the surface

Fig. 1

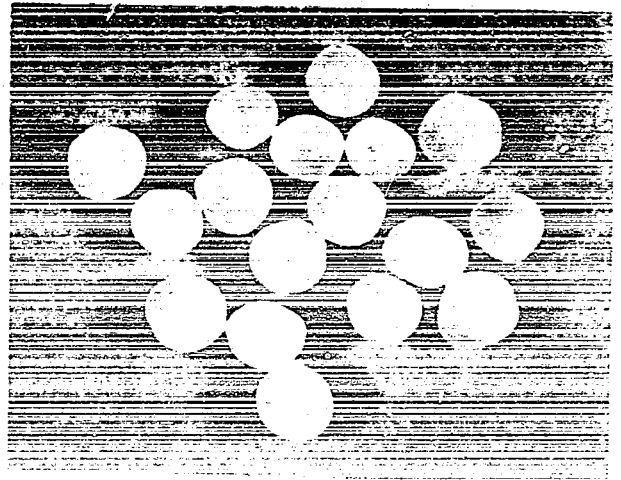
METHOD OF WAXING GLASS BEADS



unwaxed



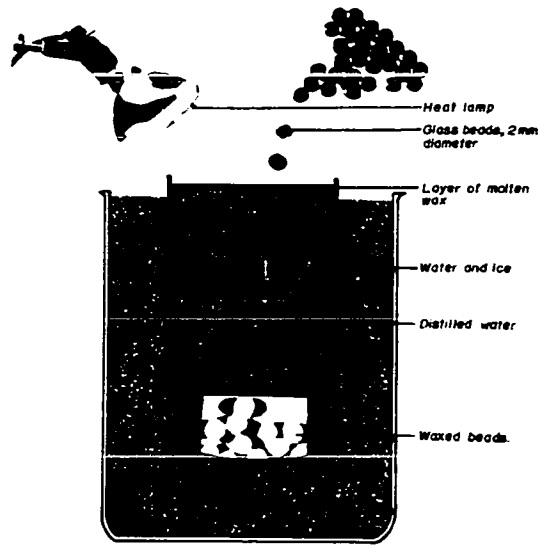
waxed



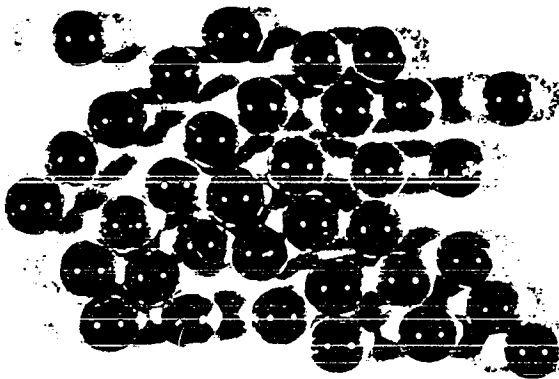
magnification x 4.50

Fig. 1

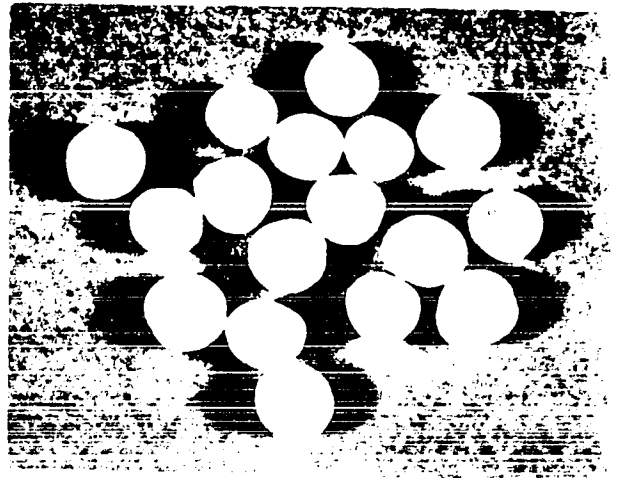
METHOD OF WAXING GLASS BEADS



unwaxed



waxed



magnification x 4.50

area of the packed column.

### Separation of Chicken Leucocyte

#### Types Using Waxed Beads

The waxed beads were carefully packed into the column, a modified condenser 1cm. in diam. x 50cm. in length. The inside of the column was waxed with eicosane by pouring a small amount of the molten wax down the previously warmed column. The column was then rotated to produce an even coat of the molten wax on the inner surface of the column. By placing the column in a cold room at 4°C and rotating, an even solid wax coating was produced.

The packed column was connected to a water bath and pump system which circulated water around the jacket of the column, which could be then maintained at any desired temperature. Chicken whole blood was circulated through the waxed bead column for 90 minutes. Phase contrast monitoring of the effluent blood revealed that about 50% of the monocytes and granulocytes had adhered to the wax coated beads by this time. Incubation for a longer period did not increase substantially the number of adhered cells. Elution of the wax bead column was carried out using HBSS/plasma 20%. Lymphocytes and erythrocytes with the unattached monocytes and granulocytes were obtained. Elution with EDTA produced a fraction containing granulocytes and thrombocytes contaminated with erythrocytes. Again no monocytes were seen on elution with EDTA. The column temperature was raised to 40°C and the molten wax collected. Fig. 2 shows the column

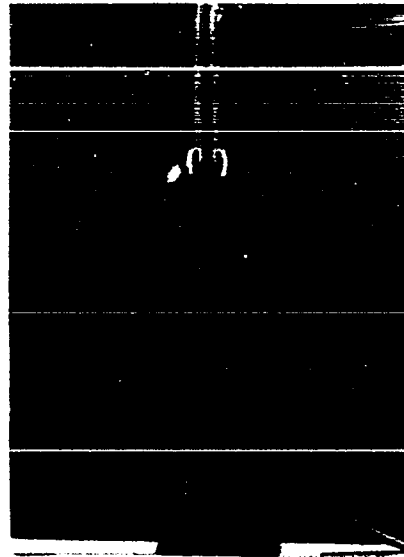


Fig. 2

## WAXED BEAD COLUMN



Incubation at 32°C.

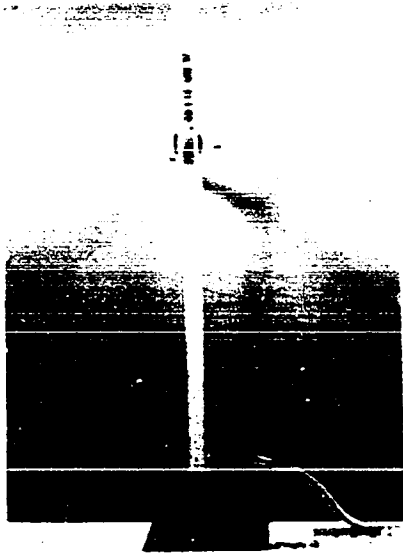


Temp. raised to 40°C.

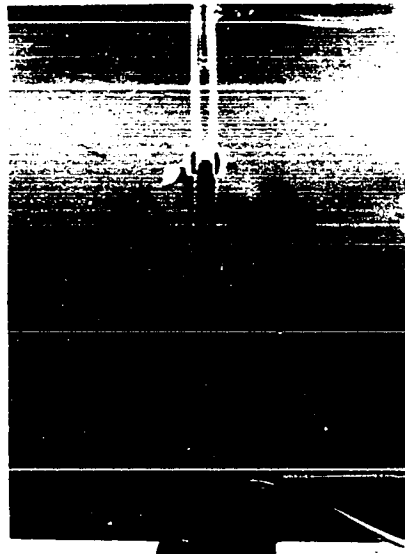
**Note the layer of molten wax which rises to the top of the column after raising the temperature.**

Fig. 2

WAXED BEAD COLUMN



Incubation at 32°C.



Temp. raised to 40°C.

Note the layer of molten wax which rises to the top of the column after raising the temperature.

before and after melting the wax. The melted wax was withdrawn and examined by phase contrast microscopy at 40°C. Monocytes were observed in the wax along with thrombocytes and a few erythrocytes.

Two problems arose from the use of wax bead columns. Firstly, approximately 50% of the granulocytes and monocytes did not adhere to the 2mm. wax beads compared with nearly 100% adherence of these cell types on the 200 $\mu$  bead column. The column volumes were similar in both instances. The surface area provided by the 2mm. waxed beads for a given column volume was far less than that provided by the 200 $\mu$  glass beads. A smaller surface area was therefore available on which the cells could attach. With this reduction in area, the area occupied by the large number of erythrocytes became increasingly important in that they could occupy a large portion of the waxed 2mm. beads, so that granulocytes and monocytes could not even come into contact with the waxed surface. With the 2mm. waxed beads it is quite possible also for monocytes and granulocytes to pass directly through the column as the blood is slowly recirculated. Stationary incubation produced the severe erythrocyte contamination that was encountered in the earlier experiments using 200 $\mu$  glass beads.

Another possibility is that there was a reduction in the ability of some of the monocytes and granulocytes to stick to wax surfaces. Crude leucocyte preparations obtained from the buffy coat were incubated on eicosane-coated petri dishes

for one hour, and the petri dishes then washed gently with HBSS. Only lymphocytes, erythrocytes and a few thrombocytes were seen in the HBSS, showing that monocytes and granulocytes readily attach to the wax surface. These results seem to exclude a decrease in the ability of these cells to adhere to wax as a major factor in the apparent decrease of granulocytes and monocytes adhering to the 2mm. waxed bead column.

Secondly, the increasing erythrocyte contamination of the leucocyte fractions obtained from the waxed bead column was due to the difficulty in producing spherical waxed beads. The slightly irregular shape of the waxed beads produced sheltered areas which were difficult to wash clear on elution. Erythrocytes remained in the sheltered areas of the column.

It was concluded from these studies that with the smaller surface area provided by the 2mm. waxed beads, the problems encountered were the result of the large numbers of erythrocytes present. Wildy and Ridley (120), using a "Vaspar" surface for separating phagocytes from blood, stressed the need for the removal of erythrocytes prior to leucocyte incubation for an efficient recovery of the phagocytes.

It was necessary, therefore, to devise a method for the removal of erythrocytes from whole blood prior to the incubation of leucocytes on the column. Removal of erythrocytes would make a larger surface area available to the leucocytes, and permit stationary incubation.

### Removal of Erythrocytes from Chicken Whole Blood

The failure of sedimenting agents to increase the sedimentation rate of chicken erythrocytes made the floatation technique appear the most promising for the separation of erythrocytes from leucocytes. Albumin has been used to separate erythrocytes from chicken blood (36), but is expensive and requires extensive reworking to produce the physiological conditions required by the leucocytes. Experiments with gum acacia were unsuccessful, due to our inability to obtain pH and osmotic pressure compatible with the recovery of intact leucocytes.

A successful separation was achieved with Ficoll, a polymer of sucrose having a molecular weight of approximately 400,000.\* It is inexpensive, stable and appears to be non-toxic to cells. Dialysing against distilled water removed the trace of sodium chloride (less than 1%) contained in the Ficoll. For dialysis, the Ficoll was made up as a 50% aqueous solution which was reduced to approximately 20% during dialysis. Concentrations much greater than 20% did not lyophilize well, and in place of a light fluffy material the Ficoll was recovered as a solid "glassy" substance which

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\* Ficoll, Pharmacia (Canada) Ltd., 100 Place Cremazie, Montreal 11.

appeared to contain water. A given percentage of this "glassy" Ficoll was much lighter than the same percentage of the light fluffy material. A 10% solution of this latter material in Seligmann's balanced salt solution (SBSS) (50) has a specific gravity of 1.039 (Fig. 3).\* Provided each batch of lyophilized Ficoll was checked against this standard, no detectable variations were observed in its performance as a density medium for the separation of cells by floatation.

Using various percentages of Ficoll dissolved in 9ml. of HBSS placed in a nitro-cellulose tube 1" diam. x 3" length\*\*, it was found that a 35% Ficoll solution gave a separation of erythrocytes from leucocytes. Five ml. of chicken blood obtained by heart puncture was layered over the Ficoll. After centrifugation at 1,000g for 30 minutes the leucocytes separated as a cohesive sheet just below the Ficoll-plasma interface, and could be removed by gentle suction using a syringe and large bore needle having a long bevel. This layer contained all the leucocyte types mixed with thrombocytes and a few erythrocytes. The clumping of the leucocytes was severe and the rise in temperature produced by centrifugation at room temperature produced damage to the cells expressed by a "blebbing" of the cell membranes. All subsequent work was carried out in a cold

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\* Determined by Copper Sulphate Method (53)

\*\* Spinco Division, Beckman Instruments Inc. Palo Alto, California, U.S.A.

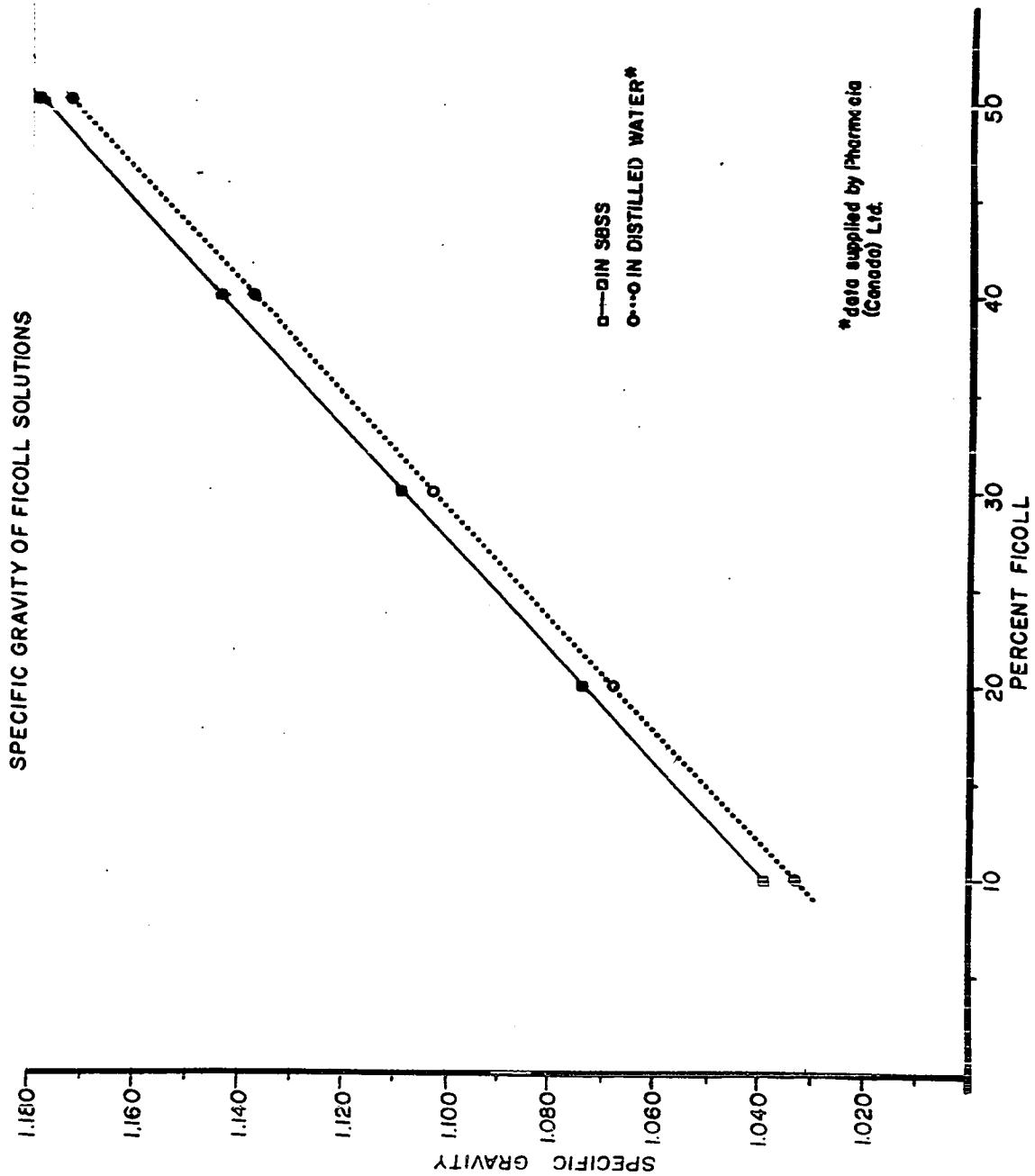


Fig. 3

room at 4°C. This resulted in good morphological preservation of the leucocytes.

Clumping of the leucocytes was so severe that a suspension of individually separate cells could not be accomplished by gentle agitation of the cells in HBSS. The degree of clumping could be alleviated to some extent by taking 5 ml. of blood into 5ml. of EDTA solution with heparin as the anticoagulant. Small clumps of cells were obtained. Attempts to achieve separation of the leucocytes into their various cell types using different density gradients were unsuccessful because these clumps, containing a mixture of cell types, acted as units so that differences in the specific gravity of the individual cells of the different cell types could not be utilized.



Separation of Leucocytes from Erythrocytes  
using Seligmann's Balanced Salt Solution

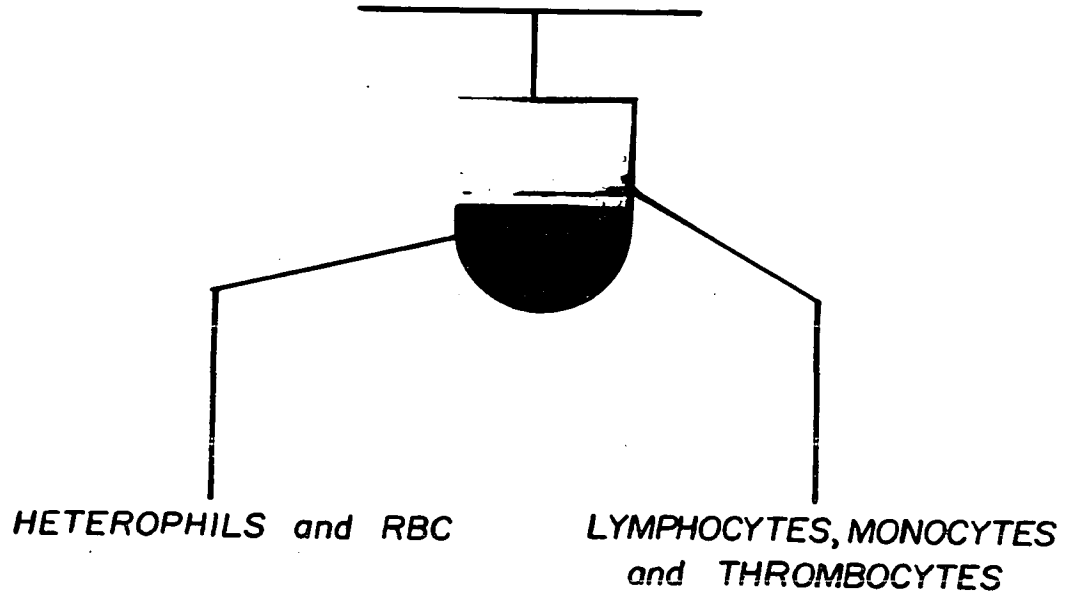
Initially heparin was used as the anticoagulant, and possibly as a result of this there was excessive clumping of the leucocytes (6, 42). To avoid clumping, 5ml. of blood were taken into the syringe which contained 5ml. of SBSS containing 5mg. of EDTA as the anticoagulant. The blood was gently mixed and layered over 9ml. of 35% Ficoll in SBSS, and centrifuged at 1,000g for 30 minutes. The entire procedure, including withdrawal of the blood sample, was carried out at 4°C, and all reagents and equipment used had been chilled previously to this temperature.

A greyish-white band of leucocytes formed just below the Ficoll-plasma interface, clearly separated from the underlying erythrocytes (Fig. 4). This layer of discretely dispersed cells could be recovered only by slicing the tube below the buffy layer, and removing the entire contents above the blade of the tube slicer. The cells were washed and resuspended in SBSS. When examined by phase contrast, the cells of this layer, shown in Figs. 2 and 3 of Plate 3, consisted of lymphocytes, monocytes and thrombocytes with only an occasional erythrocyte. The granulocytes remained intimately mixed with the red cells of the upper portion

Fig. 4

**LEUCOCYTE SEPARATION USING SELIGMANN'S  
BALANCED SALT SOLUTION**

*Blood taken into SBSS containing 5 mg EDTA.  
Layered on 9 mls Ficoll, centrifuged at 1000 g  
for 30 min. at 4°C*



of the erythrocyte layer. Attempts to separate granulocytes from erythrocytes using Ficoll floatation proved unsuccessful. It appeared that the granulocytes and some erythrocytes have the same or very close specific gravities so that no distinct band of granulocytes was ever seen. Lymphocytes could be separated from monocytes and thrombocytes using the waxed bead column. The failure to obtain granulocytes led to further modification of the method.

### Legend for Plate 3

- Fig. 1 Peripheral blood from the chicken showing lymphocytes, heterophils and thrombocytes. Phase contrast X125. Chicken heterophils contain refractile rod-shaped granules which made the cell difficult to photograph.
- Figs. 2 and 3 Leucocytes isolated by Ficoll Flootation using Seligmann's solution. Note the absence of clumping and the loss of granulocytes and erythrocytes. Phase contrast X125.
- Fig. 4 A clump of leucocytes isolated by Ficoll floatation using HBSS and heparin. Phase contrast X1125.
- Fig. 5 The same cells as in Fig. 4, showing the clumped nature of the cells. Note that granulocytes (heterophils) are present in large numbers, along with lymphocytes, monocytes and a few erythrocytes. Phase contrast X125.
- Figs. 6 to 8 Morphological features of leucocytes isolated by Ficoll floatation.
- Fig. 6 Monocytes. One cell is shown engulfing a thrombocyte (arrow). Phase contrast X1125.
- Fig. 7 Large monocyte. Phase contrast X1125.
- Fig. 8 Heterophils. These cells show typical refractile rod-shaped granules. Phase contrast X1125.
- Fig. 9 Human granulocytes isolated by Ficoll floatation using HBSS. Phase contrast X1125.

In order to show cell detail more clearly, the preparations were crushed slightly to aid photography. The legend is the same for all Figures. M = monocyte, L = lymphocyte, H = heterophil, Th = thrombocyte.

Plate 3

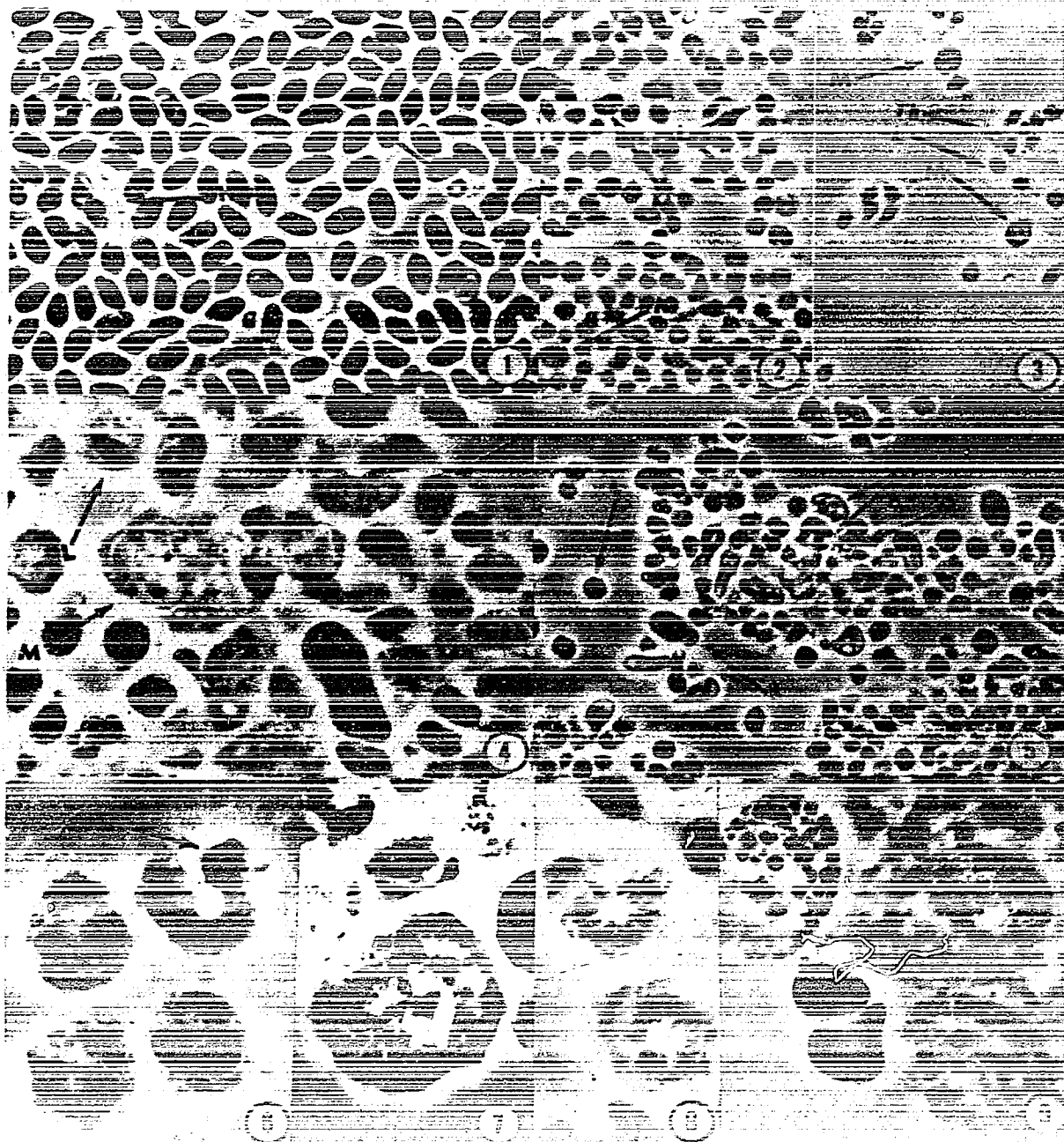
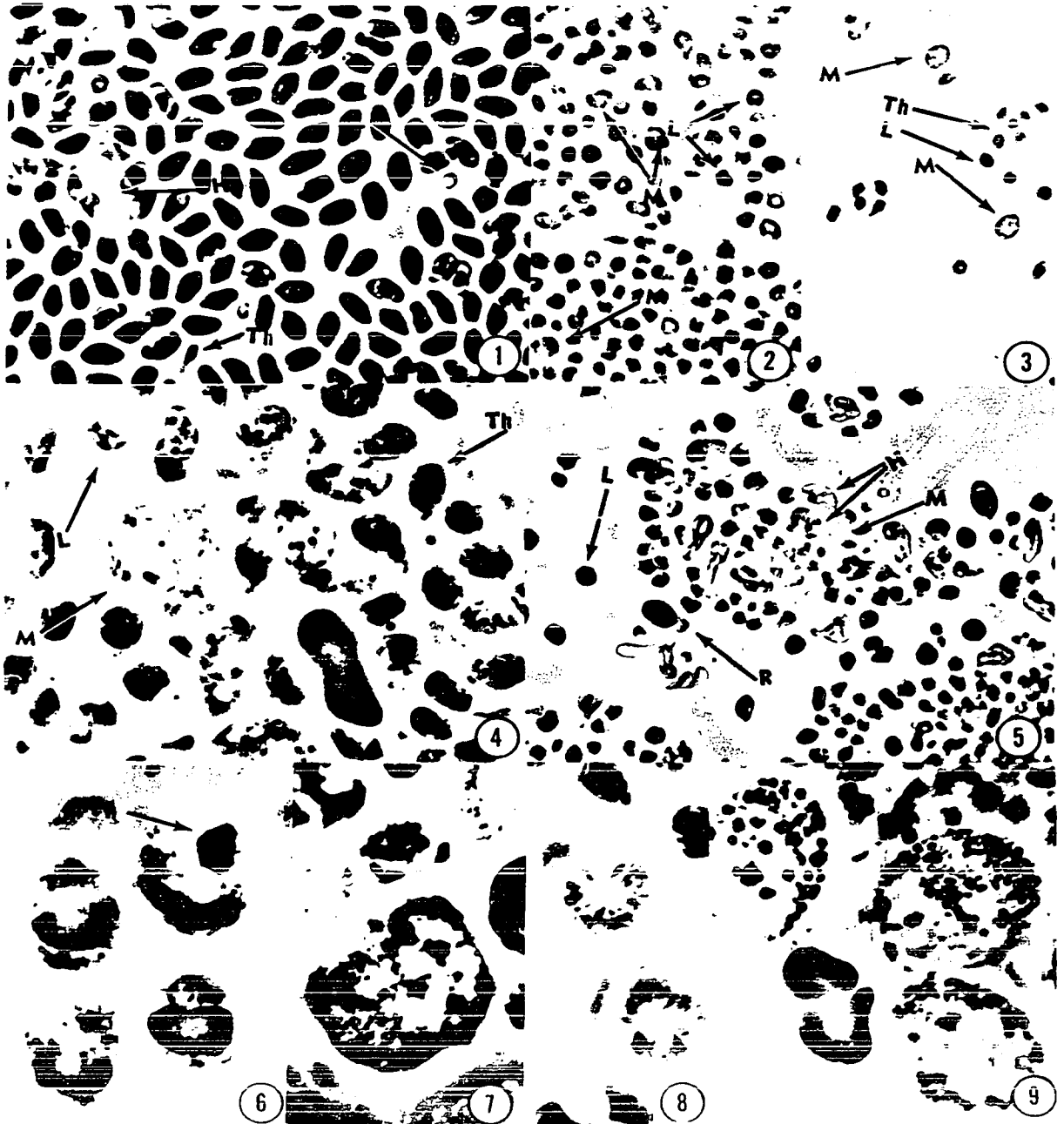


Plate 3



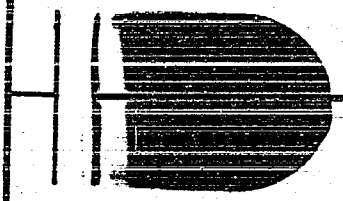
Leucocyte Separation Using Hank's Balanced  
Salt Solution and Heparin

It was noted in the earlier studies that Hank's BSS and heparin produced a layer of leucocytes containing all leucocyte cell types. Heparin promoted clumping of all cell types so that the heavier granulocytes became enmeshed in these clumps and remained with the lymphocytes, monocytes and thrombocytes rather than sedimenting with the erythrocytes. 5ml. of blood were withdrawn into 1ml. of HBSS containing 100 units of heparin and layered over 9ml. of 35% Ficoll in SBSS. Centrifugation at 1,000g for 30 minutes at 4°C produced a cohesive sheet of white cells clearly separated from the erythrocytes (Fig. 5 upper portion). These cells could be removed by gentle suction using a syringe and large bore needle having a long bevel (Plate 3, Figs. 4 and 5).

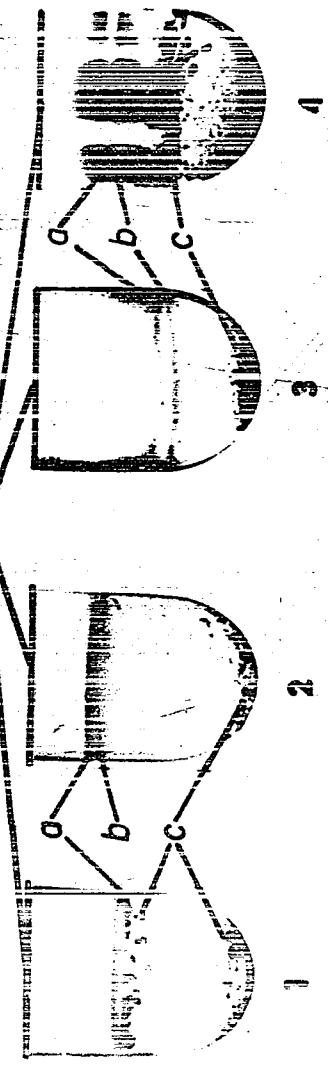
The cells were washed once in SBSS to remove the Ficoll and then resuspended in 5ml. of SBSS. Gentle agitation for approximately 10 to 15 minutes using a Pasteur pipette produced a suspension of completely dispersed cells (Plate 4). It was now possible to investigate further the separation of leucocyte types using Ficoll.

# LEUCOCYTE SEPARATION USING HANKS BSS AND HEPARIN

Blood taken into HBSS containing 100 units of heparin. Layered on 9 mls. Ficoll, centrifuged at 1000 g for 30 min. at 4° C.



Lymphocytes, heterophils, monocytes, thrombocytes. Dispersed in HBSS layered on 9 mls. Ficoll, centrifuged at 300 g for 15 min. at 4° C.



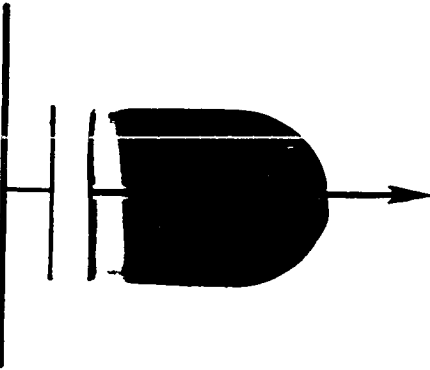
a- lymphocytes and monocytes  
b- light erythrocytes and heterophils  
c- heavy erythrocytes and heterophils

Fig. 5

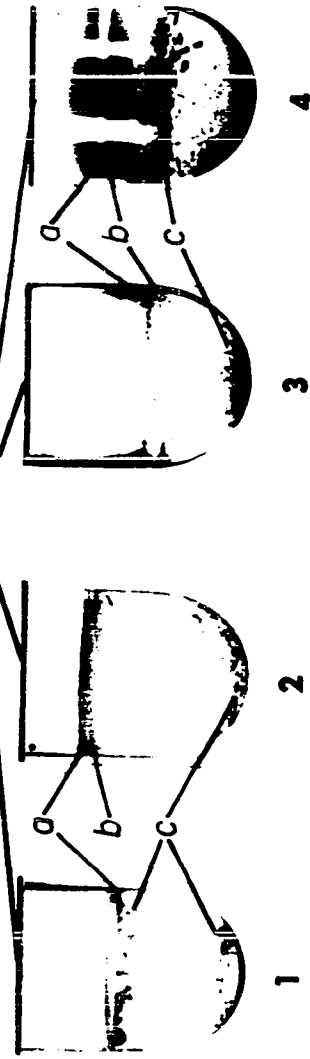


# LEUCOCYTE SEPARATION USING HANK'S BSS AND HEPARIN

Blood taken into HBSS containing 100 units of heparin. Layered on 9 mls. Ficoll, centrifuged at 1000 g for 30 min. at 4° C.



Lymphocytes, heterophils, monocytes, thrombocytes. Dispersed in SBSS layered on 9 mls. Ficoll, centrifuged at 300 g for 15 min. at 4° C.



a- lymphocytes and monocytes  
b- light erythrocytes and heterophils  
c- heavy erythrocytes and heterophils

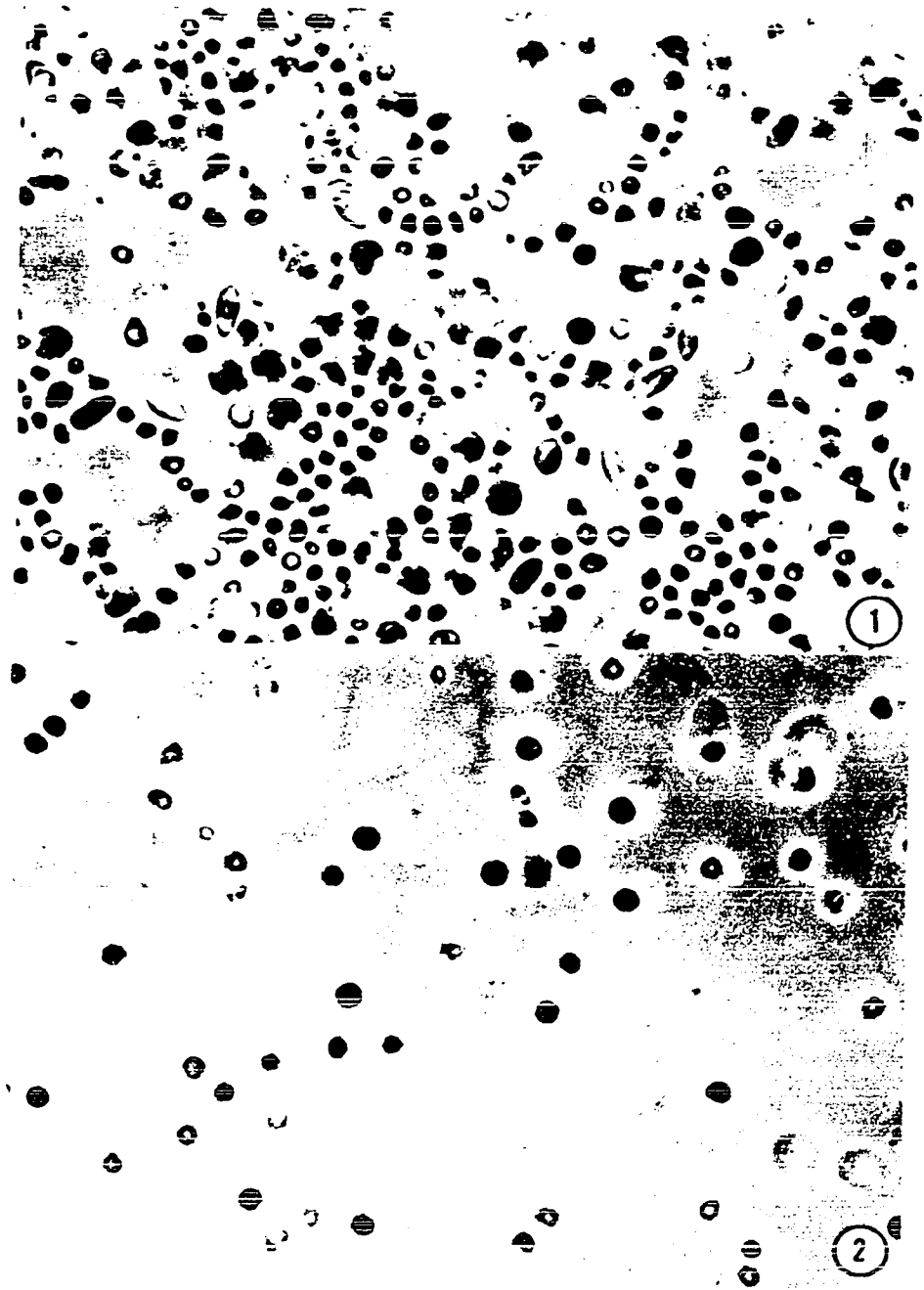
Fig. 5

Separation of Individual Leucocyte Types using  
Ficoll and Waxed Bead Column

The lower portion of Fig. 5 shows the various Ficoll preparations used to bring about further separation of the leucocyte types. The cells were washed and resuspended in SBSS, and layered over 9ml. of 28% Ficoll in SBSS (Tube 1 Fig. 5). After centrifugation at 300 to 400g for 15 to 20 minutes at 4°C, lymphocytes, monocytes and thrombocytes formed a layer at the Ficoll interface (Tube 1 (a)). Granulocytes and the few contaminating erythrocytes and thrombocytes clumped at and just below the interface and could not be separated. Although some of the granulocytes, contaminated with a few red cells and thrombocytes, were present in the lower portions of the tube, additional centrifugation served only to increase the contamination of the granulocyte clumps with lymphocytes and monocytes, so that individual leucocyte type separation became impossible.

In order to overcome the abrupt interface between the Ficoll and the overlying cell suspension, further modifications were tried. Three ml. of SBSS were layered over 9ml. of 28% Ficoll in SBSS and allowed to equilibrate for 4 hours or 24 hours at 4°C. These are shown as tubes 2 and 3 respectively,

Plate 4



in Fig. 5. Non-linear continuous gradients were prepared also, by layering 5 ml. portions each of 30%, 25% and 20% Ficoll in SBSS in successive layers, and allowing them to equilibrate for 48 hours at 4°C (Tube 4, Fig. 5). The cells dispersed in SBSS were layered over the gradients and centrifuged at 300g for 15 minutes at 4°C.

Good separation of lymphocytes and monocytes from granulocytes and erythrocytes was achieved by any of the latter three preparations. The separated cells are shown in Figs. 1 and 2, Plate 5. Discontinuous Ficoll gradients separated an additional distinct band of lighter erythrocytes, not previously seen. This band contained a number of granulocytes discretely dispersed throughout the red cells. The remainder of the granulocytes settled with the heavier erythrocytes to the bottom of the tube and were present in coarse, loose clumps. The granulocytes could be freed from the few contaminating red cells by "paradoxical" sedimentation (78, 94), as shown in figure 3 plate 5.

Tube 3 figure 5 was sliced between the bands a and b and the layer a, consisting of lymphocytes, monocytes and thrombocytes was washed once in HBSS to remove the Ficoll and then resuspended in HBSS containing 20% chicken plasma. The cell suspension was loaded on the column from the bottom of the tube, thus avoiding formation of air pockets which formed if the column was loaded from the top. After incubation for one and a half hours at 32°C the column was eluted with 2 column volumes (2 x 16ml.) of HBSS which removed the

Legend for Plate 5

Cells isolated by Ficoll floatation  
(See Fig. 5 for band designation)

Fig. 1

Band a. L = lymphocyte, M = monocyte,  
Th = thrombocyte. Phase contrast X125.  
Insert shows a monocyte. Phase contrast  
X1125.

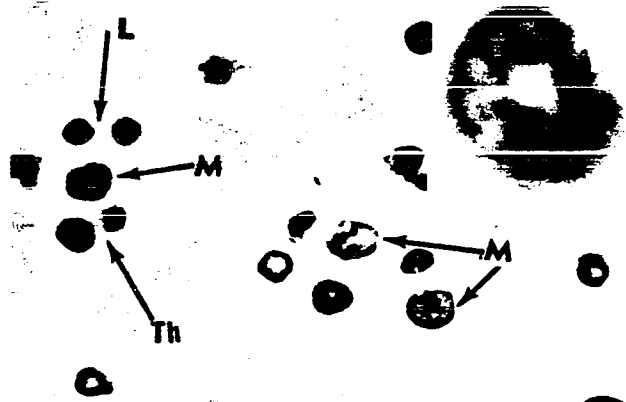
Fig. 2

Band c. Phase contrast X125. H =  
heterophils, R = erythrocytes, Th =  
thrombocytes.

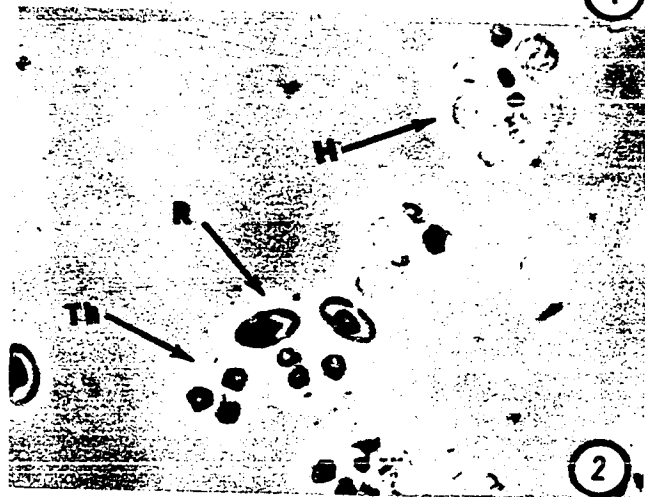
Fig. 3

Band c after paradoxical sedimentation.  
Note the lack of erythrocytes and  
reduction of thrombocytes. Phase contrast  
X125. H = heterophils, Th = thrombocytes.  
Insert shows two heterophils. Phase  
contrast X1125.

Plate 5



1



2



3

lymphocytes (Plate 6, Figs. 5 and 6).

Raising the column temperature to 40°C melted the wax which floated to the top of the column. The wax was removed with a warm pipette and was centrifuged in a clinical centrifuge placed in a cabinet maintained at 40°C. The monocytes, with thrombocyte contaminants, are shown in Plate 6, Figs 1 - 4. A summary of the whole procedure is shown in Fig. 6.

It was not possible to wash all the wax from the monocytes, even when Tween 80 was incorporated into the balanced salt solution used for washing. On cooling from 40°C the remaining wax solidified and it became impossible to work with the isolated cells. Attempts to spin the wax suspension of cells over SBSS at 40°C were also unsuccessful as the cells would not penetrate the wax-BSS interface.

The possibility of separating monocytes from lymphocytes by Ficoll floatation was therefore investigated.

Legend for Plate 6

Fig. 1 Monocytes in wax recovered from the column after raising the temperature to 40°C. Note thrombocyte contamination. Phase contrast X1125.

Figs. 2, 3 & 4 Monocytes in wax at 40°C. Phase contrast X1125.

Figs. 5 & 6 Lymphocytes obtained by elution of waxed bead column with HBSS. Phase contrast X125.  
Insert. Lymphocyte. Phase contrast X1125.



Plate 6

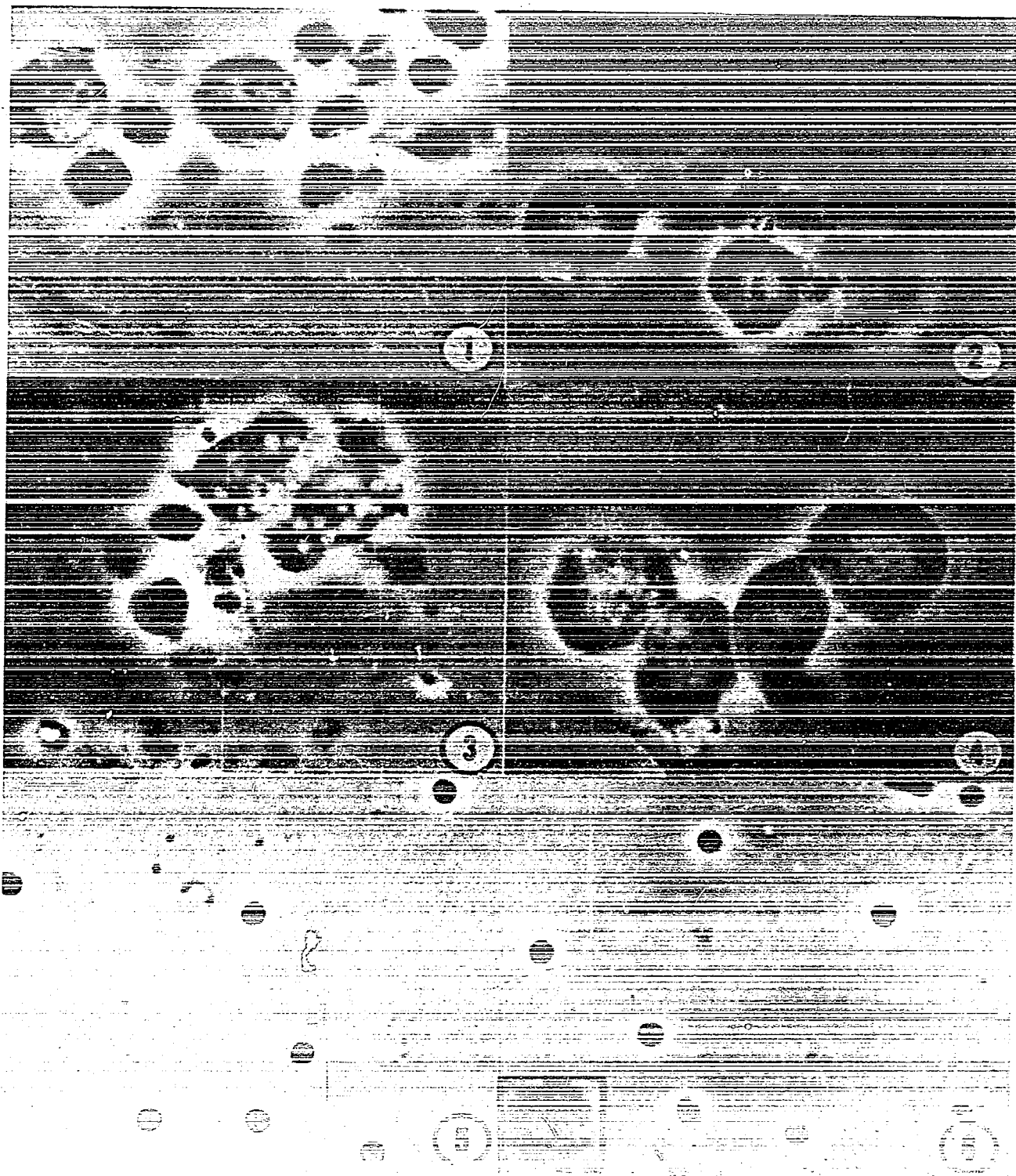


Plate 6

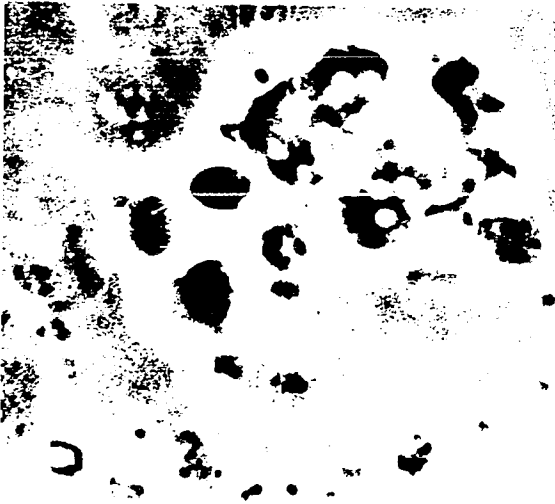
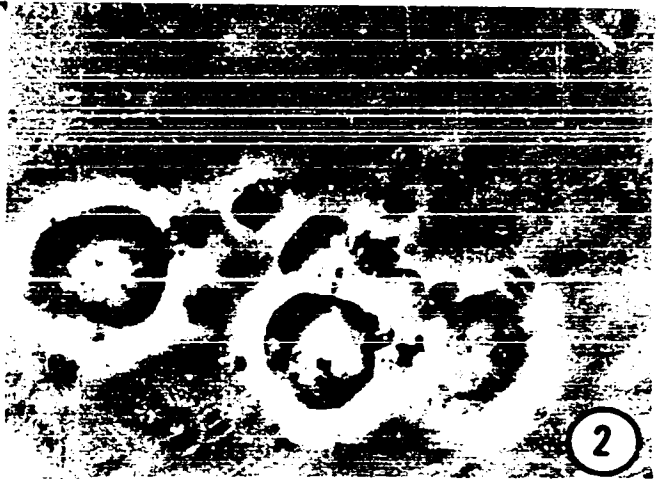
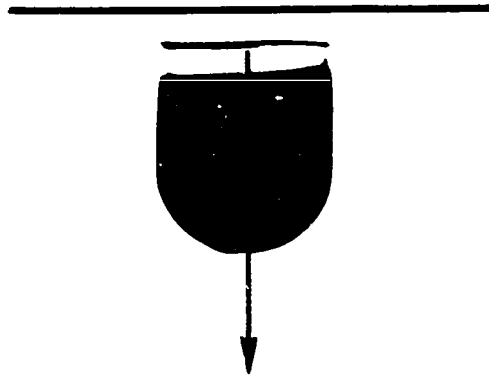


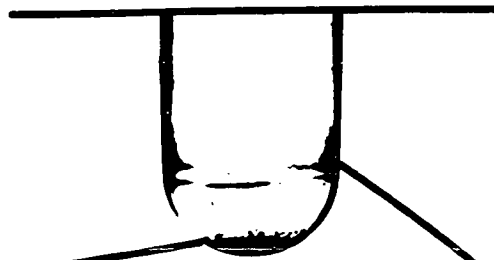
Fig.6

## LEUCOCYTE SEPARATION USING HANK'S BSS AND HEPARIN

5 mls of blood taken into 1 ml. HBSS containing  
100 units of heparin, layered over 9 mls of 35 %  
ficoll in SBSS, centrifuged at 1000g for 30 min.  
at 4°C.



cell layer washed and dispersed in SBSS, layered  
over 9 mls of 28 % ficoll in SBSS, centrifuged  
at 400g for 20 min. at 4°C.



HETEROPHILS, RBC

paradoxical  
sedimentation

HETEROPHILS

LYMPHOCYTES, MONOCYTES

washed, resuspended in  
HBSS + 20% plasma,  
incubated on waxed-bead  
column at 32°C.

wash, 2 column  
vols. HBSS

LYMPHOCYTES

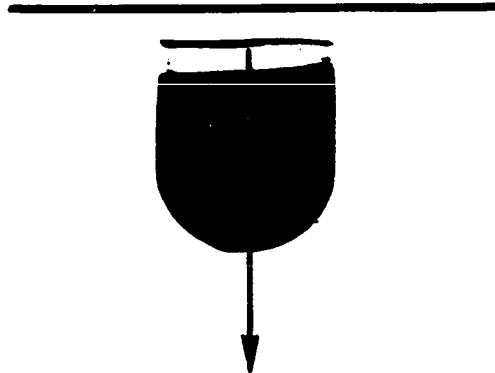
temp. to 40°  
removed wax,  
centrifuged

MONOCYTES

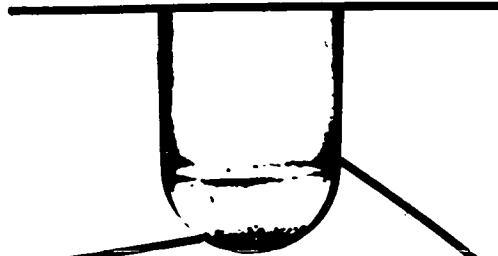
Fig.6

## LEUCOCYTE SEPARATION USING HANK'S BSS AND HEPARIN

5 mls of blood taken into 1 ml. HBSS containing 100 units of heparin, layered over 9 mls of 35 % ficoll in SBSS, centrifuged at 1000 g for 30 min. at 4°C.



cell layer washed and dispersed in SBSS, layered over 9 mls of 28 % ficoll in SBSS, centrifuged at 400g for 20 min. at 4°C.



**HETEROPHILS, RBC**

paradoxical sedimentation

**HETEROPHILS**

**LYMPHOCYTES, MONOCYTES**

washed, resuspended in HBSS + 20% plasma, incubated on waxed-bead column at 32°C.

wash, 2 column vols. HBSS

**LYMPHOCYTES**

temp. to 40° removed wax, centrifuged

**MONOCYTES**

The Separation of the Different Chicken Leucocyte  
Types by Ficoll Floatation

The work described previously using Ficoll floatation resulted in the separation of granulocytes from lymphocytes and monocytes (Fig. 5, Tube 3). Attempts to separate further lymphocytes from monocytes using fine "step" gradients were generally unsatisfactory due to the cells clumping on passage through the abrupt changes in specific gravity which occurred at each interface.

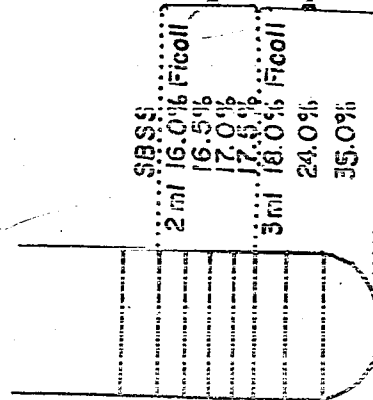
A gradient of the composition shown in Fig. 7A and allowed to equilibrate for the times given in the diagram, resulted in the separation of the different types of leucocytes. Tween 80 was added to the upper portion of the gradient to prevent clumping of the cells, particularly granulocytes, on passage through the Ficoll. Tween 80 was omitted from the lower portion of the gradient so that the granulocytes would clump facilitating the paradoxical sedimentation procedure used to remove the contaminating erythrocytes.

Fig. 7B shows the reproducibility of the method. The position of the bands is constant but the intensity of a band varies from experiment to experiment due to

## SEPARATION OF CHICKEN LEUCOCYTES

Leucocytes separated from erythrocytes by Ficoll floatation, leucocytes removed and placed over a second Ficoll gradient.

A



B

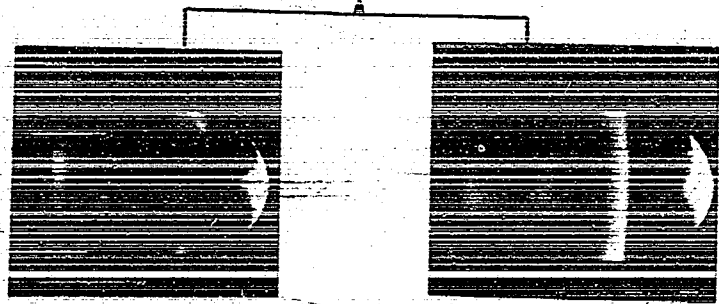
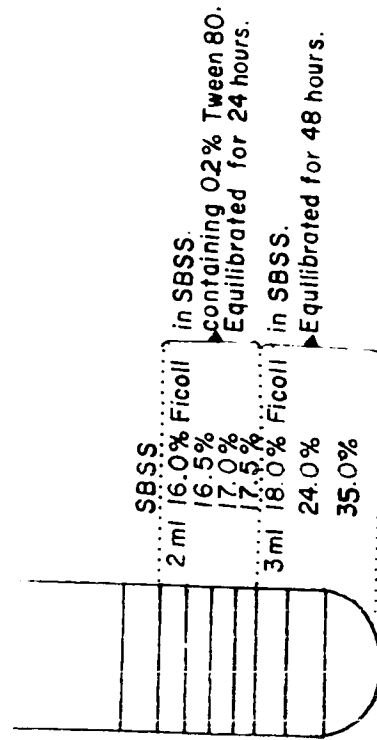


Fig. 7

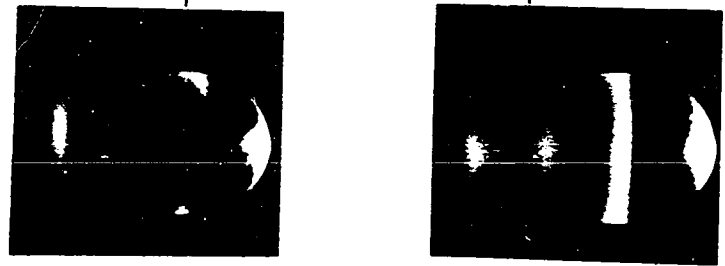
## SEPARATION OF CHICKEN LEUCOCYTES

Leucocytes separated from erythrocytes by Ficoll floatation; leucocytes removed and placed over a second Ficoll gradient.

A



B



Appearance of tubes after centrifugation at 50g for 5 min., followed by 300g for 10 min.

Fig. 7

the different numbers of leucocyte types obtained from different birds.

This gradient was further modified to increase the distance between the two uppermost bands in order to make recovery of these bands easier. The modification is shown in Fig. 8.

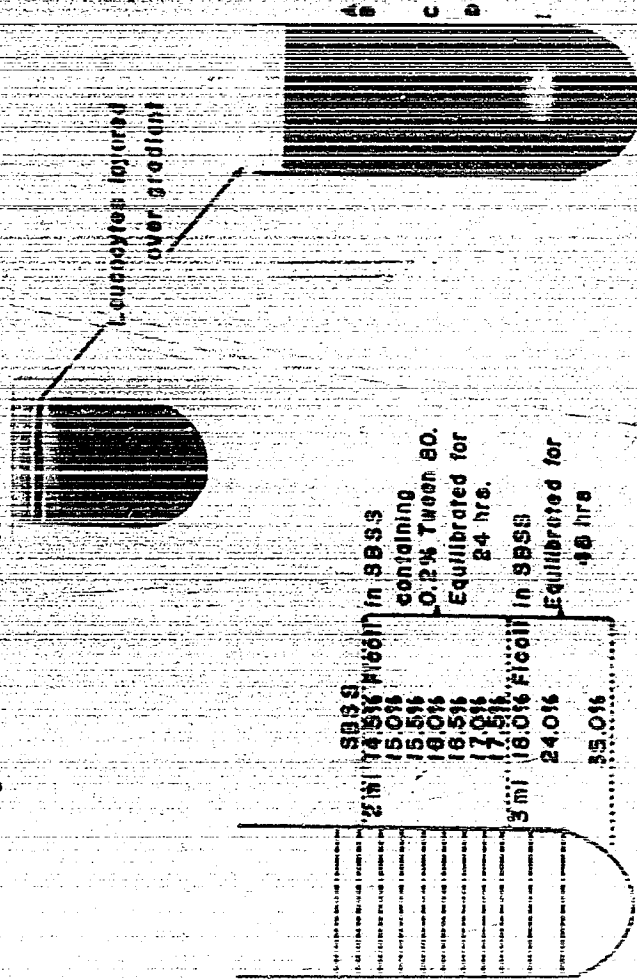
Two problems associated with this work were the visualization and recovery of the separated cells. Our first concern was that bands of cells such as monocytes which are relatively few in number might not be visible under normal lighting conditions and so go undetected. After examining various lighting sources and directions of incident light, it was found that a light source directed through the bottom of the tube revealed bands of cells which were not visible under normal lighting conditions. Various microscope oculars were placed between the light source and the tube to correct for the convex lens effect produced by the rounded tube. The bands were observed and photographed in a dark room.

The second problem, that of recovering the bands of cells, was overcome by piercing the nitro-cellulose tube with a syringe and needle just below the layer of cells required. This was done while the bands were made visible using the lighting system just described. Plate 7 shows the cell types associated with the different bands.



**SEPARATION OF THE VARIOUS TYPES OF LEUCOCYTES FROM CHICKEN BLOOD**

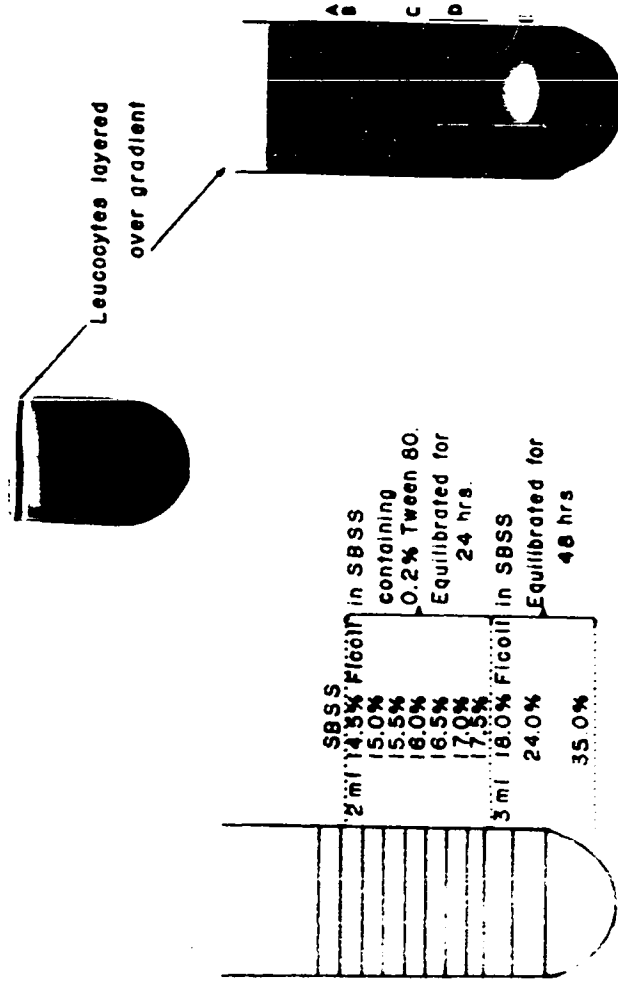
5 ml. of blood taken into 1 ml. of HBSS containing 100 units of heparin per ml. Layered over 9 ml. of 35% Ficoll in SBSS, centrifuged at 1000g for 30 min. at 4°C.



**Fig 8**

**SEPARATION OF THE VARIOUS TYPES OF LEUCOCYTES FROM CHICKEN BLOOD**

5 ml of blood taken into 1 ml. of HBSS containing 100 units of heparin per ml. Layered over 9 ml. of 35% Ficoll in SBSS, centrifuged at 1000g for 30 min. at 4°C.



Composition of Ficoll gradient

**Fig 8**

### Cellular Composition of the Bands

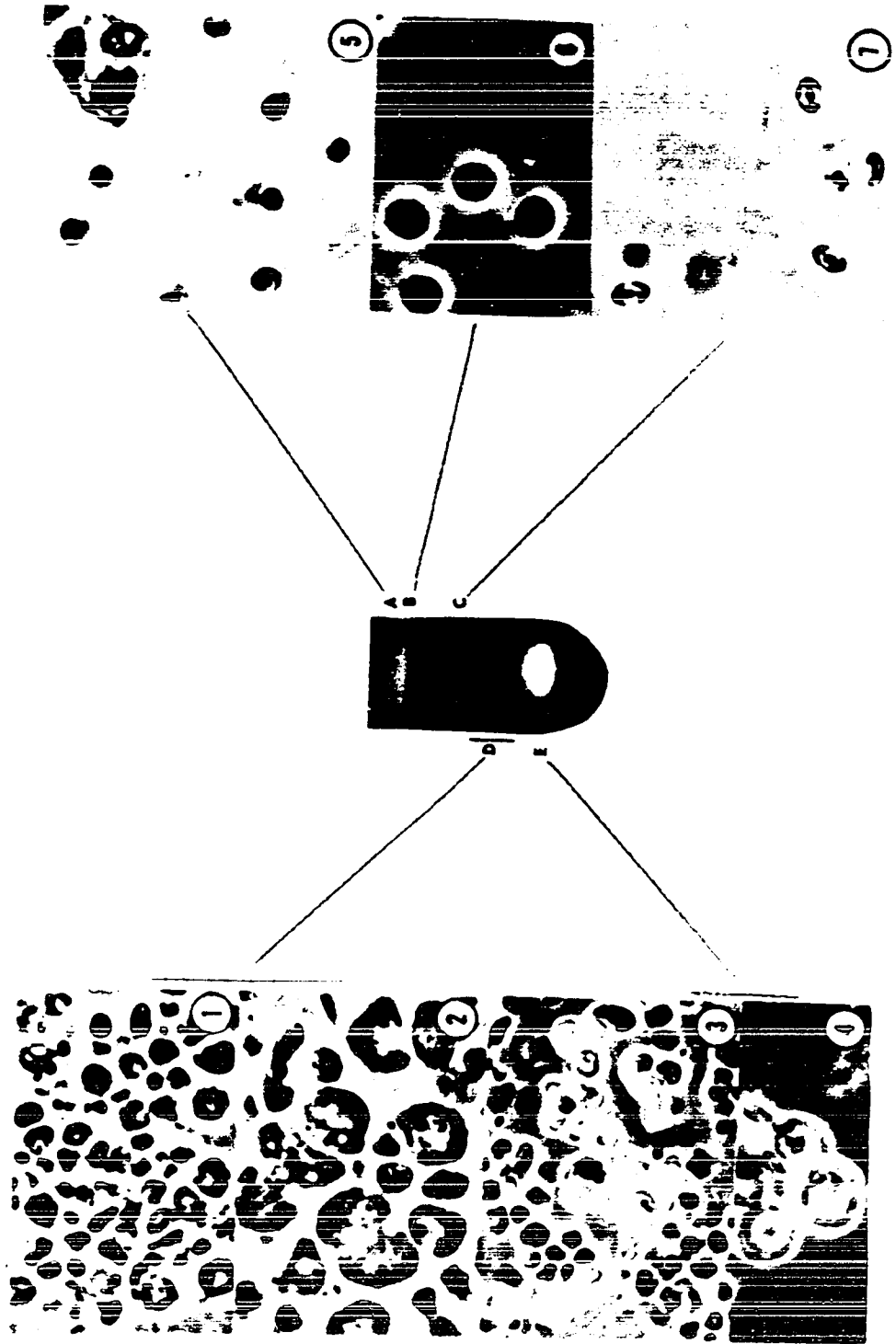
Only the differential count of the cells in each band was determined. Total counts were attempted using a haemocytometer, but at the low magnification required by this method no distinction could be made between thrombocytes and lymphocytes and also between thrombocyte clumps (Plate 7, Fig. 5 inset) which could easily be mistaken for larger cells e.g. granulocytes or monocytes. Thrombocytes could be distinguished from other cell types with certainty only by observation under oil immersion phase contrast. Thrombocytes readily break down, especially during centrifugation, and form clumps which after they have been centrifuged a second time are not readily dispersed even by SBSS. Thrombocytes contaminated each band to varying degrees. The ability of thrombocytes to slowly break down, form clumps of varying numbers of cells resulted in a wide range of specific gravity shown by this cell type. Szenberg and Shortman (107), using albumin density gradients, found thrombocytes to have a range in specific gravity of 1.05 to 1.09.

This irreversible clumping made total counts using a haemocytometer so inaccurate that recovery data could

Legend for Plate 7

All Photographs Phase Contrast

- Fig. 1 Band D: Monocytes. Note the extent of thrombocyte contamination.  
x 300
- Fig. 2 Band D: Higher power showing monocytes in greater detail.  
x 1125
- Fig. 3 Band E: Granulocytes with contaminating thrombocytes after  
paradoxical sedimentation. x 300
- Fig. 4 Band E: Granulocytes. Cells refractile making photography  
difficult. x 1125
- Fig. 5 Band A: Thrombocytes in various stages of degeneration.  
x 300  
Insert. Three thrombocytes from peripheral blood.  
Note the clump formation and signs of degeneration  
i.e. vacuoles in the cytoplasm. x 1125
- Fig. 6 Band B: Lymphocytes. x 300
- Fig. 7 Band C: "Light erythrocytes". x 300



not be obtained.

Differential counts were performed under oil immersion phase contrast with at least 100 cells, other than thrombocytes, being counted (Table 1).

#### Estimation of Viability

Whilst oxygen consumption is the most sensitive means of estimating viability, the large number of cells required in this method made it impracticable for use in this study. Phagocytosis was not used to assess viability as the results of Vaughan (113) show that the extent of phagocytosis depends upon the material presented to the cell. Therefore this method does not give the true estimate of the total number of viable cells.

Dye exclusion was used to estimate the number of viable cells. The method of Holmberg, using lissamine green, was used (58). To the cell suspension was added two drops of concentrated dye solution in SBSS. Wet preparations were made, sealed with vaseline and the number of stained cells determined after half an hour at room temperature. A minimum of 100 cells were enumerated. The results are shown in Table 2.

Percent Composition of Chicken Leucocyte

Types within a given Band

Cell Type	Expt. No.	Band B	Band D	Band E*
Granulocytes	1	-	17	80
	2	-	10	76
	3	-	19	91
	4	-	18	90
	5	-	14	87
Lymphocytes	1	91	10	2
	2	93	6	1
	3	96	9	1
	4	88	3	2
	5	90	7	3
Monocytes	1	1	70	1
	2	-	81	3
	3	2	68	2
	4	1	76	4
	5	3	78	5

Balance to 100% in each band consists of erythrocytes.

\* After paradoxical sedimentation.

Thrombocytes contaminated each band. Heavy contamination occurring in bands D and E. (Plate 7, Figs 1 - 3).

Table 1

## Estimation of the Viability of Leucocyte Types

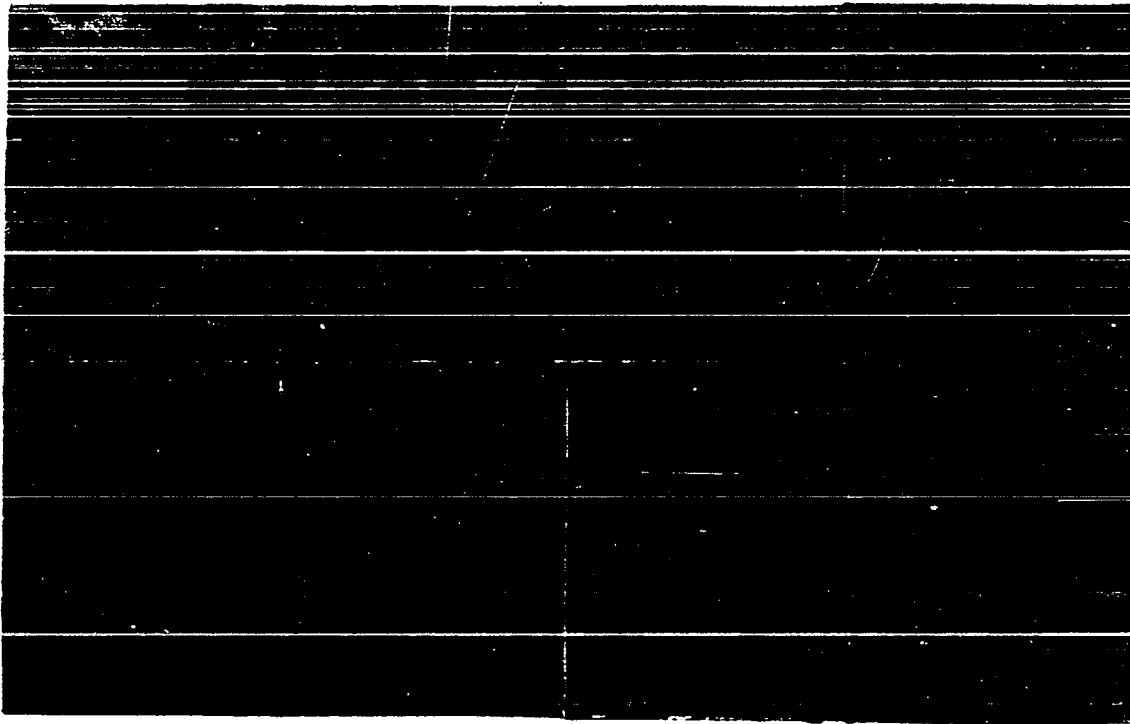
	% Cells Stained by Dye
	Mean of 5 expts. $\pm$ S.D.
Lymphocytes	1.4 $\pm$ 0.28
Monocytes	2.0 $\pm$ 1.00
Granulocytes	5.4 $\pm$ 2.29

Viable cells are not stained by dye.

Table 2



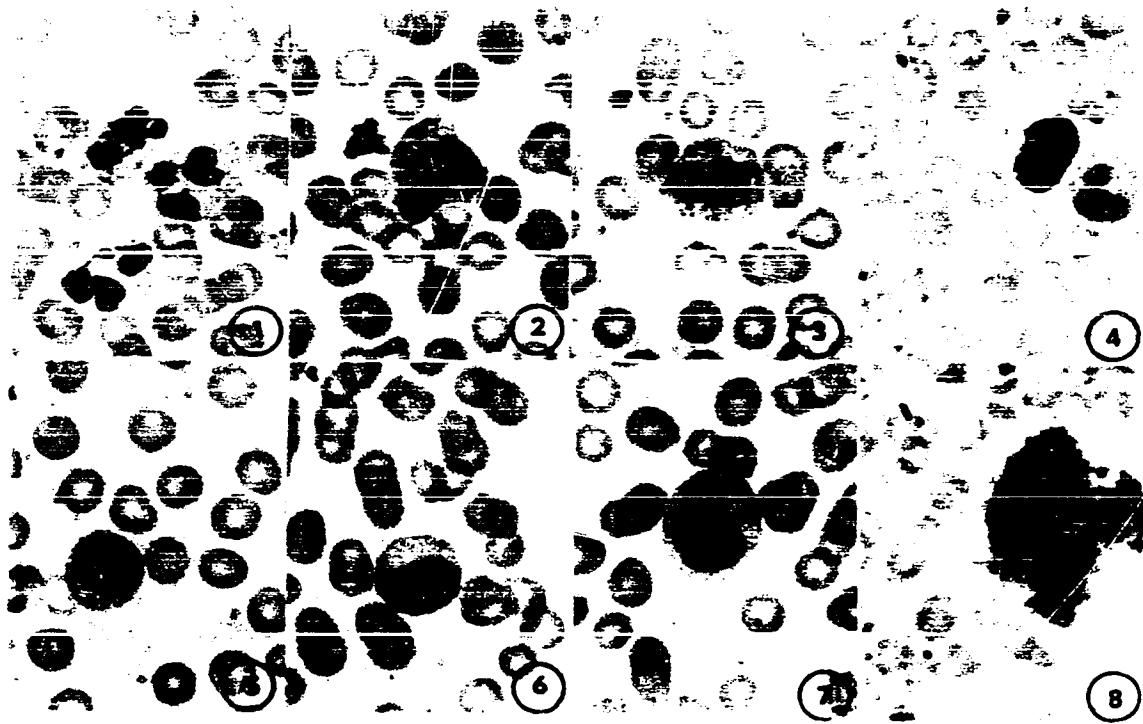
68  
Plate 8



**Leucocytes in Human Blood**

- Figs. 1-4 Granulocytes**  
    **1 Neutrophils**  
    **2,3 Eosinophils**  
    **4 Basophil, Eosinophil**
- Figs. 5-7 Agranulocytes**  
    **5,6 Lymphocytes**  
    **7 Monocytes**
- Fig. 8 Mass of platelets**

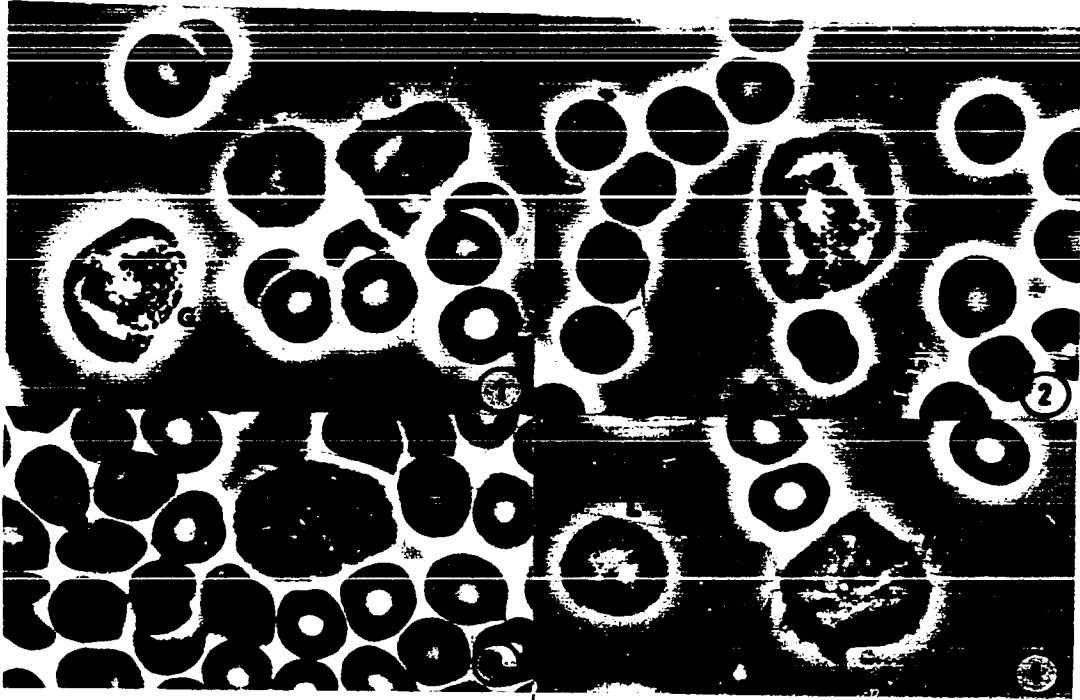
Wright's stain x1200



Leucocytes in Human Blood

- Figs. 1-4    Granulocytes  
    1        Neutrophils  
    2,3      Eosinophils  
    4        Basophil, Eosinophil
- Figs. 5-7    Agranulocytes  
    5,6      Lymphocytes  
    7        Monocytes
- 8        Megakaryoblasts

Plate 9



Human leucocytes

Fig 1. Granulocytes, Lymphocyte

2. Granulocyte

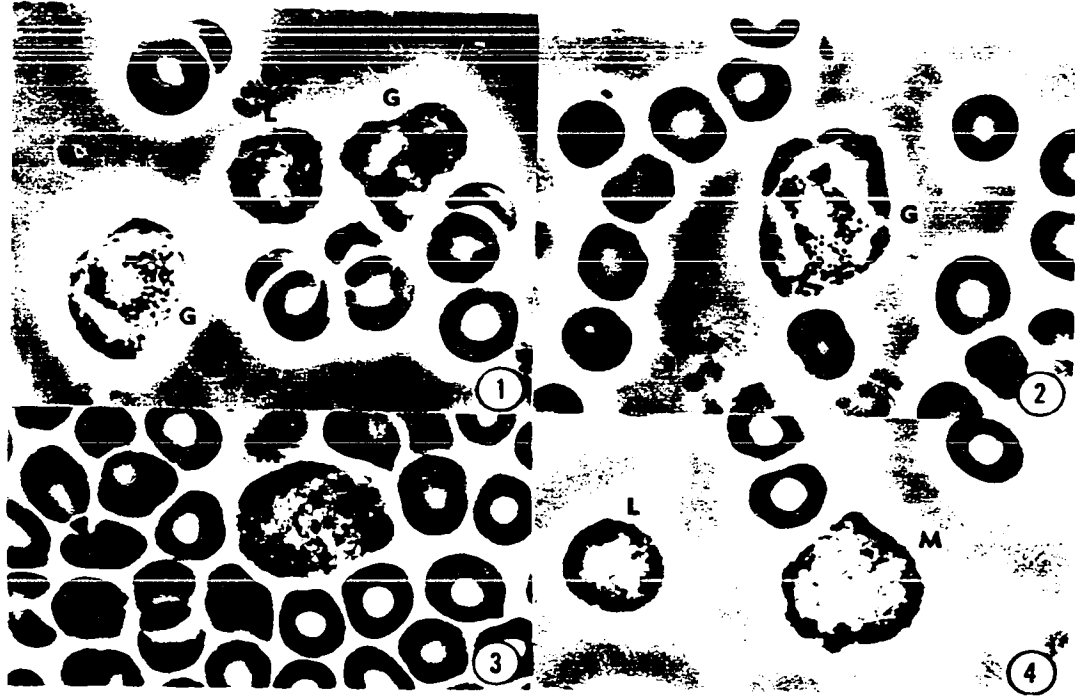
3. Monocyte

4. Monocyte, Lymphocyte

G = Granulocyte, M = Monocyte, L = Lymphocyte.

Phase Contrast x 1200

Plate 9



Human leucocytes

Fig 1. Granulocytes, Lymphocyte

2. Granulocyte

3. Monocyte

4. Monocyte, Lymphocyte

G = Granulocyte, M = Monocyte, L = Lymphocyte.

Phase Contrast x 1200

The Separation of the Various Leucocyte Types  
from Human Peripheral Blood

The methods used to separate chicken leucocyte types can also be applied, with minor modifications, to the separation of human leucocyte types.

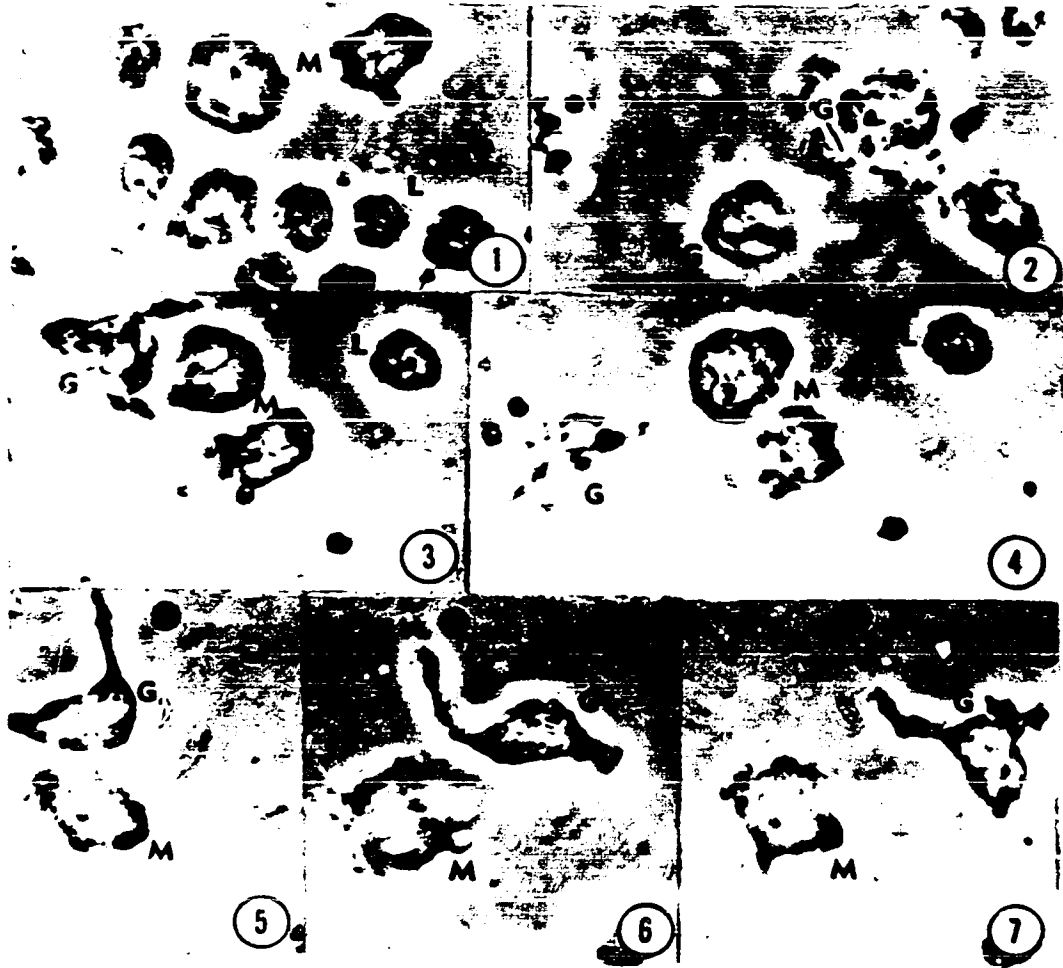
5ml. of human blood were taken into an equal volume of SBSS with EDTA as the anticoagulant, and layered over 12ml. of 35% Ficoll in HBSS. Centrifugation for 30 minutes at 1000g and 4°C resulted in the separation of lymphocytes, monocytes and platelets from granulocytes which remained intimately mixed with erythrocytes.

When 5ml. of blood were taken into 1ml. of HBSS containing 100 units of heparin; layered over 12ml. of 35% Ficoll in HBSS and centrifuged as mentioned above, the layer of separated cells contained all leucocyte types (Plate 10).

The 35% Ficoll was dissolved in HBSS. Seligmann's BSS contains sodium acetate which has been used to prevent non-specific erythrocyte aggregation. It was thought rouleaux formation of the erythrocytes would increase the distance between the layer of separated leucocytes and erythrocytes.

The leucocytes were removed using a pasteur pipette

Plate 10



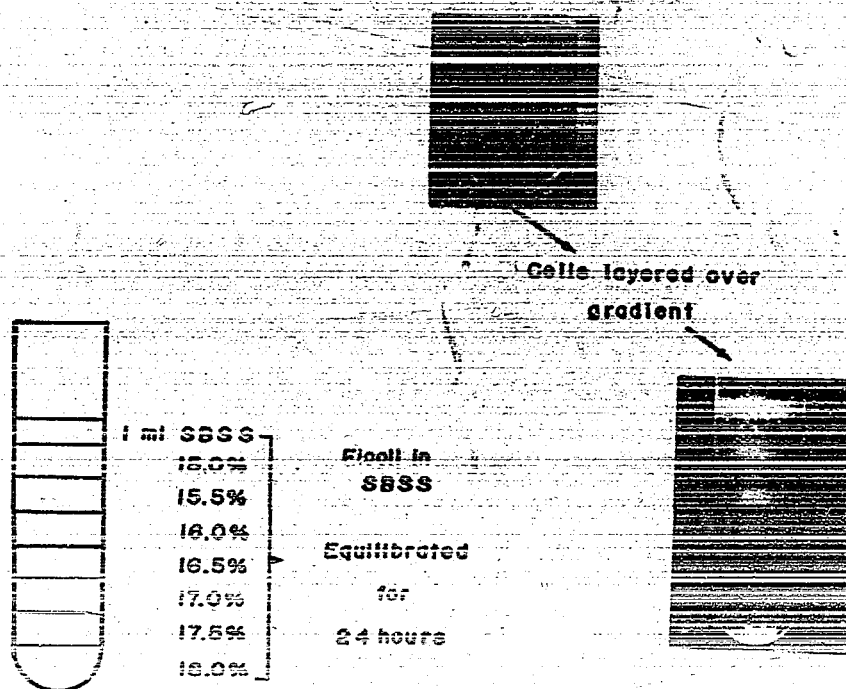
taking care not to disturb the erythrocytes. The separated leucocytes were washed once in SBSS and carefully layered over a gradient of the composition shown in Fig. 9\*. The appearance of the tube after centrifugation is also showed in Fig. 9. The bands of cells were observed and removed using the same procedure described for chicken.

\* Gradient made up in a nitro-cellulose tube, 5/8" diam. x 3" length. Spinco Division, Beckman Instruments Inc. Palo Alto, California, U.S.A.

Fig. 9

### SEPARATION OF THE TYPES OF LEUCOCYTES FROM HUMAN BLOOD

5 ml. of blood taken into 1 ml. HBSS containing 100 units of heparin. Layered over 12 ml. of 35% Ficoll in HBSS, centrifuged at 1000g for 30 min. at 4°C



Composition of Ficoll gradient

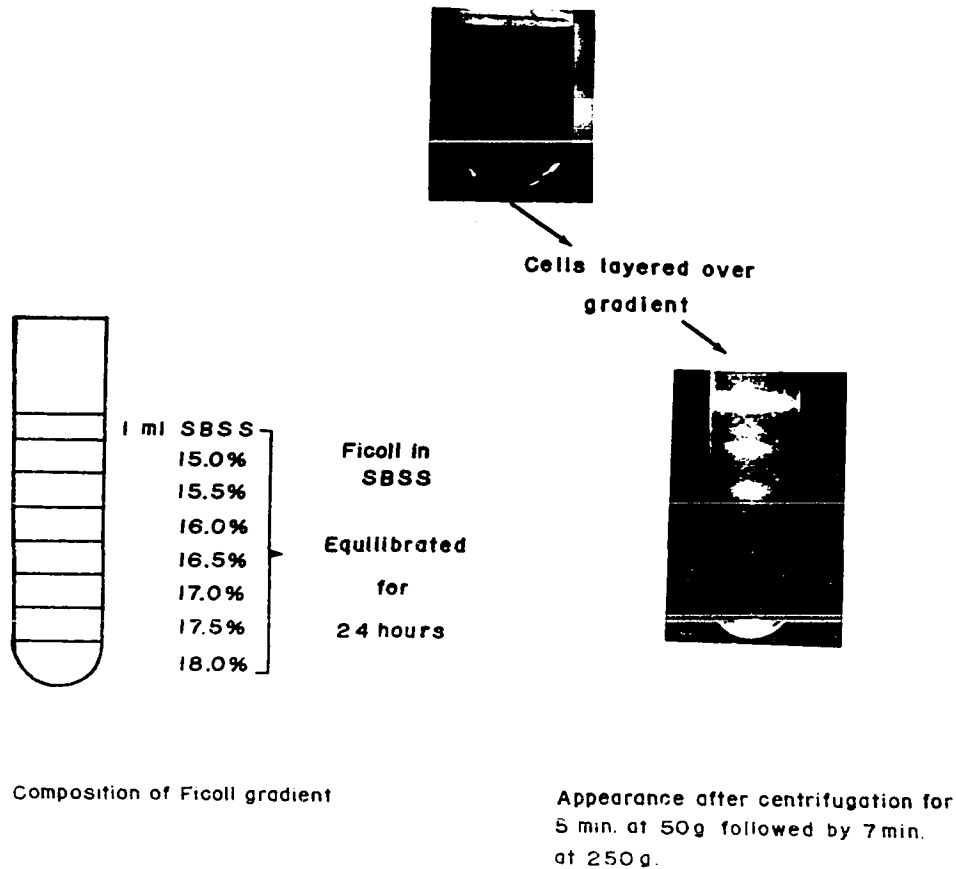
Appearance after centrifugation for 5 min. at 50g followed by 7 min. at 250g.



Fig. 9

### SEPARATION OF THE TYPES OF LEUCOCYTES FROM HUMAN BLOOD

5 ml. of blood taken into 1 ml. HBSS containing 100 units of heparin. Layered over 12 ml. of 35% Ficoll in HBSS, centrifuged at 1000g for 30 min. at 4°C



The Distribution of Leucocytes  
within the Bands

The predominant cell type in each band is shown in Plate 11. Platelets tended to contaminate slightly each band. This is probably due to overloading the gradient with respect to platelets. Cells in the order of  $10 \times 10^6$  were placed on the gradient to keep the platelet contamination to a minimum while placing adequate monocyte numbers on the gradient to enable a recovery of this cell type to be made. A blind assay was performed on the cellular components of the bands.

Legend for Plate 11

All Photographs Phase Contrast

- Fig. 1 Band A. Platelets x 300.
- Fig. 2 Band B. Lymphocytes x 1125.
- Fig. 3 Band C. "Light erythrocytes" x 300.
- Fig. 4 Band D. Monocytes and contaminating lymphocyte x 1125.
- Fig. 5 Band E. Granulocytes: cells motile accounting for the blurred granules x 1125.

Plate 11

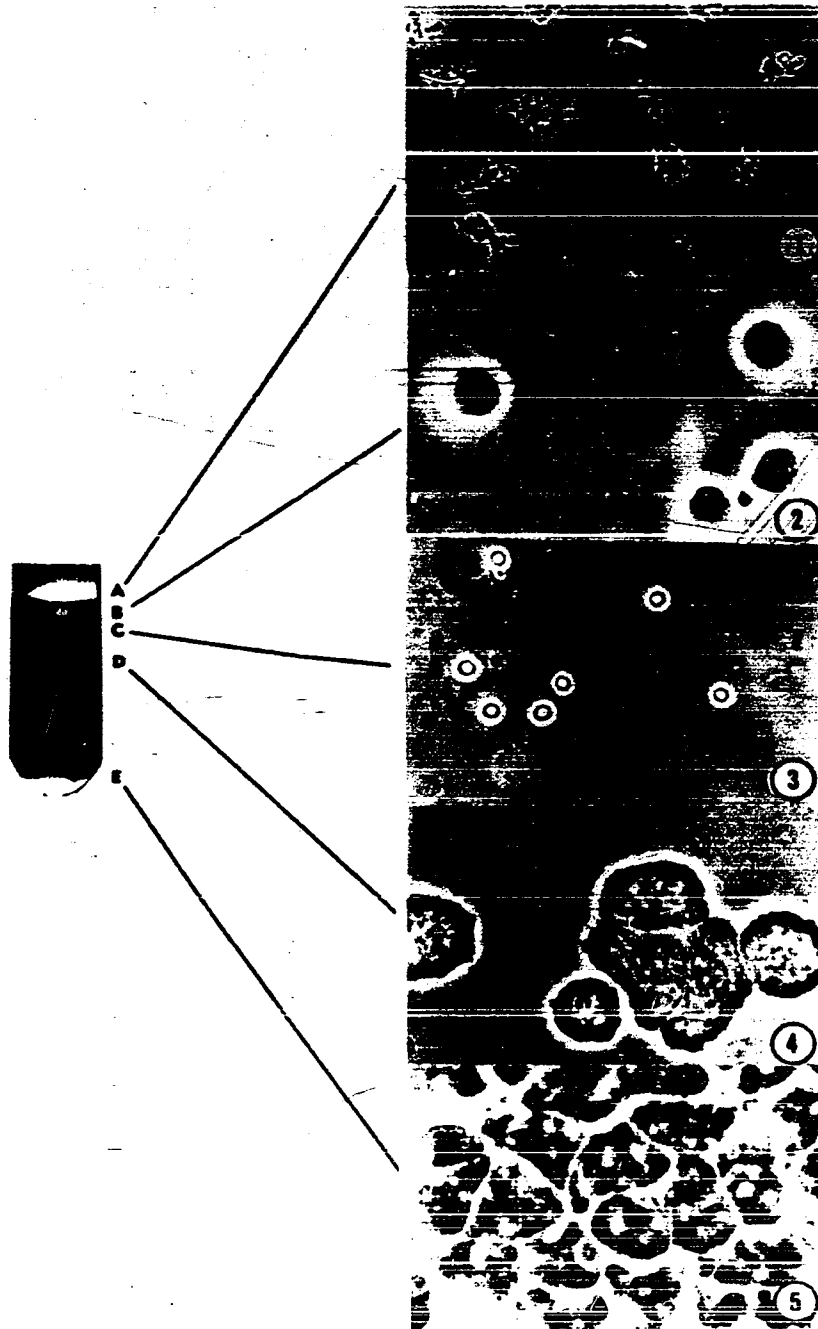
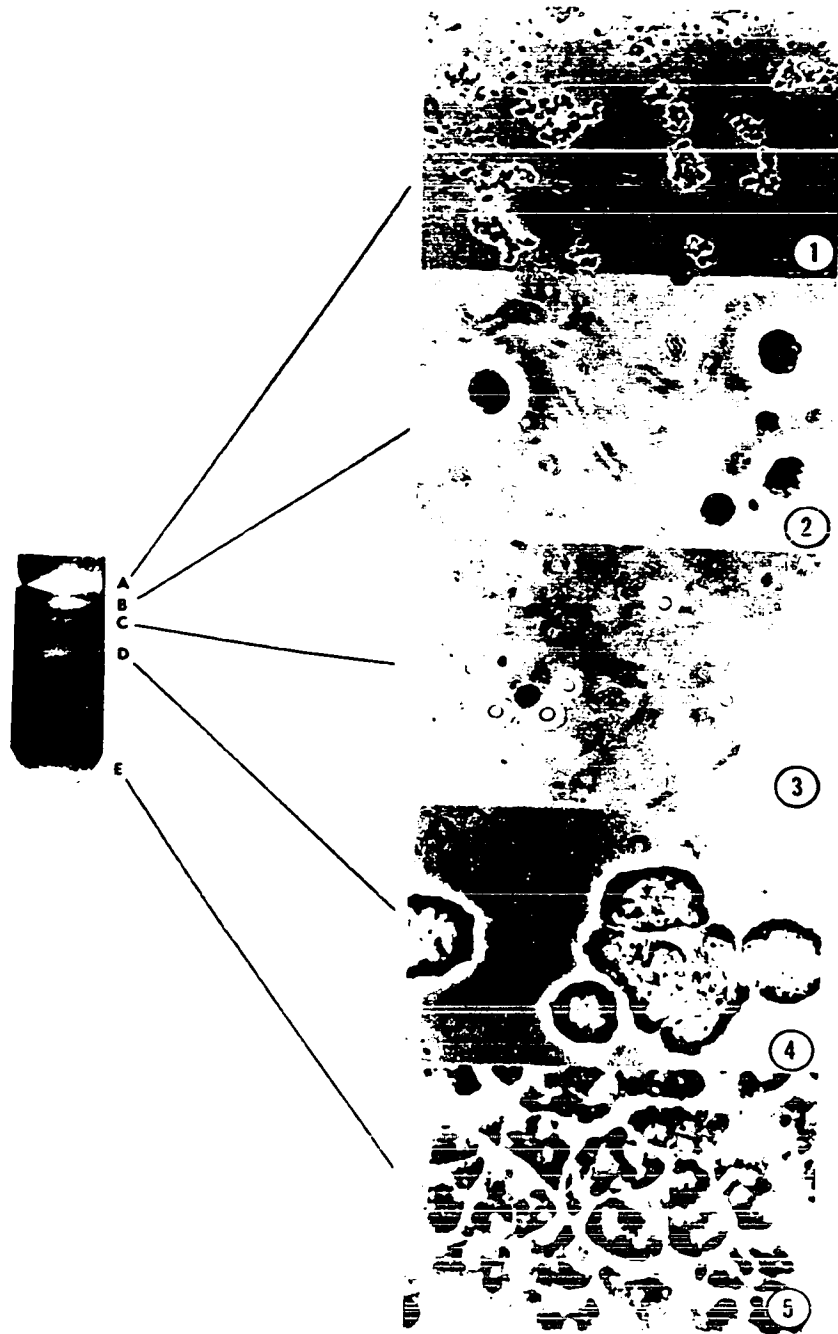


Plate 11



## Recovery and Viability Studies on Separated Human Leucocyte Types

Peripheral differential counts were performed on Wright's stained smears. All total counts were estimated, in duplicate, using a haemocytometer. The differential counts on leucocytes separated from erythrocytes and on separated individual leucocyte types were performed under oil immersion phase contrast. A minimum of 100 cells were counted. The cells from the bands were washed once with SBSS and resuspended in 1ml. of SBSS for counting and viability estimations. Band A, platelets and Band C, light erythrocytes were not enumerated. Blood was taken from 5 different individuals for this study.

### Estimation of Viability

The criteria used for estimating cell viability have already been presented in the section on the viability of chicken leucocyte types.

The method of Holmberg (58) using lissamine green was used. To the cell suspension was added two drops of

the concentrated dye solution in SBSS. Wet preparations were made, sealed with vaseline and the number of stained cells recorded after half an hour at room temperature. A minimum of 100 cells were counted.

### Morphology

Morphology of the isolated cells, as judged by phase contrast microscopy, was excellent (Plate 11).

The Separation of Leucocytes from Erythrocytes  
Recovery Data (Table 3)

Leucocytes can be separated from erythrocytes using Ficoll floatation. The low erythrocyte contamination and adequate leucocyte recovery compare very favourably with sedimentation techniques (102). It is interesting to note that the loss of leucocytes is due mainly to a corresponding loss in granulocytes. Lymphocyte and monocyte recoveries being, except in one instance, appreciably higher. This is in keeping with the context that granulocytes are trapped in the clumping induced by HBSS and heparin, enabling a separation of all leucocyte types. The use of SBSS and EDTA as the anticoagulant which prevents clumping result in separation of lymphocytes, monocytes and platelets only. Granulocytes which were not clumped with other leucocyte types would therefore pass through with the red cells.

The range in the percent recovery of granulocytes probably reflects the varying ability of leucocytes, from different individuals, to clump.



Separation of Leucocytes from Erythrocytes

Expt.	Rbc x 10 <sup>9</sup>	Wbc x 10 <sup>6</sup>	Granulocytes x 10 <sup>6</sup>	Lymphocytes x 10 <sup>6</sup>	Monocytes x 10 <sup>6</sup>
1) Peripheral Blood	26.22	33.00	20.79	9.57	2.64
After Rbc Removal	0.006	24.00	12.60	9.00	2.40
% Recovery	0.02	72.7	60.6	94.0	90.9
2) Peripheral Blood	26.64	39.50	26.07	10.27	3.16
After Rbc Removal	0.003	18.92	9.68	7.04	2.20
% Recovery	0.01	47.9	37.1	68.5	69.7
3) Peripheral Blood	27.40	40.00	17.60	20.00	2.40
After Rbc Removal	0.008	30.30	14.20	14.20	1.90
% Recovery	0.04	75.7	80.7	71.0	79.1
4) Peripheral Blood	22.80	28.00	16.20	9.80	2.00
After Rbc Removal	0.0009	17.10	8.50	7.20	1.40
% Recovery	0.004	61.1	52.5	73.5	70.0
5) Peripheral Blood	24.50	35.00	18.20	14.70	2.10
After Rbc Removal	0.005	25.60	10.37	13.40	1.80
% Recovery	0.02	73.1	56.9	91.1	85.7
6) Peripheral Blood	24.05	40.50	27.54	9.32	3.60
After Rbc Removal	0.011	28.80	18.00	8.00	2.80
% Recovery	0.05	71.2	65.5	85.8	79.7

Table 3

The Separation of Individual  
Leucocyte Types (Table 4)

Bands B and E, consist of lymphocytes and granulocytes respectively in a fairly high degree of purity. There was some platelet contamination but it was not as pronounced as the thrombocyte contamination found in chicken. Band D, consisting of mainly monocytes, is not as pure; and probably reflects on the close specific gravities of large lymphocytes, monocytes and granulocytes; in particular eosinophils which formed a high proportion of the contaminating granulocytes.

Viability estimations on the separated cell types are shown in Table 6. Mean recovery values for the whole procedure are summarized in Table 5.

The Separation of Individual Leucocyte Types

Cell Type	Expt. No.	Cells to Gradient x 106	Band B		Band D		Band E	
			% cells x 106	Total cells x 106	%	Total cells x 106	%	Total cells x 106
Granulocytes	1	6.30	-	-	10	0.104	95	4.56
	2	4.84	-	-	14	0.098	90	2.79
	3	8.55	-	-	16	0.192	88	5.28
	4	5.10	-	-	14	0.140	95	3.80
	5	5.07	-	-	17	0.136	70	2.95
	6	9.00	-	-	15	0.133	88	5.10
Lymphocytes	1	4.50	92	3.20	8	0.083	2	0.096
	2	3.52	95	2.66	10	0.070	3	0.093
	3	8.55	90	4.14	6	0.072	5	0.300
	4	4.32	96	2.98	12	0.120	3	0.120
	5	6.70	89	3.47	17	0.136	3	0.126
	6	4.00	90	2.69	13	0.115	1	0.058
Monocytes	1	1.20	2	0.070	80	0.832	2	0.096
	2	1.10	3	0.084	71	0.497	5	0.155
	3	1.14	-	-	70	0.840	2	0.120
	4	0.84	1	0.031	69	0.690	1	0.040
	5	0.90	2	0.073	64	0.512	3	0.126
	6	1.40	-	-	65	0.578	1	0.059

The balance to 100% in each band consists of erythrocytes as contaminants.

Table 4

Summary

Separation of Leucocytes from Erythrocytes

	WBC	Granulocytes	Lymphocytes	Monocytes
Mean % Recovery + S.D. (6 Determinations)	66.9 ± 10.5	50.5 ± 17.1	78.9 ± 11.0	79.2 ± 8.4

Separation of Individual Leucocyte Types

	Granulocytes	Lymphocytes	Monocytes
Mean % Recovery + S.D. Cells layered on Gradient (6 Determinations)	63.7 ± 8.0	63.9 ± 11.4	61.4 ± 16.3
Mean % Recovery + S.D. Cells in Peripheral Blood (6 Determinations)	37.8 ± 9.1	52.6 ± 12.4	49.4 ± 15.6

Table 5

## Estimation of the Viability of Isolated Leucocyte Types

	% Cells Stained by Dye
	Mean of 6 expts. $\pm$ S.D.
Lymphocytes	1.8 $\pm$ 0.27
Monocytes	1.3 $\pm$ 0.28
Granulocytes	6.0 $\pm$ 1.94

Viable cells are not stained by dye.

Table 6

## Discussion

The problem of identifying leucocytes, other than the typical mature cell, by morphological criteria has led to a great deal of controversy. In our study of the haematological effects produced by L. monocytogenes and MPA, difficulties arose in cell classification. Many cells appeared in the peripheral blood with morphological characteristics somewhat between those of lymphocytes and monocytes. It would be interesting to see whether or not the various types of leucocytes could be distinguished from one another immunologically. However, the immediate problem was that no method was available for the separation of leucocyte types from peripheral blood. It was essential that leucocytes from the peripheral blood of a normal animal be used as cells obtained by exudate techniques are different morphologically from those in the circulating blood. In an initial comparative study of the antigens of the different leucocyte types it is preferable to study the normal rather than the abnormal.

Chicken blood was used as it has a high leucocyte count and adequate quantities (20ml) can be obtained easily.

The starting point for this study on leucocyte separation was the method of Rabinowitz published in 1964. Attempts to apply this method to the separation of chicken leucocytes met with difficulty due to the fact that dextran does not promote rouleaux formation in chicken erythrocytes. The initial step in the method of Rabinowitz could not be used.

Experiments using 200 $\mu$  glass bead columns resulted in a partial separation of the various leucocyte types. The large numbers of erythrocytes present produced heavy contamination of the leucocyte fraction obtained by elution with EDTA. Monocytes were not eluted by EDTA. This failure of EDTA to detach adhered monocytes from glass has been noted by others (21, 109). Even in Rabinowitz's method, nearly two thirds of the monocytes applied to the column were unaccounted for. Presumably they were either lysed or remained attached to the glass beads even after elution with EDTA. In either event, EDTA was capable of eluting only a small percentage of adhered monocytes. Trypsin, neuraminidase and lysosyme were tried, quite at random, as a means of dislodging adhered monocytes from 200 $\mu$  glass beads without success. Freezing and thawing (21) and cooling the column to 4°C were also unsuccessful.

Chicken monocytes were not eluted by EDTA and in this regard it is interesting to compare the speed with which monocytes and granulocytes, both human and chicken, move

over glass at 37°C and the ability of EDTA to elute these cell types from glass. Human granulocytes and monocytes along with chicken granulocytes travel in the range of 30-40  $\mu$  per minute (12,88) and are to a certain extent detached from glass by EDTA. Chicken monocytes move at about 1  $\mu$  per minute (88) and are not eluted by EDTA from a glass surface. Whether this slow movement of chicken monocytes compared to other cell types implies a stronger or different mode of attachment to glass is open to speculation.

Using wax coated beads packed in a column, it was possible to obtain monocytes by melting the wax. However the large numbers of erythrocytes present appeared to occupy a large area of the column seriously reducing the area available to which the monocytes and granulocytes could adhere. Therefore it seemed necessary to remove the erythrocytes from leucocytes before attempting the separation of individual leucocyte types.

#### The Separation of Leucocytes from Erythrocytes

The ability of certain substances to increase rouleaux formation of erythrocytes and thus increase their sedimentation rate, has been used to prepare leucocyte rich suspensions. This technique is however limited to those species whose erythrocytes form rouleaux. Cow and goat erythrocytes do not form rouleaux (96). It is our experience that chicken erythrocytes do not form rouleaux either.



The floatation technique appeared to be the best means available for the separation of leucocytes from erythrocytes. Albumin has been used, with a measure of success, in this regard. However the cost and time involved in its preparation are serious disadvantages.

Ficoll appears to offer a medium suitable for the separation of leucocytes. Compared to albumin it is cheap and does not require extensive reworking. Ficoll was dialysed against distilled water and freeze-dried before use. Providing the specific gravity was 1.039 for a 10% Ficoll solution in SBSS no differences were observed in its performance as a floatation medium from batch to batch.

Anderson (3) and Hilal (56) have discussed some of the problems associated with density gradient techniques. In our experience the greatest problem was the prevention of non-specific clumping produced by centrifugation and also possibly by the floatation medium itself. This clumping, along with the cost of albumin, has seriously limited the usefulness of the floatation technique. Consequently, in our early floatation studies, it was thought that the separation of leucocytes could best be achieved by preventing non-specific clumping of leucocytes. This clumping could be prevented to some extent by using an EDTA BSS and our first separations of leucocytes from erythrocytes were accomplished using Ficoll dissolved in this solution (86). However chicken granulocytes were adversely affected by EDTA BSS

if left in this solution at room temperature for even a short period of time. Subsequent work was carried out using SBSS.

Seligmann's BSS, containing sodium acetate, prevented the clumping of chicken leucocytes which readily occurs even in freshly drawn blood. The mechanism by which sodium acetate prevents clumping is unknown. By taking the blood directly into an equal volume of SBSS, clumping was prevented. The use of EDTA as anticoagulant, 4°C and the dilution of the blood by SBSS are probably additive factors in the prevention of leucocyte clumping. Centrifugation resulted in the separation of lymphocytes, monocytes and thrombocytes only. The granulocytes remained mixed with erythrocytes and it was not possible to separate further these two cell types by Ficoll floatation. It appears that a proportion of the erythrocytes have the same or lighter specific gravity when compared with granulocytes.

When clumping of the leucocytes was encouraged by heparin (6, 42) and HBSS, a layer of leucocytes was obtained which now contained all leucocyte classes. These clumped cells were easily dispersed in SBSS.

The method of separating human leucocytes from erythrocytes was essentially the same as that given for the chicken. Using SBSS and EDTA as the anticoagulant, the separated cells consisted of lymphocytes, monocytes and platelets, while with HBSS and heparin all leucocyte types were obtained. Due possibly to the larger number of erythrocytes

per cu. mm. in human blood, the distance separating the layer of leucocytes from erythrocytes was not as great in human compared to chicken. The success of the separation of all leucocytes types from erythrocytes appears to depend on the initial degree of clumping of the leucocytes. In the chicken, the large number of leucocytes per cu. mm. and the relative ease with which they clump lends itself to such a separation. Human blood, on the other hand, contains only about one quarter of the leucocytes and nearly twice as many erythrocytes per cu. mm. Leucocyte separation from erythrocytes in human was improved if the blood was mixed with heparin at room temperature to promote clumping, then layered over the Ficoll gradient. The isolated leucocytes could be dispersed easily in SBSS.

A 35% Ficoll solution in SBSS has a specific gravity of 1.126 which is higher than the value quoted for the human erythrocyte (112). The apparent paradox of cells passing through a solution of specific gravity greater than their own can be tentatively explained. On the initial 35% Ficoll solution the leucocytes separated out as a band of cells a few mm. below the Ficoll: plasma interface. These leucocytes when redispersed in SBSS would not penetrate a 35% Ficoll solution when centrifuged under the same previous conditions. It appears therefore that as the erythrocytes passed through the interface, small amounts of plasma were carried along also, thus modifying the original Ficoll solution. This would account for the band of

leucocytes forming below the Ficoll: plasma interface. We have withdrawn from the bottom of the tube 5ml. of the Ficoll containing the separated erythrocytes, and after removal of the latter the Ficoll has a specific gravity that was less than that of the original 35% Ficoll solution. The extent of this dilution effect is worthy of further investigation. Attempts to use Ficoll solutions of the same specific gravity as the albumin solutions used by Vallee, Hughes and Gibson (112) proved unsuccessful in that leucocytes passed through the interface along with the erythrocytes. Possibly the difference in viscosity between Ficoll and albumin is relevant in this context. Fawcett and Vallee (36) made the suggestion that viscosity was of importance as blood floated on albumin yet sank when layered over sucrose of the same specific gravity. The problem of high osmotic pressure of sucrose might have been a factor in the blood sinking through this solution. Biggers and McFeely used a 26% Ficoll solution to effect a separation of bovine leucocytes from erythrocytes, although they stated that an incomplete separation of leucocytes from erythrocytes resulted. Ficoll solutions much below 35% have resulted, in our experience, in incomplete separation of leucocytes from erythrocytes.

#### The Separation of Individual Leucocyte Types

The dispersion of clumped leucocytes by SBSS made it possible to attempt further separation of leucocyte types

by Ficoll floatation.

Single step floatation failed to achieve a separation sufficient to allow recovery of leucocyte types. Lymphocytes and monocytes separated as a band at the Ficoll interface immediately above and in contact with a layer of erythrocytes and granulocytes. The absence of large numbers of erythrocytes prevented the establishment of a linear gradient at the interface, so that an abrupt change in density occurred at the Ficoll: medium junction. As the leucocytes met this interface they tended to collect and form clumps of mixed cell types thus giving an incomplete separation of individual cell types. Abrupt changes in density at the medium: Ficoll interface could be avoided by allowing the Ficoll: SBSS to equilibrate for varying lengths of time. This type of preparation separated in addition to bands of leucocytes, a band of lighter erythrocytes. Vallee, Hughes and Gibson (112) have noted the separation of lighter erythrocytes from the blood of patients with anemia, after floatation on albumin. Ferrebee, using dense solutions of plasma to obtain differential floatation of erythrocytes parasitized by malaria, found the parasites in a layer of lighter red cells, and presented evidence to show that these lighter cells were young forms of erythrocytes (37). Studies on the separation of cells from bone marrow using Ficoll floatation have shown that reticulocytes separated out above the mature granulocytes (28). The presence of lighter erythrocytes explains our inability to separate granulocytes from

erythrocytes obtained by using SBSS and EDTA in the initial separation of leucocytes from erythrocytes.

The simple gradient produced by allowing SBSS to equilibrate with a 28% Ficoll solution for 24 hours, resulted in the separation of lymphocytes, monocytes and thrombocytes from granulocytes. A few thrombocytes and erythrocytes remained as contaminants in the granulocyte fraction. The wax bead column effectively separated lymphocytes from monocytes and thrombocytes but unfortunately the wax could not be completely removed from the monocytes, making it very difficult, if not impossible, to proceed further with the isolated cells. The granulocytes could be freed from the few contaminating erythrocytes by paradoxical sedimentation, a procedure which proved to be very effective (78, 94).

Various gradients were investigated to separate lymphocytes from monocytes and it was soon seen that these two cell types, particularly in the chicken, have close specific gravities. It was found that centrifugation first at 50g. and then at a higher centrifugal force resulted in a better separation of the cell types. Possibly the initial lower centrifugal force pulls the heavier granulocytes into the gradient slightly ahead of the other leucocyte types. Thus the layers of lighter lymphocytes and monocytes which separate out higher up the gradient are not disturbed by granulocytes passing through to the lower portion of the gradient.

The use of the gradients presented problems of which the greatest was that certain conditions tended to make the cells clump on passage through the gradient during centrifugation. Step gradients produced by layering various percentages of Ficoll over one another and used immediately, readily induced clumping of the leucocytes. Each time the cells confronted an abrupt change in specific gravity, albeit even a small change, the degree of clumping increased and this eventually led to incomplete separation of leucocyte types. This was much more pronounced with chicken blood which, in our experience, clumps much more readily than human. Subsequent separation of the various types of leucocytes was accomplished using fine step gradients which were left to equilibrate for 24 hours. This softened the abrupt change occurring at each interface and greatly reduced the incidence of clumping.

Overloading the gradient with cells led to clumping and the resulting incomplete separation. In the case of chicken leucocytes, the long path distance through a solution of gradually increasing specific gravity tended to produce aggregates of cells; an effect which could be kept to a minimum by the addition of Tween 80. Killmann (63) has used Tween 80 to prevent non-specific clumping during leucoagglutination tests. Centrifugation, beyond the time required to effect a separation, also tended to produce clumping of the cells already separated so that

the cells would sink lower and contaminate other bands. Using discontinuous and continuous gradients it was possible to separate the various types of leucocytes.

Two other problems encountered were the visualization and recovery of the bands of separated cells. Cells as few in number as the human monocyte could separate as a band which would not be visible under normal lighting conditions. After trying various light sources and directions of incident light, it was found that a light source directed through the bottom of the tube revealed faint bands of cells which otherwise went unnoticed. Visualization of these bands in a dark room using the light source made photography of the bands and recovery of the cells possible.

Initial recovery of cells had been performed with a tube slicer which proved to be somewhat temperamental in our hands, with a few samples ending up on the floor. Forcing the contents of the tube upward using a high density sucrose solution, or draining the contents of the tube from the bottom tended to produce gross contamination of the bands of separated cells. It was found that simple piercing of the tube immediately below the cell layer to be recovered made for an efficient and easy recovery with a minimum of contamination by other cell layers. This process was performed whilst visualizing the bands using the previously described light system. This method of recovery has the added advantage that the bands of cells



need only be separated by the thickness of the needle to enable a recovery.

Thus leucocyte types could be separated and collected using Ficoll floatation. Throughout the various stages of cell separation contamination of the different cell fractions by thrombocytes (platelets) was a problem. Thrombocytes constantly altered their specific gravity as they formed small clumps or broke down and shed their cytoplasm. In human blood, this problem was not as acute due to the great difference in specific gravity between platelets and the other leucocyte types. In chicken blood, thrombocytes are present as nucleated elements closely approximating lymphocytes in size. The specific gravity of chicken monocytes, lymphocytes and thrombocytes appear to be very close, making separation of these cell types more difficult. Mammalian blood with much lighter platelets does not present the same problem.

## Summary and Conclusions

1. Attempts to use the method of Rabinowitz to separate chicken leucocyte types were unsuccessful due to the inability of dextran to encourage rouleaux formation in chicken erythrocytes. A leucocyte rich suspension could not be obtained therefore by this method. EDTA would not release chicken monocytes from 200 $\mu$  glass beads. Monocytes could be obtained from waxed bead columns but the difficulty of removing the wax from the cells limited the usefulness of this method.
2. Ficoll, a polysucrose material, is suitable for the separation of leucocytes from erythrocytes and for the separation of individual leucocyte types by density gradient technique. It is particularly useful for leucocyte separation from erythrocytes in those species whose erythrocytes do not form rouleaux, as the sedimentation technique cannot be applied to produce leucocyte rich suspensions in these cases.
3. Ficoll has the following advantages over albumin as a density gradient medium. It is cheap, requires little reworking and is stable indefinitely. As far

as can be determined it is non-toxic to cells.

4. The main problem associated with the floatation technique and one which has severely limited its use; that of severe cell clumping can be overcome by the use of Seligmann's balanced salt solution.
5. The separation of leucocytes from erythrocytes is dependent upon the balanced salt solution and the anticoagulant used. Seligmann's balanced salt solution with EDTA as anticoagulant results in the separation of monocytes, lymphocytes and thrombocytes (platelets) while Hank's balanced salt solution with heparin as the anticoagulant results in the separation of all leucocyte types.
6. The composition of the gradients used to separate individual leucocyte types are presented and a method of observing and recovering the bands of isolated cells was developed. A separation of all leucocyte types was achieved as well as a separation of reticulocytes from erythrocytes.
7. The viability of the isolated leucocytes is comparable to that obtained by other methods where centrifugation has been used. There is greater cell damage than that produced by the sedimentation technique for the separation of leucocytes from erythrocytes, due probably to the initial high centrifugal force required. However the higher leucocyte yield and lower erythrocyte contamination

obtained using Ficoll floatation compensates for this loss of viability. The slight loss of approximately 5% in viability is not a serious disadvantage of the method. The morphology of the isolated cells was excellent as judged by phase contrast microscopy.

8. Further development, modification and application of the method is possible, e.g. the separation of the various types of granulocytes; the separation of cell types from haemopoietic organs and other tissue cells in general.
9. It is now possible to study the specificity of the antigens to certain leucocyte types and to see if an immunological classification, based on specific cell antigens, is at all possible.

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## Appendix I

- 1) All glassware was siliconized with Siliclad, Clay Adams Inc. New York.  
Stainless steel needles, 3" long # 18 gauge, used for chicken heart puncture treated with Monocote-E, Armour and Co., Kankakee, Illinois.
- 2) For chicken heart puncture, the needle was inserted medially through the dorsal opening of the clavicles, direction posterior and downward in line with the carina of the sternum.
- 3) Human blood samples were taken from the vena cubiti, using 5ml. disposable syringes. Tomac, American Hospital Supply, Evanston, Illinois.
- 4) The Ficoll solutions differing by  $\frac{1}{2}\%$  were accomplished by making the appropriate dilution with SBSS of a stock 18% Ficoll in SBSS. All Ficoll solutions were filtered through a millipore filter of  $0.45\mu$  pore size (Millipore Filter Corp., Bedford, Mass.) and the pH readjusted with sterile  $\text{CO}_2$  if necessary.

## Appendix II

## Composition of Balanced Salt Solutions

## EDTA Balanced Salt Solution

EDTA	0.20gm.
NaCl	8.00gm.
KCl	0.20gm.
Na <sub>2</sub> HPO <sub>4</sub>	1.15gm.
KH <sub>2</sub> PO <sub>4</sub>	0.20gm.
Glucose	0.20gm.

per litre of double distilled water.

## Seligmann's Balanced Salt Solution

NaCl	7.65gm.
KCl	0.20gm.
NaCO <sub>2</sub> CH <sub>3</sub>	1.50gm.
NaH <sub>2</sub> PO <sub>4</sub>	0.05gm.
KH <sub>2</sub> PO <sub>4</sub>	0.10gm.
NaHCO <sub>3</sub>	0.70gm.
Glucose	1.00gm.
Ascorbic Acid	0.003gm.

per litre of double distilled water.

## Hank's Balanced Salt Solution

NaCl	8.00gm.
KCl	0.40gm.
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.20gm.
CaCl <sub>2</sub>	0.14gm.
Na <sub>2</sub> HPO <sub>4</sub> .2H <sub>2</sub> O	0.06gm.
KH <sub>2</sub> PO <sub>4</sub>	0.06gm.
Glucose	1.00gm.

per litre of double distilled water.

pH adjusted with 25ml., 1.4% NaHCO<sub>3</sub>.

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All BSS were filtered through a millipore filter of 0.45 $\mu$  pore size. pH readjusted with sterile CO<sub>2</sub>, if necessary.