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The Use of Human Blood Cells in the Study of Lipid Metabolism

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Abstract

Certain aspects of the use of human blood cells in the study of lipid metabolism are reviewed. A discussion of the different cells and their varied functions illustrates the complex nature of the formed elements of the blood, and that studies of these cells must take into account this heterogeneity in planning experiments and in interpreting results. Investigation of blood cell lipids requires either the use of anticoagulants or defibrination for the separation of the cells from the fluid part of the blood. Some of the problems resulting from these procedures are reviewed. Available methods for separating the heterogeneous cell mixture of normal blood into homogeneous cell populations are presented. It is stressed that the large number of methods attests to their limitations and that none is completely satisfactory. The preparation of the separated cells for lipid analysis is discussed, but methods of lipid analysis are not presented. A brief presentation of the lipid synthesis and content of human blood cells in normal and abnormal conditions is given with occasional supplementary data from the study of other species.

Introduction

ALTHOUGH BLOOD CELL LIPIDS have been investigated for a number of years, the recent development of newer techniques and approaches have greatly broadened the research possibilities in this area. Because investigators in this field may not have had previous experience in working with blood cells, a review of current knowledge in the processing of blood for study of cell lipids may prove helpful.

Blood Composition

The fluid part of the blood, the plasma, contains many diverse molecules ranging in size from water and electrolytes to lipids and proteins. Normally, in an adult man, plasma constitutes about 53% of the blood volume, the cellular elements comprising the other 47% (1). Erythrocytes constitute most of the cell mass. A cubic millimeter of normal blood contains the following average number of cells: 5,400,000 erythrocytes (red blood cells) in the male (4,800,000 in the female) (1); 7,000 leukocytes (white blood cells) (2); and 250,000 thrombocytes (platelets) (3).

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The plasma as a whole serves as a fluid medium in which the cellular elements are transported through

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the blood vessels to the various parts of the body. The exact function of many of the individual plasma components is still not known, however. The plasma lipids are almost always found in association with protein and have been identified as: cholesterol, phospholipids, glycerides, cerebrosides, carotenoids and unesterified fatty acids. It is believed that the plasma lipids have no specific function per se in the plasma but are in transit from one anatomical location to another, although their possible influence on the circulating blood cells themselves remains to be clarified. Serum is the fluid portion remaining after blood or plasma has been permitted to clot. It is similar to plasma, but lacks fibrinogen, prothrombin and certain other clotting factors which are consumed in the coagulation process.

The various cellular components of the blood can be readily identified by special stains (for example, Wright's stain); however, morphologic identification does not permit functional characterization. Other studies have provided further information concerning their various functions. It is known that in normal man the bone marrow is the site of production of most of the blood cells found in the circulation; lymphatic tissue such as lymph nodes and spleen serve as primary sources of production for some of the leukocytes known as lymphocytes (4).

The mature circulating erythrocyte is a highly specialized cell with a well-defined lifespan. It has lost its nucleus by the time it enters the blood stream, but it may still contain some RNA (ribonucleic acid) which can be demonstrated as a reticulum with special stains. Accordingly these young cells are known as reticulocytes (5). As the cell ages, this reticulum is lost. Although the mature erythrocyte lacks a nucleus, it is metabolically active. On the average, the lifespan of an erythrocyte is 120 days (6). Certain changes are known to occur with increasing age of the cell, but for the most part these are not reflected in the appearance of the mature erythrocyte. Normally, only 0.5–1.5% of the total circulating erythrocytes are reticulocytes (5); however, their proportion can be greatly increased, as in response to blood loss or destruction.

The lipid content of red blood cells (erythrocytes) can be expressed in different ways, such as a given amt of lipid per 10^{10} red blood cells or per gram of hemoglobin or per surface area of the red cell or per volume of red blood cells or per weight of erythrocytes. Table I may, therefore, be of help in converting results from one mode of expression to others when the size, shape, and hemoglobin content of the erythrocytes are normal.

Aside from the differences related to age, erythrocytes are essentially a homogeneous population, while leukocytes are divided into five subgroups which are readily recognized by stains which reveal a varied morphology. These cell types in their average relative proportions are as follows: polymorphonuclear neutrophils (neutrophilic granulocytes, "polys") 57–67%; lymphocytes 25–33%; monocytes 3–7%; eosinophils 1–3%; and basophils 0–0.75% (2). It seems clear that the cell types have different functions, and the main reason for grouping them together is that they contain nuclei, lack hemoglobin and are quite distinct from platelets which will be discussed later.

The main function of the polymorphonuclear leukocytes seems to be related to the body's acute response to infection. During various types of infection, the number of these cells circulating in the blood is increased, and they may accumulate at the point of a localized infection. It is important to recognize that the circulating polymorphonuclear leukocytes may constitute only 1/60 of the total body content of such cells (9); it is still not clear whether the circulating leukocytes are truly a representative sample of the noncirculating cells or whether they are distinctive either by age or in regard to a specialized function. Attempts to determine the lifespan of the polymorphonuclear neutrophils have met with frustration due to a lack of knowledge concerning their fate after they leave the circulating blood and enter the body tissues. Nevertheless, studies indicate that they have a circulating lifespan of 6½ to 11 days prior to the time they enter the tissues (10). The age of polymorphonuclear leukocytes is partly reflected in the degree of segmentation of the nucleus, young forms (sometimes called stab cells) having an unsegmented nucleus and older forms normally having up to five segmented lobules to the nucleus.

Lymphocyte function is incompletely understood, although the cell appears to play an important role in the immune response of the body. While information concerning the kinetics of lymphocyte production and function is limited, DNA labeling experiments indicate a lifespan of 100–200 days for most of the cells although about 20% of them seem to have a mean age of only 3–4 days (11).

Monocytes may act as scavenger cells, inasmuch as they are found in increased numbers in areas of inflammation. The eosinophils, containing distinctive large granules, may play a role in detoxification processes and often are increased in allergic reactions. The stained basophils are also easily identified by distinctive granules which seem to contain heparin, an anti-

TABLE I

Conversion Table for Expression of Results for Measurements of Red Blood Cells Normal in Size and Shape

	mm^3	No. of RBC	Gm Hb	RBC surface area (cm^2)	Ml	Gm RBC
1 mm^3		1.15×10^{7} RBC	3.40×10^{-4} Gm	1.61×10^{1} cm^2	1.00×10^{-3} ml	1.10×10^{-3} Gm
1 RBC	8.70×10^{-8} mm^3		3.04×10^{-11} Gm	1.4×10^{-6} cm^2	8.70×10^{-11} ml	9.55×10^{-11} Gm
1 Gm Hb	2.94×10^{3} mm^3	3.38×10^{10} RBC		4.74×10^{4} cm^2	2.94 ml	3.22 Gm
RBC Surface Area (1 cm^2)	6.20×10^{-2} mm^3	7.13×10^{5} RBC	2.11×10^{-5} Gm		6.20×10^{-5} ml	6.80×10^{-5} Gm
1 ml	1.00×10^{3} mm^3	1.15×10^{10} RBC	3.40×10^{-1} Gm	1.61×10^{4} cm^2		1.10 Gm
1 Gm RBC	9.15×10^{2} mm^3	1.05×10^{10} RBC	3.11×10^{-1} Gm	1.47×10^{4} cm^2	9.11×10^{-1} ml	

Based on the following values taken from *Clinical Hematology* (5th ed.) by M. M. Wintrobe.
RBC count = $5.4\times10^{6}/mm^3$ whole blood (1)
Hemoglobin (Hb) = 16.0 Gm% whole blood (1)
Hematocrit (Hct) = 47% whole blood (1)
RBC area = 140 square microns per RBC (7)
RBC specific gravity = 1.095 (8)

coagulant, as well as histamine and serotonin (12).

The third distinctive cell group in the circulating blood is the blood platelets. These spherical bodies are considerably smaller than the leukocytes or erythrocytes, do not have a nucleus, and seem to be mainly concerned in the control of bleeding. They may absorb many proteins to their surface, such as serotonin and various clotting factors.

Processing of Blood

The preceding discussion should make it clear that the investigation of the formed elements of the blood as a single group can lead to confusion, and that even work limited to a specific cell type may also prove troublesome because of the variation in age and lipid content of the cells examined. Nevertheless, techniques for the separation of these cells and analysis of their contents have been developed.

After removal from the body, blood normally clots. Coagulation results in the formation of an insoluble protein network (fibrin) through a sequential series of reactions (13). The cellular elements of the blood are trapped in the net, and this process may aid in sealing a break in the wall of a blood vessel. However, in the study of blood cells this phenomenon can prove troublesome and many different substances have been used as anticoagulants to prevent clotting. The effect of these anticoagulants on the cells and more specifically on the cell lipids, is not known. Therefore, it may be hazardous to make comparison between different investigations using different anticoagulants. An ideal coagulant should not alter the blood composition or its characteristics at all, but in reality, all that can be hoped for is one which has a minor and relatively well-defined effect. One must consider not only the chemical aspects of the problem, but also the physical characteristics such as changes in osmolarity and pH of the plasma caused by the anticoagulant.

Because anticoagulants present such problems, attempts have been made to separate cells by permitting the blood to clot in a way that the fibrin which is formed is removed by absorption on glass beads or wooden sticks. This process, known as defibrination, removes the fibrin as it is formed, but also removes some cells which adhere to the fibrin clot. Carbon dioxide is lost from the blood as defibrination is carried out, and thus, the pH rises to high levels, approximating pH 7.9 (14). Platelets, which are directly involved in the clot formation, are necessarily removed, at least in part, by this procedure. In addition, some leukocytes and erythrocytes become entangled in the fibrin clot and will be lost to analysis. Prothrombin (Factor II) has also been converted to thrombin in this process. Although unlikely, it is possible that certain lipids of these cells may be altered in the clotting process; also the cells may be broken and destroyed by this procedure. The cells so lost are probably older and in some ways less resistant to physical trauma (15). When the fibrin clot is removed the remaining cells can be separated by methods which will be described later. Thus, although defibrination has the advantage of not adding any foreign substances to the blood, there is no doubt that this process does produce morphological and biochemical changes in the sample which must be taken into account. Defibrination is, in general, a satisfactory means for the collection of red cells or of serum, but unsatisfactory for the other formed elements.

Heparin (16,17) is an anticoagulant which may occur in small amts naturally in the blood (18). It is a mucoitin polysulfuric acid having an estimated mol wt of 20,000 with a very strong electronegative charge which is considered responsible for its anticoagulant property. Heparin has been demonstrated to act at four different stages of the blood clotting sequence. Interestingly, it has also been found to have a clearing effect on lipemia (elevated plasma lipids) in vivo (19) although it may lack this effect on plasma in vitro; however, it is not clear whether this effect occurs in vitro in the presence of blood cells, an important consideration if heparin is used as anticoagulant in the study of blood cell lipids. In routine blood work heparin is used infrequently, because it is relatively expensive and prevents coagulation for only a limited period (20). Under carefully controlled conditions, 1 unit of heparin prevents the coagulation of 1 ml of plasma. In practice, however, it is usual to employ a 10–25 fold excess of the anticoagulant. Most commercial preparations of heparin contain a small amt of preservative such as phenol or benzyl alcohol and although only small amts of heparin are needed for anticoagulation the effects of the preservative in the given test system would need to be defined. At least two studies suggest that heparin may not be suitable for use in blood cell lipids. Hanahan et al. (21) found considerable alteration of the phospholipid fraction of the erythrocyte, and injury to the erythrocyte membrane (where the lipids occur) was found by Anderson and Turner (15) when heparin was used. In addition, Frei (22) has noted that leukocytes behave differently from normal in heparin.

The majority of the other anticoagulants act by binding calcium which is required for the formation of fibrin. Ethylene diamine tetraacetic acid (EDTA, Sequestrene, Versene) chelates calcium as well as apparently having some inhibitory effect on the conversion of fibrinogen to fibrin. It is generally considered to have a minimal effect on blood cells including the platelets and frequently is used, usually at a concn of 1 mg/ml whole blood, in biochemical studies of blood cells (20). In studying enzymes in the blood, it may be important to remove excess EDTA by washing, since some metal-requiring enzymatic reactions are inhibited by it.

Potassium oxalate is frequently used in routine hematological work. One advantage of this anticoagulant is its modest expense and the fact that it can be employed as a solution or in the dried state. Although a 1.6% solution is said to be isotonic with blood (23), a balanced oxalate mixture is frequently used to prevent alteration in the size of the erythrocytes through osmotic imbalance. A balanced mixture contains 3 parts ammonium oxalate (alone, it causes swelling of the cells) and 2 parts potassium oxalate (alone, it causes shrinkage of the cells); 2 mg of this mixture are used for each milliliter of whole blood. Anderson and Turner (15) found that 1.4% potassium oxalate compared to ACD (see below) resulted in a considerable diminution of membrane substance. It would be of interest to know if a balanced oxalate mixture has similar effects, because a 1.4% potassium oxalate solution may have this effect by hypotonicity alone. Oxalate is extremely toxic to many enzymes found in the blood cells, and therefore, is unsuitable for use in metabolic studies.

Numerous investigations have been carried out to develop suitable solutions for storage of whole blood in which the cells, especially the erythrocytes, can be kept viable for prolonged periods. Since these solutions contain anticoagulants, consideration should be

given to their use in the study of cell lipids. In point of fact some studies of blood cell lipids have been carried out on blood cells collected in ACD solution (acid-citrate-dextrose) (24). Two different ACD solutions are available. Solution A is used in a ratio of 15 ml to 100 ml of blood and contains 22 g sodium citrate, 7.4 g anhydrous citric acid, and 24.5 g of dextrose, all dissolved in water to make 1000 ml. Solution B is used in a ratio of 25 ml to 100 ml of blood and contains 13.2 g sodium citrate, 4.4 g anhydrous citric acid and 14.7 g dextrose all dissolved in water to make 1000 ml. The citrate serves as an anticoagulant, the low pH (5.0) of the solution results in a relatively physiological pH (7.4–7.5) at 4C (25), and the glucose serves as substrate for red cell metabolism. While unsuitable for preservation of white blood cells and platelets, a high proportion of red cells remain viable on reinfusion into a compatible recipient after they have been stored for several weeks in the ACD solution. The effect of this anticoagulant preservative on blood cell lipids has not been defined clearly.

Blood Cell Separation

Once the blood is suitably prevented from clotting or the clot removed, the next step is the separation of the cells from the plasma or serum. This also requires the separation of the various types of cells from each other so that a fairly homogeneous sample is available for study. This matter of separation is somewhat more difficult than it might seem superficially, but is quite important. One recent example of its importance in the study of blood cell lipids has been well demonstrated (26,27) and discussed by Buchanan (28) who showed that active lipid metabolism had been attributed incorrectly to erythrocytes because of contaminating leukocytes.

Four main approaches or combinations of these have been utilized in attempts to improve cell separation: 1) differential centrifugation with and without special tubes; 2) use of sedimenting agents which increase the speed or rate of erythrocyte settling through rouleaux formation; 3) separation with and without centrifugation using solutions of different density; and 4) lysis of unwanted erythrocytes. The great variety of described methods attest to the difficulty of achieving good cell separation since none gives complete partition. A short review of previously reported methods may prove useful in demonstrating their limitations.

The different blood cell types have different specific gravities: 1) erythrocytes 1.092; leukocytes 1.065; and platelets 1.030 (29). These differences form the rationale for centrifugation as a means of separating the cells. The denser cells are found at the bottom and the lighter at the top of the centrifuge tube. By utilizing different degrees of centrifugal force it is possible to obtain a fair separation of the cells. For example, if one is interested in obtaining only erythrocytes, centrifugation packs these at the bottom of a centrifuge tube and withdrawal of the leukocyte-platelet layer, the "buffy coat," removes 60–80% of the leukocytes (30). By removing the contaminating lighter leukocytes and platelets with a pipette and with subsequent washing of the separated erythrocytes with isotonic salt solution followed by recentrifugation, the purification can be increased with each washing. There is undoubtedly some loss of younger erythrocytes with the buffy coat, especially if adequate care is not taken to avoid aspirating the top of the red cell layer. Although some studies (15,31) have indicated a loss of erythrocyte lipids upon repeated saline washing, others (21,32,33) have not confirmed this observation.

If a sample of anticoagulated blood is left to stand in a tube, a separation of cellular elements eventually occurs due to the varied specific gravities of the cells. Complete sedimentation occurs in about 14 hr but does not give good cellular separation (34). Several agents have been found to cause clumping of red cells, thus enhancing their sedimentation in a tube (35,36). Such substances can be used either alone or before centrifugation of the sample, to aid in the separation of erythrocytes from leukocytes and platelets. Among the sedimenting agents used have been dextran, polyvinylpyrrolidone and phytohemagglutinin; these agents cause rouleaux formation or adherence of the flat surfaces of the discoid erythrocytes through an unknown mechanism. One major problem with the use of such agents is that they may affect the cellular lipids which are to be studied later. Seabright (34) compared the results obtained with these substances and reported an erythrocyte lysing method using a mixture of acetic and tartaric acids, which in his hands gave the best results for isolating the leukocytes.

In separating the polymorphonuclear leukocytes from other white blood cells, Cassen et al. (37) used the phagocytic activity of these cells to advantage. By permitting in vitro phagoctyosis of gum arabic coated iron spherules these cells became heavier and could be spun down in a centrifuge. The method was apparently well suited for obtaining relatively pure suspensions of lymphocytes.

Others have resorted to ultracentrifugation for enhanced separation of cells without apparent alteration of cellular morphology (38).

Vallee et al. (39) and Prankerd (40) centrifuged blood in bovine albumin and found an improvement in separation; however, it is also noted that the leukocytes have a semipermeable membrane so that their volume and density will be altered by solutions of varying density (39). Agranoff et al. (41) layered anticoagulated blood over two iso-osmotic solutions of albumin of different densities. The two albumin solutions did not mix on centrifugation and the cells settled out in the different solutions according to their densities; they were able to isolate cell types in purity exceeding 90% with this method. Spear (42) used gum acacia in a similar fashion and PVP has also been used in this manner (43).

Lysis of red blood cells by hypotonic solutions or saponins (9,28) can selectively separate leukocytes from erythrocytes as well as separate erythrocytes of different ages from each other, inasmuch as older red blood cells are more easily lysed than the younger red cells (45–48). The effects of this treatment on the lipids of the unlysed cells is still unknown (48).

Passing blood through columns of glass wool (49) or cotton wool (28,50) is said to selectively adsorb out leukocytes and platelets, which can then be eluted in a concd state. Two recent methods have achieved leukocyte separations with a glass bead column using special washes to elute the leukocytes from the glass beads (51,52). Tullis (29) also noted that platelets can be separated from the other blood cells by using resin beads. Singer et al. (53) reported that lymphocytes were obtained in animals in a relatively pure state by cannulating the thoracic lymph duct, a procedure not practical for human work.

Levine (54) tried to collect red blood cells by the use of magnetism. He reasoned that these cells contained iron in a paramagnetic form and might therefore be attracted to a magnet; however, the cells could not be separated with this technique probably due in

part to the loss of paramagnetic properties of the hemoglobin when the ferrous iron couples with certain molecules, such as oxygen. Nevertheless, he was able to separate polymorphonuclear neutrophils with a magnetic field after permitting these cells to first phagocytize small iron particles.

Still another approach is described by Wildy (55) who found that leukocytes attached preferentially to siliconized or greased glassware. The sample was placed to a 2 mm depth in a flat dish whose surface has been coated with silicone. It was then incubated at room temp for 1 hr to permit the leukocytes to attach to the surface. Other cells were washed away and the leukocytes were removed from the silicone by gentle agitation with sodium EDTA solution; the leukocyte to erythrocyte ratio was changed from 0.0014 to greater than 1,800 with this method.

Cell Lysis and Preparation

Once a reasonable separation has been successfully achieved the next step requires that lipids of the cells be available for analysis in an unaltered form. The lipids can be released by lysis or disruption of the cell by fairly simple procedures. If the cell is not broken up, lipids inside the cell may not be released for analysis.

Exposure of red blood cells to concd salt solutions increases the permeability of the cells and then dissolution, possibly due to dispersion of the lipids from the cell membrane (56). Such an effect is undesirable here. Red blood cells can also be lysed by exposure to hypotonic solutions; and distilled water is often used. This removes most of the hemoglobin and other water soluble constituents leaving an insoluble residuum, often referred to as the membranes, the stroma, or the red cell ghost. Anderson and Turner (15) found that varying the hypotonic solutions introduces the possibility of denaturation and fragmentation of the cell membrane, whereas, repeated use of the same hypotonic solution did not produce this effect. However, several authors (57–59) describe a gradual osmotic hemolysis which more effectively removes hemoglobin from the red blood cells and produces electron microscopically intact red cell membranes. Dodge et al. (32) showed there was essentially no loss of lipids in preparing red cell ghosts by hypotonic tests. Freezing and thawing of red cells also leads to lysis of the cells, but results in greater fragmentation of the stroma and the release of some constituents (for example ATPase) which are not released on osmotic lysis. Sonication should also be an effective means of disrupting blood cells without alteration of the lipids. Condrea et al. (60) describe lysis of red cells by snake venom which, however, also alters the lipids in susceptible cells. Turner et al. (61) prepared the red cell stroma for lipid analysis by lyophilization.

Leukocytes are more resistant to osmotic lysis, and although freezing and thawing is more effective, Elsbach and Rizack (62) noted a change in the lipase and phospholipase activities with this treatment. They described the production of a leukocyte homogenate using a motor drive Teflon pestle. However, leukocytes tend to form viscous masses when they are disrupted, a problem which has not been satisfactorily resolved to our knowledge. Platelets can be handled in a manner similar to the leukocytes. A good method for extracting platelet lipids is given by Marcus et al. (63).

Cellular Lipids

A brief summary of the current information on the blood cell lipids themselves will help to illustrate the extent of our present knowledge on the subject. For the most part discussion will be limited to human blood cells and will be concerned primarily with erythrocytes as these are the cells most extensively studied.

In the red blood cell the lipid constituents are located mainly in the cell membrane and together with proteins, form an organized layer (64). After lysis of the cell only 2–5% of the wet weight of the cell membrane remains as insoluble material, the stroma or ghost, with lipid content reported from 10% (65) to 45% (32). This lipid consists of phospholipids, cholesterol, cholesterol esters, glycerides, free fatty acids, and smaller amts of other lipids including glycolipid. There is not good agreement as to the exact proportions of these (21,32,66,67) but phospholipids and cholesterol make up most of the lipids. Ponder (68) indicates that the lipids are highly oriented, forming a layer about 40 Å thick on or near the surface of the cell; at present no adequate data are available concerning lipids within the cell. Because of the apparent surface location of the lipids, and results of other studies (64), it has been suggested that the lipids may be related to permeability properties of the red cell membrane. Ponder (68) suggested and Lovelock (31) has demonstrated that repeated washings of the erythrocytes remove some of the lipids possibly due to their relative superficial position on the cell surface; compatible with this impression is the observation that repeated washings reduce the volume of the red cell ghost (68). However, Hanahan et al. (21) found 90–92% of the total red cell lipid still to be present in the stroma and to have the same distribution as in the intact cell, suggesting minimal alteration of the lipids in preparation of the stroma; Reed et al. (33) also reported no loss of cholesterol or phospholipids on washing the cells.

Extensive analyses of the lipids of the red blood cell have been published (21,33,66,70–74) but probably due to methodological differences, there is no consensus regarding the lipid composition in normal red cells.

Work by Erickson et al. (75) and Bentley (76) indicated that the red blood cells of infants and children have qualitatively and quantitatively the same lipids as those of adults.

Munn and Crosby (77) treated the red blood cells with carbon monoxide prior to analysis to prevent subsequent oxidation of unsaturated fatty acid linkages by oxygen from oxyhemoglobin. Lovelock (31) who found loss of cellular lipid with repeated washing of the cells, also reported that lipid loss was increased with storage of the cells.

Although some early work on blood cell lipid synthesis (78–80) suggested that mature erythrocytes may be actively involved in lipid synthesis, subsequent studies (26–28,81) indicated that this cell is not able to synthesize lipids, except possibly at the reticulocyte stage (82). It seems reasonable that a young erythrocyte can form its own lipids; the question of when in the life of the cell this synthesis ceases remains unanswered. Most studies of lipid synthesis have utilized radioactive labelled precursors such as C^{14}-acetate. A more recent report indicates red blood cells in vitro incorporate P^{32} phosphate mostly into phosphatidic acid; it is also noted that the content of the incubation medium affects this incorporation (83).

It is difficult to distinguish experimentally between synthesis of lipids by cells and merely exchange of lipids. Some studies indicate there are lipid binding sites on the red blood cell and its ghost (84,85). Others suggest that at least some lipids may have the capacity

of exchanging with the plasma or other medium: cholesterol (86,87); phospholipids, neutral fat, and unesterified fatty acids (88); lecithin and sphingomyelin (89); and lysolecithin (90). Lovelock et al. (91) found that there was no exchange of saponifiable lipid between whole blood cells and plasma lipids although there was an exchange of "unsaponifiable lipid" and phospholipid in the same system.

On the other hand, Oliveira and Vaughn (92,93) showed incorporation of C^{14}-labelled fatty acids into phospholipids by washed human red blood cell ghosts and that ATP was required for the process which was markedly stimulated by $MgCl_2$ and coenzyme A. Further studies by the same authors suggest that lecithin in the red cell ghosts is necessary for fatty acid incorporation into phospholipids to occur.

As the erythrocyte ages there seems to be an alteration in its lipid content. The work of Marks et al. (82,94) suggests that the reticulocyte can still synthesize a small amt of lipid. As the cell ages or matures it tends to lose total lipids (95). Prankerd (40) suggests that this loss may be mainly phospholipid. Further work by Munn (96) indicates a decrease in saturated acids and an increase in unsaturated acids, while the total fatty acids are also decreased in direct proportion to the reduction of the red blood cell surface area with age. He also noted that the free cholesterol increased as the cells matured. Because of such alterations with age, it seems that comparable studies require that cells of the same age be used.

Since a major source of the body's lipids is dietary, it may be reasonable to ask if it can affect the lipid content of the red blood cells. This problem has been explored, and it seems that dietary fats may reflect themselves in the lipids of the erythrocytes. Horwitt et al. (97) showed that the ingestion of linoleic-acid-containing fats increases the deposition of linoleic acid in the erythrocytes. Farquhar and Ahrens (98) noted that an equilibrium between dietary fats (fed at 40% of calories) and erythrocyte fatty acids and aldehydes occurs in 4–6 weeks. They interpreted this finding to mean that exchanges occur between mature erythrocytes and certain precursor pools. Incorporation of dietary fats into maturing erythrocytes in the marrow would result in equilibrium values at about 17 weeks, the lifespan of the erythrocyte. It was also found that all four major phospholipid classes participate in the changes at equal rates. The rate at which changes in fatty acid composition occur in the red blood cells is slower than in the plasma lipids but more rapid than in adipose tissue (98).

While animal studies can only occasionally be applied directly to man, it is of interest to look briefly at related experiments performed in other mammals. De Gier and Van Deenen (99) found only small differences in the total amts of red cell ghost lipids of sheep, ox, pigs, man, rabbit, and the rat, but there was considerable variation in the amts of individual types of phosphatides. Hallinan and Eden (100) reported a larger amt of phospholipid in reticulocytes than mature rat erythrocytes. Kögl et al. (101) studied palmitic-oleic acid ratios in different mammals and concluded there may be a possible relationship between fatty acid composition and cell permeability. The content of lecithin in erythrocytes was related to susceptibility to lysis by cobra venom (61,102). O'Donnell et al. (26) reported that rabbit reticulocytes and normoblasts can synthesize fatty acids and cholesterol, whereas mature erythrocytes from the chicken and rabbit have lost this capacity. Canine red blood cells have considerably more diglyceride and less NEFA than human erythrocytes (103).

Several studies carried out chiefly in rats (104–109) indicate that dietary changes in fats are reflected in the erythrocytes.

Boyd was one of the first to investigate the human leukocyte lipids (110), particularly in pregnancy (111) and surgery (112). More recent determination of the leukocyte cholesterol content indicates a wide variation in values which was considerably greater than in the erythrocytes; it was noted that there was no relationship of the content to the subject's age or sex.

In apparent contrast to the erythrocytes, the leukocytes engage in active lipid synthesis. This concept is supported by several studies using radioactive lipid precursors (26,28,81,82). Marks et al. (82) reported that normal leukocytes incorporate acetate into triglycerides and phospholipids and transfer these lipids into plasma lipids. Rowe et al. (81) found that leukocytes incorporate acetate into both saponifiable and unsaponifiable lipid fatty acids, as well as phospholipid fatty acids. Malamos et al. (113) demonstrated that ionizing radiation did not seem to affect the incorporation of acetate into leukocyte lipids. The work of Miras et al. (114) indicated that dietary fat and protein can affect the leukocyte lipid synthesis probably as an alteration of the turnover rate of the lipids. An observation which may be of considerable importance is that the content of the incubation medium for in vitro studies seems to affect the results of studies, both qualitatively as well as quantitatively (115).

Using Sudan Black B which stains neutral lipids, phospholipids and sterols, Sheehan reported that the granules of mature polymorphonuclear neutrophils stain heavily while the immature forms stain less and lymphoblasts did not take up the stain (116). Kidson (117) reported a low rate of total lipid synthesis in the leukocytes in infection although the ratio of phospholipid synthesis to neutral lipid synthesis did not differ markedly from normal controls; the lowest rate of synthesis occurred in the more immature cells. The findings were considered to represent dependence of lipid synthesis rate on cell age, with slow synthesis in the young cells of the myeloid series. Miles (118) noted that leukocytes which appear similar, but taken from different parts of the body (e.g., blood vs. peritoneal exudate) may be different, suggesting an environmental effect. In this regard Evans and Mueller (119) found that circulating leukocytes differ from exudate leukocytes in the guinea pig with respect to their metabolic behavior in the presence of palmitate.

Studies have been carried out on lipid metabolism in rabbit leukocytes. Burt (120) found that the polymorphonuclear neutrophils contained more total lipid than the red blood cells. O'Donnell et al. (26) demonstrated that rabbit leukocytes showed little or no capacity to synthesize cholesterol and fatty acids.

Elsbach (62,121–125) has extensively studied lipid metabolism of rabbit leukocytes from peritoneal exudates, removing the leukocytes at a time when most of them are polymorphonuclear neutrophils. Their lipid content constituted 8.7% of the dry weight and were characterized as: 60% phospholipids; 20% triglycerides; 17% cholesterol and cholesterol esters; and 3% nonesterified fatty acids. Although the lipid content and composition were not affected by phagocytosis, this process resulted in an increase in the rate of turnover of the lipids. Free fatty acids were rapidly

incorporated into cell lipids, whereas complex lipids did not seem to enter the cell but adhered to the surface. Other studies by the same author suggest that incorporation of nonesterified fatty acids by the leukocytes depends upon esterification and, therefore, upon the availability of glycerophosphate derived from anerobic glycolysis. Additional findings suggested that the esterification of free fatty acids occurs at the cell surface during uptake. Studies of the enzymes of these cells indicated that they contain three lipid splitting activities; lipase, phospholipase, and lysophospholipase.

Evans and Mueller (119) investigated the effect of palmitate on guinea pig exudate polymorphonuclear neutrophils and found that the respiratory quotient decreased from 1.34 to 0.82 when palmitate was added. Comparative results from these exudate cells and circulating leukocytes indicated that they differ with respect to their metabolic behavior in the presence of palmitate.

Platelet lipids appear to have been less thoroughly investigated than those of the erythrocytes and leukocytes. Following the early work of Erickson et al. in 1939 (126) considerable time elapsed before further reports appeared (127–129). The findings seem in general agreement regarding the lipid content of platelets and the close qualitative likeness of the platelet lipids with those of the erythrocytes. The following representative values are taken from Troup et al. (128): 17% phosphatidyl ethanolamine; 6% phosphatidyl serine; 32% lecithin; 13% sphingomyelin; 5% inositol phosphatide; 19% cholesterol; and 8% other lipids.

Marks et al. (82) found that platelets, like leukocytes, incorporate acetate into triglycerides and phospholipids, which can be transferred into plasma. Platelet lipids seem to be important in normal blood coagulation (130). Studies by Rouser et al. (131,132) indicate that phosphatidyl ethanolamine and phosphatidic acid greatly accelerate clotting of recalcified low platelet plasma. However, this effect of phosphatidyl ethanolamine was not confirmed by others (63, 133).

As with erythrocytes and leukocytes, Paysant et al. (134) found that the results of in vitro platelet lipid metabolism is dependent on the content of the incubation medium.

Blood Cell Lipids in Disease

In view of the incomplete state of knowledge of normal blood cell lipids, a considerable amt of work has been done in various disease states. Early work includes the study of anemias by Erickson et al. (135) and Williams et al. (136). Erythrocytes in pernicious anemia were found to have an excessive content of cholesterol esters and a deficiency of phospholipid and free cholesterol. They also reported abnormalities in the concn and distribution of erythrocyte lipids in hemolytic and hypochromic anemias of childhood. These findings need confirmation using newer methods and techniques.

Phillips and Roome (137) reported that the total lipid phosphorus concn was increased in patients with intermediate thalassemia, although there was no consistent difference from normal in the distribution of the individual phospholipid fractions. De Gier et al. (64) found a normal phospholipid distribution in congenital elliptocytosis, congenital hemolytic anemia, thrombotic thrombocytopenic purpura, and autoimmune hemolytic anemia with thrombocytopenia.

Another disease of erythrocytes, hereditary spherocytosis, has been extensively studied. Phillips and Roome (137) and De Gier et al. (138) found no abnormalities in these cells. However, Kates et al. (139) reported that the phosphatides of spherocytic cells contained a lower proportion of lysophosphatidyl ethanolamine; the latter component produced sphering of normal cells. These authors speculated that the primary genetic abnormality in this condition lies in a partial block in the enzyme system for conversion of lysophosphatidyl ethanolamine to phosphatidyl ethanolamine. Robertson and Lands (140) noted that the synthesis of diacyl glycerophosphoryl choline and diacyl glycerophosphoryl ethanolamine proceeded at equal rates in normal and spherocytic erythrocytes, and that both types of cells had similar amts of acylating enzyme activity and similar amts of endogenous monoacyl phosphatides available for acylation. They noted in passing that a possible factor controlling phosphatide composition may be differences in the protein portion of the cellular lipoproteins that lead to more stable binding of one phosphatide over another; this might be a critical factor in spherocytic cells having relatively normal metabolic activities but still appearing to have altered lipoprotein membranes. Reed (89) found that hereditary spherocytosis erythrocytes exchange significantly less lipid phosphorus than normal cells during several hours of incubation in vitro.

The erythrocytes from four patients with paroxysmal nocturnal hemoglobinemia have been found to have a normal phospholipid distribution (141).

Acanthocytosis is an inherited disease in which the plasma lipids are altered. An early study by Mier et al. (138) indicated that the total lipids and total lipid hexose of the erythrocytes differed from normal. Phillips (143) and Ways et al. (144) investigated the lipids of the red blood cells from affected individuals and agree that there is an increase in sphingomyelin and a decrease in lecithin. Phillips (143) observed little or no decrease in total phospholipid and cholesterol. Ways et al. (144) noted that the esterified linoleic content of the erythrocyte total phospholipid and individual red blood cell phospholipids were decreased to 25% or less of the amt normally found.

In some diseases of the central nervous system abnormalities occur in the lipids of the brain. Balint et al. (145–148) reported their findings in erythrocyte lipids in such diseases. The first of these was Niemann-Pick disease (145,146). The red cells showed a decreased content of cephalin. The decrease affected the phosphatidyl ethanolamine and serine fractions equally. In addition it was noted that the sphingomyelin in the red cells had fallen from a normal value of 26% to 10% of the phospholipid. In Tay-Sachs disease and in Gaucher's disease (147,148) results were found similar to Niemann-Pick disease: a significant decrease of both sphingomyelin and cephalin. The authors interpreted their findings as suggestive of a factor common to all three conditions.

It has been reported that erythrocytes of splenectomized calves infected with Anaplasma marginale have a decrease in concn of lecithins and cephalins (149).

Leukocyte lipids have been investigated in infection and leukemia. Kidson (117) found that the leukocytes of patients with infections synthesized lipids at a reduced rate although there seem to be no alteration from normal in the ratio of phospholipid to neutral lipid synthesis. It has been shown (26) that leukocytes from a patient with chronic myelogenous leukemia and another with monocytic leukemia were

capable of utilizing labeled acetate for lipid synthesis, whereas the leukocytes from patients with chronic lymphocytic leukemia were devoid of this capacity. Kidson (150) also reported a high rate of lipid synthesis in acute myelogenous leukemia and a low rate in acute lymphocytic leukemia. Malamos et al. (151) found that chronic leukemic (myelogenous and lymphatic) leukocytes incorporated the highest percentage of radioactive acetate into the phospholipid fractions, whereas normal leukocytes incorporated the highest percentage into the glyceride fractions. An early report on the lipid content of leukemic cells by Boyd (152) indicated that the per cell content of total lipids and phospholipids was high in chronic myeloid leukemia and low in chronic lymphatic leukemia, although his range of values for total lipids of leukocytes of normal adults was sufficient to include all the values found in leukemia. More recently Firkin and Williams (153) found the phospholipid composition of platelets and leukocytes from patients with acute and chronic leukemia to be similar. In vivo P^{32} labelling was present mainly in phosphatidyl ethanolamine, phosphatidyl choline and phosphoinositide, contrasting with in vitro studies which showed the major radioactivity to be in the phosphoinositide and a component believed to be phosphatidic acid.

Lipid synthesis in polycythemia vera was found to be low although a normal proportion of lipids was found; the leukocytes had the appearance of being young which may have accounted for this (154). In the same report three patients with myelosclerosis were found to have normal lipid synthesis and one had elevated lipid synthesis, especially of the phospholipids.

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Preparative Gas-Liquid Chromatography of Lipids

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Abstract

Applications of preparative gas-liquid chromatography of lipids are presented and discussed. Various designs of preparative units are reviewed, including those commercially available. A general discussion of preparative gas-liquid chromatography is presented with emphasis on the practical problems involved.

Introduction

THE ADVENT OF GAS-LIQUID chromatography (GLC) presented the field of analytical chemistry with a unique and valuable tool for the analysis of organic materials. The uniqueness of GLC lies in the fact that a highly efficient separation process and a highly sensitive detection process are combined into one system allowing the analysis of very small quantities of complex mixtures with relative ease and speed.

Although conceived as an analytical tool, GLC's function as a separation process naturally led to interest in it as a preparative tool in the purification of relatively large quantities of a mixture. The range of sample sizes, micrograms to milligrams, for analytical GLC can not be increased significantly on analytical size columns without an appreciable loss of resolution. This led to the scaling up of GLC systems by the use of columns of larger diameter with proportionately larger vaporizers. However, it was generally found that the high separating power of small diameter columns could not be obtained with large diameter columns. Despite the drawback of limited resolution, numerous examples of successful applications of large scale GLC have been published. Most of these reports deal with relatively volatile, stable materials, e.g. low molecular weight hydrocarbons and fluorocarbons. Relatively little has been published on the purification of lipids by preparative GLC, and a sizeable proprotion of the published work deals with the purification of small amounts of material on analytical size columns.

The collection of relatively small samples from analytical size columns can also be thought of as preparative GLC. This technique is often used in cases where only small quantities of a compound are required. There are no serious problems involved in such an application except in the collection system. The term "preparative GLC" is more commonly used to denote the purification of relatively large quantities of materials with columns of larger diameter than normally used in analytical work and as such, provides the basis for this paper.

Application of Preparative GLC to Lipids

Most of the published reports dealing with preparative GLC of lipids has been with relatively small scale systems with analytical columns. Kaneshiro and Marr (15) used small scale GLC with an unspecified instrument as one of their steps in isolating and identifying methyl esters derived from bacterial phospholipids. Methyl myristate, palmitate, palmitoleate, oleate, cis vaccenate, and lactobacillate were separated and collected from an 8 ft x ¼ in. column containing 25% diethylene glycol succinate (DEGS) on firebrick. Details of the collection procedure for the methyl esters were not presented nor was data included for the per cent recovery of the methyl esters by the procedure employed.

Chang and Sweeley (3) used preparative GLC as part of their procedure for isolating and identifying polyenoic acids from canine adrenal glands. Methyl