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Metabolism of acetate by human leukocytes

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METABOLISM OF ACETATE BY HUMAN LEUKOCYTES

by

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TO MY PARENTS
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I. GENERAL STATEMENT

Historically our knowledge of the blood elements began almost three hundred years ago with Anton Van Leeuwenhoek’s microscopical observations(82). His use of the microscope to observe a drop of blood ushered in a new era of understanding of a subject in which little was known. This accomplishment is a prime example of the application of a new technique to an old subject with consequent alteration of men’s ideas.

A more modern example of a technological advance which changed our concept of living tissue was Rudolph Schoenheimer’s use of isotopic tracers leading to the fundamental discovery of the dynamic nature of many of the chemical compounds making up living tissue. It was his and David Rittenberg’s experiment with Urey’s recently-discovered deuterium which suggested that "all constituents of living matter, whether functional or dynamic, of simple or of complex constitution, are in a steady state of rapid flux."(68)

This thesis deals with a particular component of the formed elements of the blood, the leukocytes, and describes the application of the isotopic techniques pioneered by Schoenheimer and Rittenberg to a study of the metabolism of these cells. The combination of developments
making this study possible include the following: the availability of the $^{14}$C-labeled substrate in large amounts at reasonably low cost; improved techniques of obtaining normal human leukocytes in good yield; and techniques for the separation of closely related compounds by filter paper chromatography, column chromatography and radioautography as well as a host of sensitive and specific colorimetric and enzymological procedures. With these new techniques it has been possible to study efficiently the metabolic fate of acetate in the human leukocyte, a cell about which relatively little is known biochemically, and to compare its metabolism of acetate with that of other tissues.
II. INTRODUCTION

A. Purpose of the Investigation

The purpose of this investigation has been to study the intermediary metabolism of leukocytes isolated from normal human blood and to compare their metabolic patterns both qualitatively and quantitatively with tissues more fully characterized biochemically.

Further knowledge of leukocyte metabolism is especially desirable not only because these cells serve important and well known biological roles, but also because these cells are excellent material for general metabolic studies. Their qualifications and advantages in this latter role are manifold. First, the leukocytes may be obtained by relatively mild isolation procedures such as simple sedimentation at room temperature (16). Second, they may be incubated under conditions approximating their in vivo environment by use of a medium consisting of homologous plasma or serum. Third, the number of cells may be estimated quantitatively by direct counting in a hemocytometer. Fourth, the cells may be repeatedly withdrawn with little inconvenience to the donor facilitating the "before and after" type of experiment. Finally, the mixed population of peripheral leukocytes may be separated into quite pure populations of the individual cells such as the
granulocytes or lymphocytes\textsuperscript{(60a)}. This separation of peripheral leukocytes into pure populations was not attempted here. On the other hand in the liver, an organ commonly used for metabolic studies, there are connective tissue, muscle and nerve cells in addition to the hepatic epithelium\textsuperscript{(50)}. These cannot be separated readily. The leukocytes therefore represent a more homogeneous material with which to study the metabolism of mammalian cells.

A clearer understanding of the metabolism of the normal human leukocytes will make possible the use of these cells to detect pathological conditions biochemically just as direct microscopy of leukocytes was used in the past for diagnosis of hematological disorders. Such knowledge might be especially suitable in the study of hereditary metabolic diseases in which the biochemical lesion is frequently manifest in all body cells. Wagner\textsuperscript{(91)} provided an example of this when he showed that the leukocytes of the peripheral blood of patients with glycogen storage disease have abnormally high levels of glycogen. Another example of leukocytes reflecting abnormal metabolism was the demonstration that leukocytes from diabetics respond to insulin with an increase of lactic acid while those of normal individuals do not\textsuperscript{(48)}.

A study of leukocyte metabolism may serve to clarify differences between normal and malignant tissue as
in leukemia. For example, differences in glycolysis and respiration have been found between leukocytes isolated from the blood of normal individuals as compared with leukemic patients\((11, 13)\). Further understanding of metabolic control mechanisms has been obtained by studying the differences in the glycolytic systems of these normal and leukemic cells\((8, 9, 10)\). This subject is discussed more fully on page 17.

Further knowledge of leukocyte metabolism is also desirable since these cells constitute a considerable percentage of the total body weight. Yoffey\((103a)\) estimated the number of granulocytes in myeloid tissue of the guinea pig to be one hundred times that in the circulating blood. Craddock et al.\((21a)\) arrived at a similar figure in dogs. If this figure is applied to humans, the granulocytes alone are comparable in weight to the liver. In disease states such as leukemia there may be a thirty fold increase in circulating leukocytes. The metabolism of this mass of cells must certainly be reflected in the overall metabolism of the body. Yet, we still know comparatively little about the metabolism of leukocytes.

Finally, knowledge of human physiology must ultimately be obtained from studies on human tissues. An example of the species variability frequently encountered
in metabolic studies is the recent finding of O'Donnell et al. (57a). These investigators have been able to demonstrate lipide biosynthesis from acetate by chicken and rabbit leukocytes but not by human leukocytes. Thus, data from experimental animals cannot always be carried over to man.

B. Scope of the Investigation

The investigation has involved the metabolism of C^{14}-labeled acetate. This compound occupies a central position in currently accepted schemes of metabolism because it enters into both anabolic and catabolic reactions within the cell. All tissues possess the capacity to metabolize acetate. This compound is readily oxidized to CO_{2} in body tissues during the tricarboxylic acid cycle. Extrahepatic tissues apparently can provide at least half of their energy needs by combustion of acetate. The metabolic fate of the two carbons of acetate is not identical(99). Depending upon the tissue, the rate of conversion of the carboxyl carbon to CO_{2} may be from one to four times the rate at which the methyl carbon is converted to CO_{2}. The acetate which does not enter the tricarboxylic acid cycle reactions may take part in various acetylations or it may condense with another molecule of acetate to form acetoacetic acid, a normal
product of liver metabolism. A polymerization of acetate accompanied by reduction results in the production of long chain fatty acids. An alternative product of polymerization and reduction is cholesterol. Finally, the acetate carbon may contribute to the formation of organic acids, amino acids and carbohydrates.

This investigation has sought to determine which of the above pathways exist and to what extent they contribute to the metabolism of human leukocytes. After establishing optimal conditions for isolation and incubation of the cells, they were allowed to metabolize the substrate for varying lengths of time. Upon termination of the incubations, the various cell components were isolated and analyzed for radioactive carbon. The estimation of the incorporation of acetate carbon into all components mentioned above was attempted.

Owing to the nature of the approach, a rigorous study of any one particular pathway has not been feasible. However, due to current intensive interest in lipide metabolism and its association with circulatory disease, the biosynthesis of lipides by leukocytes was pursued rather extensively. The biosynthesis of fatty acids was characterized for cofactor and substrate requirements in homogenates or cell-free systems. The incorporation of radioactivity into proteins and carbohydrate was brought
to a satisfactory completion. The section dealing with
the formation of amino acids leaves several radioactive
compounds as yet unidentified. No organic acids have
been positively identified although a preliminary
chromatographic separation has shown that at least four
compounds contain appreciable amounts of the label.
Experiments are now in progress to identify these
compounds.
III. REVIEW OF THE LITERATURE

B. Historical

The literature dealing with white blood cells is extraordinarily extensive and covers a variety of broad subdivisions such as: proliferation and differentiation; release, storage and destruction; discrete cellular function; and metabolism. The present work, however, concerns itself entirely with the last category. Nevertheless, a few selected general references covering the hematological literature have been provided as a framework for the present study. Key references to the literature of the other subdivisions have been included in the bibliography (34a, 50, 56, 101).

With Leeuwenhoek's description of the red blood cell began the history of the formed elements of the blood. The importance of this discovery was not thoroughly appreciated at the time. The slow and frustrating progress of the subject beyond this point, a lesson in negative thinking, is admirably presented by Dreyfus (25). Almost one hundred years passed before Schwenke (25) recorded observing a whitish layer due to lighter material above the blood clot, the white cells, and an additional period of almost one hundred years elapsed before Donne's (25) description of the elevated levels of these cells in
leukemia. At this time, the middle of the nineteenth century, attempts were being made to apply quantitative procedures to blood examination. Such advances were dependent upon the development of diluting solutions which would allow counting of the cells while not hemolysing them and certain technological advances including improvements of the microscope. These improvements made possible use of the microscope in clinical observation and incorporation of microscopic investigation into medical thinking.

The next development is a most noteworthy one forming the foundation of modern hematology. This was Ehrlich's use of dyes to stain the blood cells thereby allowing a differentiation between the various forms existing in blood and bone marrow. Until this time the leukocytes were simply colorless masses. This discovery did more than allow the differentiation of the formed elements of blood as we know them today. It brought forth the important principle of the chemical specificity of cytological structure, a concept important for the rational approach to chemotherapy. Following these advances the subject subsequently expanded along the lines of origin, proliferation, differentiation, release, storage and destruction. Although much has been learned, studies along these lines are still in progress; and, it is perhaps worth pointing out that, after having been studied for approximately one hundred years, the leukocytes still do not have a definite unequivocal role assigned
to them in peripheral blood. Further details bearing upon this subject will be found in the references included at the beginning of the section. The remainder of this review will be concerned with the development of the biochemical knowledge of the individual cell.

B. Biochemical Composition of Leukocytes

The biochemical aspects of leukocytes have been reviewed fairly recently (12, 47, 86) and these papers should be consulted for a complete treatment of the older literature. Much of this early work drew upon histochemical procedures to describe the composition of the leukocytes. This large, dispersed body of knowledge is hardly more than a list, there being little insight into the biological role of but a few of the components identified. These components include enzymes such as the acid and alkaline phosphatases, beta-glucuronidase, esterase, β-pase, proteases, catalase, nucleotidase, adenosine-triphosphatase, transaminase and lysozyme as well as such non-enzymatic constituents as histamine, heparin, glycogen, glucuronic acid and sulfhydryl compounds.

Acid and alkaline phosphatases have been studied by several different groups (84, 95) and both enzymes have been shown to be present in normal leukocytes in much higher concentration than in plasma. The activity of these enzymes is labile and has been shown to fluctuate with disease states, with the maturity of the cell and also with stress in some
species. The variation of the alkaline phosphatase is much greater than the acid phosphatase. It has been found to be increased several fold in a wide variety of pathological conditions including infection, trauma, myocardial infarction, diabetic acidosis, gout and others. However, active disease does not universally evoke this elevation in leukocyte alkaline phosphatase activity. A three-fold increase in unit leukocyte alkaline phosphatase activity may be brought about by administration of ACTH or 17-OH corticosteroids (84). Here again it is not always possible to elicit the response. The situation is complex and it may be that further insight into these phenomena await a better understanding of the role played by this enzyme in normal cell metabolism.

Beta-glucuronidase is another well-studied but ill-understood enzyme of human leukocytes. The activity of this enzyme in whole blood is almost completely attributable to these cells (3, 66). Although it seems to be a constant finding that there is a variation of activity with disease, the magnitude - and sometimes even the direction - of this change is not agreed upon (31, 86). The role of this enzyme may be one of importance in detoxification reactions although most evidence favors a hydrolytic role rather than a synthetic one.

The lysozyme content of whole blood is contained wholly in the leukocytes (29). The enzyme activity is demon-
strable only upon cell lysis and this, in fact, may be used to assess blood storage and handling techniques. The enzyme may contribute to the defense role of leukocytes owing to its lytic powers upon certain bacteria. Although this function appears attractive, its importance should be minimized in light of knowledge that most of the bacterial species on which its lytic activity has been demonstrated are non-pathogenic(96).

Glyoxylase is another prominent leukocyte enzyme. Its activity has been shown to be more than 700 times greater in mature granulocytes than in erythrocytes(52). There is a decrease to about one third of this level in leukemic cells (53). Much importance has been attributed to this enzyme as a means of explaining differences in lactic acid production between normal and leukemic cells. Further discussion of this interesting point will be continued under the section on metabolism.

Additional enzymes found in leukocytes are not well characterized and their roles in leukocyte physiology are even less well understood than the above. References to them may be found in the review articles cited. It is anticipated that more thorough characterization of these enzymes will be carried out with the superior technological devices presently available and that pieces of this puzzle may begin to be assembled.
The non-enzymatic constituents of interest found in leukocytes include glycogen, lipide, histamine, heparin, glucuronic acid and glutathione. The glycogen content has been studied in leukocytes by both histochemical(103, 63) and biochemical procedures(85). The results indicate that the glycogen is present probably entirely in the granulocytic leukocytes. The mean value expressed per $10^8$ human granulocytes is 0.75 mgs.(91). As mentioned previously (see introduction) this white cell glycogen has been shown to be elevated in glycogen storage disease. Variations in cell glycogen have also been reported in other diseases. Elevations have been found with polycythemia vera while depressions to about one half of normal have been found in cases of chronic myelocytic leukemia. Valentine(86) makes the interesting point that this biochemical difference occurs in these two disease processes in cells which are morphologically indistinguishable - a good example of the extension of hematological studies by biochemical procedures.

The lipide content of human leukocytes is high in comparison with the other formed elements of the blood and with the body tissues in general. For the formed elements of the blood, the total lipide, expressed as per cent of dry weight, is as follows: leukocytes - 22; platelets - 15; erythrocytes - 1.3; and erythrocyte stroma - 11(28). Rheingold and Wislocki(63), using sudan black B and histochemical
techniques, have shown this lipide to be present pre­
dominantly in the polymorphonuclear leukocytes while the
small and large lymphocytes were always quite free of
staining granules. Erythrocytes were found to stain only
lightly with this dye.

Histamine and heparin are also found in leuko­
cytes. Valentine(86) has found the histamine content of
white cell preparations to increase as the per cent of
basophils increase and he concludes that this cell is the
most important repository for white cell histamine.
C. Metabolism

Dynamic aspects of leukocyte metabolism have been under study for approximately fifty years. Several good reviews covering this period are available (43, 74). Much interest in the metabolic machinery and metabolism of these cells has been generated by the observance of their oxidative ability occurring in the presence of a strong aerobic glycolysis. This combination of high aerobic glycolysis and moderate respiration is characteristic of the metabolism of cancer tissue (97) and embryonic tissues. According to Beck (9), statements that this "cancerous" metabolism is due to injury inflicted upon leukocytes during isolation procedures are probably invalid inasmuch as the metabolism of homogenates is comparable to the intact cell while the cells of the various leukemias actually have a lower level of aerobic glycolysis than do the normal cells (8). In addition certain tissues, presumably normal, e.g., retina and kidney medulla, have a high aerobic glycolysis. There apparently exists considerable overlap between normal and cancer tissue and it is hardly worthwhile to dwell upon this controversial point¹ in a discussion of the metabolism of normal leukocytes.

¹For a discussion of the role of glycolysis in cancer, see Warburg (97).
The leukocytes are able to degrade their stores of glycogen readily *in vitro*, and Wagner(93) has shown this catabolism to be dependent upon phosphorylating glycolysis. The following enzymes were identified: phosphorylase, phosphoglucomutase and phosphohexoisomerase. He has also identified intermediates between glucose and lactic acid such as glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, fructose-1, 6-diphosphate and phosphoglyceric acid. The phosphatase which he characterized in horse blood leukocytes has a pH optimum between 8.0 and 9.0. It is interesting to note that Cori and Cori(21) found human glucose-6-phosphatase to have a pH optimum near 6.8. Apparently, therefore, at least one of the differences between liver and white blood cell carbohydrate metabolism has been shown.

Beck has undertaken a rigorous characterization of all the glycolytic enzymes in an attempt to learn more about the control of this multienzyme system(8,9). The only enzyme whose activity approached the rate of the overall process was hexokinase. That is, only this enzyme had a velocity maximum approaching that of the overall glycolytic system. The diminished hexokinase content in leukemic cells is the cause of the lower levels of their glycolysis. Beck's results show that the addition of either hexokinase or glucose-6-phosphate increased the production of lactic acid in normal cells. The percent increase, moreover, was much greater when hexokinase
was added to leukemic preparations. The percent increase was not as large as expected when glucose-6-phosphate was added to the leukemic preparations. The reason for this was attributable to a lack of ADP since the leukemic preparations, in addition to being deficient in hexokinase, are deficient in ADP-generating systems(9).

The pentose phosphate (phosphogluconate) pathway has also been studied in leukocytes. Beck(10) has found this pathway to account for less than ten percent of the glucose utilized in normal human cells while the percent of utilized glucose which traverses this pathway in leukemic cells is higher than in the normal cells. Villavicencio and Barron(89), studying these two pathways in normal and cancerous rabbit tissues, have arrived at similar conclusions. They compared lymphatic cells of rabbit appendix with lymphosarcoma cells and found that the former metabolized glucose almost exclusively via the Embden-Meyerhof glycolytic scheme while the pentose phosphate pathway played an important role in the latter. The increased metabolism via the pentose phosphate pathway in leukemic cells is interpreted by Beck(10) as being due to their lower levels of glucose-6-phosphate. As the level of glucose-6-phosphate is increased, a larger proportion is metabolized via straight glycolysis leading to an apparent diminished percent proceeding by the alternative pentose phosphate pathway. This occurs, according to Beck,
because the pentose phosphate pathway's glucose-6-phosphate dehydrogenase is saturated at relatively low levels of substrate.

An interesting relationship between these two pathways has been brought out by Stahelin et al.(77). They have displayed an increase in the pentose phosphate pathway in rabbit peritoneal exudate cells when these cells are actively phagocytosing heat-inactivated bacteria. This preferential stimulation of a particular pathway during increased cellular activity leads to interesting conjecture concerning the significance of these paths within the cell.

Martin, McKinney and Green(47) have recently suggested an additional pathway based upon the action of an enzyme, glyoxylase, which converts methylglyoxal to D-lactic acid. The evidence is based upon two very interesting pieces of data. First, they have observed a discrepancy in lactic acid levels in leukocytes when using a non-specific colorimetric method and an enzymatic method specific for L-lactic acid(49). The higher levels obtained with the colorimetric method were believed to be due to D-lactic acid which they attributed to the action of glyoxylase. Second, using an inhibitor of lactic acid dehydrogenase, oxamic acid, they observed only a 20 to 30 percent decrease in lactic acid production. This again supports the idea that a large amount, as much as 80 percent, of lactic acid may be derived by a
pathway not involving reduction of pyruvate. The data are also in agreement with the fact that leukemic cells have lowered levels of aerobic glycolysis since these cells also have been shown to have lowered levels of the enzyme, glyceraldehyde-3-phosphate dehydrogenase (53). Further work on this interesting point is awaited.

Reviews containing references to the earlier work on leukocyte respiration were included at the beginning of this section. The results indicate that intact cells and homogenates consume oxygen and that this oxygen consumption is significantly lowered in leukemic cells. Chronic myelocytic and chronic lymphocytic cells have approximately one-third and one-quarter, respectively, of the oxygen consumption of normal cells. Martin et al. (47) found that only pyruvate increased the oxygen consumption of intact cells while a variety of substrates were active on broken cells. The largest increase, over one hundred percent, was obtained with succinate. These results suggest a functioning tricarboxylic acid cycle. Wagner (92) found alpha-glycerol phosphate to be even more potent than succinate as a respiratory stimulant for intact cells. The respiration of leukocytes has been shown to increase during phagocytosis (77). In addition Blecher and White (14) have shown that the respiration of rat lymphocytes in vitro may be inhibited by a variety of steroids. In conclusion it may be stated that the respira-
tion of leukocytes is a labile process and that any inferences based upon data from different laboratories must take this fact into consideration.

Studies of biosynthetic mechanisms in leukocytes are few although recently there has been a sharp increase in studies on this aspect. The work of Blecher and White (14) has demonstrated the incorporation of glycine-2-Cl\textsubscript{14} into the protein and nucleic acids of rat lymphocytes in vitro. They showed that steroids, in addition to inhibiting respiration, also inhibited this process. Winzler (102) has studied the incorporation of Cl\textsubscript{14}-labeled formate, glycine and adenine and P\textsuperscript{32} into nucleic acids of leukocytes. His data have shown that formate incorporation into the gross protein fraction is markedly elevated in leukemic cells and, furthermore, that this incorporation can be inhibited by various chemotherapeutic agents such as amethopterin. His data concerning incorporation of substrates and the action of inhibitors points out other interesting relationships which may be exploited in the chemotherapy of leukemia.

Additional studies involving incorporation of inorganic P\textsuperscript{32} into ester phosphates and nucleic acids have been carried out by Zakrzewski et al. They have shown that the specific activities of ribonucleic acids reached a level of 3 to 4 percent that of the ester phosphates. No incorporation was detectable in the desoxyribonucleic acids. An interesting
point in their work was the finding that during phagocytosis
ribonucleic acid turnover is increased but no change in the
ester phosphates turnover could be detected (104).

In addition to the glycine incorporation cited
above, it has also been demonstrated that both normal and
leukemic leukocytes can incorporate cystine in vitro (98).
In contrast only chronic myeloid leukemic leukocytes readily
incorporate sodium sulfate. Their data indicate that dif­
ferences exist in the utilization of l-cystine and inorganic
sulfur by normal and leukemic leukocytes. The present work
displays the incorporation of carbon from C\textsuperscript{14}-labeled acetate
into glucose, glycogen, lipides, proteins, amino acids and
organic acids by normal human leukocytes.
IV. EXPERIMENTAL

A. General Experimental Procedure

The experimental procedure was that basic to most tracer studies. In the words of a pioneer in the field, Martin D. Kamen (40), it consists of "...simple experiments in which a compound, suitably labeled, has been introduced into the biological system from which, at some later time, various biochemical fractions have been prepared and the location and nature of the label determined".

In this study the biological material consisted of human leukocytes isolated from heparinized human blood collected by venipuncture and separated partially from accompanying red blood cells by fibrinogen sedimentation techniques (72). Platelets were removed by differential slow-speed centrifugation of the resulting cell suspension.

The cells were then washed several times at the centrifuge and brought to a convenient volume. At this point, the yield of white cells as well as the number of contaminating red blood cells was determined by direct counting of suitably diluted aliquots according to standard hematological procedure. The volume of the original suspension was then modified to permit convenient transfer, by pipetting, of the desired number of cells into incubation vessels. This was followed by the addition of substrates and cofactors, gassing of flasks and incu-
bation at 37° C for the desired length of time. The incubation vessels were either standard, double side arm Warburg flasks or Erlenmeyer flasks equipped with center wells. The latter were stoppered with rubber serum bottle caps which allowed for the addition of substrates and for gassing of the flasks by means of hypodermic needles.

Incubations were terminated in a variety of ways depending upon the analyses to be performed. Addition of acid, protein precipitants or immersing the flasks in boiling water were most frequently employed. The substrate converted to CO₂ was collected in center wells. The CO₂ bound by the biological material was displaced by addition of acid. Finally, the incubation media and cells were subjected to the various fractionation procedures and the major end products of metabolism were isolated, purified and assayed for radioactivity. In addition to CO₂, these included glycogen, glucose, proteins, lipides, amino acids, some di- and tri-carboxylic acids and acetoacetic acid. A flow diagram of a typical fractionation is shown in FIG. 3.

The results are generally expressed as specific activity (S.A.)¹, or as total incorporation in terms of CPM or umoles of substrate.

¹ Specific Activity, (S.A.), equals counts per min. per mg. This will be abbreviated in the text as S.A. = CPM/mg.
B. Biological Material

1. Blood Collection

Blood was collected from young, adult male medical students by venipuncture of the antecubital vein and collected aseptically into sterile Fenwal bags containing 0.5 ml. (500 units) of heparin per 100 ml. of whole blood. The blood was then allowed to cool slowly to approximately 20° C and during this time, the leukocyte count (WBC) and hematocrit were performed on aliquots. Only blood having a WBC of between 5,000 and 10,000 per cubic millimeter was used in order to insure that normal, mature leukocytes were being isolated. It is well established (74, 84) that immature and pathological cells, even though indistinguishable morphologically, may have distinct metabolic differences.

Serum used in these experiments was prepared from the same blood from which the leukocytes were obtained. This was done by drawing an additional volume of blood into a clean, dry tube, allowing twenty to thirty minutes for clot retraction and then removal of the clear serum by centrifugation. Glucose determination (See Methods) were performed on the serum when glucose levels were a factor in the experiments.
2. **Separation of Leukocytes**

The method of isolating leukocytes was that of Skoog and Beck (72). This involved the use of bovine fibrinogen, Armour Fraction I, to speed the sedimentation of the erythrocytes. It was found necessary to establish optimal conditions, e.g., concentration of fibrinogen and time of sedimentation of red cells, for each batch of fibrinogen. This was accomplished by performing a series of small scale separations with varying fibrinogen concentration. At ten minute intervals white and red cell counts were performed and both the white cell yield and the red cell-white cell ratio were calculated. The optimal red cell-white cell ratios did not usually coincide with the best leukocyte recoveries. The percent recovery is an important factor when working with normal leukocytes because of their low concentration in normal blood. Since red blood cells were found not to interfere with the metabolism of acetate by white cells under the experimental conditions described here, optimum leukocyte recoveries were preferred. The usual procedure involved mixing whole blood with an equal volume of 5-6 percent fibrinogen in physiological saline. The average suspension yielded approximately 70 percent recovery of leukocytes with a red cell-white cell ratio of between four and eight to one.
Volumes of blood from 200 to 550 mls. were routinely processed.

The resulting supernatant leukocyte-platelet suspension was then subjected to slow-speed differential centrifugation (60 x g, 20 minutes) which served to separate the leukocytes from the platelets. It was found necessary to exercise care during this part of the procedure to avoid clumping of the cells. Precautions found beneficial included the use of siliconized glassware, the removal of any foam produced in mixing and pouring of fluids, the use of heparin in wash buffers, and the immediate resuspension of sedimented cells. Other details are given in the above reference. Strict adherence to this procedure has allowed repeated isolation of viable leukocytes with little clumping. It is possible to further reduce this erythrocyte contamination of these suspensions but this usually has the disadvantage of lowering the leukocyte yield as well as introducing additional extraneous reagents into the suspensions.

3. **Assessment of Condition of Isolated Leukocytes**

Direct observation by phase microscopy was used routinely to assess the morphological integrity of the isolated leukocytes. Carefully isolated cells characteristically possessed sharp outlines and remained as discrete cells, while cells which had a tendency to clump
usually presented a fuzzy appearance. If desired, the clumping could be reduced by gentle agitation or pipetting. Even though it was found that these clumped cells still possessed the ability to respire and to utilize acetate, they were not used because of the large errors which are incurred in counting them.

Alternative methods of assessing cell viability are available. A standard in vitro procedure is the determination of the phagocytic index (82). For this purpose, either bacteria or inert starch granules may be used. The particles which are ingested by the leukocytes are observed and counted by direct microscopy. In addition to phagocytosis, viable white blood cells are also capable of amoeboid motion. This may also serve as a means of evaluating isolation procedures. Other criteria included by Tullis (82) as being useful in distinguishing "live" cells from "dead" cells are oxidative metabolism, resistance to impermeable dyes and Brownian movement. Although, most of these have not been employed in the present study, the combination of morphological integrity by phase microscopy coupled with repeated observation of an unaltered oxidative metabolism, stated to be the most labile of the leukocytic functions, is believed to be sufficient.
4. Quantitation of Leukocytes

The washed, leukocyte-rich suspension obtained from the combined sedimentation and centrifugation procedure was routinely brought to a volume approximating that necessary to give the desired cell concentration. This was estimated from the original WBC, the total volume of blood being used and the factor of 70%, which is the average yield of leukocytes isolated from whole blood by this method.

Leukocyte counts were then performed on the suspension. Counting was done in a hemocytometer of sufficient thinness to allow use of the phase microscope on the same preparation. Owing to the increased concentration of leukocytes, a standard red blood cell pipette was used to make the proper dilution while the cells were counted in the large squares of the hemocytometer. For the calculations involved, see Appendix I.

Routinely four white blood cell dilutions were made on each suspension and two hemocytometer chambers were counted for each dilution. In addition, a red blood cell count (RBC) was performed to allow calculation of the red cell-white cell ratio.

Care must be exercised in counting of leukocyte-rich suspensions. These suspensions should be thoroughly mixed by inverting the container, usually a 25 ml. gradu-
ated cylinder, between each sampling. Any clumps present may be detected by holding the graduate cylinder horizontally to a light source and rocking it gently back and forth. Inasmuch as clumped cells invoke serious errors in cell counts as well as interfere with accurate pipetting of aliquots, they should be removed from the suspension. This may be accomplished by allowing the clumped cells to settle and decanting the unclumped cells into a second cold, siliconized graduate cylinder. The process of quantitation should then be performed on the uniform suspension.

5. Preparation of Homogenates

After enumeration, the cells were spun down at 2000 rpm in a refrigerated centrifuge and the supernatant was removed with a pipette.

Frozen-thawed homogenates were prepared by alternately freezing and thawing the suspensions directly in incubation vessels. An ethanol-dry ice bath was used. Three cycles were sufficient to adequately rupture the cells.

Homogenized cells were also prepared by grinding in a cold mortar and pestle with several volumes of alundum. The alundum was extracted with isotonic KCl, sucrose solutions or phosphate buffer. The method finally resorted to, and which gave the best results in
lipide synthesis involved grinding the cells in a motor-driven Potter-Elvehem homogenizer with a teflon pestle.

The homogenate was taken to a volume sufficient to allow the desired number of flasks to be run rather than to a prearranged concentration, e.g. ten percent, as is customary when working with other tissues. This was found more convenient for white cell studies where the material is limited and, in addition, both the donor's initial WBC as well as the percentage recovery are additional variables. The concentration of material in each flask was, therefore, expressed as homogenate equivalent to a given number of cells as determined from the initial count. Here it was tacitly assumed that one-hundred percent of the cells were broken. In actual practice, better than ninety percent of the cells were found to be broken when whole homogenates were examined microscopically.

6. Fractionation of Leukocyte Homogenates

Further division of whole homogenates into mitochondrial, microsomal and particulate-free fractions was accomplished by differential centrifugation according to established procedures (67). The whole homogenate, \( H_w \), was spun in the International Centrifuge, Model PR-2, at 600 x g to remove unbroken cells, nucleii and cellular debris. This slow-speed supernate, \( H_{sp} \), was removed
carefully, transferred to the Spinco Model L Preparative Ultracentrifuge, Rotor D, and spun at 10,000 x g for ten minutes. This step yielded a tan-colored mitochondrial pad, $M_t$, and a clear, pink supernate. This supernate was then respun at 100,000 x g for forty-five to sixty minutes to bring down the submicroscopic particles, the microsomes, P. Both particulate fractions were resuspended in isotonic sucrose by homogenizing with a loose-fitting pestle directly in the lusteroid tubes. They were taken to a volume equivalent to that from which they were spun.

C. Methods

1. Counting of Radioactive Samples

Radioactive samples containing $^{14}C$ were counted in a windowless gas-flow proportional counter of the Robinson type (65). Planchets were made of aluminum having a well of 1 mm. x 14.3 mm. Samples were routinely plated to approximately 5 mgs. by taking appropriate aliquots or by adding sufficient carrier material. All counts were then corrected for self-absorption to the 5 mg. level. The self-absorption of lipides and glucosazones is the same as that of an equal weight of Ba CO$_3$ throughout the range of 1 to 7 mg. (41).
2. **Combustion Method**

Water-soluble compounds were combusted to \( \text{CO}_2 \) with potassium persulfate\(^{(59)} \) and counted as \( \text{Ba CO}_3 \). For the collection of \( \text{CO}_2 \) the oxidations were carried out in 50 ml. Erlenmeyer flasks equipped with center wells containing a piece of fluted filter paper and 0.5 ml. of 30\% potassium hydroxide. The flasks were stoppered with serum bottle caps. Samples to be oxidized were placed in the flasks containing approximately 700 mg. of potassium persulfate. The contents was adjusted to approximately 15 ml., mixed and acidified with a few drops of 5N sulfuric acid. Then 1 ml. of 2N \( \text{AgNO}_3 \) was added and the flasks were capped and immediately evacuated by plunging a hypodermic needle connected to a water aspirator through the stoppers. The reaction was allowed to go to completion by placing the flasks in a water bath and slowly bringing the temperature to 70\(^\circ\) C. Care must be taken at this point since the increase in pressure may blow off the rubber cap. It has been found helpful here to secure the cap with a snug-fitting rubber band.
3. **Preparation of Protein for Counting**

The procedure is essentially that of Allfrey et al. (2) with the exception that experiments were terminated with perchloric acid (HClO₄) instead of trichloroacetic acid (TCA). The protein precipitate was removed by centrifugation and was washed three times with 5% TCA. The protein pellet was extracted by refluxing once with approximately ten volumes of each of the following: ethanol (95%); ethanol-ether (3:1); ethanol-ether (1:1); and ether. This procedure served to remove the lipides coprecipitated with the protein. The protein precipitate was then suspended in acetone and homogenized in an all-glass homogenizer of the Potter-Elvehjem type. An aliquot of the protein dispersion was deposited on filter paper for counting while the remainder was transferred to a weighed watch glass and dried to constant weight. Counting was performed and self-absorption corrections applied as mentioned above. The material so obtained represents the lipide-free gross protein fraction. No attempt was made to extract nucleic acids.
4. Glycogen

Glycogen was isolated by essentially the same method as described by Boxer and Stetten (76). To the incubation flasks was added 0.5 ml. of a hot, saturated potassium hydroxide solution (approximately 200%) and the flasks were placed on a steam bath until the tissue was completely disintegrated. Ethanol was added to bring the final concentration to 60%. The solution was brought briefly to boiling and cooled. After washing the precipitate several times with 60% ethanol, the residual ethanol was driven off by placing the tubes briefly in a water bath. The glycogen was extracted several times with 5% TCA and further purified by reprecipitation from TCA followed by reprecipitation from water. The purified glycogen was hydrolysed by boiling with 1N H$_2$SO$_4$ for one hour. Glucose determinations and glucosazone formation were performed as described below.

5. Glucose

Glucose was determined on Somogyi (75) filtrates or neutralized acid hydrolysates by the ferricyanide method of Park & Johnson (60). Glucose was isolated for counting by formation of the phenylglucosazone (78). The filtrates were first concentrated under
reduced pressure at $50^\circ$ to a volume of 2.0 mls. For every 10 mg. of glucose 0.1 ml. of liquid phenylhydrazine and 0.15 ml. of glacial acetic acid were added. The mixtures were heated on a steam bath for thirty minutes and allowed to cool. The yellow crystals were washed first with water and then with warm ether to remove aniline and excess phenylhydrazine. The precipitate was recrystallized from 1 ml. of ethanol by the addition of fifteen volumes of water. The yellow crystals were plated directly, dried and counted as usual.

6. Lipides

Several different methods were used to extract lipides from tissues. Initially, tissue and media were taken to dryness and then extracted with lipide solvents according to the procedure of Folch et al.(30). In this procedure the dehydrated lipide was taken up in chloroform-methanol and washed by submerging beneath a large volume of water. Alternatively the lipide was precipitated along with the protein by perchloric acid and extracted from the lipo-protein complex in a manner similar to the former method. After extraction, total lipide was taken up in chloroform and washed with dilute acid. The total lipide was plated, counted and corrected to 5 mgs. for self-absorption. The total lipide was fractionated by ethanolic potassium hydroxide hydrolysis.
The saponified lipides were acidified with 5N H₂SO₄ and extracted with a known volume of CHCl₃. After washing with 0.1N HCl, an aliquot was taken to dryness and dissolved in ether. The fatty acids were extracted with 10% aqueous KOH. After acidification, the fatty acids were reextracted with pentane, washed, plated and counted as usual.

Cholesterol was precipitated from the nonsaponifiable fraction with digitonin(7). Inasmuch as there was no significant activity in this fraction, it was not further purified. An aliquot of the digitonin mother liquor was also taken to dryness and counted.

7. Analysis of C¹⁴-Labeled Water-Soluble Compounds

For the analysis of water-soluble compounds other than glycogen and glucose, several different procedures were used depending upon the purpose of the experiment. Initially, when it was intended to ascertain only the approximate extent of the formation of C¹⁴-labeled compounds by the leukocytes, a modification of the method of Katz and Chaikoff(42) was used. After termination of the incubation by addition of acid or heat, the flasks' contents were transferred to 15 ml. centrifuge tubes and spun down. After removing the supernatant, the tissue was extracted several times with one or two ml. portions of hot water. The washings and supernatant were
combined and taken to volume. The solution was desalted electrolytically as described below. The volume was reduced by heating to $50^\circ C$ and applying suction. This was necessary to facilitate application of the solution to the paper chromatogram.

An alternative method was used to obtain the amino acids more directly after it was found, by the above procedure, that there was significant labeling of the amino acid pool. In this procedure a perchloric acid extract was prepared. The extract was partially neutralized and desalted by ion exchange chromatography. Details of this method are included under Desalting. This procedure not only served to desalt the extracts, but it also separated the amino acids from unutilized substrate, organic acids and non-ampholytes.

Prior to paper chromatographic separation of the amino acids, their level was estimated quantitatively by the ninhydrin method(54). Organic acids were separated on paper also, but here it was necessary to add carrier acids prior to this step.

8. Acetate

Acetate has been determined titrimetrically (20) and by a specific colorimetric method(37). In addition, since the acetate involved was radioactive, a factor which allowed accurate detection far beyond
that of colorimetric or titrimetric methods, a hybrid procedure was evolved. This consisted essentially in coupling the microdiffusion procedure of Conway with the sensitive methods for detection of radioactive atoms. In this method, both the decrease in the volatile substrate, acetic acid, as well as the increase in non-volatile compounds may be followed simultaneously in the same sample by counting the distilled activity for the former and the residual center well activity for the latter. This procedure has served especially well for time studies involving large numbers of analyses which would have been time-consuming by standard steam distillation-titration procedures.

Incubations were terminated by addition of 0.4 ml. of 60% HClO₄, and the precipitate was washed twice with approximately 1 ml. of 3% HClO₄.

The extracts were neutralized to phenolphthalein endpoint with concentrated KOH. After removing the HClO₄ precipitate by centrifugation in the cold and washing the precipitate twice with cold water, the combined extracts and washings were transferred to 10 ml. volumetric flasks and taken to volume.

For the diffusion analysis 0.5 ml. aliquots were run in No. 1 Conway microdiffusion units in duplicate according to the standard procedure. Diffusion was allowed to take place overnight at room temperature.
The outer chamber containing the unused substrate was oxidized to CO₂ by the persulfate method and sufficient carrier was added to give a planchet weight of approximately 5 mg. The center wells containing the non-volatile C¹⁴ activity were also oxidized and counted for total CPM. Although the combustions are not strictly necessary, they were performed to facilitate comparison of activity from different sources. The control flasks containing equivalent amounts of C¹⁴-acetate were subjected to the above procedure and all recoveries were referred to this value as 100%. When the C¹⁴O₂, volatile C¹⁴ and non-volatile C¹⁴ of various samples was summed, recoveries were better than 95%.

9. Acetoacetic Acid

Acetoacetic acid was determined by the method of Edson (27). This method is based on the decarboxylation of beta-keto acids by primary amines in an acid medium to yield CO₂. The CO₂ evolved was measured manometrically and then assayed for C¹⁴ activity by precipitation as Ba CO₃ after collecting in base.

10. Desalting

Salts were removed from extracts either electrolytically or by ion exchange. The electrolytic de-
salting apparatus was that of Consden, Gordon & Martin (19) as modified by Astrup, Stage & Olsen (4). This device effectively deionized 5 ml. of physiological saline in fifteen to twenty minutes.

For ion exchange desalting the procedure of Mueller et al (55) was used. This method makes use of Dowex 50 in the acid cycle. Amino acids, reacting as cations, are bound by the resin while non-ampholytes and organic acids are washed through with water. All the amino acids except arginine, histidine, lysine and cystine are eluted with 0.8N HCl in 55% ethanol. Salts are next removed by aqueous 1N HCl and then the remaining amino acids not removed by the first eluant are removed with aqueous 6N HCl. The elution of the amino acids was followed by spotting aliquots on filter paper and spraying with 0.2% ninhydrin - 5% pyridine in 95% ethanol.

11. Paper Chromatography

Amino acids and organic acids were resolved by paper chromatographic separation by the ascending technique of Williams & Kirby (100). Both one- and two-

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1 An apparatus was built in collaboration with Dr. M. A. Derow, of the Microbiology Department, and Dr. Norman C. Telles.
dimensional procedures were used; however, for samples partially fractionated as described above, one-dimensional chromatograms were found adequate. Whatman Nos. 1, 3, 3MM and 4 filter paper prepared for chromatography were used. A variety of solvents was used and positive identification was accepted if $^{14}C$ activity coincided with ninhydrin color through three different solvents, the usual literature requirements. These solvents for amino acids included phenol:water (80:20, v/v), phenol buffered at pH 12(51) and methanol:pyridine:water (160:40:8).

For organic acids ether:acetic acid:water (156:36:12) was used as solvent(23). The spray reagent was 1% xylose in methanol containing 1 ml. of aniline(73). Organic acids were run on Whatman 3MM filter paper.

Elution of radioactive areas from chromatograms was accomplished by descending chromatography using very dilute HCl. The solvent was allowed to drip off the end which was cut into a point. Less than 0.5 ml. was sufficient to completely elute the activity.
12. **Radioautographic Techniques**

Dried chromatograms were routinely checked for activity using a Chicago nuclear strip scanner coupled to a rate meter. Since this method does not accurately locate the C\(^{14}\) spots, it was used mainly to determine the length of time necessary for radioautography. Spots containing as little as 200 CPM usually gave good radioautographs within two to three weeks.

The dried radioautograms were stapled to Ansco Non-Screen X-Ray Film and were left in contact under sufficient weight to insure proper contact between film and chromatogram. After developing, film and chromatogram were superimposed, using the staple holes as guides, and viewed with a standard X-Ray film illuminator. The areas of radioactivity were traced onto the chromatogram, the spots were cut out, and then elution of the substance performed as described above.
V. RESULTS

A. The Effects of Cell Concentration and Number Upon the Oxidative Metabolism of Leukocytes

1. Cell Concentration

The blood leukocytes are said to be fragile cells whose metabolism is quite sensitive to changes in their environment(74). The older idea that the characteristic metabolism of isolated cells, i.e., high aerobic glycolysis and moderate respiration, is the "metabolism of injury"(43) has been challenged(11). Very recently, however, Warburg, et al. (97) and Burk(16) have presented evidence that this characteristic tumor-like metabolism is a result of damage inflicted upon these cells during isolation procedures. These latter investigators have presented evidence that cells obtained without cooling and centrifugation and which are incubated in serum display an oxygen consumption comparable with normal non-cancerous tissues. It is not intended to enter into this highly controversial subject although it is understood that this matter is pertinent to the present investigation. Rather, the first few experiments have been designed to assess the effects of environmental conditions on the cells during incubation after they have been isolated by a standard procedure. A recent review (82) places the oxidative
metabolism of leukocytes at the top of a list of physiological functions progressively lost as the cell dies. Conflicting reports have appeared in the literature (35, 6) describing detrimental effects of cell crowding on leukocyte respiration. For example, it was observed that respiratory rates did not increase linearly with an increase in cell number. In order to assess the fitness of our experimental conditions, several experiments were devised. One was designed to demonstrate the effect of cell concentration on oxygen consumption throughout the range anticipated in this work. The second experiment was designed to test oxidative capacity of the cells for our substrate with extreme variation in size of tissue sample. In the latter experiment any decrease in rate due to substrate disappearance or to the accumulation of inhibitory products of metabolism would be detected. These data would be essential in order to justify correction of experimental results obtained from a variety of cell concentrations to a standard number of cells.

The results of the first experiment are shown in Fig. 1. The cells were incubated in standard Warburg flasks and the oxygen consumption measured by the direct method (24). The plotted data comprise results obtained from leukocytes isolated from three different blood samples run on different days. Each sample is identified
FIGURE 1

THE DEPENDENCE OF LEUKOCYTE RESPIRATION ON CELL NUMBER
The graph illustrates the relationship between cell count (CELL COUNT, \( \times 10^6 \)) and the volume of oxygen (\( \mu \)liters \( O_2 \) / 3 HOURS). The data points are represented by different symbols, with a trend line indicating a positive correlation between the two variables.
Leukocytes were incubated in Warburg flasks in a total volume of 2.9 mls. The incubation medium was phosphate buffer, \(0.067\text{M}, \text{pH} 7.4\) and 30\% homologous serum. Glucose concentration was \(0.003\text{M}\). The points represent three different samples of blood. Respiratory \(\text{CO}_2\) was collected in center wells containing 0.3 ml. of 30\% KOH.
by its characteristic symbol, \( O, O, O \). The microliters of oxygen consumed over a three-hour period are plotted against time as abscissa. The curve demonstrates that there is a direct relation between cell concentration and oxygen consumption under our experimental conditions between 40 and 155 \( \times 10^6 \) leukocytes per flask. Since the volume of the cell suspension was 2.7 mls., this corresponds to a cell concentration range of 15 to 60 \( \times 10^6 \) leukocytes per ml. of incubation medium. These data\(^1\) give a \( Q_0 \) value of approximately 3. This value falls well within the range, 1 to 6, established in a recent critical reevaluation (16) of this aspect of normal leukocyte metabolism.

2. Cell Number

A second experiment was run measuring the conversion of acetate-1-\( ^{14}C \) to \( ^{14}CO_2 \) by two side-by-side differing amounts of cells over a three-hour time interval. The cells were obtained and washed by the usual procedure, and the incubation was carried out in modified Erlenmeyer flasks equipped with center wells for the collection of radioactive \( CO_2 \). The incubations were carried out in constant temperature shaker bath. Ten \( \mu \)moles of

\(^1\)To convert cell number to mgs. dry weight the factor \( 7.5 \times 10^6 \) = 1 mg. dry weight was used (16).
acetate-1-C\textsuperscript{14} per flask were used in each experiment. This experiment differs from the previous one in that the cell concentrations were similar while the amount of tissue varied. The results show that the conversion of acetate carbon to CO\textsubscript{2} is directly proportional to cell number in the range studied. The percent of the acetate utilized has been plotted against time in Fig. 2. It is seen that the conversion progresses steadily during the three-hour period. The results normalized to one hundred million cells are shown in Table I. As seen in the last column of this table, the specific utilization (\textmu moles acetate converted per 10\textsuperscript{8} cells) for the low and the high amounts of tissue are 0.85 and 0.88\textmu moles, respectively. These results show that the conversion of acetate to CO\textsubscript{2} is directly proportional to cell number and that no error is incurred by converting results obtained with different amounts of tissue to a standard number of cells. The results also indicate that the substrate has not become a limiting factor. This last point will be useful in interpreting later experimental results.
FIGURE 2

CO$_2$ PRODUCTION FROM ACETATE
AS A FUNCTION OF CELL NUMBER
% ACETATE CONVERED TO CO₂

(370 x 10⁶ CELLS)

(170 x 10⁶ CELLS)

TIME (HOURS)
FIGURE 2

- Legend -

Cells were incubated in .067M phosphate, pH 7.4 with 30% homologous serum containing .01M glucose. Gas phase was 95% O2-5% CO2. After equilibration each flask received 10 μmoles of acetate-1-C14 (S.A. 1.98 X 10^5 CPM/μmole).

Final cell concentrations were 61 X 10^6 leukocytes/ml. for lower curve and 77 X 10^6 leukocytes/ml. for the upper curve.
TABLE I

$^{14} \text{C}_2 \text{O}_2$ PRODUCTION FROM ACETATE-$1-{^{14}\text{C}}$

AS A FUNCTION OF CELL NUMBER*

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Leukocytes ($X 10^8$)</th>
<th>Acetate Utilized ($\mu$moles)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Per ml.</td>
</tr>
<tr>
<td>1</td>
<td>1.70</td>
<td>0.61</td>
</tr>
<tr>
<td>2</td>
<td>3.70</td>
<td>0.77</td>
</tr>
</tbody>
</table>

* The experimental conditions for this experiment are identical with those given in the legend for Fig. 2. The $^{14} \text{C}_2 \text{O}_2$ data represent the cumulative production over the three-hour incubation period.
B. The Relative Production of CO₂ from Carboxyl- and Methyl-Labeled Acetate

The oxidation of acetate by tissues is known to require an exceedingly complex series of reactions involving an initial condensation of its active form, acetyl coenzyme A, with oxaloacetic acid. The location of the radioactive atoms of acetate after condensing and traversing the cycle is presented in Appendix 3. The citric acid formed proceeds by a series of transformations involving the loss of two carbons and finally is complete with the formation of a new molecule of oxaloacetic acid. The interesting point here is that the molecule of oxaloacetic acid is not the one which originally condensed with the acetate molecule. Rather, this four carbon compound now contains only two of its original carbons while its other two carbons have been lost as CO₂. The oxaloacetic acid now contains two carbons which originally belonged to the acetate molecule. Furthermore, although citrate is a symmetrical molecule, it proceeds through the tricarboxylic acid cycle series of reactions asymmetrically. Therefore, the two carbons of the original acetate molecule are not equivalent.

Insight into the cycle in this tissue may be gained by studying the relative C¹⁴O₂ production from acetate labeled in the methyl or the carboxyl carbon.

Table II includes data obtained from incubating leukocytes with acetate-1-C¹⁴ (carboxyl-labeled) and acetate-2-C¹⁴ (methyl-labeled). The CO₂ was collected and the total activity
## TABLE II

**RELATIVE PRODUCTION OF CO₂ FROM CARBOXYL- AND METHYL-LABELED ACETATE BY HUMAN LEUKOCYTES**

<table>
<thead>
<tr>
<th>Flask</th>
<th>Incubation Time (Hrs.)</th>
<th>Carbon Labelled</th>
<th>Total Recovery (CPM)</th>
<th>Specific Activity (CPM/μmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5</td>
<td>-COOH</td>
<td>7710</td>
<td>165</td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>-COOH</td>
<td>9280</td>
<td>170</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>CH₃⁻</td>
<td>4350</td>
<td>75</td>
</tr>
<tr>
<td>4</td>
<td>1.5</td>
<td>CH₃⁻</td>
<td>4070</td>
<td>75</td>
</tr>
<tr>
<td>5</td>
<td>3.0</td>
<td>-COOH</td>
<td>18200</td>
<td>390</td>
</tr>
<tr>
<td>6</td>
<td>3.0</td>
<td>CH₃⁻</td>
<td>8990</td>
<td>182</td>
</tr>
</tbody>
</table>

### CO₂ RATIO*

<table>
<thead>
<tr>
<th>Time (Hrs.)</th>
<th>COOH/CH₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>2.02</td>
</tr>
<tr>
<td>3.0</td>
<td>2.02</td>
</tr>
</tbody>
</table>
TABLE II

- Legend -

Incubations were carried out in standard Warburg flasks containing approximately 125 x 10⁶ leukocytes in a final volume of 2.7 mls. The incubation medium was Krebs-Ringer bicarbonate, 0.07M, pH 7.4, containing 30% homologous serum. Each flask received 10 μmoles of acetate-C₁⁴ (S.A. 3.85 x 10⁴ CPM/μmole) and 5.6 μmoles of glucose. The center well contained 0.2 ml. of 2N H₂SO₄. The reaction was terminated by inverting the flasks and displacing the C⁴O₂ through the side arm vent into 7 mls. of a dilute solution of BaCl₂-NH₄Cl to precipitate BaC¹⁴O₃.

\[ \text{* CO}_₂ \text{ RATIO} = \frac{\text{C}¹⁴ \text{ recovered in CO}_₂ \text{ from carboxyl-labeled acetate}}{\text{C}¹⁴ \text{ recovered in CO}_₂ \text{ from methyl-labeled acetate}} \]
calculated for incubations of 1.5 and 3.0 hours. The results show that the amount of radioactivity obtained from the carboxyl-labeled acetate is twice that obtained from the methyl-labeled compound. For an exact definition of the CO₂ Ratio, see the legend of Table II. This term, CO₂ Ratio, will be used subsequently without further explanation. The CO₂ Ratios obtained here are well within the range found for other tissues. CO₂ Ratios obtained for tissues other than leukocytes are as follows: rat liver slices, 1.3 - 3.6 (78); lactating rat mammary gland, 1.3 - 2.3 (26); and lactating cow mammary gland, 1.5 - 3.3 (44). The constant CO₂ Ratio and the linear production of CO₂ with time (Fig. 2) indicate that a steady state exists in the leukocyte experiments. Under conditions in which there is a steady production of CO₂ from the tricarboxylic acid cycle, i.e., infinite turns of the cycle, the cycle compounds have a constant isotope pattern (78). With this steady influx of acetate this pattern is obtained after relatively few turns of the cycle (78). As soon as the labeling is constant, it does not matter which positions in the intermediates are labeled. The constant passage of labeled compounds through the cycle reactions will result in a steady production of C¹⁴O₂. This means that there should be no difference between the activity derived from methyl- and carboxyl-labeled acetate. However, the results obtained with a variety of tissues - and those
recorded here - are clearly in disagreement with this statement.

The solution to this conflict between theory and experimental fact is apparently resolved. It is based upon Krebs' original observation(45) that the component reactions of the cycle may serve to supply intermediates for organic synthesis. This point has been further clarified for mammalian tissues by Strisower et al.(78). CO₂ Ratios greater than one are interpreted as definite evidence that there is an exchange of tricarboxylic acid intermediates with unlabeled compounds and that the magnitude of this ratio is an indication of the amount of this dilution. This exchange of labeled cycle intermediates would result in the production of a variety of labeled compounds all derived from acetate. Several compounds would arise directly from the cycle intermediates as illustrated in Appendix 4. The data presented in Table II indicate that there should be a biosynthesis of labeled compounds also in the leukocyte preparations. It was therefore decided to begin a systematic isolation and identification of all radioactive compounds produced by the incubation of leukocytes with labeled acetate.

C. The Utilization of Acetate by Leukocytes

In order to follow the fate of acetate carbon, larger numbers of leukocytes were incubated in 25 ml. Erlenmeyer flasks equipped with center wells as described under Methods. The flasks were temperature equilibrated and gassed with a mixture of 95% oxygen-5% CO₂ by passing a pair of hypodermic
needles through the rubber serum bottle caps. At various time intervals KOH was added to the center wells containing fluted filter paper and the reactions were terminated by addition of perchloric acid to the cell suspension by means of a needle. After allowing 30 minutes for adsorption of bound CO$_2$, the flask contents was fractionated according to the flow diagram shown in Fig. 3. After removing the center well contents for CO$_2$ assay, perchloric acid filtrates were prepared at the centrifuge and the lipo-protein pad was washed several times with dilute perchloric acid. Aliquots of the filtrates were assayed for unused acetate and non-volatile compounds by a modification of Conway's microdiffusion procedure. The results of a time study are shown in Tables III, IV and Fig. 4.

The results in Table III show the gross distribution of the C$^{14}$ activity in the CO$_2$, volatile and the non-volatile fractions as well as the total activity (CPM) recovered. In the last column percent recovery is based upon the total recovery from three controls consisting of unincubated samples. Complete recovery of the original activity put in was not obtained. This is not unusual in that acetate is volatile in acid solutions such as those used to terminate the reactions. Acetic acid "distilled" into the center well during the time necessary to absorb bound CO$_2$ would be washed out of the Ba CO$_3$ precipitate and discarded. The results recorded in Table III are the actual experimental values. Perhaps, a more meaningful expression of the data is presented in Table IV.
FIGURE 3

ACETATE UTILIZATION BY HUMAN LEUKOCYTES
- FRACTIONATION SCHEME -

GLYCOGEN $\xrightarrow{\text{KOH Digestion}}$ Ethanol $\xrightarrow{\text{WBC SUSPENSION}}$ center well $\text{CO}_2$ $\xrightarrow{\text{BaCO}_3}$

$\xrightarrow{\text{HCIO}_4}$

FILTRATE
- Conway Microdiffusion

NON-VOLATILES

VOLATILES

AMINO ACIDS

ORGANIC ACIDS & NON-AMPHOLYTES

PROTEINS

LIPIDES

PRECIPI TATE
- Ethanol- Ether Extraction

FATTY ACIDS

NON-SAPONIFIABLES

KOH Digestion
TABLE III
ACETATE-1-C\textsubscript{14} UTILIZATION BY HUMAN LEUKOCYTES*

<table>
<thead>
<tr>
<th>Flask</th>
<th>Incubation Time (Hrs.)</th>
<th>CO\textsubscript{2}</th>
<th>Total Radioactivity (CPM X 10\textsuperscript{3})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Non-volatile</td>
<td>Volatile</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>44</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>146</td>
<td>296</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>297</td>
<td>293</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>474</td>
<td>328</td>
</tr>
<tr>
<td>1</td>
<td>3.0</td>
<td>644</td>
<td>350</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>30</td>
<td>1,550</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>33</td>
<td>1,256</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>24</td>
<td>1,428</td>
</tr>
</tbody>
</table>

(Avg. 1,411) (Avg. 1,460)

* Flasks contained 3.67 X 10\textsuperscript{8} leukocytes incubated at 37\textdegree C in a total volume of 5.0 mls. Incubation medium was 0.067M phosphate buffer, 0.01M glucose and 30% homologous serum (final concentrations). Gas phase was 95\% O\textsubscript{2}-5\% CO\textsubscript{2}. In addition, each flask received 10 \mu moles Acetate-1-C\textsubscript{14} (S.A. 1.987 X 10\textsuperscript{5} CPM/\mu mole).

\textdagger Not included here is the activity appearing in lipides and proteins.

\textdoubledagger Recoveries are expressed as percent of the average control value, 1,460 X 10\textsuperscript{3} CPM.
TABLE IV

THE UTILIZATION OF ACETATE-C\textsuperscript{14} BY HUMAN LEUKOCYTES*

<table>
<thead>
<tr>
<th>Incubation Time (hrs.)</th>
<th>Total Radioactivity as % of Initial Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CO\textsubscript{2}</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>10.4</td>
</tr>
<tr>
<td>1.0</td>
<td>19.9</td>
</tr>
<tr>
<td>2.0</td>
<td>33.2</td>
</tr>
<tr>
<td>3.0</td>
<td>45.7</td>
</tr>
</tbody>
</table>

* Experimental conditions are the same as those in Fig. 2.
FIGURE 4

TIME COURSE OF ACETATE UTILIZATION

BY HUMAN LEUKOCYTES
Each flask contained $370 \times 10^6$ intact leukocytes incubated in a total volume of 4.8 mls. Incubation medium was \(0.067\text{M}\) phosphate buffer, \(\text{pH} 7.4\) and 30% homologous serum containing \(0.1\text{M}\) glucose. After equilibration each flask received 10 \(\mu\)moles acetate-1-\(^{14}\text{C}\) (S.A. 1.98 \(\times 10^5\) CPM/\(\mu\)mole).

Non-volatile activity was equivalent to residual activity after removal of unutilized substrate by microdiffusion.

\[
\%\text{ Acetate Utilized} = \frac{\text{C}^{14}\text{O}_2\text{ CPM} + \text{Non-Volatile CPM}}{\text{Total CPM Recoverable}} \times 100
\]
activity in the various fractions is expressed as a percentage of the original activity (CPM) placed in each flask. It is again seen that the CO₂ production is linear with time. In contrast are the data obtained for the non-volatile fraction. The data show that 80% of the total three-hour incorporation occurred in 30 minutes. These data are presented graphically in Fig. 4. The upper curve shows the utilization of substrate over the time course of the experiment. This curve represents the combined utilization of acetate by two different processes as shown in the bottom plot. Additional experiments were designed to determine the nature of this fraction.

D. The Incorporation of Acetate Carbon into Various Metabolites

1. Glycogen and Glucose

The first fractions to be studied were glycogen and glucose. The glucose was isolated directly as the glucosazone while the glycogen was hydrolysed prior to formation of this derivative. The cells were incubated for three hours with acetate-2-C¹⁴ with and without substrate glucose. The results of this experiment are presented in Table V. The results show no striking activity in either the glucose or the glycogen and since no attempt was made to exhaustively purify these fractions, the incorporation may be said to be negligible.
TABLE V

INCORPORATION OF ACETATE-2-C\textsuperscript{14} CARBON INTO GLUCOSE & GLYCOGEN BY HUMAN LEUKOCYTES

GLUCOSE

<table>
<thead>
<tr>
<th>Flask*</th>
<th>Glucose added (mg.)</th>
<th>Glucose Recovered</th>
<th>Glucose CPM/mg. Utilized</th>
<th>Total CPM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Substrate</td>
<td>Carrier</td>
<td>Total</td>
<td>Total</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>14.40</td>
<td>14.40</td>
<td>8.60</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>14.40</td>
<td>0</td>
<td>14.40</td>
<td>7.88</td>
</tr>
<tr>
<td>4</td>
<td>14.40</td>
<td>0</td>
<td>14.40</td>
<td>6.68</td>
</tr>
<tr>
<td>5</td>
<td>14.40</td>
<td>9.60</td>
<td>24.00</td>
<td>13.57</td>
</tr>
<tr>
<td>6</td>
<td>14.40</td>
<td>9.60</td>
<td>24.00</td>
<td>12.67</td>
</tr>
</tbody>
</table>

GLYCOGEN

<table>
<thead>
<tr>
<th>Flask*</th>
<th>Glycogen Carrier (mgs.)</th>
<th>Glycogen Recovered</th>
<th>Glycogen Equivalents Recovery</th>
<th>CPM/mg. Osazone</th>
<th>CPM/mg. Glucose in Glycogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>9.24</td>
<td>8.60</td>
<td>86</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0.1</td>
<td>--</td>
<td>--</td>
<td>5**</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>7.98</td>
<td>7.98</td>
<td>74</td>
<td>21</td>
</tr>
</tbody>
</table>
* Each flask contained $2 \times 10^8$ leukocytes in a total volume of 3.0 mls. containing .067M phosphate buffer and 30% homologous serum. Flasks were incubated at 37°C for 3 hours with 95% O2-5% CO2 as gas phase. Each flask received 20 μmoles Acetate-2-C14 (S.A. 3.77 × 105 CPM/μmole). Substrate glucose was added at the beginning while carrier glucose was added prior to isolation of fractions. Incubation was terminated by plunging flasks into boiling water bath. Control flasks were identical in all respects except that incubation was terminated immediately upon addition of labeled substrate.

Activity in glucose was calculated by multiplying the S.A. of glucose (determined from the S.A. of glucosazone) by the total mgs. glucose determined colorimetrically on an aliquot.

Glucose was converted to glycogen equivalents by the factor 0.93.

** Carrier glucose was added after hydrolysis and prior to formation of the osazone.

Glucose was isolated from Ba-Zn filtrates as the phenylosazone. Glycogen was isolated and hydrolysed to glucose from which the phenylosazone was prepared.
It is well established that the glycolytic pathway can be reversed and that acetate carbon can be converted into glucose \textit{in vitro}(78). The labeling of the glucose units are consistent with the participation of the Krebs Cycle. The negative results obtained here do not prove that this process does not occur in this tissue. However, owing to the strong glycolytic system present in these cells as compared with their oxidative metabolism, the results are not surprising. A study of glycogen biosynthesis from acetate-$\text{C}^{14}$ by rabbit peritoneal exudate cells has recently appeared(57). These investigators were able to isolate radioactive glycogen and, by assaying the activity in the individual carbons of the derived glucose, they concluded that the Krebs Cycle was involved. It must be kept in mind that rabbit exudate cells are a different population of cells which are obtained by grossly differing isolation procedures. The results are, therefore, not strictly comparable to those obtained here.

2. **Protein**

Protein was isolated from perchloric acid precipitates and purified by extraction with lipide solvents. No attempt was made to extract the nucleic acids. This gross protein fraction was plated directly and counted as described under Methods. The results are shown in Fig. 5.
FIGURE 5

INCORPORATION OF Carbon FROM
ACETATE-1-\textsuperscript{14} INTO PROTEIN
BY HUMAN LEUKOCYTES*
Each flask contained $370 \times 10^6$ intact leukocytes incubated in a total volume of 4.8 mls. Incubation medium was 0.067M phosphate buffer, pH 7.4 and 30% homologous serum containing 0.01M glucose. After equilibration each flask received 10 μmoles acetate-1-C$^{14}$ (S.A. 1.98 $\times$ 10$^5$ CPM/μmole).

Reactions were terminated by addition of HClO$_4$. Protein was isolated and purified as described under methods.
The incorporation of radioactivity is plotted against time over a three-hour interval. It is seen that the incorporation proceeds continuously over this time interval at a decreasing rate. The total activity incorporated represents but a small fraction of the added substrate. The total incorporation expressed as per cent of the original activity is approximately 0.3%. Expressed as per cent of the acetate utilized, which is probably the more useful figure, the incorporation becomes 0.5%. The amount of substrate acetate incorporated is roughly .03 μmoles.
3. **Lipides**

   a) **Intact Cells**

   The next polymeric leukocyte component to be analyzed for $^{14}C$ activity was the cell lipide. A general flow diagram of the isolation of lipides from the cell suspensions is shown in Fig. 6. In practice several different preparatory steps were employed depending upon the purpose of the experiment. The experimental details are given under Methods. In the first experiment to be described the flasks' contents were dehydrated by heating to 60 degrees and applying gentle suction. Since the flasks had been acidified to drive off bound $CO_2$, this step also served to remove most of the unused radioactive acetate. The lipide was extracted from the residue by refluxing with the standard organic solvents as described. After removal of the solvents, the lipide was taken up in chloroform-methanol (2:1, v/v). Contaminants were removed by carefully submerging the crude lipides beneath a large volume of water according to Folch et al (30). The gross lipide fraction was plated and counted directly. This total lipide was then saponified in ethanolic KOH and the long chain fatty acids (LCFA) were removed from the acidified solution with aqueous KOH. The fatty acids were reextracted with hexane, washed, plated and counted. This complete procedure was applied to flasks
**FIGURE 6**

**LIPIDE EXTRACTION FROM MIXED HUMAN LEUKOCYTES**

**LEUKOCYTE SUSPENSION**

Centrifuge, 600 x g.

**CELLS**

- Ethanolial KOH
- Hydrolysis Acidification
- Chloroform Extraction

**SUPERNATANT**

(Substrates Utilized)

**CHLOROFORM EXTRACT**

- Dilute acid wash 3 times. Extract with 10% Aqueous KOH

**AQUEOUS SUPERNATANT**

(Discarded)

**TOTAL LIPIDE**

(Count)

**FATTY ACIDS**

- Acidify
- Extract with Pentane

**NON-EXTRACTABLE LIPIDES**

**LONG CHAIN FATTY ACIDS**

(Count)

**CHOLESTEROL - PRECURSORS**

(Wash DIGITONIN PRECIPITATE)

(Count)

**SUPERNATANT**
incubated for various time intervals. The results are shown in Fig. 7. It is seen that the incorporation continues over the four-hour time interval. It also may be seen that the labeling is not uniform throughout this fraction as is indicated by the higher specific activity of the fatty acids.

A more complete fractionation of the lipides of leukocytes was performed in order to determine which lipides contained most of the acetate carbon. The isolation of the total lipides was carried out in the same manner as described for Fig. 7. The rest of the fractionation procedure is described in Fig. 6. The results are shown in Table VI. In this experiment the lipide from several flasks was pooled in order to have sufficient material for the analysis. Each fraction is expressed as percent of the total lipide activity. It is seen that the long chain fatty acids account for most of the activity incorporated into lipides. Apparently the distribution is the same whether methyl- or carboxyl-labeled acetate is used. In Fig. 8 are presented quantitative data (total CPM incorporated/370 million cells) for acetate-1-C\textsuperscript{14}.

It is well known that there is a relationship between carbohydrate metabolism and lipide synthesis (70, 71). These authors showed that the livers from fasted animals had a decreased ability to synthesize fatty acids
FIGURE 7
INCORPORATION OF ACETATE-1-C$^{14}$ INTO
LONG CHAIN FATTY ACIDS BY MIXED
HUMAN LEUKOCYTES
TOTAL LIPIDE

LCFA

S. A. (CPM / MG)

TIME (HOURS)

250

150

50
FIGURE 7

- Legend -

INCORPORATION OF ACETATE-1-Cl4 INTO LONG CHAIN FATTY ACIDS BY MIXED HUMAN LEUKOCYTES

Incubations were of 250 x 10^6 leukocytes in a total volume of 6.6 mls. of .067M phosphate buffer, pH 7.4, and 30% homologous serum. In addition each flask received 20 μmoles of acetate-1-C14 (S.A. 7.7 x 10^4 CPM/μmole). Center wells contained 0.3 ml. of 30% KOH.
### TABLE VI

**PARTITION OF RADIOACTIVE LIPIDES ISOLATED FROM LEUKOCYTES INCUBATED WITH ACETATE-C$^{14}$**

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Carbon Labeled</th>
<th>Lipide Recovered (CPM)</th>
<th>% of C$^{14}$ Lipide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Digitonide Supernatant</td>
</tr>
<tr>
<td>1</td>
<td>CH$_3$</td>
<td>963</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>CH$_3$</td>
<td>1401</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>-COOH</td>
<td>746</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>-COOH</td>
<td>771</td>
<td>10</td>
</tr>
</tbody>
</table>

Approximately 45 mgs. of total lipide, obtained by pooling the contents of 3 flasks, were used for each fractionation.
FIGURE 8
INCORPORATION OF ACETATE-1-\textsuperscript{14} CARBON INTO FATTY ACIDS BY NORMAL HUMAN LEUKOCYTES

![Graph showing the incorporation of acetate-1-\textsuperscript{14} carbon into fatty acids by normal human leukocytes. The graph plots total activity (CPM x 10\textsuperscript{3}) against time (hours). The activity increases over time, reaching a peak at around 3.0 hours.]
from acetate. The studies were extended to cell-free extracts by Shaw and Gurin(70) and later by Siperstein and Fagan(71). The latter clearly showed that a stimulation of glucose metabolism either by the Embden-Meyerhof or the pentose-phosphate pathway increased the incorporation of acetate into fatty acids. The leukocytes used in the present studies have been washed free of glucose. In addition the length of time required to isolate the leukocytes was sufficient to allow breakdown of their glycogen(91). It was, therefore, decided to test the effects of glucose on fatty acid biosynthesis from acetate in these experiments. Cells isolated in the usual manner were incubated in the presence and absence of .025M glucose. The results are shown in Fig. 9. It is observed that there is a moderate increase in incorporation in the glucose containing flasks. Although the magnitude of this change is not great, experiments conducted with several different blood samples always showed the same response to added glucose. Furthermore, since the suspensions were incubated in 30% serum, glucose was not entirely absent from the "no glucose" flasks. Since leukocyte incubations were always carried out in serum containing buffers, this point was not pursued. Fig. 9 also includes data obtained from controls on the extraction procedure and on the red blood cells present in our preparations. The number of red cells in these
FIGURE 9

EFFECT OF GLUCOSE ON FATTY ACID BIOSYNTHESIS
FROM ACETATE-1-\(^{14}\)C BY LEUKOCYTES
FIGURE 9

- Legend -

THE EFFECT OF GLUCOSE ON THE INCORPORATION
OF ACETATE-1-C\textsuperscript{14} INTO LONG CHAIN FATTY ACIDS

Incubations were of approximately $300 \times 10^6$ leukocytes in a total volume of 5.0 mls. Flasks contained 20 μmoles of acetate-1-C\textsuperscript{14} (S.A. $3.5 \times 10^5$ CPM/μmole).

The final concentration of glucose was .025M.

Controls contained twice the number of erythrocytes present in leukocyte flasks.

The extraction control was a zero time sample.
controls was twice the number ordinarily found in white cell suspensions. Neither control yielded fatty acids containing appreciable amounts of activity.

Another variable factor in leukocyte experiments is the length of time required for preparation of the cells. The average time required for the collection, sedimentation and washing of cells is approximately 90 minutes. This time may vary substantially from one run to another. Since the whole blood-fibrinogen mixture is allowed to sediment at room temperature, this operation is equivalent to a pre-incubation period. An experiment was set up to see whether some of the variability observed between various samples of blood might be due to this process. A sample of blood was drawn aseptically and divided into two portions. One half was sedimented immediately while the second half was allowed to stand four hours at room temperature before the sedimentation procedure was initiated. After washing the cells, they were incubated with acetate-C\textsuperscript{14} and glucose as previously described. The results are shown in Fig. 10. It is seen that there is an increase in both the specific activity and the total activity in the fatty acids. This increase in the utilization of acetate for the formation of fatty acids by a preincubation is most likely related to depletion of endogenous substrates of the leukocytes. The high lipide and glycogen content of these cells has been
FIGURE 10

THE EFFECT OF PREINCUBATION OF WHOLE BLOOD ON THE INCORPORATION OF ACETATE-1-C\textsuperscript{14} INTO LONG CHAIN FATTY ACIDS
FIGURE 10

- Legend -

EFFECT OF PREINCUBATION OF WHOLE BLOOD ON THE INCORPORATION OF ACETATE-1-Cl\textsubscript{14} INTO LONG CHAIN FATTY ACIDS

Incubations were of $95 \times 10^6$ leukocytes in a total volume of 3.3 mls. for 3.0 hours at 37° C. Each flask received 10 μmoles of acetate-1-Cl\textsubscript{14} (S.A. $7.7 \times 10^4$ CPM/μmole).
referred to under Biochemical Composition. However, since the glycogen is rapidly depleted from these cells (91), they may have been using lipide stores as a source of energy. This may have made the cells more dependent upon exogenous substrates.

b) Homogenate

The observation that normal peripheral human leukocytes contained a complete multienzyme system capable of synthesizing substantial amounts of lipide from acetate was especially interesting both in the light of current interest in fatty acid synthesis per se and also because of the important circumstantial association of blood lipides with degenerative disease. It was decided to characterize the system more fully in this human tissue in order to compare it with similar systems from other tissues. The leukocyte homogenates were prepared from cells harvested by the usual procedure. The cells were ground in a motor-driven Potter-Elvehjem unit equipped with a teflon pestle. Twenty to twenty-five passes served to rupture more than 90% of the cells as observed by direct microscopy. Intact cells, nuclei and cellular debris were removed by spinning for 10 to 20 minutes at 600 x g in the cold. The homogenates were routinely prepared in isotonic sucrose or KCl. Pertinent data on the incubation medium is given in Table VII. Incubations
**TABLE VII**

**INCUBATION MEDIUM FOR LEUKOCYTE HOMOGENATES**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium phosphate</td>
<td>30 μmoles</td>
</tr>
<tr>
<td>Potassium bicarbonate</td>
<td>30 μmoles</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>15 μmoles</td>
</tr>
<tr>
<td>Acetate-1-(^{14})C</td>
<td>20 μmoles</td>
</tr>
<tr>
<td>Potassium citrate</td>
<td>15 μmoles</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>15 μmoles</td>
</tr>
<tr>
<td>Hexose diphosphate</td>
<td>15 μmoles</td>
</tr>
<tr>
<td>Glucose-6-P dehydrogenase</td>
<td></td>
</tr>
</tbody>
</table>

Homog. equivalent to 150-300 × 10⁶ cells (30-60 mgs. dry wt.) prepared in 0.25M sucrose containing 0.01M nicotinamide and 0.001M cysteine

<table>
<thead>
<tr>
<th>Incubation volume</th>
<th>3 mls.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation time</td>
<td>2-3 hrs.</td>
</tr>
</tbody>
</table>
were carried out in the usual Erlenmeyer flasks containing center wells for the collection of $^{14}\text{O}_2$.

When an homogenate capable of incorporating acetate into the long chain fatty acids had been obtained, an attempt was made to determine the cofactor and substrate dependencies of the system. This was accomplished by the stepwise removal of the various additions and a comparison of the results with the complete system. The results of a typical experiment are shown in Table VIII. At the termination of experiments of this type, 10 mgs. of carrier palmitic acid were routinely added to minimize losses during fatty acid isolation procedures. The results are expressed as actual activity isolated per 10 mgs. of fatty acid as shown in the second column and as mumoles of acetate incorporated in the third column. The last two columns express the relative incorporation as percent of the control and complete flasks. The control flask contained all additions except ATP, CoA, DPN and TPN. It is seen from Table VIII that the control flask incorporated a small amount of acetate while the boiled homogenate showed no incorporation. These two flasks served to check the efficiency of the isolation procedure. The addition of ATP, CoA, DPN and TPN, in concentrations indicated in the legend, stimulated the incorporation approximately seven fold. Upon removal of ATP there was no loss in activity. Removal of TPN in this experiment
### TABLE VIII

**FATTY ACID BIOSYNTHESIS FROM AC-1-C$_{14}$**

**BY LEUKOCYTE HOMOGENATES**

- **SOME REQUIREMENTS OF THE SYSTEM** -

<table>
<thead>
<tr>
<th>Flask</th>
<th>LCFA (CPM/10 mgs.)</th>
<th>Acetate Incorporated (mumoles)</th>
<th>% of Control</th>
<th>% of Complete Homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>1660</td>
<td>4.47</td>
<td>719</td>
<td>100</td>
</tr>
<tr>
<td>-ATP</td>
<td>1763</td>
<td>4.76</td>
<td>763</td>
<td>106</td>
</tr>
<tr>
<td>-CoA</td>
<td>420</td>
<td>1.13</td>
<td>182</td>
<td>25</td>
</tr>
<tr>
<td>-DPN</td>
<td>848</td>
<td>2.28</td>
<td>367</td>
<td>51</td>
</tr>
<tr>
<td>-TPN</td>
<td>2002</td>
<td>5.39</td>
<td>867</td>
<td>121</td>
</tr>
<tr>
<td>Control</td>
<td>231</td>
<td>0.62</td>
<td>--</td>
<td>14</td>
</tr>
<tr>
<td>Boiled homog.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

-
TABLE VIII

- Legend -

Homogenate was prepared in isotonic sucrose containing nicotinamide (.01M) and cysteine (.001M).

Each flask contained homogenate equivalent to $300 \times 10^6$ leukocytes plus the following additions: potassium citrate, 15 μmoles; potassium phosphate, 30 μmoles; potassium bicarbonate, 30 μmoles; MgCl₂, 15 μmoles; hexose diphosphate, 15 μmoles; acetate-$1^{-14}C$, 20 μmoles (S.A. $3.7 \times 10^5$ CPM/μmole) and units of glucose-6-phosphate dehydrogenase.

Additions were made in the following final molar concentrations: ATP, .002; DPN, .001; CoA, .0002; and TPN, .001.

Incubations were carried out in 25 ml. Erlenmeyer flasks in a final volume of 3.15 mls. for three hours at 37° C. At the termination of the experiment 10 mgs. of carrier palmitate were added.
seemed to result in a greater incorporation than the complete homogenate. That ATP was not a necessary factor was a consistent finding in all further experiments. The results with TPN showed some degree of variability. The removal of DPN in this experiment dropped the activity to 50 percent of the complete homogenate. This finding has been confirmed in further experiments. The withdrawal of CoA dropped the incorporation to 25 percent. This factor then is the most potent one studied to date.

In Table IX are presented results from a similar experiment. It is seen that the activity of this system derived from 250 million cells is higher than the previous one containing 300 million cells. The variation, however, is not great and the values are certainly of the same level of magnitude. The greater incorporation of the control flasks in this experiment as compared to the previous one probably means that there are more endogenous cofactors present here. This is supported by the fact that the activity of the controls in this experiment are approximately twice that in the previous experiment while the complete flasks differ by only 10 to 15 percent.

The removal of ATP from this preparation reduces the incorporation by only 10 percent. Approximately the same effect is observed with the removal of TPN. Both these results are in agreement with the previous data of Table VIII. The removal of DPN results in a decrease in
TABLE IX

FATTY ACID BIOSYNTHESIS FROM AC-1-C\textsuperscript{14}

BY LEUKOCYTE HOMOGENATES

-SOME REQUIREMENTS OF THE SYSTEM-

<table>
<thead>
<tr>
<th>Homogenate</th>
<th>LCFA (CPM/10 mgs.)</th>
<th>Acetate Incorporated (mmoles)</th>
<th>% of Complete Homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>2186</td>
<td>5.91</td>
<td>100</td>
</tr>
<tr>
<td>-ATP</td>
<td>1966</td>
<td>5.30</td>
<td>90</td>
</tr>
<tr>
<td>-CoA</td>
<td>824</td>
<td>2.22</td>
<td>38</td>
</tr>
<tr>
<td>-DPN</td>
<td>1545</td>
<td>4.17</td>
<td>71</td>
</tr>
<tr>
<td>-TPN</td>
<td>2018</td>
<td>5.44</td>
<td>92</td>
</tr>
<tr>
<td>Control + CoA</td>
<td>1323</td>
<td>3.57</td>
<td>61</td>
</tr>
<tr>
<td>Control</td>
<td>573</td>
<td>1.55</td>
<td>26</td>
</tr>
<tr>
<td>Control</td>
<td>506</td>
<td>1.36</td>
<td>23</td>
</tr>
<tr>
<td>Boiled homog.</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
TABLE IX

- Legend -

Homogenate was prepared in isotonic sucrose containing nicotinamide (0.01M) and cysteine (0.001M).

Each flask contained homogenate equivalent to 250 X 10^6 leukocytes plus the following additions: potassium citrate, 15 μmoles; potassium phosphate, 30 μmoles; potassium bicarbonate, 30 μmoles; MgCl₂, 15 μmoles; hexose diphosphate, 15 μmoles; acetate-1-C₁₄, 20 μmoles (S.A. 3.7 X 10⁵ CPM/μmole) and units of glucose-6-phosphate dehydrogenase.

Additions were made in the following final molar concentrations: ATP, 0.002; DPN, 0.001; CoA, 0.0002; and TPN, 0.001.

Incubations were carried out in 25 ml. Erlenmeyer flasks in a final volume of 3.28 mls. for three hours at 37° C. At the termination of the experiment 10 mgs. of carrier palmitate were added.
activity to 70 percent of the complete homogenate. CoA removal results in a decrease to less than 40 percent. Although the absolute figures vary somewhat from the previous experiment, the relative decrease is the same. That is, CoA has the largest stimulatory effect while DPN is somewhat less active. The addition of CoA alone to the homogenate resulted in an incorporation of acetate 60 percent that of the complete homogenate. These data, therefore, establish CoA and DPN as essential for maximal activity of this system. The results showing a CoA and DPN dependence of the fatty acid biosynthesizing system are in agreement with other systems present in the literature. Van Baalen and Gurin (87) were unable to show a dependence on TPN for a high-speed supernatant system isolated from pigeon liver. Porter and Tietz (61) also failed to show a clear-cut need for TPN in a purified pigeon liver system. Hele and Popjak (36), using a purified system from rabbit mammary gland were likewise unable to show a TPN requirement. It seems, therefore, that the results obtained here are not contradictory or unusual. Most of the systems mentioned do have an ATP requirement. However, this is not a universal finding either. A recent report by Langdon (46) using a preparation from rat liver indicated that ATP was not necessary for fatty acid biosynthesis from acetate.
A further requirement of many of the systems cited in the literature has been the presence of an oxidizable substrate or a TPNH-generating system. Our complete system contained glucose-6-phosphate, hexose diphosphate and citrate. A further experiment was designed to see whether these were necessary for the activity of this system. The results of such an experiment are shown in Table X. The activity of this system, equivalent to 150 million cells per flask, was in good agreement with the preparations run in Tables VIII and IX. The removal of CoA here again resulted in a decrease to approximately 20 percent of the activity of the complete flask. Removal of citrate caused a marked decrease in incorporation of acetate. The removal of the glucose-6-phosphate and hexose diphosphate also resulted in a marked drop in activity. The addition of fumarate did not stimulate further the incorporation of acetate. It, therefore, appears that this system of human leukocytes has similar requirements as the pigeon liver, rat liver and rabbit mammary gland. Additional data in Table X show a complete inhibition of the system by 0.1M fluoride and a partial inhibition by the same concentration of cyanide. Similar results showing a depressing effect of fluoride on acetate incorporation into fatty acids by lactating guinea pig mammary glands was obtained by Terner(80). This inhibition by fluoride was only partial when pyru-
TABLE X

FATTY ACID BIOSYNTHESIS FROM AC-1-C\textsuperscript{14}

BY LEUKOCYTE HOMOGENATES

-SOME REQUIREMENTS AND INHIBITORS OF THE SYSTEM-

<table>
<thead>
<tr>
<th>Homogenate</th>
<th>LCFA (CPM/10 mgs.)</th>
<th>Acetate Incorporated (μmoles)</th>
<th>% of Complete Homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>707</td>
<td>3.03</td>
<td>100</td>
</tr>
<tr>
<td>Complete</td>
<td>781</td>
<td>3.35</td>
<td>100</td>
</tr>
<tr>
<td>+F\textsuperscript{-}</td>
<td>46</td>
<td>0.20</td>
<td>6</td>
</tr>
<tr>
<td>+CN\textsuperscript{-}</td>
<td>254</td>
<td>1.09</td>
<td>34</td>
</tr>
<tr>
<td>+fumarate</td>
<td>601</td>
<td>2.58</td>
<td>81</td>
</tr>
<tr>
<td>-citrate</td>
<td>305</td>
<td>1.31</td>
<td>41</td>
</tr>
<tr>
<td>-CoA</td>
<td>137</td>
<td>0.59</td>
<td>18</td>
</tr>
<tr>
<td>-CoA</td>
<td>144</td>
<td>0.62</td>
<td>19</td>
</tr>
<tr>
<td>Complete*</td>
<td>1573</td>
<td>6.75</td>
<td>100</td>
</tr>
<tr>
<td>-G6P, HDP and G6P-dehydrogenase</td>
<td>182</td>
<td>0.78</td>
<td>11</td>
</tr>
<tr>
<td>-Control</td>
<td>107</td>
<td>0.46</td>
<td>7</td>
</tr>
<tr>
<td>-Control</td>
<td>89</td>
<td>0.38</td>
<td>6</td>
</tr>
</tbody>
</table>

* These results were obtained from a different sample of blood.
TABLE X

- Legend -

Homogenate was prepared in isotonic sucrose containing nicotinamide (.01M) and cysteine (.001M).

Each flask contained 1.8 mls. of homogenate equivalent to approximately 150 X 10^6 leukocytes and the following in final molar concentrations: potassium phosphate buffer, pH 7.4, .01; potassium bicarbonate, .01; MgCl₂, .005; hexose diphosphate, .005; glucose-6-phosphate, .005; potassium citrate, .005; DPN, .001; glucose-6-phosphate dehydrogenase, units; and sufficient H₂O to bring the final volume to 3.05 mls.

The various additions had the following final molar concentrations: CoA, 2 X 10⁻⁴; fluoride, .01 and cyanide, .01.

Each flask also contained 20 µmoles of acetate-1-C¹⁴ (S.A. 2.33 X 10⁵ CPM/µmole).

Incubations were for three hours. The gas phase was 95% oxygen-5% CO₂. Reactions were stopped by brief immersion of the flasks in boiling water. 10 mgs. of carrier palmitate were then added followed by 0.2 ml. of 0.2N HCl to displace bound CO₂.

The average incorporation of the complete homogenates is set equivalent to 100% activity.
vate-2-C$_{14}$ was used as the precursor of fatty acids. This would implicate the acetate-activating step as the point sensitive to fluoride. Aisenberg and Potter(1) have shown that there are two acetate-activating systems, one mitochondrial and the other supernatant. Of these the supernatant system is less fluoride sensitive than is the mitochondrial. The system in leukocytes is quite sensitive to fluoride although further data will show it to be a soluble system. However, it is known that fluoride also inhibits glycolysis(79). Both phosphoglucomutase and enolase are inhibited by this ion. Results obtained in the present work indicate a dependence of acetate incorporation upon active glycolysis. The strong inhibitory effect of fluoride could, therefore, be attributed to its ability to block the system at more than one point. The partial inhibition of the leukocyte system by cyanide would indicate a dependence upon oxidative processes. However, additional experiments to be presented show an increased incorporation of acetate carbon into long chain fatty acids under anaerobic conditions. This increased incorporation under anaerobic conditions argues against an interference with oxidative processes although the inhibitory effects of cyanide may be exerted on systems other than those concerned with cell respiration.
The finding of a strong dependence of fatty acid biosynthesis in leukocyte homogenates on DPN and CoA suggests that this tissue is normally deficient in these cofactors. Results in the literature have shown that there are optimal levels of cofactors (62) and higher levels may severely inhibit the biosynthetic process. It appeared desirable to study the effects of varying levels of DPN and CoA in these preparations to find out what the optimal concentrations might be. The results of such an experiment are shown in Table XI. It is seen that there is a steady increase in acetate incorporation when DPN is increased up to 6 umoles per flask. The stimulation at this point is two-fold over the control flasks. Increasing CoA up to 1.2 umoles per flask increased the incorporation approximately six-fold over controls. The combination of 3 umoles of DPN and 0.6 umoles of CoA resulted in a stimulation of over ten fold. It is apparent from these results that optimum levels of these two cofactors have not yet been reached. Further experiments to determine the optimal levels are needed. It is of interest that such high levels of CoA (4 X 10^{-4} M) are stimulatory. Brady et al (15) using a crude system and Porter et al (62) with a purified preparation have shown CoA levels beyond optimal to be strongly inhibitory to the activity of this system. In fact the former investigators have shown fatty acid
TABLE XI

INFLUENCE OF CoA AND DPN CONCENTRATION ON FATTY ACID BIOSYNTHESIS BY HOMOGENATES OF NORMAL HUMAN LEUKOCYTES

<table>
<thead>
<tr>
<th>Flask</th>
<th>Cofactor (umoles)</th>
<th>Acetate Incorporated (umoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoA</td>
<td>0.2</td>
<td>1.70</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>1.92</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>2.43</td>
</tr>
<tr>
<td>DPN</td>
<td>1</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.88</td>
</tr>
<tr>
<td>CoA</td>
<td>0.6</td>
<td>6.75</td>
</tr>
<tr>
<td>DPN</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0.46</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0.38</td>
</tr>
</tbody>
</table>
TABLE XI

- Legend -

Each flask contained homogenate equivalent to 220 x 10^6 leukocytes. Incubations were for 2.0 hours at 37° C in a total volume of 2.92 mls.

Each flask received 20 µmoles of acetate-l-C¹⁴ (S.A. 2.33 x 10⁵ CPM/µmole).

All other conditions were the same as in previous homogenate experiments.
oxidation to be stimulated by high levels of CoA. Further evidence that the leukocyte preparations are relatively deficient in CoA is the fact that stimulatory effects are obtained in the crude homogenates without dialysis. With the pigeon liver system a requirement for CoA could not be demonstrated until CoA was removed by dialysis or ion exchange resins (62).

The conversion of acetate to fatty acid is not only an endergonic reaction, but it also is a reductive process. That is, electrons must be made available for the production of an acyl chain containing from 7 to 9 acetyl units. This reductive biosynthesis should be favored under anaerobic conditions. A comparison of fatty acid biosynthesis under aerobic and anaerobic conditions is shown in Table XII. The flasks contain the usual additions of previous experiments except that oxidized and reduced pyridine nucleotides are varied. Using the DPN flasks as controls, it can be seen that the substitution of nitrogen for oxygen in the presence of 5 percent CO₂ results in a three-fold increase in acetate incorporation. Under aerobic condition little effect was obtained by substituting reduced for oxidized cofactors. Under anaerobic conditions the substitution of TPNH for TPN apparently resulted in a decrease in acetate incorporation. Little can be concluded from this single experiment except that the addition of TPN, DPNH or TPNH to the system under
TABLE XII

THE EFFECT OF GAS PHASE AND PYRIDINE NUCLEOTIDES ON BIOSYNTHESIS OF LONG CHAIN FATTY ACIDS BY HOMOGENATES OF HUMAN LEUKOCYTES

<table>
<thead>
<tr>
<th>Flask</th>
<th>Gas Phase</th>
<th>Additions (.001M)</th>
<th>Acetate Incorporated* (mmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>O₂-CO₂</td>
<td>DPN</td>
<td>71</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>DPN, TPN</td>
<td>64</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>DPNH, TPN</td>
<td>69</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>DPN, TPNH</td>
<td>81</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>DPNH, TPNH</td>
<td>80</td>
</tr>
<tr>
<td>6</td>
<td>N₂-CO₂</td>
<td>DPN</td>
<td>193</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>DPN, TPN</td>
<td>192</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>DPNH, TPN</td>
<td>218</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>DPN, TPNH</td>
<td>156</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>DPNH, TPNH</td>
<td>145</td>
</tr>
</tbody>
</table>

* Results are expressed for homogenate equivalent to 10¹⁰ cells.
TABLE XII

- Legend -

Results expressed per $10^{10}$ leukocytes.

Each flask contained $150 \times 10^6$ leukocytes plus the usual additions in a volume of 3.0 mls. Incubations were for three hours.

Other additions, where indicated, were made in final concentration of .001M.
aerobic or anaerobic conditions had no marked effect on the incorporation of acetate into long chain fatty acids. In the crude system used up to this point, it is difficult to determine absolute requirements necessary for activity. In addition to glycolysis and the pentose-phosphate pathway the manifold reactions of the mitochondria and microsomes are present. As a first step at simplification the whole homogenate was fractionated by high-speed differential centrifugation. This step would attempt to localize this multienzyme system in one of the subcellular fractions. A slow-speed supernatant fraction was prepared by spinning the whole homogenate at 600 x g for 15 to 20 minutes. This preparation was free of intact cells, nuclei and cellular debris. The supernatant fluid was transferred to the Spinco Model L Preparative Ultracentrifuge and spun at 10,000 x g for 10 minutes to remove mitochondria. A further spinning at 100,000 x g for 50 minutes was used to take down the microsomes. These particulate fractions were resuspended in an equivalent volume of isotonic sucrose containing salts, cofactors and substrates and incubated with $^{14}$C-acetate. The results are shown in Table XIII. Removal of mitochondria resulted in a partial decrease in activity. The mitochondria alone had little activity. The microsomes also were essentially without activity. The high-speed
TABLE XIII

LONG CHAIN FATTY ACID BIOSYNTHESIS FROM ACETATE-$\text{C}^{14}$
BY SUB-CELLULAR FRACTIONS OF HUMAN LEUKOCYTES

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Long Chain Fatty Acids (CPM/10 mgs.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow-speed Supernate</td>
<td>266</td>
</tr>
<tr>
<td>High-speed Supernate plus Microsomes</td>
<td>146</td>
</tr>
<tr>
<td>&quot;                               &quot;</td>
<td>121</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>48</td>
</tr>
<tr>
<td>&quot;                               &quot;</td>
<td>66</td>
</tr>
<tr>
<td>Microsomes</td>
<td>27</td>
</tr>
<tr>
<td>&quot;                               &quot;</td>
<td>19</td>
</tr>
<tr>
<td>High-speed Supernate</td>
<td>217</td>
</tr>
<tr>
<td>&quot;                               &quot;</td>
<td>215</td>
</tr>
</tbody>
</table>
TABLE XIII

- Legend -

Each flask contained material equivalent to 200 $\times 10^6$ leukocytes. Incubations were for 2.5 hours in a total volume of 3.0 mls. with 95% $O_2$-5% $CO_2$ as gas phase.

All other conditions, cofactors and substrates are the same as those used in whole homogenate incubations.
particulate-free supernatant fluid was found to retain most of the activity of the whole homogenate. This finding places the leukocyte system along with the fatty acid biosynthesizing of pigeon and rat liver and mammary gland. The location of all enzymes in this soluble portion of the cell will facilitate further work on the purification and determination of requirements of this interesting system.

A further attempt at characterizing this system involved dialysis of the crude homogenate against isotonic sucrose. The results are presented in Table XIV. Neither the dialysed nor the undialysed controls were capable of incorporation of much acetate. The addition of ATP, CoA and DPN increased the undialysed flask to approximately twice the activity of the dialysed flask. Apparently essential components other than those replaced were necessary for maximal activity. The removal of CoA and DPN resulted in the usual decrease in activity, i.e., approximately 30 and 60 percent, respectively, of the complete system. Even with this period of dialysis no requirement for ATP could be demonstrated.

4. Amino Acids

The results presented in Table IV showed that 25 percent of the initial radioactive acetate was con-
TABLE XIV

EFFECT OF DIALYSIS ON LONG CHAIN FATTY ACID BIOSYNTHESIS FROM ACETATE BY HOMOGENATES OF HUMAN LEUKOCYTES

<table>
<thead>
<tr>
<th>Flask</th>
<th>LCFA (CPM/10 mgs.)</th>
<th>Acetate Incorporated (μmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undialysed Complete</td>
<td>291</td>
<td>1.25</td>
</tr>
<tr>
<td>Undialysed Control</td>
<td>37</td>
<td>0.16</td>
</tr>
<tr>
<td>Dialysed Complete</td>
<td>158</td>
<td>0.68</td>
</tr>
<tr>
<td>-CoA</td>
<td>65</td>
<td>0.28</td>
</tr>
<tr>
<td>-DPN</td>
<td>97</td>
<td>0.46</td>
</tr>
<tr>
<td>-ATP</td>
<td>198</td>
<td>0.85</td>
</tr>
<tr>
<td>Dialysed Control</td>
<td>29</td>
<td>0.12</td>
</tr>
</tbody>
</table>
Homogenates were prepared and dialysed with .25M sucrose containing .01M nicotinamide and .001M cysteine. Dialysis was for 4 hours at 4°C.

Each flask contained homogenate equivalent to 290 $\times$ 10$^6$ leukocytes plus 20 µmoles of acetate-1-C$^{14}$ (S.A. 2.33 $\times$ 10$^5$ CPM/µmole). Other additions were the same as in previous homogenate experiments.

When present, the cofactors were of the following final molar concentrations: CoA, 2 $\times$ 10$^{-4}$; DPN, 1 $\times$ 10$^{-3}$ and ATP, 1 $\times$ 10$^{-3}$. 

---

**TABLE XIV**

- Legend -
verted to non-volatile compounds. Of this fraction the activity contributed by glucose and glycogen was seen to be negligible (Table V). The activity found in protein and lipid, although significant, also accounted for only a small percentage of the original substrate, i.e., less than 1 percent (Figs. 5 and 8). To determine the nature of this fraction aqueous extracts of cell suspensions incubated with acetate-2-C\textsuperscript{14} were prepared and subjected to electrolytic desalting. A preliminary two-dimensional chromatogram was run and a radioautograph was prepared from it. The developed film showed quite extensive labeling including approximately 10 radioactive spots. In order to isolate and identify these compounds a series of streak chromatograms were run in one dimension and the zones of C\textsuperscript{14} activity were located by radioautography. Spraying with ninhydrin indicated that the radioactive spots contained amino acids. Using acetate-2-C\textsuperscript{14}, 6 distinct bands of radioactivity were detectable after approximately 1 week exposure to X-ray film. Five bands were obtained with acetate-1-C\textsuperscript{14}. The intensity of the bands also varied with the different acetates. Photographs of these chromatograms are shown in Fig. 11. The radioactive areas were traced onto the chromatograms. These spots were cut out, stitched together and eluted by descending chromatography. Aliquots of the eluted
FIGURE 11

RADIOAUTOGRAPHS OF WATER-SOLUBLE COMPOUNDS EXTRACTED FROM LEUKOCYTES INCUBATED WITH C\textsuperscript{14}-ACETATE
material were combusted to CO₂ and counted as Ba CO₃ to
determine the total activity of the eluted material. Ad-
ditional aliquots were removed for the quantitative de-
termination of amino acids. These data are presented in
Table XV.

It is seen that spot 2 accounts for most of the
activity. The number 1 spot was the next most intense
zone. Based upon Rf values in the literature and with
values obtained in this laboratory, a tentative identifi-
cation of zones 1 and 2 was aspartic and glutamic acid,
respectively. Confirmation of this identification was
obtained by running the eluted compounds in a second and
third solvent. Radioautographs were prepared, the spots
were eluted, carrier acids were added and chromatography
in a third solvent was carried out. Upon spraying with
ninhydrin only one spot was detectable indicating that
the original material had traveled with the carrier amino
acid. Also the darkened areas on the radioautographs co-
incided exactly with the ninhydrin-positive spots. A
photograph of a chromatogram prepared after the third
solvent is shown in Fig. 12. On the basis of this evi-
dence, it is felt that positive identification of spots
1 and 2, as aspartic and glutamic acid, has been attained.
The identification of the remaining zones of activity has
not been completed. On the basis of Rf values they may
be serine, threonine and hydroxy proline. A radioactive
TABLE XV

PAPER CHROMATOGRAPHIC SEPARATION OF RADIOACTIVE WATER-SOLUBLE COMPOUNDS SYNTHESIZED FROM ACETATE-2-C\textsubscript{14} BY HUMAN LEUKOCYTES

<table>
<thead>
<tr>
<th>Spot</th>
<th>Rf*</th>
<th>Amino Acid (mg.)**</th>
<th>Activity (CPM X 10\textsuperscript{3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>.08</td>
<td>19.9</td>
</tr>
<tr>
<td>1</td>
<td>.09</td>
<td>.10</td>
<td>31.0</td>
</tr>
<tr>
<td>2</td>
<td>.18</td>
<td>.20</td>
<td>131.0</td>
</tr>
<tr>
<td>3</td>
<td>.26</td>
<td>.23</td>
<td>14.0</td>
</tr>
<tr>
<td>4</td>
<td>.41</td>
<td>.10</td>
<td>26.0</td>
</tr>
<tr>
<td>5</td>
<td>.59</td>
<td>.16</td>
<td>24.0</td>
</tr>
</tbody>
</table>

* Solvent was buffered phenol, pH 12.
** Valine was used as a standard for quantitative colorimetric estimation of amino acid with ninhydrin.
TABLE XV

--Legend--

Flasks contained $500 \times 10^6$ leukocytes. Incubations were carried out in 25 ml. Erlenmeyer center well-equipped flasks in a total volume of 5.5 mls. of Krebs-Ringer phosphate buffer and 30 percent homologous serum. In addition each flask received 15 μmoles of potassium fumarate and 30 μmoles of acetate-2-C$^{14}$ (S.A. $3.85 \times 10^5$ CPM/μmole). Incubation time was 3 hours.

The leukocyte tissue was extracted with boiling water and the solutions were desalted electrolytically. Separations were accomplished by ascending chromatography with buffered phenol, pH 12, on Whatman No. 3 filter paper. Radioautograms were prepared as described under Methods.
FIGURE 12

CHROMATOGRAPHIC-RADIOAUTOGRAPHIC IDENTIFICATION OF ASPARTIC AND GLUTAMIC ACIDS
ninhydrin-positive spot which did not move in the phenol solvent is as yet completely unidentified. Further experiments will have to be carried out to positively identify these other products of acetate metabolism by leukocytes.

The results in Table IV showed a rapid rise in the non-volatile in the first 30 minutes followed by a plateau. Since considerable activity was present in this amino acid fraction, a rapid rise here could possibly account for this observation. Incubations of leukocytes were conducted as usual and the reactions were terminated at various time intervals by the addition of perchloric acid. After removal of most of the perchloric acid by partial neutralization with KOH, the amino acids were separated from unutilized substrate, other organic anions and non-ampholytes by adsorption on Dowex-50(H). The amino acids were eluted with dilute ethanolic HCl and an aliquot was combusted for the determination of $^{14}$C activity. The results of this experiment are shown in Table XVI. These data show that the amino acid fraction comprises the largest portion of the non-volatile radioactivity. Furthermore, it is seen that almost 70 percent of the activity of this fraction is present at 30 minutes. A plot of these data is shown in Fig. 13. It is clear, therefore, that the sharp rise in activity of the non-volatile compounds is due predominantly to this amino acid fraction.
### TABLE XVI

**INCORPORATION OF ACETATE-1-\textsuperscript{14}C CARBON INTO AMINO ACIDS BY NORMAL HUMAN LEUKOCYTES**

<table>
<thead>
<tr>
<th>Flask</th>
<th>Time (hrs.)</th>
<th>Total CPM (x 10\textsuperscript{3})</th>
<th>% of Non-Volatile CPM</th>
<th>% of Total CPM Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>184</td>
<td>62.3</td>
<td>12.6</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>224</td>
<td>76.7</td>
<td>15.3</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>278</td>
<td>84.5</td>
<td>19.1</td>
</tr>
<tr>
<td>1</td>
<td>3.0</td>
<td>281</td>
<td>80.3</td>
<td>19.2</td>
</tr>
</tbody>
</table>

* Experimental conditions are the same as those in Table IV. Average amino acid recovery was 3.2 mgs. per flask.
FIGURE 13

INCORPORATION OF ACETATE-1-C\textsubscript{14} CARBON INTO AMINO ACIDS BY NORMAL HUMAN LEUKOCYTES
5. **Organic Acids**

It would be expected that a tissue containing an active tricarboxylic acid cycle would contain labeled di- and tricarboxylic acids if incubated with $^{14}C$ acetate. The experimental results obtained so far would indicate that this fraction could comprise no more than 20 percent of the non-volatile compounds (Table XV). It is also known that the tricarboxylic acid cycle intermediates turnover rapidly and that their concentration in animal tissues is quite low (32). Initial attempts to locate these compounds on paper chromatograms by spraying failed although some radioactivity was detectable. In further experiments in which it was desired to extract these acids, carrier compounds were added at the termination of the incubations. By combined chromatographic-radioautographic procedures it was possible to locate acids which showed $^{14}C$ activity. The tentative identification of these spots based upon Rf values alone in an ether-acetic acid solvent was succinic, fumaric and alpha-ketoglutaric acids. In order to positively identify these spots they must be eluted and rechromatographed as described for the amino acids. These preliminary data support the existence of a complete Krebs cycle in this tissue.
VI. DISCUSSION

The idea of using human leukocytes as model cells is not a new one; however, most biochemical investigations on these cells have been devoted to a search for differences between normal and leukemic metabolism usually with the hope of establishing a basis for chemotherapy. The present investigation is concerned solely with the metabolism of mixed leukocytes isolated from normal human blood.

The in vitro metabolism of leukocytes apparently is not affected over wide ranges of cell concentration as indicated by their oxygen consumption. Also, oxidative metabolism, determined by ability to convert acetate to CO₂, is directly proportional to the number of leukocytes present. This linear response makes possible the comparison of results obtained from incubating samples varying in cell concentration and number. The observations in this paper apply to isolated populations of cells. Whether in vitro experiments with leukocytes are concerned with the "metabolism of injury" or not is a question which remains in dispute at the time of this writing (9, 16). However, the results of the above experiments indicate that the isolated cells used in this study are viable and reasonably normal.
In the present study, the data show that leukocytes isolated from normal human blood are capable of utilizing acetate by a variety of paths including conversion to CO₂, and incorporation into lipide, protein, and a group of water-soluble non-volatile compounds such as amino acids and organic acids. Since normal leukocytes possess the ability to utilize considerable amounts of acetate, it is of interest to compare the intermediary metabolism of these cells with that of other tissues(42). For purposes of comparison data on leukocytes (Table II) were converted to μmoles of acetate utilized per hour per 100 mgs. of tissue dry weight starting with the following incubation conditions:

10 μmoles acetate/3 hrs./370 x 10⁶ leukocytes.

Converting number of cells to weight¹ of leukocytes:

10 μmoles acetate/3 hrs./170 mgs. leukocytes

or

20 μmoles acetate/3 hrs./100 mgs. dry weight leukocytes.

¹The conversion factor, 7.5 x 10⁶ cells = 1 mg. dry weight leukocytes, obtained by Burk et al., (16) has been used here. It has been assumed that all cell types compared are 70 per cent water.
The per cent of acetate converted to CO₂ and non-volatile compounds is shown in Table IV. From this the µmoles of substrate acetate in each of the main subdivisions may be calculated with the following:

- total acetate utilized, 4.7; CO₂, 3.0; and non-volatiles, 1.6 µmoles/hr./100 mgs. dry weight leukocytes.

In an experiment with rat liver slices Katz and Chaikoff(42) used the following conditions:

- 50 µmoles acetate/3 hrs./1000 mgs. wet weight liver
- or
- 17 µmoles/3 hrs./100 mgs. dry weight liver.

Their experimental conditions including gas phase, buffer, temperature and time were similar to those used in the present study. Their results for liver slices were as follows:

- total acetate utilized, 2.5; CO₂, 1.1; and non-volatiles, 1.4 µmoles/hr./100 mgs. dry weight liver.

It may be seen from these data that acetate utilization by liver tissue is quite similar to that of leukocytes on a quantitative basis. On the other hand a difference does exist between the two tissues in the
relative fate of the acetate carbons. The leukocytes converted twice as much acetate to CO$_2$ as to non-volatile compounds while the liver converted approximately equal amounts into these two major subdivisions. This finding is comparable with the higher CO$_2$ ratios found in liver(78). The higher CO$_2$ ratio indicates a high degree of dilution of tricarboxylic acid cycle intermediates with a corresponding increase in tricarboxylic acid cycle-linked biosynthesis.

Of interest is a comparison of the fate of labeled carbon in the non-volatile fractions of these two types of cells. The leukocytes converted small and equal quantities of acetate carbon into lipide and protein. As previously seen on page 70, this amounted to 0.3 per cent of the initial substrate for each. In the liver slice experiment of Katz and Chaikoff(42) considerably more substrate was converted to lipide and protein; but here too, the amount of substrate appearing in each of these fractions was almost equal. With the liver the values were 2.2 per cent in lipide and 1.8 per cent in protein.

The incorporation of acetate has been calculated on a weight basis. We have the following initial conditions for each tissue:
20 μmoles/3 hrs./100 mgs. dry wt. leukocytes and
17 μmoles/3 hrs./100 mgs. dry wt. liver.

The leukocytes converted 0.3 per cent into protein:

\[20 \text{ μmoles} \times 0.3\%/100/3 \text{ hrs.} = \text{0.02 μmoles/hr./100 mgs. dry wt. leukocytes.}\]

The liver slice converted 1.8 per cent into protein:

\[17 \text{ μmoles} \times 1.8\%/100/3 \text{ hrs.} = \text{.10 μmoles/hr./100 mgs. dry wt. liver.}\]

The ratio of incorporation of acetate into protein by leukocytes and liver, .02 μmoles/.10 μmoles, is 1 to 5. Leukocytes, therefore, have an activity 20 per cent of that of liver which has a relatively fast turnover rate estimated to be 10 times greater than that of muscle(33). On the basis of this approximation the leukocyte has the ability to incorporate acetate carbon into protein at a rate between that of muscle and liver.

The ratio between leukocyte and liver for the incorporation of acetate into lipide is .02 μmoles/.12 μmoles or 1 to 6. Thus liver has a relatively active turnover of lipide, but this includes the formation of large amounts of cholesterol(71). On the other hand, most of the radioactivity incorporated by leukocytes into
lipides is found in fatty acids (Table VI). Block and Kramer(14a) have studied fatty acid biosynthesis from acetate by liver slices. Their data for acetate incorporation expressed on a dry weight basis is:

\[ 0.05 \text{ μmoles/hr.}/100 \text{ mgs. dry weight liver.} \]

Since the corresponding value for leukocytes, as stated above, is \( 0.02 \) μmoles, this gives a ratio of 1:2.5. Thus there is less discrepancy between liver and leukocyte values when cholesterol formation is eliminated. In the above comparison of the fate of acetate in incubations of leukocytes on one hand and liver slices on the other it was shown that leukocytes actually converted more acetate carbon to \( \text{CO}_2 \) than did an equal weight of liver.

Additionally, the data of Pardee et al. (59a), recalculated to a dry weight basis, yielded similar results. Their data for the conversion of acetate to \( \text{CO}_2 \), obtained under similar experimental conditions were as follows:

\[ 2.6 - 4 \text{ μmoles acetate/hr.}/100 \text{ mgs. dry weight liver.} \]

The value obtained with leukocytes was:

\[ 3.0 \text{ μmoles/hr.}/100 \text{ mgs. dry weight leukocytes.} \]

Thus the findings in this paper on the utilization of
acetate by leukocytes are in good agreement with similar finding for liver tissue as determined by several groups of investigators.

Pardee et al. (59a) have studied the oxidation of acetate by a variety of tumors and found that tumors oxidize less acetate than did normal liver and kidney tissue. Leukocytes have often been described as having a metabolism similar to that of cancer tissue (11) since they are said accumulate lactic acid under aerobic conditions. This finding is disputed by Burk et al. (16) and Warburg (97). It is interesting that the data presented here show that leukocytes oxidize to CO₂ an amount of acetate comparable to that oxidized by normal tissue. Although one aspect of leukocyte metabolism, aerobic glycolysis, may be similar to that of cancer tissue, the data obtained in the present study show that other metabolic functions of leukocytes are quantitatively similar to normal tissues.

The data presented in Table XVI show that 25 per cent of the acetate utilized by leukocytes was converted to compounds other than CO₂, lipide and protein. An analysis of this fraction indicated that 80 per cent of the activity was present in amino acids and, while a substantially smaller portion was present in organic
acids, there was virtually none present as glucose or glycogen. The amino acid fraction contained glutamate as the most highly labeled component while the second most highly labeled amino acid was aspartate. These two amino acids contained 65 per cent of the activity incorporated into the water-soluble fraction. This type of labeling is found in tissues possessing an active tricarboxylic acid cycle. As shown in Appendix 4, these amino acids may arise directly from the cycle intermediates. The results of Busch and Balthrush(17) obtained with heart tissue serve as an example of the type of labeling obtained via the tricarboxylic acid cycle. Their preparations contained most of the acetate radioactivity in glutamate. These investigators showed that labeling takes place rapidly when acetate oxidation is proceeding at a linear rate. The results present in Fig. 13 show that the leukocytes also rapidly label their dicarboxylic amino acids. Eighty per cent of the total radio activity in amino acid fraction is present after 30 minutes (Fig. 13). This suggests that the tricarboxylic acid cycle is quite active in leukocytes.

The distribution of activity incorporated into other non-volatile compounds by liver differs from that found with leukocytes. One of the striking differences is the high degree of labeling of glucose by liver(42).
A second component highly labeled by liver is acetoacetic acid. Neither of these compounds contained radioactive carbon in the leukocyte experiments. Both leukocytes and liver form large amounts of radioactive glutamate from acetate. It is thus seen that the pattern of distribution of radioactivity from labeled acetate is quite different for these two cell types although their capacity for total utilization of this substrate is of the same order of magnitude.
VII. SUMMARY

A study of the metabolism of acetate in a population of leukocytes isolated from normal human peripheral blood was made. The results obtained are summarized as follows:

1. The oxygen consumption of leukocytes is independent of cell concentration within the range of 15 to 60 X 10^6 cells per ml.

2. The oxidation of acetate to CO₂ proceeds linearly with time over a three hour period; and the amount of acetate converted is proportional to the number of cells present.

3. Approximately twice as much C^{14}O₂ is derived from carboxyl-labeled acetate as from methyl-labeled acetate.

4. One hundred million leukocytes utilize 0.5 μmoles of acetate per hour.

5. The fate of this acetate, after three hours incubation and expressed as per cent of the original radioactivity added to the cell suspension, is as follows: CO₂, 45%; non-volatile compounds, 25%; unused acetate, 30%.

6. Fractionation of the non-volatile compounds indicates that leukocytes possess enzyme systems capable
of incorporating acetate carbon into protein, lipide, amino acids, and organic acids. Negligible amounts of radioactivity are detectable in glucose and glycogen.

7. Eighty per cent of the radioactivity in the non-volatile fraction is identified as amino acids. Less than 1 per cent of the initial radioactivity is incorporated into protein and lipide. The balance of the radioactivity remains in water-soluble compounds as yet unidentified.

8. Glutamate and aspartate account for approximately 65 per cent of the labeled amino acids.

9. Eighty-five to ninety per cent of the lipide synthesized from acetate by intact cells is in the form of long-chain fatty acids. The amount of incorporation is the same with either carboxyl or methyl-labeled acetate.

10. Whole homogenates prepared from leukocytes are capable of incorporating acetate carbon into long-chain fatty acids. For maximal synthesis the following are necessary: a glycolytic intermediate; citric acid; CoA; DPN; and anaerobic conditions.

11. The complete multienzyme system capable of incorporating acetate carbon into long-chain fatty acids is located in the particulate-free high-speed supernatant.
A comparison of acetate metabolism by leukocytes with that of the liver indicates that both cell types utilize approximately the same amounts of acetate. Qualitatively, however, significant differences exist. These differences and similarities are presented in the text (p. 120).
APPENDIX 1

QUANTITATIVE ESTIMATION OF LEUKOCYTES

Leukocyte-rich suspensions are conveniently enumerated by diluting them in a red blood cell pipette and counting the cells in the large squares of a standard hemocytometer as with normal whole blood. The suspension is taken up in the pipette to the 0.5 mark and dilute acetic acid is taken to the 101 mark. This accomplishes a 200-fold dilution. Cells are counted in 4 large squares in both hemocytometer chambers. The cells per large square are equal to a volume of 0.1 mm$^3$. To convert this number to the number of cells in 1 mm$^3$ of the original suspension, the following calculation is performed:

$$\text{cells per large square} \times 10 \times 200 = \text{leukocytes per mm}^3 \text{ of the original suspension}$$
APPENDIX 2

EFFICIENCY OF C₁⁴ COUNTING

The activity of radioisotopic compounds as obtained from commercial sources is designated as a fraction of a curie, e.g., millicurie or microcurie. The curie is defined as $3.7 \times 10^{10}$ disintegrations per second (DPS). This is converted to the more useful term, the microcurie ($\mu$C), as follows:

$$\frac{3.7 \times 10^{10}}{10^6} = 3.7 \times 10^4 \text{ DPS.}$$

Converting to disintegrations per minute (DPM),

$$3.7 \times 10^4 \times 60 = 2.22 \times 10^6 \text{ DPM.}$$

Owing to losses normally incurred during counting procedures, this activity is not realized. In practice the activity of a sample is measured in "counts" which are related to disintegrations. It is frequently advantageous to know how many counts will be realized from a given amount of radioactive material in order to be able to accurately calculate the needed amount of radiosubstrate. The efficiency of the flow counter used in the present study was determined by accurately weighing out radioacetate, combusting it completely to CO₂ and counting the
carbon as BaC\textsubscript{14}O\textsubscript{3} at 5 mgs. The counts observed were then expressed as per cent of the theoretical DPM as follows:

\[
\text{counts obtained} \times \frac{100}{2.22 \times 10^6} = \text{absolute counting efficiency}
\]

The value obtained was 17 per cent. Therefore, with the counting equipment available for the present study, 1 \(\mu\text{c}\) of C\textsuperscript{14} yields 17 per cent of the theoretical 2.22 \(\times\) \(10^6\) DPM or 3.77 \(\times\) \(10^5\) CPM.
APPENDIX 3

FATE OF THE RADIOACTIVE CARBON OF ACETATE-1-C\textsuperscript{14}
IN ONE TURN OF THE KREBS CYCLE*
APPENDIX 3

FATE OF THE RADIOACTIVE CARBON OF ACETATE-1-C\textsuperscript{14} IN

ONE TURN OF THE KREBS CYCLE *

OXALOACETIC

\begin{align*}
\text{HO-} & \quad \text{C-} \quad \text{COOH} \\
\text{C} & \quad \text{C} \\
\text{C}^* \text{OOH} & \quad \text{C}^* \text{OOH}
\end{align*}

ACETIC

\begin{align*}
\text{HO-} & \quad \text{C-} \quad \text{COOH} \quad \rightarrow \quad \text{HO-} \quad \text{C-} \quad \text{COOH} \\
\text{C} & \quad \text{C} \\
\text{C}^* \text{OOH} & \quad \text{C}^* \text{OOH}
\end{align*}

CITRIC

\begin{align*}
\text{HO-} & \quad \text{C-} \quad \text{COOH} \\
\text{C} & \quad \text{C} \\
\text{C}^* \text{OOH} & \quad \text{C}^* \text{OOH}
\end{align*}

\begin{align*}
\text{COOH} & \quad \text{COOH} \\
\text{C} & \quad \text{C} \\
\text{C}^* \text{OOH} & \quad \text{C}^* \text{OOH}
\end{align*}

\begin{align*}
\text{HO-} & \quad \text{C-} \quad \text{COOH} \\
\text{C} & \quad \text{C} \\
\text{C}^* \text{OOH} & \quad \text{C}^* \text{OOH}
\end{align*}

\begin{align*}
\text{COOH} & \quad \text{COOH} \\
\text{C} & \quad \text{C} \\
\text{C}^* \text{OOH} & \quad \text{C}^* \text{OOH}
\end{align*}

\begin{align*}
\text{HO-} & \quad \text{C-} \quad \text{COOH} \quad \rightarrow \quad \text{HO-} \quad \text{C-} \quad \text{COOH} \quad \rightarrow \quad \text{HO-} \quad \text{C-} \quad \text{COOH} \quad \rightarrow \\
\text{C} & \quad \text{C} \quad \text{C}^* \text{OOH} & \quad \text{C}^* \text{OOH}
\end{align*}

\begin{align*}
\text{COOH} & \quad \text{COOH} \quad \rightarrow \quad \text{CO}_2 \quad \rightarrow \quad \text{COOH} \\
\text{C}^* \text{OOH} & \quad \text{C}^* \text{OOH}
\end{align*}

\begin{align*}
\text{OXALOSUCCINIC} & \quad \text{ALPHA-KETOGLUTARIC} \\
\text{C} & \quad \text{C} \quad \text{C}^* \text{OOH}
\end{align*}

\begin{align*}
\text{C}^* \text{OOH} & \quad \text{SUCCINIC}
\end{align*}
APPENDIX 4

THE DIRECT RELATION OF TCA CYCLE INTERMEDIATES TO SOME METABOLITES OF INTEREST
APPENDIX 4

THE DIRECT RELATION OF TCA CYCLE INTERMEDIATES TO SOME METABOLITES OF INTEREST

\[
\begin{align*}
\text{PYRUVIC} & \quad \text{OXALOACETIC} \\
\text{ACETIC} & \\
\text{ASPARTIC} & \quad \text{GLUTAMIC} \\
\text{GLUTAMINE} & \\
\end{align*}
\]
BIBLIOGRAPHY


X. ABSTRACT

The purpose of this investigation was to study the metabolism of normal human leukocytes. Leukocytes were incubated in vitro with $^{14}C$-labeled acetate, and the fate of the radioactive carbon was determined by fractionation and analysis of the major, cell components.

Leukocytes were obtained from whole blood by fibrinogen sedimentation and differential centrifugation. Optimal conditions for isolation and incubation of viable cells were developed and assessed using phase microscopy for direct observation of their morphological integrity and by their oxidative metabolism. Control experiments to determine the possible effect of erythrocyte utilization of acetate were run using twice the number of cells ordinarily found in leukocyte suspensions. No utilization of acetate by red blood cells was observed.

Respiration studies were performed using standard Warburg manometry. Otherwise, incubations were carried out in modified Erlenmeyer flasks equipped with center wells for $CO_2$ collection and stoppered with serum bottle caps. Flasks were equilibrated and various additions were made to the suspensions using hypodermic needles. Total cells per flask varied from $1$ to $5 \times 10^8$ with concentrations ranging from $60$ to $80 \times 10^6$ cells per ml. of suspension. Response
of respiration and combustion of acetate were directly proportional to cell number and no detrimental effects due to cell crowding were detectable within this range.

All C\textsuperscript{14}-containing samples were counted in a gas flow proportional counter and corrected for self-absorption. Respiratory CO\textsubscript{2} was collected at the termination of incubations and counted as BaCO\textsubscript{3}. Hydrolysed glycogen and glucose were counted as the osazones while lipide and protein were counted as such. Other water-soluble compounds were converted to CO\textsubscript{2} by persulfate oxidation and counted as BaCO\textsubscript{3}. Glucose was determined colorimetrically by the ferricyanide method, acetate by the lanthanum method and amino acids with ninhydrin. Glycogen was isolated by KOH digestion, ethanol precipitation and TCA purification. Glucose was isolated from Somogyi filtrates. Protein was precipitated with perchloric acid and purified by extraction with ethanol-ether. Lipides were obtained from dehydrated samples by ethanol-ether extraction. Direct isolation of fatty acids was performed by sodium ethylate saponification followed by direct extraction. Homogenates of leukocytes were prepared in a standard motor-driven unit with a teflon pestle.

Cells were shown to utilize approximately 0.5 μmoles of acetate per 10\textsuperscript{8} cells per hour. At three hours approximately two-thirds of the substrate was in the form of CO\textsubscript{2} and one-third was present as non-volatile compounds.
Of the latter, amino acids, mainly glutamic and aspartic, accounted for most of the activity. The activity in protein and lipide amounted to approximately 0.4 per cent of the acetate utilized. Of the lipide activity 80 per cent was in the long-chain fatty acids.

Homogenates of leukocytes were found to incorporate acetate carbon into long-chain fatty acids. This cell-free system required CoA, DPN, citrate and a glycolytic intermediate such as glucose-6-phosphate or hexose diphosphate for maximal activity. No requirements for ATP or TPN could be demonstrated. Optimal incorporation was obtained under anaerobic conditions. Fluoride inhibited the incorporation. The complete fatty acid biosynthesizing system was found to reside in the particulate-free, soluble, supernatant fraction of the homogenate.

The metabolic pattern of intact leukocytes has been compared both qualitatively and quantitatively with other mammalian tissues.
BIOGRAPHY

Name: Edward J. Pastore

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Education:

B.S.  1950  University of Rhode Island, Kingston, R.I.
A.M.  1952  Boston University, Boston, Mass.
Ph.D. 1959  Boston University, Boston, Mass.

Societies and Awards:

Recipient of an Italian Government Fellowship for the study of biochemistry in Italy during the academic year 1954-55.

Sigma Xi
New York Academy of Science
American Association for the Advancement of Science

Positions:

1955-56  Assistant in Biochemistry, Boston University School of Medicine
1956-57  Junior Teaching Fellow in Biochemistry, same
1957-58  Junior Teaching Fellow in Biochemistry, same
1958-59  Senior Teaching Fellow in Biochemistry, same