The Efficiency of Oxidative Phosphorylation by Normal and Leukemic Human Leukocytes

By Virginia E. Davis, William L. Wilson and Charles L. Spurr

Numerous reports have been published on the glycolytic and respiratory metabolism of leukocytes. However, the literature is practically devoid of information relating to the efficiency of oxidative phosphorylations by leukocytes, although it has been suggested that these cells possess an active tricarboxylic acid cycle. The labile nature of the link between oxidation and phosphorylation, even under ideal conditions, is well known. A major impediment in the investigation of oxidative phosphorylation in leukocytes has been the lack of a method for the rapid isolation of sufficient quantities of viable, uninjured cells suitable for the study of the efficiency of aerobic phosphorylations.

Modifications of the technics of Skoog and Beck and Ellison provided a procedure for rapid separation of leukocytes from whole blood without deleterious effects to the oxidative phosphorylation system of anticoagulants, standing at room temperature, or high centrifugal forces. This technic for isolating leukocytes was employed in the study of the efficiency of oxidative phosphorylations by normal and leukemic leukocytes.

Materials and Methods

Source of Leukocytes

Data were obtained on 14 normal individuals whose leukocyte counts were between 6,000 and 11,000/cu.mm.; 6 patients with chronic myelocytic leukemia had counts ranging from 98,000 to 207,000/cu.mm.; 7 chronic lymphocytic leukemia patients had counts with a range of 80,000 to 492,000/cu.mm.; and 2 patients with acute monocytic leukemia had counts of 13,000 and 88,000/cu.mm. Most cases were studied repeatedly as indicated in the text.

Procedure for Isolation of Leukocytes

Ten ml. of fresh human blood collected in silicone-coated syringes were delivered into silicone-coated centrifuge tubes containing 0.3 ml. of 5 per cent Versene* solution and 5 ml. of 3 per cent dextran† solution. The dextran and Versene solutions were made up in 0.013 M tris** buffer pH 7.4 in isotonic sucrose. Versene was selected as the anticoagulant because it has been demonstrated that this compound removed calcium from heart-muscle sarcosomes and that calcium was responsible for the instability of the oxidative phosphorylation system.

The blood, Versene, and dextran were mixed by inverting the tubes 10 times and allowing them to stand at room temperature for 2 minutes. This permitted coating of

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* Ethylene-diamine-tetra-acetic acid.
† Supplied by Commercial Solvents Corporation, New York, N. Y., M. W. 650,000.
** Tris (hydroxymethyl) aminomethane, obtained from Sigma Chemical Co.
the red cells with dextran and facilitated more rapid sedimentation of the erythrocytes. The tubes were then placed in an ice bath and all further procedures were carried out at temperatures of 2 C. to 4 C.

After the blood, dextran, and Versene had been in the cold for 20 minutes, the red cells were usually well sedimented. If good sedimentation of the red cells had not occurred, the samples were centrifuged for 2 minutes at 500 r.p.m. in the refrigerated centrifuge before the leukocyte-rich plasma was aspirated.

To facilitate sedimentation of the white cells, the leukocyte-containing plasma from each 10 ml. volume of blood was diluted with 5 ml. of 0.013 M tris pH 7.4 in isotonic sucrose solution. The plasma and leukocyte mixture was then centrifuged at 2000 r.p.m. for 4 minutes in a refrigerated centrifuge.

The plasma was then aspirated from the sedimented leukocytes and 0.013 M tris pH 7.4 in isotonic sucrose added to adjust the leukocyte count of the suspension to approximately 300 million cells per ml. Cell counts on these suspensions were made in triplicate. The leukocytes suspended in tris-sucrose solution were then broken in an Omni-mixer. Counts of the remaining, intact leukocytes were again made on this suspension to determine, by difference, the number of cells broken.

In all experiments, broken cell preparations rather than intact cells were used because preliminary experiments demonstrated that the addition of selected tricarboxylic acid cycle-intermediates to reaction mixtures containing intact cells did not increase the rate of respiration above the endogenous level. Activity was found to remain linear with time and was also proportional to the amount of broken cells incubated. All data, therefore, are presented on the basis of broken cells.

Manometric Technic

The basic incubation mixture in the reaction vessels, modified from that used by Maley and Lardy, was as follows: main compartment, 0.03 μM of cytochrome c; 15 μM of MgCl₂; 6 μM of ATP*; 21 μM of substrate; 30 μM of KF; 1.0 ml. of leukocyte broken-cell preparation; 30 μM of malonate when α-ketoglutarate was used as a substrate; 3 μM of DPN and 100 μM of nicotinamide, when indicated; and 0.013 M tris buffer pH 7.4 to adjust the final volume to 3.0 ml. One side arm contained 1 mg. of purified hexokinase†; 50 μM glucose; and 50 μM of potassium phosphate. The other side arm contained 0.3 ml. of 50 per cent perchloric acid. The center well contained 0.2 ml. of 20 per cent KOH and a fluted filter paper. All flask components were made up in 0.013 M tris and adjusted to pH 7.4.

All flasks were kept in an ice bath until the leukocyte broken-cell preparations were added, and then immediately removed, placed on manometers, and transferred to the Warburg bath, kept at 30 C. An equilibration period of 3 minutes was routinely employed. The contents of the side arms which contained glucose were then added to the main compartment and equilibration allowed to proceed for 2 minutes. The stop-cocks were closed during this time. Zero time flasks were quickly deproteinized and initial readings were taken. Oxidation in the experimental flasks was allowed to proceed for 20 minutes. After the final readings, the flask contents were deproteinized. All zero time and experimental flasks were run in duplicate.

Analytical Procedures

The flask contents were analyzed for orthophosphate by the method of Lowry and Lopez after deproteinization with perchloric acid. Net phosphorus uptake was calculated as the difference in inorganic phosphorus content between the zero time flasks and the corresponding experimental flasks. From these data the phosphorus uptake values

*The following abbreviations are used: ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; DNP, 2,4-dinitrophenol; G-6-P, glucose 6-phosphate; and α-KG, alpha-ketoglutarate.
†Obtained from Sigma Chemical Company, St. Louis, Mo.
and the oxygen consumption during the same time interval were used to calculate the P:O ratios. Nitrogen was determined on the original broken-cell preparations by the standard micro-Kjeldahl method.

Glucose 6-phosphate formation was measured on aliquots of the Warburg reaction mixtures by a modification of the enzymatic assay of Seegmiller and Horecker. The reaction mixture for the assay of G-6-P consisted of 1.0 ml. 0.19 M tris buffer pH 8.0, 0.1 ml. 0.3 M MgCl₂, 0.2 ml. 0.005 M TPN, an aliquot of the Warburg reaction mixture, 0.1 ml. of freshly prepared G-6-P dehydrogenase* in 0.1 per cent sodium bicarbonate, and water to make a volume of 3.0 ml. This mixture was immediately transferred to a 1 cm. wide silica cuvette, and the optical density read at 340 mJ with a Beckman DU spectrophotometer against a reagent blank. The incubation was continued until the optical density remained constant with time. This constant optical density, corrected for any absorption due to G-6-P in the aliquot of the original Warburg reaction mixture, was used to calculate the amount of TPN which was reduced. A molecular extinction coefficient of 6.22x10⁻⁴ sq. cm./mole of reduced TPN was used. For each molecule of TPN which was reduced, one molecule of G-6-P was oxidized and, therefore, the amount of G-6-P present in the assay system could be readily calculated. Control experiments using purified G-6-P resulted in 97 per cent recovery of the added G-6-P and confirmed the specificity of the assay method.

RESULTS

Efficiency of Oxidative Phosphorylation by Normal and Leukemic Leukocytes

Data were collected to determine the rate and efficiency of aerobic phosphorylation by normal and leukemic leukocytes in the presence of selected Kreb's cycle intermediates. Average values for the level of oxygen and phosphorus uptake by normal and leukemic leukocytes with succinate and alpha-ketoglutarate as substrates in the presence and absence of DPN are given in table 1. These results indicate that leukemic leukocytes, but not normal leukocytes, were capable of oxidative-linked phosphorylations under the conditions used in these experiments. Determinations of total nitrogen content of normal, myelocytic leukemic, lymphocytic leukemic, and acute monocytic leukemic leukocytes gave average values of 293.4, 141.3, 79.6, and 146.3 mg. of nitrogen per 10¹⁰ cells, respectively.

Effect of Dinitrophenol on Oxidative Phosphorylation by Leukemic Leukocytes

Experiments were conducted to determine whether the uptake of oxygen and phosphorus by leukocytes takes place by mechanisms indistinguishable from those occurring in normal liver mitochondria. Sensitivity of these phosphorylations to low levels of dinitrophenol and their dependence upon substrate addition would exclude the possibility that glycolytic mechanisms were solely responsible for the decrease in inorganic phosphorus. Table 2 presents the summarized results of these experiments.

Effect of Iodoacetate on Oxidative Phosphorylation and Glucose-6-Phosphate Formation in Leukemic Leukocytes

Table 3 presents the summarized results of experiments designed to determine both the P:O ratio and G-6-P formation by leukemic leukocytes in the presence and absence of 0.001 M iodoacetate, a known inhibitor of

*Obtained from Sigma Chemical Company, St. Louis, Mo.
TABLE 1.—Comparison of normal and leukemic leukocyte aerobic phosphorylation efficiency in the presence of selected Krebs's substrates. Each flask contained in the main compartment 0.03 μM cytochrome c, 15 μM MgCl₂, 6 μM ATP, 21 μM substrate, 30 μM KF, 1.0 ml leukocyte preparation, 30 μM of malonate when α-ketoglutarate was used as substrate, 3 μM DNP and 100 μM nicotinamide when indicated, and 0.013 M tris buffer pH 7.4 to adjust the final volume to 3.0 ml. One side arm contained 1 mg purified hexokinase, 50 μM glucose, and 50 μM potassium phosphat. The other side arm contained 0.3 ml 50 per cent perchloric acid. The center well contained 0.2 ml 20 per cent KOH and a fluted filter paper. Incubated 20 minutes at 30°C in air. The numbers in parentheses indicate the total number of individual experiments. Average values total experiments in duplicate.

<table>
<thead>
<tr>
<th>Incubation System</th>
<th>Type of Leukocyte</th>
<th>Myelocytic Leukemic</th>
<th>Lymphocytic Leukemic</th>
<th>Acute Monocytic Leukemic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔO</td>
<td>ΔP</td>
<td>P:O</td>
<td>ΔO</td>
</tr>
<tr>
<td>Succinate Blank</td>
<td>0.96</td>
<td>0.00</td>
<td>0.00</td>
<td>0.76</td>
</tr>
<tr>
<td>Succinate</td>
<td>2.00</td>
<td>0.00</td>
<td>1.63</td>
<td>2.91</td>
</tr>
<tr>
<td>α-KG Blank</td>
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<td>0.00</td>
<td>0.47</td>
<td>0.00</td>
</tr>
<tr>
<td>α-KG only</td>
<td>1.37</td>
<td>0.00</td>
<td>0.68</td>
<td>1.56</td>
</tr>
<tr>
<td>DNP only</td>
<td>0.27</td>
<td>0.00</td>
<td>1.53</td>
<td>2.46</td>
</tr>
<tr>
<td>α-KG + DNP</td>
<td>1.39</td>
<td>0.00</td>
<td>1.63</td>
<td>3.13</td>
</tr>
</tbody>
</table>

TABLE 2.—Effect of dinitrophenol (DNP) on oxidative phosphorylations by leukemic leukocytes. Flask contents and experimental conditions the same as given in table 1. DNP added at final molarity of 1 x 10⁻⁴. Data expressed as μ atoms O/mg N/hr. and μM Pi/mg N/hr. Average values of three experiments in duplicate.

<table>
<thead>
<tr>
<th>Incubation System</th>
<th>Type of Leukocyte</th>
<th>Myelocytic Leukemic</th>
<th>Lymphocytic Leukemic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔO</td>
<td>ΔP</td>
<td>P:O</td>
</tr>
<tr>
<td>Succinate Blank</td>
<td>0.27</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>DNP only</td>
<td>1.37</td>
<td>0.00</td>
<td>0.68</td>
</tr>
<tr>
<td>Succinate only</td>
<td>1.06</td>
<td>2.04</td>
<td>1.92</td>
</tr>
<tr>
<td>α-KG + DNP</td>
<td>0.98</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

glycolysis. Alpha-ketoglutarate was used as the substrate and the other components of the reaction mixtures were the same as used in the standard reaction mixture for the measurement of oxidative phosphorylation. Iodoacetate at a level of 0.001 M was used in flasks where glycolysis was inhibited. This
concentration of iodoacetate has been shown to be a potent inhibitor of aerobic lactic acid production by leukocytes.\textsuperscript{14}

\textbf{In Vivo Effect of Nitrogen Mustard Therapy on Lymphocytic Leukemic Leukocyte Oxidative Phosphorylation}

Three patients with chronic lymphocytic leukemia, previously maintained on hydrocortisone therapy, were given 0.2 mg./Kg. nitrogen mustard intravenously. The effect of a single intravenous injection of nitrogen mustard on the oxidative phosphorylations mediated by leukocytes from these patients was tested before nitrogen mustard therapy and at 1 and 22 hours after nitrogen mustard was administered. These data are summarized in table 4.

Although nitrogen mustard therapy did not result in an uncoupling of the oxidative-linked phosphorylations mediated by the lymphocytic leukemic leukocytes, there was a marked decrease in the net oxygen and phosphorus uptake, and, therefore, the net yield of high energy phosphate was decreased 22 hours after administration of nitrogen mustard with all substrates tested.

\textbf{DISCUSSION}

The tissue reference unit most commonly used for expression of data on leukocyte metabolism has been the unit cell basis. However, as the data presented in table 1 indicates, the unit cell basis may not be the ideal reference unit for expression of leukocyte metabolic data. Data obtained in this labora-

\textbf{Table 3.} Effect of iodoacetate and dinitrophenol on oxidative phosphorylation by leukemic leukocytes and \textit{db}a mouse liver mitochondria. Flask contents and experimental conditions the same as given in table 1. Data expressed as \( \mu \text{ atoms/mg. N/hr.}, \mu \text{ M P/mg. N/hr.}, \) and \( \mu \text{ M G-6-P/mg. N/hr.} \). Dinitrophenol was added at a final molarity of \( 1 \times 10^{-4} \) and iodoacetate at a final molarity of \( 1 \times 10^{-5} \). The values for chronic myelocytic leukemic leukocytes represent duplicate determinations on a single patient; the values for chronic lymphocytic leukemic leukocytes were obtained from duplicate determinations on two patients; and the values for mouse liver mitochondria are mean values from duplicate determinations of two separate experiments.

\begin{table}
\centering
\begin{tabular}{|l|l|c|c|c|c|}
\hline
\textbf{Tissue} & \textbf{Incubation System} & \textbf{Substrate} & \textbf{Additions} & \textbf{\( \Delta O \) \( \mu \text{ atoms/mg. N/hr.} \)} & \textbf{\( \Delta P \) \( \mu \text{ M P/mg. N/hr.} \)} & \textbf{\( \Delta G-6-P \) \( \mu \text{ M G-6-P/mg. N/hr.} \)} & \textbf{P-O} & \textbf{G-6-P:O} \\
\hline
\textbf{a-KG} & Chronic & & & -2.38 & -5.98 & +6.87 & 2.51 & 2.89 \\
\textbf{a-KG} & Lymphocytic & & + DNP & -3.26 & -0.57 & +1.46 & 0.18 & 0.45 \\
\textbf{a-KG} & Leukemic & & + Iodoacetate & -1.77 & -3.78 & +4.62 & 2.14 & 2.61 \\
\textbf{a-KG} & Leukocytes & & + DNP & -2.58 & -0.75 & +1.60 & 0.29 & 0.62 \\
\hline
\textbf{a-KG} & Chronic & & + DNP & -0.62 & -1.25 & +0.80 & 2.02 & 0.79 \\
\textbf{a-KG} & Myelocytic & & + Iodoacetate & -0.62 & -1.25 & +0.80 & 2.02 & 0.79 \\
\textbf{a-KG} & Leukemic & & + Iodoacetate & -0.50 & -0.34 & +0.38 & 0.68 & 0.76 \\
\hline
\textbf{db} & \textbf{Mouse} & \textbf{Succinate} & & -11.47 & -14.34 & +16.49 & 1.25 & 1.44 \\
\textbf{db} & \textbf{Liver} & \textbf{a-KG} & & -7.15 & -23.14 & +25.25 & 3.24 & 3.53 \\
\hline
\end{tabular}
\end{table}
TABLE 4.—In vivo effect of nitrogen mustard on oxidative phosphorylation by chronic lymphocytic leukemic leukocytes. Flask contents and experimental conditions the same as given in table 1. All data was calculated on basis of μ atoms O/10⁶ cells/hr. and μM P/10⁶ cells/hr. Average values of duplicate determinations on three patients.

<table>
<thead>
<tr>
<th>Time After Therapy</th>
<th>Substrate</th>
<th>Succinate</th>
<th>Alpha-Ketoglutarate</th>
<th>P:O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔO</td>
<td>ΔP</td>
<td>P:O</td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>300</td>
<td>607</td>
<td>2.02</td>
<td>248</td>
</tr>
<tr>
<td>1 Hour</td>
<td>280</td>
<td>521</td>
<td>1.86</td>
<td>186</td>
</tr>
<tr>
<td>22 Hours</td>
<td>186</td>
<td>386</td>
<td>2.08</td>
<td>140</td>
</tr>
</tbody>
</table>

The higher rate of oxygen and phosphorus uptake by lymphocytic leukemic leukocytes than by myelocytic leukemic leukocytes is emphasized when data are calculated per mg. of nitrogen because of the significantly smaller size of the lymphocytic leukemic leukocytes. Data on the morphologically obvious size differences of normal and leukemic leukocytes has been presented previously.

The high endogenous uptake of oxygen and phosphorus by myelocytic leukemic leukocytes under conditions optimal for the use of succinate as substrate suggests a comparatively higher endogenous succinate level in these leukocytes as compared with normal or other leukemic leukocytes. This conclusion is substantiated by the data obtained when the oxygen and phosphorus uptake is followed under conditions optimal for the determination of the efficiency of oxidative phosphorylation with alpha-ketoglutarate as substrate. As the data in table 1 demonstrate, the endogenous oxygen and phosphorus uptake by myelocytic leukemic leukocytes was at the same level as the other leukemic leukocytes when malonate was present in the reaction mixture.

The increased endogenous uptake of oxygen and phosphorus by myelocytic leukemic leukocytes in the presence of additional DPN in contrast with lymphocytic leukemic leukocytes, and the more marked stimulation of oxidative phosphorylation by myelocytic leukemic leukocytes by the addition of DPN to the reaction mixture, suggests the possibility that the DPN level of myelocytic leukemic leukocytes may be limited in the cells because of diffusion during isolation and homogenization. There is also the possibility of the existence of DPN-splitting enzymes associated with the leukocytes which could lower the effective level of DPN to limiting amounts in the isolated leukocytes. Pyridine nucleotide levels have been shown to be lower in chronic myelocytic leukemic leukocytes than in chronic lymphocytic leukemic leukocytes.

The lack of any demonstrable oxidative phosphorylation under the present experimental conditions by normal leukocytes, even with the less labile succinic oxidase system, in contrast with the aerobic phosphorylations mediated by leukemic leukocytes, is of particular interest in relation to the difficulties...
involved in obtaining adequate amounts of normal leukocytes for oxidative phosphorylation determinations. In agreement with the report of Skoog and Beck, leukocyte-clumping usually occurred during isolation of normal leukocytes. This difficulty did not present itself in the isolation of leukemic leukocytes. The clumping of normal leukocytes during isolation could exert a deleterious effect on the labile oxidative phosphorylation system.

The uncoupling of the endogenous phosphate uptake of myelocytic leukemic leukocytes in the absence of malonate by dinitrophenol (DNP) suggests that these particular leukemic cells have a higher endogenous level of succinate in contrast to normal and lymphocytic leukemic leukocytes. The phosphorylations mediated by leukemic leukocytes were extremely sensitive to the classic uncoupler of oxidative phosphorylation, dinitrophenol, and were dependent upon the addition of substrate. Dinitrophenol had little effect upon the oxygen consumption.

The response of leukemic leukocytes to the uncoupling action of dinitrophenol strongly suggests that these cells are capable of true oxidative phosphorylation as defined by accepted criteria. However, glycolysis is a possible side reaction which, if present, under conditions of oxidative phosphorylation could cause a high estimate of the P:O ratio, since it could lead to the formation of G-6-P without oxygen uptake. Although the aerobic and anaerobic glycolytic capabilities of leukocytes have been well established, the formation of G-6-P in stoichiometric amounts in the presence and absence of iodoacetate is additional evidence of the esterification of inorganic phosphate associated with the oxidation of selected tricarboxylic acid cycle intermediates by leukemic leukocytes.

**Summary**

1. A new modification of existing methods has been described for the separation of leukocytes from whole blood which provides a procedure for the rapid isolation of uninjured cells suitable for the study of oxidative phosphorylations.

2. This method has been employed in a study of the relative efficiency and yield of oxidative-linked phosphorylations mediated by normal and leukemic or immature leukocytes. The maximum aerobic phosphorylating capacity was exhibited by chronic lymphocytic leukemic leukocytes, followed in decreasing order of activity by acute monocytic leukemic leukocytes and chronic myelocytic leukemic leukocytes. Oxidative phosphorylation was not demonstrated with normal leukocytes.

3. Results of this study suggest that expression of leukocyte metabolic data on a unit nitrogen basis more accurately reflect the morphologically obvious size differences among the various leukocytes than presentation of data on a unit cell basis.

4. The aerobic phosphorylations mediated by leukemic leukocytes were found to be dependent upon substrate addition and were depressed by low levels of dinitrophenol. Under the experimental conditions employed in this study, glucose-6-phosphate was formed in stoichiometric amounts. These re-
Results indicate that leukemic leukocytes are capable of the aerobic esterification of inorganic phosphate accompanying the oxidation of selected Kreb's cycle intermediates.

**Summario in Interlingua**

1. Es describite un modification del existente methodos pro le separation de leucocytos ab sanguine integre. Illo provide un technica pro le rapide isolation de cellulas non-lesionate que es appropriate pro le studio de phosphorylationes oxydative.

2. Iste technica esseva usate in un studio del relative efficacia e rendimento de phosphorylationes oxydative mediate per leucocytos normal e leucemic. Le maximo del capacitate de phosphorylation aerobie esseva exhibite per leucocytos a chronic leucemia lymphocytic, sequite—in ordine descendente per leucocytos a acute leucemia monocytic e leucocytos a chronic leucemia myelocytic. In le caso de leucocytos normal, phosphorylation oxydative non esseva demonstrate.

3. Le resultatos de iste studio suggere que le expression de valores pertinente al metabolismo leucocytic reflecte plus accuratemente le morphologicamente obvie differentias del dimensiones del varie leucocytos si illo se refere al unitate de nitrogeno que si illo se refere al unitate cellular.

4. Esseva trovate que le phosphorylationes aerobie mediate per leucocytos leucemic dependeva del addition de substrato e esseva deprimite per basse nivellos de dinitrophenol. Sub le conditiones experimental usate in le presente studio, glucosa-6-phosphato esseva formate in quantitates stoicheiometric. Iste resultatos indica que leucocytos leucemic es capace de effectuar le esterification aerobie de phosphato inorganic accompaniante le oxydation de seligite intermediarios in le cyclo de Krebs.

**REFERENCES**


OXIDATIVE PHOSPHORYLATION BY HUMAN LEUKOCYTES

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