Studies on the Energy Metabolism of Human Leukocytes

1. Oxidative Phosphorylation by Human Leukocyte Mitochondria

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REPEATED INVESTIGATIONS on the nature of the carbohydrate metabolism of mammalian leukocytes have resulted in a large body of conflicting data. Until recently it was generally felt that leukocytes derived most of their energy from glycolysis. This conclusion was drawn because a high rate of aerobic glycolysis was usually observed in leukocytes and because their cellular ATP levels were much more sensitive to substances like iodoacetate, which inhibit glycolysis, than to those which inhibit respiration, such as cyanide. More recent studies, however, have demonstrated a well-defined Pasteur effect in leukocytes and have suggested that its absence in previous studies was not an inherent property but resulted from isolation and incubation procedures. Warburg, Gawehn, and Geissler went so far as to suggest that high aerobic glycolysis resulted from damage to the respiratory pathway during isolation. Whatever the mechanism, a cell which exhibits such a profound alteration in its metabolism is an interesting system to study. Recently developed concepts of metabolic control, together with new methods for studying control mechanisms, now provide new ways to approach the problem.

In those cells studied, respiration appears to control glycolysis through the effects of mitochondrial oxidative phosphorylation on the ATP/ADP ratio. Therefore, the ability to demonstrate a Pasteur effect in leukocytes implies that their respiratory activity under physiologic conditions must be quantitatively comparable to their glycolytic rate. Leukocytes are believed to have a functional citric acid cycle, although only a few of the enzymes have actually been demonstrated. Moreover, Chance has shown that leukocytes contain a normal pattern of respiratory carriers. However, mitochondrial oxidative phosphorylation has not yet been demonstrated in leukocytes from normal blood. Some years ago, Davis, Wilson, and Spurr were able to demonstrate a dinitrophenol-sensitive uptake of orthophosphate by homogenates of leukemic leukocytes, but not of normal ones. They attributed the latter to low yields of material and to extreme lability of the phosphorylation systems in normal...
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leukocytes. Since then many improvements in procedures for isolating mitochondria have taken place, and highly sensitive specific methods for measuring ATP have been developed. Recently we have developed a polarographic method suitable for measuring the very low oxygen consumption of leukocyte preparations.16 As will be described in this paper, these new methods now make it possible to demonstrate oxidative phosphorylation by cell-free preparations from normal human leukocytes.

MATERIALS AND METHODS

A leukocyte-rich suspension was first prepared by drawing blood (usually 100 ml.) from a healthy donor directly through plastic tubing into a siliconized bottle. A solution containing heparin* and dextran† in normal saline was added as the blood was being collected to give final concentrations of 8 U./ml. and 0.01 Gm./ml., respectively. By adding the heparin-dextran solution in small increments, these concentrations were approximately maintained throughout the collection process, which took 3 to 5 minutes. The blood was then placed in the cold room at 4 C. and allowed to chill slowly while settling for 30 minutes. All succeeding steps were carried out at 4 C. The supernatant plasma was drawn off with a plastic pipette, placed in conical centrifuge tubes, and centrifuged gently (100 g for 10 min.) to recover the cells. The cell pellets were then resuspended for homogenization, combined, and counted. Microscopic examination of the suspension showed approximately equal numbers of leukocytes and erythrocytes and a variable number of platelets. Differential counts of the leukocytes showed no change in the proportions of cell types from the original blood sample. The erythrocytes and platelets subsequently vanished during homogenization.

Satisfactory homogenization of leukocytes requires special procedures. Despite their reputation for fragility, leukocytes require prolonged homogenization with conventional tissue grinders.17 More drastic procedures, in addition to possibly damaging the mitochondria, release large amounts of free nuclear material, so that the resulting homogenate has a strong tendency to agglutinate. This seriously interferes with subsequent fractionation procedures. Cohn and Hirsch17 found that vigorous pipetting in 0.34 M sucrose would readily rupture polymorphonuclear leukocytes from guinea pig intraperitoneal exudates. However, they found the method did not work with rat eosinophils, and we have found it did not work with human leukocytes. The following procedure was therefore adopted to preserve the nuclei and to minimize the length of time a given cell was subjected to mechanical trauma: The cell pellet obtained after sedimentation with dextran was suspended in 5 ml. of a medium containing 0.25 M sucrose, 1 mM EDTA, 1 per cent serum albumin, and adjusted to pH 7.2. The suspension was passed quickly through a small pressure homogenizer, similar to the one described by Davoren and Sutherland.18 Under the phase microscope the resulting homogenate showed large numbers of intact nuclei, or the lobes of polymorphonuclear nuclei, together with free granules and mitochondria. The nuclei can also be preserved by adding Mg or Ca ions to the medium, but the use of serum albumin gave better results in subsequent experiments. The procedure also ruptures the erythrocytes, eliminating the need for any further steps to remove them. The homogenate was then centrifuged for 10 minutes at 1000 g to remove the nuclei, after which the granules and mitochondria were sedimented by centrifuging for 15 minutes at 8500 g. The pellet was washed once with fresh homogenizing medium and resuspended in 0.2-0.5 ml. medium for use. Respiration was measured polarographically, using an apparatus designed for measuring very low respiration rates.16 Adenine and pyridine nucleotides were assayed in neutralized perchloric acid filtrates by fluorimetric procedures,19,20 using a Zeiss spectrophotometer equipped with fluorescence attachment, accessory amplifier, and recorder.

*“Panhepin”, Abbott Laboratories, N. Chicago, Ill.
†Pharmachem Corp., Bethlehem, Pa. Mol. wt. 227,000.
Crystalline enzymes, coenzymes, and substrates were purchased from C. F. Boehringer and Son, P-L Biochemicals, or Sigma Chemical Co.

RESULTS AND DISCUSSION

In previous work on the effect of citric acid cycle substrates, McKinney et al. found most of them increased the respiration of leukocyte homogenates by less than 20 per cent, whereas succinate doubled the rate. The behavior of our preparations was similar. Nearly all substrates had some effect on respiration, but a really pronounced effect was limited to only three. Typical respirometer tracings are shown in Figure 1. A and B. Succinate showed the largest response, in some cases as much as 20 times the endogenous rate, followed by \( \alpha \)-glycerophosphate and malate. Rapid oxidation of malate required supplementation with NAD. Fluorimetric assays of several samples showed that their endogenous pyridine nucleotide levels were extremely low. Whether or not this is an inherent property of mitochondria from leukocytes, similar to that observed in tumor mitochondria by Wenner and Weinhouse, remains to be determined. In any event, because of their ability to promote rapid respiration, succinate, malate, and \( \alpha \)-glycerophosphate were used as substrates for further experiments.

The effect of inhibitors of electron transport is shown in Figures 1C and D. The oxidation of succinate was inhibited by malonate (Fig. 1C). Respiration could then be restored by adding the NAD-linked substrate malate. In Figure 1D the experiment was done the other way around by inhibiting malate oxidation with rotenone or amytal, and then reversing the inhibition with succinate. The oxidation of all substrates tested was inhibited by antimycin. A. Such results are typical of what would be expected of a conventional type of mitochondrial electron transport system. In addition, all the traces in Figure 1 show a considerable endogenous respiration, which nearly always occurred as soon as a sample was injected into the respirometer chamber. The initial rate then usually declined to a more or less constant value, and it was customary to wait until this rate had been established before adding substrates or inhibitors. Occasionally, a completely fresh preparation had very low endogenous respiration, but the respiration rate would subsequently increase steadily during storage in the ice bath. The nature of this endogenous respiration is not known. Zatti and Rossi have suggested that part of the oxygen uptake of leukocytes does not involve the electron transport system. Evans reached a similar conclusion on the basis of spectrophotometric data. In Figure 2A are shown the effects of inhibitors active at various points along the electron transport chain on the endogenous respiration of our mitochondrial preparations. The only one which had any effect was cyanide, which is not specific for electron transport, since it will inhibit other metal-containing oxidases besides cytochrome oxidase. These results support the above conclusion that the endogenous respiration is not electron transport-linked.

Incubation of leukocyte mitochondrial preparations with ADP and orthophosphate leads to the synthesis of ATP. For these experiments it was necessary to add 10 mM fluoride to inhibit appreciable ATPase activity. Much
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Fig. 1.—Oxygen electrode traces showing the effects of various substrates and inhibitors on the respiration of mitochondrial preparations isolated from human leukocytes. The reaction mixture contained, in a volume of 0.32 ml.: 85 mM Na⁺, 60 mM K⁺, 110 mM Cl⁻, 5 mM Mg²⁺, 5 mM F⁻, 25 mM Tris, and 10 mM P₄, pH 7.4. All samples except those in B also contained 1 mM NAD. Incubation temperature, 37 C. For each trace, 0.1 ml. of sample, containing particles from ca. 10⁸ leukocytes, was brought to temperature by warming 1 min. in a small test tube in the water bath. It was then drawn into a small syringe and injected into the respirometer chamber at the point indicated ("part."). The preincubation did not usually permit the previously cold sample to equilibrate completely with the air. The sample therefore contained a slightly higher oxygen concentration than the medium, and produced an upward deflection of the trace when injected. Longer preincubation generally led to loss of activity.

Fig. 2A.—Effects of inhibitors of electron transport on the endogenous respiration of leukocyte mitochondrial preparations. Conditions were the same as in Fig. 1.

Fig. 2B.—Effects of oligomycin and dinitrophenol (DNP).

of the latter is undoubtedly due to the presence of cytoplasmic granules, which contain a variety of hydrolytic enzymes.²² Typical data, showing the effect of several substrates, are given in Table 1. It is evident that appreciable stimulation occurred again only in the presence of succinate, malate, or α-glycerophosphate. No significant stimulation of ATP synthesis could be seen with any
Table 1.—Production of ATP by Mitochondrial Preparations from Human Leukocytes in the Presence of Various Substrates

<table>
<thead>
<tr>
<th>Substrate (10 mM)</th>
<th>ATP Formed (μmoles/20 min./10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>63</td>
</tr>
<tr>
<td>Succinate</td>
<td>123</td>
</tr>
<tr>
<td>Malate</td>
<td>104</td>
</tr>
<tr>
<td>α-glycerolphosphate</td>
<td>119</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>50</td>
</tr>
<tr>
<td>Pyruvate ± 1 mM malate</td>
<td>77</td>
</tr>
<tr>
<td>β-OH butyrate</td>
<td>58</td>
</tr>
<tr>
<td>a-ketoglutarate + 1 mM malonate</td>
<td>59</td>
</tr>
</tbody>
</table>

Samples containing mitochondria from ca. 10⁶ cells were incubated in a total volume of 0.8 ml in small test tubes for 20 min. at 37°C, then deproteinized with perchloric acid and analyzed for ATP. A sample was also deproteinized at zero time to correct for endogenous ATP. Values are averages of duplicate or triplicate analyses.

other substrate. The extra ATP synthesis induced by the three substrates responded to inhibitors in a manner typical of oxidative phosphorylation. As shown in Table 2, dinitrophenol, which uncouples respiration and phosphorylation, and oligomycin, which inhibits mitochondrial phosphorylating enzymes, reduced the phosphorylation by leukocyte preparations to the endogenous level. The remaining one-third of the ATP synthesis, on the other hand, was unaffected by either substance; nor was it affected by cyanide, as shown in the last column of Table 2. It thus appears that the endogenous ATP synthesis, as is the case with the endogenous oxygen uptake, is not linked to electron transport. The nature of this endogenous activity is also not known. The presence of fluoride in the incubation medium makes it unlikely that glycolytic enzymes are responsible, as does the fact that iodoacetate had no effect on the results. Another possibility is contamination with nuclear material, since our preparations still show a strong tendency to agglutinate when centrifuged. Nuclei have been shown by several workers to possess phosphorylative activity whose response to inhibitors is different from mitochondria. However, incubation of our material with DNAase either before or after fractionation had no effect on the amount of endogenous ATP formed. We also attempted to fractionate homogenates by sucrose density gradient centrifugation, but no separation of granules and mitochondria could be obtained.

The presence of persistent endogenous respiration and phosphorylation has made it difficult to assess directly the physiological state of the mitochondria isolated by our procedure. In particular, it is of interest to know whether the fact that they responded to only three substrates was the result of damage, leading to loss of soluble enzymes, or resulted from restricted permeability of intact mitochondria. We have, however, obtained indirect evidence that the mitochondria are intact. Rat liver was pulped by passing through a wire screen, then homogenized and fractionated by our procedure. The mitochondria so obtained showed good respiratory control and the expected P/O ratios with several substrates. One can also calculate P/O ratios for the leukocyte mitochondria by assuming that, since the endogenous respiration and phosphorylation appear to involve other pathways than the electron transport
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Table 2.—Effects of Inhibitors on ATP Production by Mitochondrial Preparations from Human Leukocytes

<table>
<thead>
<tr>
<th>Substrate (10 mM)</th>
<th>ATP formed (μmoles/20 min./10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control*</td>
</tr>
<tr>
<td>None</td>
<td>30</td>
</tr>
<tr>
<td>Succinate</td>
<td>114</td>
</tr>
<tr>
<td>Malate</td>
<td>75</td>
</tr>
<tr>
<td>α-glycerophosphate</td>
<td>131</td>
</tr>
</tbody>
</table>

Conditions were the same as in Table 1.
*Because absolute values obtained from different preparations varied considerably, data from individual representative experiments are given.
† 2,4-dinitrophenol.

system, these values may be subtracted from the data. This leads to P/O ratios of 1.8, 2.6, and 0.6 for succinate, malate, and α-glycerophosphate, respectively, and indicates that phosphorylation in the presence of these substrates proceeds with reasonable efficiency.

Another criterion of mitochondrial integrity is respiratory control. This could not be demonstrated directly with leukocyte mitochondria because the samples always contained appreciable amounts of adenine nucleotides. Therefore, conditions could not be found in which the respiration would depend upon adding ADP to the medium. However, the data in Figure 2B suggest that leukocyte mitochondria do exhibit respiratory control. In this particular experiment succinate was used as the substrate, causing a threefold increase in respiration. Oligomycin, which is a specific inhibitor of the phosphorylating enzymes, brought the respiration back to the endogenous rate. Dinitrophenol, which is believed to act between the oligomycin-sensitive site and the electron transport chain, then restored respiration to 70 per cent of its value before adding oligomycin. This is consistent with a respiratory pathway, which is closely coupled to the phosphorylating enzymes and therefore dependent on their activity. The response to a limited number of substrates thus appears to result from the restricted permeability of intact mitochondria, which is well-known in mitochondria from other tissues.

It would also explain why NADH, to which intact mitochondria are impermeable, is not rapidly oxidized while an NAD-linked substrate like malate is.

Various writers have remarked on the sparse numbers of mitochondria in leukocytes, implying that this may have something to do with their high glycolytic rate. However, the data presented here show that these mitochondria are capable of oxidative phosphorylation. Moreover, under those experimental conditions which most closely approach physiological (i.e., when serum is used as the incubation medium) aerobic conditions substantially reduce the glycolytic rate. It is therefore reasonable to conclude that in the intact, circulating leukocyte, as is true with most mammalian cells, the electron transport system plays a major role in providing energy for the cell.

**Summary**

1. Oxidative phosphorylation has been studied in mitochondrial preparations
from human leukocytes, using recently developed methods for homogenization, measuring respiration, and assaying for ATP.

2. Appreciable stimulation of both respiration and phosphorylation was limited to 3 substrates: succinate, malate, and α-glycerophosphate. The effects of other substrates were minimal.

3. The stimulating effects of these 3 substrates responded to inhibitors in a manner typical of mitochondrial oxidative phosphorylation. There was also considerable endogenous activity which, however, was insensitive to inhibitors. It is concluded the endogenous respiration and phosphorylation are not associated with electron transport. Subtracting their values from the data, P/O ratios consistent with good phosphorylation with the 3 substrates are obtained.

4. Studies with oligomycin and dinitrophenol suggest the presence of respiratory control. This indicates the mitochondria are intact. It is concluded that in the intact leukocyte the mitochondria are a major source of ATP.

SUMMARIO IN INTERLINGUA

1. Phosphorylation oxydative esseva studiate in preparatos mitochondrial ab leucocytos human, con le uso de recentemente disveloppate methodos de homogenisation pro mesurar respiration e pro essary triphosphato de adenosina.


3. Le effectos stimulatori del tres mentionate substratos respondeva a inhibitores in tin maniera typic (IC mitoehondrial phosphorylation oxydative. Esseva etiam notate un considerable activitate endogene le qual, tamen, non esseva sensibile pro inhibitores. Es conclusionate que respiration e phosphorylation endogene non es associate con transporto a electrones. Per subtraher lor valores ab le datos, proportiones de P/O es obtenite que es ben de acordo con bon phosphorylation con le tres substratos.

4. Essayos con oligomycina e dinitrophenol suggestiona le presentia de un regulation respiratori. Isto indica que le mitochondrios es intacte. Es conclusionate que in le intacte leucocyto, le mitochondrios es un major fonte de triphosphato de adenosina.

REFERENCES


Studies on the Energy Metabolism of Human Leukocytes: I. Oxidative Phosphorylation by Human Leukocyte Mitochondria

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