

# Enzymatic Basis for Bioenergetic Differences of Alveolar Versus Peritoneal Macrophages and Enzyme Regulation by Molecular O<sub>2</sub>

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**ABSTRACT** Alveolar macrophages (AM) and peritoneal macrophages (PM) originate from common precursor cells, but function in different O<sub>2</sub> environments. In the present studies, the impact of different O<sub>2</sub> tensions on cell metabolism has been quantitatively determined, an enzymatic basis for these differences established, and a mechanism which regulates enzymatic differences demonstrated.

O<sub>2</sub> consumption and lactate production were compared in rabbit AM and PM in air and nitrogen. In air, AM demonstrate significantly greater O<sub>2</sub> utilization. In nitrogen, (where glycolysis is the major source of energy provision) lactate production is two- to threefold greater in the PM.

A comparison of several enzymes of energy metabolism in AM and PM indicate that one basis for the differences in cell energetics is a difference in activity of key enzymes of both the oxidative phosphorylative and the glycolytic sequences.

Exposure of cultivated AM to hypoxic conditions results in changes in the activity of these enzymes such that the AM closely resembles the PM. A key enzyme in oxidative phosphorylation (cytochrome oxidase) shows decreased activity and reaches values similar to those found in the PM. A key enzyme in glycolysis (pyruvate kinase) shows increased activity to values resembling those found in the PM. These alterations in enzyme pattern occur in isolated cell systems, suggesting that molecular O<sub>2</sub> modifies the

intrinsic cellular regulation of some enzymes of energy metabolism.

Alterations in O<sub>2</sub> tension may lead to alterations of the rate of biosynthesis and (or) the rate of biodegradation of key enzymes involved in oxidative phosphorylation and glycolysis. In turn, the alteration of enzyme patterns leads to a more suitable bioenergetic pattern as a function of O<sub>2</sub> availability.

## INTRODUCTION

Peritoneal macrophages (PM)<sup>1</sup> and substantial numbers of alveolar macrophages (AM) originate from common precursor cells in the bone marrow (1-3) and share common functional characteristics (4, 5). One major difference is that the AM develops and functions in a relatively aerobic environment (PO<sub>2</sub> ~ 100 torr), whereas the PM develops and functions in a relatively anaerobic environment (PO<sub>2</sub> ~ 5 torr). Comparison of various metabolic functions in the two cell types thus provides an excellent model for analyzing the impact of different O<sub>2</sub> tensions on various aspects of cell function.

Oren et al. demonstrated that under resting conditions, AM had relatively high O<sub>2</sub> consumption as compared to PM (6). Dannenberg et al. showed, by histochemical techniques, higher succinic dehydrogenase and cytochrome oxidase activities in AM than PM (7). Ultrastructural studies have shown the AM to have greater numbers of mitochondria and increased density of cristae than the PM (8). Depression of respiration by glucose (Crabtree effect) is seen

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<sup>1</sup> Abbreviations used in this paper: AM, alveolar macrophage(s); Cy Ox, cytochrome oxidase; PFK, phosphofructokinase; PM, peritoneal macrophage(s); Py Ki, pyruvate kinase; QO<sub>2</sub>, oxygen consumption; SOD, superoxide dismutase.

only in PM (6). Phagocytosis in the PM appears to be independent of aerobic energy provision (9). The present studies extend these comparative observations. The amount of O<sub>2</sub> utilized by the mitochondrial electron transport chain (which determines O<sub>2</sub>-dependent energy provision) has been measured in the two cell types.

The rate of lactate production under aerobic and anaerobic conditions has been determined, thus providing data on glycolytic energy provision.

The activities of two key glycolytic enzymes, pyruvate kinase (Py Ki) and phosphofructokinase (PFK), and of cytochrome oxidase (Cy Ox), the terminal electron transport chain enzyme, were also compared to investigate a possible enzymatic basis for the differences seen in AM and PM bioenergetics. In addition Py Ki and Cy Ox activities have been determined in AM cultivated under aerobic and anaerobic conditions to examine the regulatory mechanisms underlying the observed enzymatic differences.

## METHODS

**Animals.** Healthy adult New Zealand White rabbits were used in the rabbit macrophage studies. Swiss Webster mice (20–30 g) of the NCS/PA strain were used in all mouse macrophage studies. This strain was derived from the NCS (pathogen-free) mice of the Rockefeller University, New York.

### *Studies on rabbit macrophages*

**Cell isolation.** AM were obtained by lung lavage according to the method of Myrvik et al. (10). Rabbit PM were harvested 72 h after the intraperitoneal instillation of 50 ml of mineral oil by previously described techniques (11). More than 95% of the cells obtained by these techniques are macrophages as judged by light microscopy.

**Estimate of total and mitochondrial rate of O<sub>2</sub> utilization.** A packed cell volume of 0.1 ml was suspended in 3 ml of "Rabbit Ringers" solution (NaCl, 137 mM; KCl, 2.66 mM; NaHCO<sub>3</sub>, 11.9 mM; NaH<sub>2</sub>PO<sub>4</sub>, 0.36 mM; MgCl<sub>2</sub> · 6H<sub>2</sub>O, 0.48 mM; CaCl<sub>2</sub> · 6H<sub>2</sub>O, 0.45 mM; and 10 mM glucose, pH 7.4) and this cell suspension was used to measure total O<sub>2</sub> consumption in the YSI 53 O<sub>2</sub> monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio). O<sub>2</sub> consumption was measured before and after the addition of 50 μg/ml antimycin A (an inhibitor of cytochrome *b*). Mitochondrial oxygen consumption (QO<sub>2</sub>) was calculated as follows: Mitochondrial electron transport chain QO<sub>2</sub> = Total QO<sub>2</sub> - antimycin A insensitive QO<sub>2</sub>. Total protein concentration was determined on an aliquot of the sample so that QO<sub>2</sub> in micromoles per milligram of cell protein per hour could be calculated.

**Estimate of aerobic and anaerobic rate of lactate production.** Rabbit AM and PM were harvested and washed twice in "Rabbit Ringers" solution. A cell pellet was obtained by centrifugation at 700 g (24°C) and suspended in a 20-fold dilution of Tris-buffered glucose-free solution. A 3-ml aliquot of this cell suspension (containing approximately 50 × 10<sup>6</sup> cells/ml) was added to an equal volume of Ringers containing 20 mM glucose (giving a final glu-

cose concentration of 10 mM). The pH of the final suspension was adjusted to 7.8 to provide favorable conditions for glycolysis. The flasks were incubated under aerobic (room air) or anaerobic conditions at 37°C for 1 h. Anaerobic conditions, achieved by repeatedly flushing stoppered incubation flasks with 100% nitrogen, consistently resulted in a PO<sub>2</sub> of <15 torr. 0.5-ml samples were removed from the flasks at the start and after 60 min of incubation and immediately added to an equal volume of chilled 3.5% perchloric acid. Preliminary studies showed the rate of lactate production to be linear over 1 h and the difference between the starting and 60-min incubation lactate concentration was used to calculate the rate of lactate production. These samples were centrifuged at 1,000 g and the resultant supernate used for lactate analysis (12). Protein determinations were performed on a sample of the cell suspension and lactate production was expressed as micromoles of lactate produced per milligram of protein per hour.

**Enzyme assays.** Rabbit AM and PM obtained by lavage were maintained at 0–4°C while being centrifuged at 700 g for 5 min, washed twice in 5-ml portions of Rabbit Ringers solution, resuspended in a 20-fold dilution of 35 mM Tris buffer (pH 7.4), and disrupted by sonication (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) for 30 s at a power output of 70 W. These sonicates were used for the assay of Cy Ox, Py Ki, and PFK activities as described below. Total protein concentrations were determined on aliquots of the cell sonicate so that enzyme activity could be calculated in units per milligram of cell protein.

### *Studies on mouse macrophages*

**Cell isolation.** Mouse PM were harvested by lavage using the method of Cohn and Benson (13). Lung lavage in mice produced an inadequate cell yield and mouse AM were therefore harvested by lung mincing (6). Cell purity during the initial isolation was determined by the property of glass adherence and confirmed by the structural characteristics of cells on light and electron microscopy.<sup>2</sup> More than 98% of the cells obtained by this technique were (alveolar) macrophages.

Harvested cells were washed and suspended at a concentration of 3–5 × 10<sup>6</sup> cells/ml in medium 199 (Microbiological Associates, Inc., Bethesda, Md.) 30% newborn calf serum containing 100 U/ml penicillin and 20 μg/ml of gentamycin. 8-ml aliquots of cell suspension were dispersed in T30 flasks and incubated for 2 h in 95% air-5% CO<sub>2</sub>. Cy Ox and Py Ki analyses were performed on harvested aliquots of these freshly explanted cells. For studies on cultivated AM, cell monolayers were rinsed with warm medium 199 after the initial 2-h incubation to remove non-adherent cells and fresh culture medium was added. The AM monolayers were then cultivated for 96 h under either aerobic or anaerobic conditions. Fresh culture medium was added at 24 and 72 h. After the 96-h incubation period, the culture medium was removed, the flasks were rinsed three times with 5-ml portions of warm physiological saline (37°C), and the cells were harvested by scraping in phosphate-buffered saline. The harvested mouse AM and PM were centrifuged at 700 g for 5 min (2–4°C), washed twice with phosphate-buffered saline, suspended in 0.6 ml

<sup>2</sup> Sachs, D. P. L., K. Bensch, J. Theodore, and E. D. Robin. Ultrastructural alterations produced in alveolar macrophages by in vitro and in vivo hyperoxia. Manuscript in preparation.

35 mM Tris buffer (pH 7.4), and disrupted by sonication as described above. Py Ki, Cy Ox, and protein analyses were performed on these cell sonicates.

**Ambient oxygen tension.** Ambient oxygen tension within culture flasks could be regulated by flushing flasks with defined gas mixtures and maintained at stable levels for time periods up to 72 h by tightly stoppering the flasks. Aerobic culture conditions, achieved by flushing with a 95% air–5% CO<sub>2</sub> mixture resulted in a PO<sub>2</sub> in the medium of 140 torr (measured by O<sub>2</sub> electrode). Anaerobic conditions, achieved by similar treatment with a 95% N<sub>2</sub>–5% CO<sub>2</sub> mixture, resulted in a PO<sub>2</sub> in the medium of 10–15 torr.

**Enzyme assay.** Py Ki activity was assayed by a modification of the spectrophotometric method of Valentine and Tanaka (14). A 0.05-ml sample of cell sonicate was added to a 2.95-ml reaction mixture containing: 0.5 ml of 50 mM triethanolamine-HCl buffer (pH 7.5), 1.45 ml of distilled water, 0.1 ml of 2.25 M KCl, 0.1 ml of 240 mM MgSO<sub>4</sub>, 0.2 ml of 6 mM ADP, 0.1 ml of lactate dehydrogenase (18 enzyme units), 0.4 ml of 1.3 mM NADH, and 0.1 ml of 45 mM phosphoenolpyruvic acid (trisodium salt). NADH oxidation at 25°C was followed by measuring the decrease in OD at 340 nm in a model DB spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Enzyme activity was calculated during a period of zero order kinetics using a molar extinction coefficient of  $6.2 \times 10^3$  for NADH. Enzyme activity has been expressed as micromoles of phosphoenolpyruvate converted to pyruvate per minute per milligram of protein.

Cy Ox was measured spectrophotometrically by techniques previously described (15). A 0.1-ml sample of cell sonicate was added to 2.9 ml of reaction mixture containing: 0.3 ml of sodium deoxycholate, 2.5 ml of 13 mM phosphate buffer (pH 7.4), and 0.1 ml of a 3% solution of cytochrome *c* (reduced by sodium hydrosulfite). Oxidation of cytochrome *c* at 25°C was followed by measuring the decrease in optical density at 550 nm in a model DB spectrophotometer. Enzyme activity was calculated using a molar extinction of  $19.1 \times 10^3$  for cytochrome *c*. Enzyme activity has been expressed as micromoles of cytochrome *c* oxidized per minute per milligram of protein.

PFK was assayed by spectrophotometrically after the conversion of fructose-6-phosphate to fructose-1, 6-diphosphate (16).

An aliquot of diluted supernate was added to a reaction mixture containing 1.735 ml 0.1 M Tris-HCl, pH 8.2, 0.05 ml 0.1 M MgCl<sub>2</sub>, 0.2 ml 0.1 M Na<sub>2</sub>PO<sub>4</sub>, 0.175 ml "enzyme mix", 0.56 ml "substrate mix", and 0.27 ml NADH (1.4 μmol/ml). (Enzyme mix: 4.92 ml 0.1 M Tris-HCl, [pH 8.2]; 0.6 ml aldolase [10 mg/ml]; 0.24 ml α-glycerophosphate dehydrogenase [10 mg/ml]; 0.012 ml triose-phosphate isomerase [10 mg/ml]). (Substrate mix: 0.1 M fructose-6-phosphate; 0.1 M ATP).

NADH oxidation was followed spectrophotometrically at 25°C and enzyme activity calculated during a period of zero order kinetics. Enzyme activity was expressed as micromoles of fructose-6-phosphate converted to fructose-1, 6-diphosphate per minute per milligram of protein.

**Protein analysis.** Protein content was determined by the method of Lowry et al. (17) using crystalline human albumin as the standard.

**Cell counts.** Cell counts on control and 96-h AM cultures were performed in standard fashion using a hemocytometer.

## RESULTS

Table I summarizes the data with respect to energy metabolism in the two cell types during air and nitro-

TABLE I  
Total and Mitochondrial Electron Transport Chain O<sub>2</sub>  
Consumption and Lactate Production (under  
Aerobic and Anaerobic Conditions) in  
Alveolar Versus Peritoneal  
Macrophages

	AM	PM	P value
	μmol/mg protein/h		
Total $\dot{Q}_{O_2}$	0.96±0.25 (SD)	0.50±0.13	<0.01
Electron transport chain $\dot{Q}_{O_2}$	0.72±0.18	0.38±0.10	<0.01
$\dot{Q}$ lactate (air)	0.29±0.10	0.77±0.27	<0.01
$\dot{Q}$ lactate (N <sub>2</sub> )	0.50±0.13	1.38±0.35	<0.001

n = 6 for all studies.

gen exposure. The aerobic AM shows approximately twice the rate of basal O<sub>2</sub> consumption as the anaerobic PM. Antimycin A produces 75% depression of total O<sub>2</sub> utilization in both cell types so that the amount of O<sub>2</sub> used in the mitochondrial electron transport chain is also twice as high in the AM as compared with the PM. Conversely, the rate of lactate production in the PM is about three times that in the AM under both aerobic and anaerobic conditions. Both cell types show a significant Pasteur effect. However, the rate of lactate production in the AM even during N<sub>2</sub> exposure is significantly lower than in the PM exposed to room air.

Mitochondrial electron transport chain O<sub>2</sub> utilization is the major source of energy provision under aerobic conditions and the data indicates that the AM is capable of significantly greater aerobic energy provision.

Under anaerobic conditions energy provision is largely dependent on glycolysis. The greater anaerobic lactate generation in the PM indicates a greater capacity for anaerobic energy provision, and suggests that the AM would be more vulnerable to acute hypoxia than the PM. It is also clear that energy metabolism in each cell type is well matched to O<sub>2</sub> availability. The AM in an O<sub>2</sub> rich environment derives essentially all energy from the reduction of molecular O<sub>2</sub>. The PM in an O<sub>2</sub> poor environment derives a significant fraction of metabolic energy from a non-O<sub>2</sub>-dependent source.

Table II summarizes data with respect to Cy Ox, Py Ki, and PFK activities in rabbit AM and PM. The AM has significantly higher Cy Ox and significantly less Py Ki and PFK activity than the PM. We have shown that Cy Ox is an excellent quantitative index for oxidative phosphorylative capacity and that Py Ki is an accurate quantitative index for glycolytic capacity. PFK is known to be the major rate-limiting enzyme in glycolysis. Thus the differences in the

TABLE II  
Activities of Cy Ox, Py Ki, and PFK in Rabbit Alveolar Versus Peritoneal Macrophages

	AM	PM
	<i>U/mg protein</i>	
Cy Ox	5.1±1.5 (SD)	3.5±1.2
Py Ki	30.3±13	327±128
PFK	9.7±1.2	32.7±2.6

*n* = 8 for all studies except *n* = 6 for AM PFK studies.  
*P* < 0.001 for Py Ki and PFK; *P* < 0.01 for Cy Ox.

activities of all three enzymes are consistent with the bioenergetic differences in the two cell types.

Table III summarizes the data with respect to Py Ki and Cy Ox activity in freshly explanted mouse AM and PM and in mouse AM exposed to normal and low O<sub>2</sub> tensions for 96 h. The control values (freshly explanted cells) of Cy Ox are significantly higher and the control values of Py Ki significantly lower in the AM than in the PM. This is in accord with the data on enzyme activities in freshly harvested rabbit AM and PM. In vitro differentiation under aerobic conditions results in a twofold increase in Cy Ox activities with no significant change in Py Ki activities. After cultivation under anaerobic conditions however, Cy Ox activity decreases to a value which is not significantly different from that found in the control PM. Concomitantly Py Ki activities show a sixfold increase and are actually higher in the hypoxic AM than in the control PM. Thus, with exposure to low oxygen tensions, the activity of an enzyme in the oxidative phosphorylative pathway decreases to values seen in freshly explanted PM and the activity of an enzyme in the glycolytic pathway increases to values more characteristic of PM. Total cell counts after 96 h of aerobic and anaerobic cultivations were not significantly different. This indicates that the changes in enzyme activity did not occur as a result of selective survival of AM with high Py Ki and low Cy Ox activity, but must have occurred through intrinsic cell regulatory mechanisms. Presumably these alterations in enzyme activity in the anaerobically cultivated AM are associated with reductions in aerobic energy provision and increases in the capacity for anaerobic energy provision.

## DISCUSSION

There are important structural and functional differences between macrophages indigenous to different organs. The AM in particular, shows impressive differences from other macrophages (peritoneal macrophage, circulating monocytes) in ultrastructure, in bioenergetics, and the effect of various metabolic in-

hibitors. The present studies demonstrate a higher rate of oxygen utilization and a lower rate of aerobic and anaerobic glycolysis in the AM compared to the PM. While the in vivo rates for  $\dot{Q}O_2$  and  $\dot{Q}$  lactate may be different from the rates measured in vitro, measurement of these parameters under the same conditions allows an examination of relative differences and suggests: (a) a high rate of aerobic energy provision in the AM; (b) a greater capacity for anaerobic energy provision in the PM with a lesser susceptibility to the effects of acute hypoxia.

The differences in AM bioenergetics may be mediated at least partially by the differences in enzyme activities demonstrated in the two cell types. Py Ki and PFK represent rate-limiting glycolytic enzymes and the increased Py Ki and PFK activities in the PM presumably play a role in the enhanced glycolytic capacity seen in the PM (18–20).

Whether Cy Ox is connected with a rate-limiting step in mitochondrial electron transport or whether it represents an excellent marker for mitochondrial mass is not known (21). In either case, the increased Cy Ox in the AM provides increased capacity for oxidative phosphorylation. While it is true of multi-enzyme pathways that many factors in addition to enzyme content are involved in metabolic regulation, it may be inferred that the enzymatic differences observed in the two cell types play an important functional role in their bioenergetic differences.

The in vitro studies in which AM are cultivated under aerobic and anaerobic conditions provide insight into the mechanism by which the differences in AM and PM enzyme activity arise. The major finding of these studies concerns alterations of enzyme activities

TABLE III  
Changes in Cy Ox and Py Ki Activities in Aerobically and Anaerobically Cultivated Mouse AM as Compared to Freshly Explanted Mouse PM

	AM		PM	
	Cy Ox	Py Ki	Cy Ox	Py Ki
	<i>U/mg protein</i>			
Control (2 h)	21.4±2.3 (SD)	45.5±17	9.8±2.6§	158±46
Aerobic (96 h)	41.5±4.4*	59.5±23‡		
Anaerobic (96 h)	11.0±3.0*	299±65*		

*n* = 6 for all studies.

\* *P* < 0.01 as compared to control values.

‡ No significant difference as compared to control values.

§ No significant difference as compared to 96-h anaerobic value.

evoked by alterations of O<sub>2</sub> supply. Hypoxic cultivation of AM results in a decrease in Cy Ox activity and an increase in Py Ki activity. Moreover, the enzyme activities found with hypoxic cultivation are quantitatively similar to those found in the PM. From the standpoint of energy metabolism enzyme activity the AM is converted to a cell type closely resembling the PM.

Cell numbers under normal and hypoxic exposure were not significantly different. The alterations in Py Ki and Cy Ox thus appear to represent a specific change in cell enzyme activity.

Enzyme activity rather than enzyme content was measured. It is possible that the changes in activity could be related to changes in co-factors or low molecular weight regulators of Cy Ox and Py Ki. This seems unlikely since: (a) enzyme activities were measured under standard conditions by in vitro assay systems and (b) a series of complex changes would be required which both decreased Cy Ox activity and increased Py Ki activity. It is therefore probable that the changes in enzyme activity under aerobic and anaerobic conditions do reflect changes in enzyme content.

Changes in the ambient oxygen tension appear to alter enzyme activity by changing enzyme content. In the lung, high O<sub>2</sub> tensions act on precursor cells to produce high activities of oxidative phosphorylative enzymes and low activities of glycolytic enzymes. In the tissues (peritoneum) low O<sub>2</sub> tensions produce the opposite changes. The present studies do not, however, indicate whether these changes reflect alterations in enzyme synthesis or degradation (22).

The pathophysiologic implications of these observations concern AM function under conditions of reduced alveolar PO<sub>2</sub> seen in a wide variety of respiratory disease processes. Acute hypoxia will depress O<sub>2</sub> dependent energy provision in the AM and significantly decrease energy dependent AM function. (e.g., phagocytosis and pinocytosis). With more chronic hypoxia decreased molecular O<sub>2</sub> would alter AM energy metabolism enzymes resulting in a more favorable pattern for energy provision in the diseased lung.

Recent studies in our laboratory support the role of molecular O<sub>2</sub> as a regulator of enzyme biosynthesis and (or) biodegradation. Superoxide dismutase (SOD) is an enzyme(s) which acts to scavenge free radicals of O<sub>2</sub> (23). It is believed to represent an important protective mechanism against O<sub>2</sub>-induced free radical damage at a cellular level (24). We have shown that AM contain significantly more SOD activity than found in PM (25). AM cultivated under hypoxic conditions (PO<sub>2</sub> ~ 15 torr) demonstrate significantly less SOD activity than AM cultivated under conditions of normal oxygen tensions (PO<sub>2</sub> ~ 150 torr). Moreover,

exposure to a high oxygen environment (PO<sub>2</sub> ~ 640) results in significant increases in SOD (26).

Molecular O<sub>2</sub> is known to participate directly in an extensive series of biochemical reactions which do not involve energy transduction (27). We propose another general function. Molecular O<sub>2</sub> appears to regulate the biosynthesis and (or) biodegradation of a number of enzymes involved in tissue O<sub>2</sub> metabolism. In turn, this regulation leads to more optimal tissue utilization of O<sub>2</sub>.

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