Metabolic Crowding Effect in Suspension of Cultured Lymphocytes

By Theodore Sand, Richard Condie, and Andreas Rosenberg

We studied the respiration and glucose utilization of suspensions of cultured lymphocytes. Respiration was measured by a coulooximetric method using closed systems without a gas-suspension interface. The sensitivity of the method allowed measurements during a time interval of less than 1 hr. We found that the respiration of lymphocytes was strongly density dependent over the range of $10^3$–$10^7$ cells/ml. Oxygen utilization per cell increased 100-fold with 1000-fold dilution of the cell suspension. Glucose utilization showed little density dependence. We showed that the phenomenon was not due to nutrient depletion, pH shifts, accumulation of lactic acid, nor damage of cells during the dilution. Since in our experiments a gas–liquid interface was absent, the previously suggested explanation of gas diffusion as a limiting factor has been excluded. The experiment was best explained by regulation of respiration by humoral factors produced by the cell suspension.

We have defined the metabolic crowding effect as the dependence of rates of cell metabolism on cell density. If simple starvation or competition for nutrients is excluded, the phenomenon was reported as early as 1930.1 During the following years the effect was observed by some workers2 but not by others.3,4 The effect was clearly shown to be present for peripheral lymphocytes when cell respiration was followed by Warburg techniques.5,6 The effect was, on these occasions, tentatively ascribed to inadequacy of oxygen transport between the gas and liquid phases.

Recent experiments using the cartesian diver method7 did not detect any crowding effect at higher lymphocyte densities. Unfortunately, the cell density ranges used in the Warburg type of experiments and those of the cartesian diver methodology showed practically no overlap, leaving the question still unanswered as to whether the effect observed in the Warburg instrument was one of metabolic regulation by nearest neighbor interaction or a deprivation of nutrients at high cell concentration; the latter alternative being less interesting from the point of cell biology. The present investigation was designed to detect a possible effect under experimental conditions where a gas–liquid interface was absent and at cell densities where nutrient depletion played a minor role.

Using a cell suspension without a gas phase and utilizing short times of observation of 1 hr or less, we have investigated the respiration of cultured lymphocytes over the density range of $10^3$–$10^7$ cells/ml and the rate of glucose utilization in the range of $10^3$–$10^6$ cells/ml; and we have made calorimetric de-
terminations of heat evolved between $10^6$ and $10^7$ cells/ml. We observed a clear-cut dependence of the respiratory rate on cell density, in good agreement with the previously published Warburg results of Hedeskov and Esmann. The heat evolution correlated with respiration, whereas glucose utilization showed little density dependence. We showed experimentally that the results could not be explained by nutrient depletion, oxygen starvation, pH shifts, accumulation of lactic acid, nor cell damage during centrifugation. The results suggested that a regulatory mechanism which allowed for the sensing of the presence of nearest neighbors was an alternative to be considered.

**MATERIALS AND METHODS**

**Cells**

The choice of cultured lymphocyte lines as a cell model instead of peripheral lymphocytes was based on our desire to avoid metabolic contributions from other leukocytes as well as from the residual red cells. Separation of peripheral lymphocytes could be carried out satisfactorily, but since the lymphocyte was not the metabolically most active blood cell, contributions from cells like polymorphonuclear leukocytes would always diminish the accuracy of analysis. We used two commercially available cell lines from ABS Laboratories, Buffalo, N.Y. The cell line 4098 originated from human lymphocytes, whereas L1210 represented a mouse lymphoma. The cells were grown in spinner cultures using RPMI-1640 media containing 10% fetal calf serum, L-glutamine, and penicillin/streptomycin. Both cell lines showed logarithmic growth characteristics as exemplified in Fig. 1. The arrows in the figure indicate dilutions of the culture. No clumping of cells could be detected for these cell lines during their growth in culture and during our experiments.

**Preparation of Samples for Measurement of Respiratory Rate**

The lymphocytes were counted in a hemocytometer, and viability was determined by the standard method of trypan blue exclusion. Viability was above 94%, in all experiments. The cells were centrifuged for 10 min at 120 g in polystyrene tubes and resuspended to the desired concentration at 37°C in fresh media, the pH of which was adjusted to 7.5. Air was bubbled into the syringe assembly depicted in Fig. 2. There was a 4-5-min delay following the resuspension of the cells after centrifugation before the first sample could be analyzed. We then proceeded to measure the decrease in dissolved oxygen concentration during the first hour (or less) by forcing the plunger down, causing the fluid to move through the nichrome tube and into the sampling loop. The oxygen content was analyzed in a Hersch cell coulometer at 37°C as described in the next section.

The medium used for dilution (representing the solution in the syringe assembly) was minimum essential medium (MEM) containing 100 mg/dl glucose (Gibco, Grand Island, N.Y.). A number of dilutions over the entire cell range were carried out utilizing fresh RPMI-1640 (ABS) with serum instead of serum-free MEM. No differences in density dependence were observed. The first run every day was a duplicate determination at $10^6$ cells/ml. Then a number of runs at different cell concentrations were carried out (Hank’s solution was that of Gibco, Grand Island, N.Y.). The following modifications of the standard procedure were utilized.

![Fig. 1. Growth characteristics of cell line L1210. Samples from growing cell culture were counted at 6- and 12-hr intervals. Variations of growth rate and doubling time during the 160-hr culture not statistically significant.](image-url)
Fig. 2. Reaction vessel for the oxygen uptake studies with the sample transfer mechanism and the Hersch analyzer for dissolved oxygen. Mode of operation is described in the text.

Runs in 100% oxygen. Instead of air, pure oxygen was bubbled for at least 30 sec through the final dilution before closing the reaction vessel.

Runs in the presence of bicarbonate. The standard medium was enriched with $2 \times 10^{-3} M$ NaHCO$_3$, with the pH again adjusted to 7.5.

Runs in the presence of excess lactic acid. The medium was enriched with 1 mg lactic acid/ml. The pH was maintained at 7.5.

Runs with varying pH. The pH of the medium was adjusted in 0.2-unit increments in both directions from the original pH of 7.5.

Runs with elimination of centrifugation. The respiratory rates of suspension with $10^4$ and $10^6$ cells were measured. The original suspensions were divided and half was centrifuged and resuspended to the desired concentration. The other half was diluted directly by the addition of new medium.

Preparation of Samples for Measurement of Glucose Utilization

The procedure for the preparation of samples in the glucose utilization study paralleled the above protocol except at the very end. Instead of placing the properly diluted suspension in the syringe assembly, the glucose reaction was carried out at constant oxygen concentration. The suspension, in an Erlenmeyer flask, was immersed in a 37°C bath and stirred gently with a glass rod. MEM or RPMI-1640, containing 100 and 200 mg/dl glucose, was used as the diluent.

Analytical Techniques

Measurement of oxygen concentration. Our oxygen analyzer, shown in Fig. 2, consists of two parts. After the sampling loop had been charged with a new sample, it was turned "in line" with the scrubber. The oxygen in the sample was washed out by a stream of nitrogen, which carried it to the second part of the analyzer, a Hersch-type coulouxiometer, or fuel cell. The cell is identical with the one described by Hersch, and is commercially available from Chemical Sensor Development, Los Angeles, Calif.

Glucose determinations. At timed intervals the samples from cell suspensions were drawn and filtered with a Millipore filter system (Millipore, New Bedford, Mass.). The cell-free filtrate was collected and frozen. The whole series was analyzed for glucose content by the hexokinase method utilizing a Gemsaec centrifugal analyzer.

Heat measurements by calorimeter. Cell suspensions prepared for the measurement of oxygen depletion were split, and part of the solution was pumped through an LKB-1080D-1-microflow calorimeter. The method of measuring metabolic heats of cell solutions was identical to that used by Levin for leukocyte preparations. Parallel determinations of the respiratory rate were carried out with the remaining portion of the suspension. Since the aim of the experiment was a comparison of two methods, only results from a single run were utilized. This procedure increased the precision considerably when compared to the studies of respiratory rate as a function of cell concentration, in which case data from more than two dozen independent experiments were combined.
RESULTS

The measured total oxygen utilization by the cell solution contained as an error the utilization of oxygen by the MEM by itself. Due to the content of oxidizable substances, this could not be neglected. A series of separate experiments showed that oxygen consumption by media at experimental conditions was constant and reproducible. It equaled $2.02 \pm 0.03 \times 10^{-2} \mu M O_2/ml/hr$. This value had a tendency to increase slowly if opened media containers were allowed to stand at 37°C. Although very reproducible within one lot, the oxygen utilization varied with different media lots. Values as high as $8.8 \times 10^{-2} \mu M/ml/hr$ have occasionally been found. In all experiments the media consumption was very reproducible with a low SD of $0.3 \times 10^{-2} \mu M/ml/hr$. This precision, a magnitude better than possible in runs with cells present, allowed us to substract the background. For example, the average of three successive runs with L1210 cells yielded $4.3 \pm 1.7 \times 10^{-2} \mu M O_2/ml/hr$ for $10^6$ cells/ml; $4.1 \pm 2.2 \times 10^{-2} \mu M O_2/ml/hr$ for $10^7$ cells/ml; and $3.1 \pm 1.5 \times 10^{-2} \mu M O_2/ml/hr$ for $10^9$ cells/ml.

Figure 3 represents the raw experimental data collected for three different cell concentrations. Each curve is a sum of at least three consecutive experiments. The initial oxygen concentration was determined by our ability to reach equilibrium by bubbling air through the suspension. We decided to accept a 10% variation in initial oxygen saturation because this reduced the time from dilution to the first measured point. The linearity of the lines in Fig. 3 shows that on our time scale there is no lag period discernible. Despite the high precision in media runs, it was unfortunate that the separation between the cell and the media consumption was not better. To achieve this, we conducted experiments in Hank's balanced salt solution. The oxygen utilization remained linear long...
Fig. 4. Disappearance of glucose as a function of time in suspensions of L1210 lymphocytes at 37°C and constant oxygen concentration.

enough to allow the rate to be determined; at longer time intervals, the consumption became as expected, nonlinear. The loss of oxygen in cell-free Hank's solution was 1.2 ± 0.3 × 10⁻² μM O₂/ml/hr, which was only slightly higher than the loss observed in pure water, i.e., 0.5 ± 0.4 × 10⁻² μM O₂/ml/hr. These losses were on the borderline of detection and could be accounted for by oxidative processes on the surfaces of glass, plastic, and o-rings in contact with the solution. The results for cells in Hank's solution (5.5 ± 2.7 × 10⁻² μM O₂/ml/hr for 10⁴ cells/ml; 6.5 ± 1.4 × 10⁻² μM O₂/ml/hr for 10⁵ cells/ml; and 7.4 ± 3.1 × 10⁻² for 10⁶ cells/ml) clearly showed that down to 10⁴ cells/ml the media consumption and cell utilization could be separated with confidence.

Figure 4 shows the glucose utilization rates at three cell concentrations. In order to achieve better precision, the measurements were carried out over a somewhat more extended time range than the respiration studies. Respiration and glucose utilization were in all cases linear over the time range studied. This finding agreed with the observation of Hedeskov and Esmann, except that they found slight nonlinearity in glucose utilization. Under our experimental conditions, linearity of respiration persisted even after 90% of the oxygen was consumed (which would only occur at very high cell densities on our time scale); a behavior expected from the values of Michaelis-Menten constant for oxygen as determined by Longmuir for mammalian cells.

The respiration rates compiled in Table 1 were for the three cell lines measured in this laboratory obtained at 10⁶ cells/ml and converted to 10⁵ cells/ml for comparative purposes. The values for peripheral human lymphocytes and rat bone marrow cells were taken from the literature. It was evident that our cell lines established in culture had a higher respiratory rate than peripheral lymphocytes. The differences in the mean respiratory rate for cell lines L-1210 and 4098 were compared using the two-tailed Student's t test. The difference was not significant at the 95% level (α = 0.05), but was significant if the 90% level (α = 0.1) was considered adequate.

The data plotted in Fig. 5 represent all the observed rates in MEM, RPMI-
Table 1. Respiratory Rates Calculated for Cell Density of 10^6 Cells/ml at 37°C in MEM

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Rate (μM/hr/10^10 cells)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>4098</td>
<td>370 ± 107</td>
<td></td>
</tr>
<tr>
<td>L1210</td>
<td>486 ± 141</td>
<td></td>
</tr>
<tr>
<td>1788</td>
<td>614 ± 340</td>
<td></td>
</tr>
<tr>
<td>Human peripheral lymphocytes</td>
<td>117</td>
<td>6</td>
</tr>
<tr>
<td>(40 x 10^6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone marrow (rat; 6-13 x 10^6/ml)</td>
<td>360</td>
<td>13</td>
</tr>
</tbody>
</table>

1640 and Hank’s solution converted to oxygen consumption per 10^10 cells. One additional correction of the data was made in order to account for the daily and weekly variations in metabolic activities of the different cell lines. The respiratory baseline for a cell culture was determined at the beginning of each experimental series by triplicate runs with 10^6 cells/ml. All values obtained at different dilutions were then corrected for the difference between the grand mean of the respiratory rate for the cell line and the value measured that day. This correction allowed us to combine data over longer time periods and average them statistically. The correction amounted to 20%-30% of the rate at most. It had no effect on the slope of the line in Fig. 5. We could not carry out glucose determinations at concentrations lower than 10^5 cells/ml. Below that point the change in a few hours was less than the standard error of measurement.

Figure 6 shows the results of a single experiment comparing the rates of respiration and metabolic heat evolution over a relatively narrow range of cell concentrations, determined on the one hand by the sensitivity of the microcalorimeter, and on the other by the difficulties in maintaining even flow through the calorimeter cell at high cell concentrations. The precision was far better than that in Fig. 5, which, however, is deceptive since we had one cell line measured on a single occasion in Fig. 6. The point of the experiment was one of a qualitative nature—to show that oxygen utilization is related to metabolic heat production.

Fig. 5. Logarithmic plot of the rate of oxygen uptake at 37°C in MEM with 100 mg/dl glucose determined as a function of cell density. All points have been corrected for oxygen consumption of the medium. Only one determination at 10^6 cells/ml was done, due to technical problems associated with such a high concentration. Points indicated by * refer to experiments in Hank’s balanced salt solution. x, glucose; o, 4098; m, v, e, □, L1210; s, average for L1210.
Figure 7 shows the results of a number of experiments designed to show the effect of increased oxygen concentration, bicarbonate, lactic acid, and pH shifts in the medium. As seen in the figure, the presence of 100% oxygen in the equilibrating gas phase, increased bicarbonate in solution, and pH shifts had little influence on the observed respiration rate. The presence of lactic acid that accumulated during the period of observation did not influence the density dependence of respiration. To ascertain that the pH interval of 0.4 units tested gave an adequate picture of the experiments, we measured the pH change in a reaction vessel during respiration at a cell density of 10^6 cells/ml. The average value for the pH change (37°C stirring) was 0.134 ± 0.029 pH units/hr (n = 4). This finding, together with the apparent linearity of the oxygen uptake curve with time, made it very implausible that lactic acid accumulation with small pH shifts would cause the observed change in respiratory rate.
DISCUSSION

The choice of cultured cells was based on our desire to minimize the effects of decreased viability and damage due to isolation procedures and contamination by other cell types, all of which were problems encountered by Hedekov and Esmann in their classical work on respiration of peripheral lymphocytes by the Warburg methodology. The sensitivity of the Hersch cell allowed the use of very short time intervals for rate determinations. Thus, a gas-liquid interface was not necessary. Hedekov and Esmann considered the oxygen diffusion function through the interface as a possible source of the crowding effect.

As found by Hedekov and Esmann, we also found that the variation of oxygen tension, CO₂ concentration, and lactic acid accumulation was not responsible for the cell concentration effects. The pH shifts during our experiments were small enough not to influence the respiratory rate. In fact, the change in nutrients at cell densities of 10⁶-10⁷ during our measurements was small, so that the possibility of nutrient starvation could be excluded. This conclusion was further supported by the linear nature of both the oxygen and glucose utilization curves. Localized nutrient gradients which might develop due to a settling of the cells were avoided through the use of a stirrer system. Furthermore, direct dilution experiments avoiding the centrifugation step showed that possible damage to the cells by centrifugation and resuspension was not influencing our measurements. Another possible error, oxygen consumption or removal by the presence of cells by mechanisms not associated with metabolism, was not supported by the calorimetry experiments, in which correlation between heat and cell respiration was obtained.

We are thus left with an experimentally reproducible phenomenon, a change of two magnitudes in the respiratory rate for a three-magnitude change in cell density. The apparent leveling off at high cell concentrations (Fig. 5) agrees well with the observation of Talstad using cartesian diver techniques and very high cell densities. Whether the curve at 10⁷ cells/ml is leveling off is difficult to answer with certainty. The respiratory rate here approaches the background loss of oxygen in cell-free media, presumably due to oxidation of organic components. Although the glucose measurements could not be carried out at lower cell concentrations than 10³ cells/ml, this in itself sets a limit for the possible increase in glucose uptake at low cell concentrations, and we have to agree with Hedekov and Esmann that glucose uptake is much less affected by cell densities than is the respiratory rate.

Using the published estimates of glucose metabolism in lymphocytes, we see that at 10⁷ cells/ml not more than 2%-3% is utilized by the oxidative pathway, whereas at 10³ cells/ml, this fraction has increased to approximately 15%, with all due respect for possible artifacts, since we have not carried out the necessary radioactive tracer experiments.

Through several years of experience with cultured cells, one of us (R.C.) has noticed that various cell lines have definite density ranges for optimal growth. When the density drops quite low, cell viability decreases. At the other extreme, with a high cell density, the medium becomes depleted more rapidly and large numbers of cells die off. The extension of these characteristics to Fig. 5 shows...
that as the cells thin out (as the density drops) on a relative basis, a cell's metabolism is quite active, whereas at the higher densities the individual cell's metabolism is lower.

We are consequently arriving at the same conclusion as Hedeskov and Esmann with regard to the reality of the observed effect. However, we have eliminated the problem of gas-liquid diffusion into solutions of varying viscosity as a possible explanation. We are at this point ready to consider an alternative—a possible regulatory mechanism by which cells sense the presence of nearest neighbors.

The suspension culture techniques provide physical environments which differ from the more classical and more widely used technique of monolayer culture. In a monolayer culture, untransformed cells will grow according to what has been called "density-dependent growth inhibition," which entails the cessation of cell multiplication after a particular cell density has been achieved (determined by cell type and the conditions of culture). In a spinner culture the chances for actual cell surface to cell surface contact (as can be seen in monolayer culture) are very low, casting doubt on the validity of the "contact inhibition of growth" hypothesis.

Since spinner culture allows one to avoid the complications of intimate cell-to-cell contact, one is left with long-distance interactions between neighboring cells. The linearity of both oxygen uptake and glucose utilization curves points toward rapid equilibration after dilution with no lag period observed on the time scale of our experiments, with the first point occurring after 5 min. This result is not surprising, since it has been shown that uridine transport in fibroblasts, for example, is turned on and off by serum factors in a time interval of less than 5 min. Linearity at low oxygen content ratios agrees with the known $K_m$ values for oxygen uptake by mammalian cells and seems to exclude passive extracellular or cytoplasmic diffusion as rate limiting, although it has been shown in bacteria that the apparent $K_m$ is a linear function of the size of bacteria, whereas the $K_m$ values for homogenates are identical. We can also easily calculate that, whereas collisions between cells in suspension in our density range are infrequent, the probability of two cells being at a distance of half their diameter is very much higher.

Thus, despite the unattractiveness of passive diffusion as a regulatory step, transport of nutrients across the membrane could control the rate of metabolism and has been often cited as a possible site for growth regulation. Regardless of which aspects of regulation the different theories stress—secondary messenger, tropic hormones, or loss of control of DNA synthesis—changes in the state of cell membranes are implied in all the suggested schemes.

Our primary objective in pursuing these experiments has been the quantitation of density-dependent inhibition over an adequately wide range of cell densities. The response of lymphocyte metabolism to dilution and the relation of optimal growth to definite densities point toward a mechanism by which cells sense their neighbors even in suspensions. At this time, however, it is not clear how much the metabolic rate of an individual lymphocyte is affected and to what extent the observed increase is due to shifts between classes of lymphocytes in different states of metabolic activity. Finally, the most important step at present is to find conditions under which the observed effect can be abolished.
and restored, otherwise, despite all controls, the suspicion of an artifact remains.

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