The Influence of Carbon Dioxide Tension on the Respiration of Normal and Leukemic Human Leukocytes*

I. Influence on Endogenous Respiration

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SUMMARY

The influence of CO₂ on the respiration of normal and malignant human leukocytes has been studied with the Cartesian diver technic. The following observations were made:

Endogenous respiration of normal leukocytes suspended in Ringer-Locke's solution was slightly but significantly higher than that of myeloid and lymphoid leukemia cells.

Normal leukocytes were able to maintain their respiratory function in vitro for a longer period of time than were malignant leukocytes.

CO₂ stimulated the respiration of both normal and malignant leukocytes, the optimal CO₂ concentration being 1–2 per cent.

Normal and malignant leukocytes did not differ significantly in their response to variations in CO₂ concentration.

Since 1930 the interest of an increasing number of investigators (3, 4, 10, 12, 15, 18–20, 23) studying tissue metabolism in vitro, has been directed toward the fact that the majority of tissues show a higher and a more stable rate of respiration in serum than in Ringer's solution.

As Canzanelli and co-workers (3, 4) have pointed out, this effect depends on numerous physiologically active substances in serum ultrafiltrate. Serum proteins also seem to be active, but to a lesser degree. In his attempts to identify the active components of serum ultrafiltrate, Warren (21) found that the stimulating effect of serum on tissue respiration is mostly due to bicarbonate.

Although bicarbonate has long been suspected to have an influence on tissue respiration, systematic investigations of this problem were not initiated until 1930–31, when Ashford and Holmes (1) showed that the oxygen consumption of rabbit brain tissue was about 50 per cent higher in a bicarbonate buffer than in a phosphate buffer. Their measurements were carried out with the aid of a modified Barcroft apparatus.

Subsequent authors have mostly used the classical Warburg technic. In 1935 Laser (15) reported a twofold increase in the oxygen consumption of rat retina and of the Crocker mouse sarcoma 189, when these tissues were incubated in a bicarbonate buffer instead of in a phosphate buffer. Laser's findings were confirmed by Craig and Beecher (6), who were the first to make a quantitative estimation of the effect of different concentrations of carbon dioxide/bicarbonate upon metabolism. Using rat retina tissue these authors demonstrated that, when the CO₂ concentration of the gas phase was raised from 1 to 5 per cent at a constant pH, there was a twofold increase in the rate of glycolysis and respiration. A further increase in CO₂ concentration from 5 to 20 per cent gave no detectable effect on the rate of glycolysis; the oxygen consumption, however, decreased.

In 1944 Craig (5) reported a 60 per cent increase...
in the oxygen uptake of brain cortex slices from the
cat, when the CO₂ concentration was raised from 1
to 5 per cent at pH 8.1. At pH 7.48 no effect of
CO₂ was seen. Warren observed that CO₂ produced
40 per cent stimulation on the respiration of rab-
bit bone marrow and guinea pig liver. A 20 per
cent stimulation was observed by the same author
in experiments with rat kidney and liver. Cat
medulla oblongata and rabbit renal cortex did not
show any respiratory response to CO₂ according to
Craig and Warren (5, 21).

Using a modified Cartesian diver technic, Danes and Kieler (7) found a stimulatory effect of
CO₂ on the respiratory metabolism of the L-strain
mouse fibroblasts grown in tissue culture and on
the Yoshida rat ascites tumor cells. The maximal
increase as compared with the respiration at room
air averaged 32 per cent in the L-strain fibroblasts
and 60 per cent in the Yoshida cells. Danes and
Kieler's investigations, with a number of CO₂ con-
centrations used between 0 and 5 per cent, showed
that maximal respiration is obtained with 0.5–2
per cent CO₂ in the gas phase corresponding to a
HCO₃⁻ concentration of 1.5–6.0 mM in the Ringer-
Locke suspension medium.

Thus, only few attempts have been made to es-

tablish the quantitative relationship between CO₂
tension and cellular respiration, and no such stud-

gies have been included in attempts to clarify the

metabolic differences which may exist between

normal and leukemic human leukocytes.

Previous studies on leukocyte metabolism have
been carried out mainly with animal cells and with
human leukemic cells. The relatively small number
of experiments with normal human leukocytes can
be explained by difficulties in obtaining these cells
in quantities sufficiently large to permit studies by
gasometric methods. Unsatisfactory methods for
the isolation of leukocytes from normal whole
blood present another difficulty, and this has not
yet been overcome. The investigations of the oxy-
gen consumption of normal human leukocytes re-
ported thus far show variations which are too
great to form a basis for the comparison of respira-
tory metabolism between normal and malignant
cells.

The purpose of the present investigation was to
demonstrate, with the aid of the Cartesian diver
 technic, the possible effect of CO₂ and HCO₃⁻ on
the respiration of normal and leukemic human
leukocytes.

MATERIALS AND METHODS

Leukocytes were obtained from the blood of
twelve normal persons, seven patients with chronic
myeloid leukemia, and from nine patients with
chronic lymphatic leukemia. None of the patients
was being treated with antineoplastic drugs, but a
few of them were receiving x-ray treatment to the
spleen. Leukocyte counts, which included differen-
tial counting, were carried out in all cases. The
counts of the normal persons varied from 6,000 to
10,000, while those of the patients varied from
30,500 to 250,000. None of the patients showed
signs of hematological remission.

**Isolation of leukocytes.**—Leukocytes were sepa-
rated from erythrocytes and thrombocytes by cen-
trifugation and sedimentation according to meth-
ods described by Bicz, Koj, and Zgliczynski (2).

Blood samples were placed in several 1.5-ml.
centrifuge tubes with a diameter of 8 mm. and
centrifuged at 1,000 r.p.m. for 15 minutes. The

![Chart 1](chart1.png)

**Chart 1.**—Microtube for blood cell sedimentation

supernatant plasma was removed and centrifuged
once more at high speed to free it of all formed
elements. The white buffy coat, still containing some
erthrocytes, was then aspirated and resuspended
in the purified plasma. Further purification of the
leukocyte suspension was obtained by spontaneous
sedimentation at 37° C. for 1–1½ hour in the micro-
tubes described by Bicz et al. (2). This tube is
shown in Chart 1. The cell suspension was placed
with a fine pipette into branch (A) of the U-shaped
tube. The open end of the descending branch was
then connected to rubber tubing, and, by careful
blowing, the cell suspension was transferred to
branch (B) of the tube, the end of which is drawn
out as a capillary. The open end of the descending
branch was finally closed with a drop of plasma,
and the tube was placed in a cork holder and
stored in the incubator.

After sedimentation, the tubes were emptied by
blowing fraction by fraction through the capillary. The first fraction contained decreasing amounts of thrombocytes; the following were made up of leukocytes with increasing quantities of erythrocytes, while the last fractions contained the erythrocytes.

The purest leukocyte fractions, those containing insignificant amounts of thrombocytes, were selected by microscopic examination. The erythrocyte contamination varied from 14 to 22 per cent, when normal blood was used, and from 4 to 12 per cent in the case of leukemic blood. Selected leukocyte samples were placed in 50–100-µl vessels and centrifuged for 2 minutes at 2,000 r.p.m. The supernatant plasma was then removed and Ringer-Locke's solution added at volumes of 20–50 µl. depending on the number of cells. The whole procedure for leukocyte separation lasted 2–3 hours.

The final leukocyte concentration was 60,000–120,000 cells per µl. Differential counts showed that normal leukocyte suspensions contained 35–50 per cent lymphocytes, thus indicating that these were able to stand the process of separation better than the polyllobular granulocytes. The suspensions of lymphatic leukemia cells contained more than 95 per cent lymphoid cells. The differential counts of the cell suspensions obtained from patients with chronic myeloid leukemia varied somewhat, but in all cases more than 60 per cent of the cells were immature.

The Ringer-Locke’s solution used as basic experimental medium was buffered with 30 mM tris-hydroxymethyl aminomethane. KCl was present at a concentration of 5.63 mM and CaCl₂ at a concentration of 2.16 mM. The concentration of sodium bicarbonate varied according to the CO₂ tension under investigation, and a constant osmotic pressure was obtained by varying the concentration of NaCl. Penicillin was added at a concentration of 500 i.u./ml, and streptomycin at 0.05 mg/ml.

All the solutions were gassed for 6 hours at 37° C. with the gas mixtures¹ to be studied. After gassing, the pH was adjusted to 7.4, and the solutions were stored in ampules. The composition of the gas mixtures used and the corresponding HCO₃⁻ concentrations are shown in Table 1.

**Gasometric technic.**—Leukocyte respiration was measured with the Cartesian diver technic developed by Linderstrøm-Lang (16) and Holter (11). For measurements at controlled CO₂ tensions the following precautions were essential:

1. Since filling the diver involves not only the introduction of the various solutions but also the introduction of the desired gas mixture, this operation must be carried out under water.
2. To prevent the loss of CO₂ by diffusion, the mouth seal must be provided with a stopper as described by Linderstrøm-Lang and Holter (17).
3. For the absorption of respiratory CO₂, bicarbonate must be used instead of sodium hydroxide.
4. Equilibrium must be carefully established between the gas mixture to be studied and the various solutions employed before these are introduced into the diver.

Divers of a total volume of about 15 µl. were made from Jena glass capillaries with a specific gravity of 2.41. The inner diameter of the necks varied from 0.8 to 0.9 mm. For filling, the divers were fixed in a holder and placed under water in a small thermostat at 37° C., and the various solutions were introduced by micropipettes. The tips of the latter and the inside of the divers were treated with silicone.

A schematic drawing of the charged diver is shown in Chart 2. A 0.15 M NaHCO₃ solution (0.5 µl.) was first placed at the bottom, after which the inside of the diver was gassed for 1–2 minutes with the gas mixture to be studied; 0.5 µl.

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¹ Purchased from Dansk Ilt og Brintfabrik, Copenhagen.
of the cell suspension was then placed at the lower end of the neck, and the gassing was repeated with the tip of the gassing pipette just above the cell suspension. A further 0.5 μl of 0.15 M bicarbonate solution was then placed in the neck above the cell suspension, and gassing was repeated. After this, 0.5 μl of paraffin oil was introduced, gassing again took place, and finally a provisional water seal was placed at the mouth of the diver.

The completed diver was then transferred to the flotation vessel, where the water seal was replaced by a seal of the same composition as the flotation medium. At the same time sufficient gas was sucked out from the gas space between the oil seal and the mouth seal to make the diver sink slowly to the bottom.

The use of a 0.15 M solution of bicarbonate instead of sodium hydroxide as a CO₂-absorbing medium in the Cartesian diver technique was introduced by Kieler (7), after it was found that constant CO₂ tension could be maintained in this way. Since it was found that the divers were very sensitive to distillation owing to differences in the osmotic concentration of the bicarbonate solution and the Ringer-Locke's solution, equilibrium between these two solutions had to be established before their introduction into the diver. Therefore, on the day before use the Ringer-Locke's solution and the bicarbonate solution to be used in the same divers were poured from the ampules into open tubes, which were placed in a larger container. This, after the introduction of the desired gas phase, was closed and left overnight at 37°C.

Control experiments with gas mixtures containing 0.03, 0.5, 1.0, 2.0, 3.0, and 5.0 per cent CO₂ showed that no measurable loss of CO₂ occurred over a period of 20 hours (see Chart 3).

Experiments were usually run over a period of 20–24 hours. During the first 7–8 hours manometric readings were made at 30–60-minute intervals. The divers were then left in the flotation vessels overnight, and the following day the measurements were continued for several hours. The oxygen consumption was calculated from the formula developed by Linderström-Lang (16) and recorded as μl O₂ consumed/10,000,000 leukocytes/hour.

Detailed results of four representative experiments as well as the average results of all experiments are shown in Table 2. It appears from the table that the CO₂ concentrations studied were not always the same in the different experiments. Furthermore, since it was found that the standard deviation of the results obtained in different experiments was significantly greater than the
standard deviation of duplicate and triplicate determinations within the same experiment, the statistical evaluation of the results had to be based on the changes in oxygen consumption rather than on the absolute values.

Thus, a significant increase in cellular respiration was found when the CO₂ concentration was raised from 0.03 per cent to 0.5 per cent and from 0.5 to 1 per cent. There was no significant difference between the increase at 1 and 2 per cent, but a significant decrease occurred when the CO₂ concentration was raised from 2 to 3 per cent and from 3 to 5 per cent.

The increase in respiration at 1 per cent CO₂ as compared with the respiration at room air averaged 25 per cent in the initial period of incubation and 41 per cent in the 5th–8th hour of incubation, thus indicating a stabilizing effect of CO₂ on the respiration curves in addition to a stimulatory one.

The respiration curves of leukemia cells are illustrated in Chart 5, and detailed results of representative experiments as well as the average results of all experiments are shown in Table 2. The effect of carbon dioxide on the malignant cells was similar to that on the normal cells, with respiration at 1–2 per cent CO₂ being significantly higher than at all other concentrations.

When the CO₂ concentration was raised from 0.03 to 1 per cent, the increase in oxygen consumption during the first 3 hours of incubation averaged 28 per cent in the case of myeloid leukemia cells.

### Table 2

<table>
<thead>
<tr>
<th>CO₂ conc. (per cent)</th>
<th>0.03</th>
<th>0.5</th>
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<td>I: Normal leukocytes</td>
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<td>6</td>
<td>2.1</td>
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<td>2.8</td>
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<tr>
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<td>1.0</td>
<td>0.9</td>
<td>1.5</td>
<td>1.4</td>
<td>2.2</td>
<td>1.1</td>
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<tr>
<td>III: Lymphatic leukemia cells</td>
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<tr>
<td>9</td>
<td>2.0</td>
<td>2.6</td>
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<td>2.8</td>
<td>2.8</td>
<td>1.5</td>
</tr>
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</table>

**Representative experiments:**

- **I: Normal leukocytes**
  - Exp. No. 1
  - 2: 2.7, 3.1, 3.3, 3.4, 3.3, 2.1
  - 3: 2.9, 2.3, 3.6, 3.5, 3.3, 2.5
  - 4: 2.3, 2.1, 2.9, 2.8, 3.0, 2.4
- **II: Myeloid leukemia cells**
  - 6: 2.1, 2.8, 2.8, 2.5, 2.0, 1.7
  - 7: 1.9, 0.9, 1.5, 1.4, 2.2, 1.2
  - 8: 1.0, 0.9, 1.5, 1.4, 2.2, 1.1
- **III: Lymphatic leukemia cells**
  - 9: 2.0, 2.6, 2.7, 2.5, 2.3, 1.8
  - 10: 1.8, 2.3, 2.4, 2.5, 2.5, 1.8
  - 11: 2.6, 2.4, 3.0, 2.8, 2.5, 1.3
  - 12: 1.9, 2.5, 2.5, 2.8, 2.8, 1.5

**Average results of all experiments:**

- **I: Normal leukocytes**
  - 2.6 (12), 2.3 (12), 2.8 (12), 2.2 (12), 2.2 (12), 2.6 (12)
- **II: Myeloid leukemia cells**
  - 2.8 (7), 2.8 (7), 2.7 (6), 2.6 (6), 2.4 (6), 2.0 (6)
- **III: Lymphatic leukemia cells**
  - 2.3 (9), 2.5 (5), 3.0 (9), 2.8 (9), 2.6 (9), 2.0 (9)

* A: Average respiration during the first 3 hours of incubation.
† B: Average respiration during the 4th–6th hour of incubation.
and 30 per cent in the case of lymphatic leukemia cells. During the 4th-6th hour of incubation, the increase averaged 68 and 72 per cent, respectively. Thus, the stabilizing effect of CO$_2$ seemed to be even more pronounced in the case of leukemia cells than in the case of normal cells, but otherwise no significant differences between the effect of CO$_2$ on normal and malignant cells could be demonstrated. However, the average oxygen consumption of normal leukocytes was significantly higher than that of malignant leukocytes at all CO$_2$ concentrations, and the normal cells were also able to maintain a linear respiration curve for a longer period of time.

DISCUSSION

In the present experiments both normal and malignant human leukocytes suspended in Ringer-Locke's solution showed a rate of respiration which was more than tenfold lower than that of the Yoshida ascites tumor cells and the L strain mouse fibroblasts previously studied in this laboratory (7, 13).

The respiratory capacity of all the leukocytes was maintained for only a limited period of time. With normal leukocytes the respiration curve showed a linear course during the first 5-6 hours and then a gradual falling off. After 20 hours of incubation the cells still showed some respiration, but it was at a very low and rapidly decreasing rate. With leukemia cells the decrease in respiratory function was already apparent after the first 2-3 hours of the experiment, and the respiration curve usually reached a horizontal level much sooner than in the case of normal leukocytes.

On an average normal leukocytes showed an initial rate of respiration which was slightly, but significantly, higher than that of malignant leukocytes. During the experimental incubation period this difference increased, owing to the more rapid decline of respiration in the myeloid and lymphatic leukemia cells. The differences in respiratory metabolism between normal and malignant leukocytes might be due to greater damage to the leukemic cells during the isolation procedures. However, this suggestion is contradicted by the fact that the malignant cells on microscopical examination after isolation looked, if anything, less damaged than the normal leukocytes. The more rapid falling off of the respiration curves of the malignant cells may be due to smaller amounts of intracellular nutrients available for combustion, but it seems doubtful whether such a lack of endogenous substrate could explain the initial difference between the respiration of normal and malignant leukocytes. The effect of added substrates should, however, be investigated before this possibility can be excluded.

Carbon dioxide had a stimulating effect on both normal and malignant leukocytes. A maximal effect was obtained at 1-2 per cent CO$_2$, which is in agreement with Kieler's studies of the Yoshida rat ascites tumor cells (7, 13) and Danes' studies of the L-strain mouse fibroblasts (7). There was no significant difference between the response of normal and malignant leukocytes to CO$_2$, but the stabilizing effect on the respiration curve was relatively more pronounced in the case of leukemia cells.

The complete mechanism of the effect of CO$_2$ is not known. According to Kieler (7) CO$_2$/HCO$_3^-$ has two effects on the metabolism of the Yoshida ascites tumor cells: an abbreviation of the Crabtree effect and, following this, a stimulation of the oxygen consumption. These two effects do not seem to be linked together, since the stimulatory effect on cell respiration appears after the complete disappearance of the Crabtree effect, i.e., after the complete breakdown of glucose to pyruvate, and it also occurs in the absence of an exogenous glucose substrate. The results of present experiments support the fact that CO$_2$ has a stimulatory effect on the endogenous respiration.

Consideration has been given to the possibility that the effect of CO$_2$/HCO$_3^-$ on cellular respiration might be due to a stimulation of the Wood & Werkman (8, 9, 14, 22) reaction during which CO$_2$ reacts with phospho-enol-pyruvate and pyruvate (7, 13, 21). The end product of this reaction is oxalacetate, and the concentration of this may be...
a rate-limiting factor in the citric acid cycle. In support of this hypothesis Kieler (13) has found that CO₂ can be replaced by oxalacetate and, moreover, that CO₂ has no further stimulatory effect on the cells as long as these are supplied with sufficient concentrations of oxalacetate. It seems probable that the effect of CO₂ on the respiration of normal and malignant leukocytes can be explained in a similar way; but further studies on the CO₂-substrate relationship, with manometric as well as isotopic techniques, are required before a final answer to the question can be given.

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