The Effect of Chloramphenicol and Other Antibiotics on Leukocyte Respiration

By JAMES H. FOLLETTE, PETER M. SHUGARMAN, JOHN REYNOLDS, WILLIAM N. VALENTINE and JOHN S. LAWRENCE

A GROWING NUMBER OF REPORTS have indicated that antibiotics may cause severe blood dyscrasias in susceptible individuals. These have been well summarized in reviews by Heilmann and Von Oettingen. In general, penicillin has shown a remarkable lack of toxicity for the blood forming organs, only one case of agranulocytosis having been reported by Spain and Clark. The incidence of agranulocytosis following streptomycin administration was found to be low in a survey conducted in 1947, and aplastic anemia has been observed only rarely after prolonged administration. Chlorotetracycline and oxytetracycline appear likewise to be relatively nontoxic since only one case of transient leukopenia has appeared in the literature.

Chloramphenicol, on the other hand, has been more seriously implicated. In 1948, Smith and associates noted varying degrees of anemia in three dogs receiving 1.0 Gm. chloramphenicol daily intramuscularly. In 1950, Voline reported two cases of transient neutropenia and one case of transient hypoplastic anemia following chloramphenicol administration. In spite of this, chloramphenicol was considered to be a drug of remarkably low toxicity until 1951. Since then, a number of reports have made it clear that prolonged or repeated use of chloramphenicol may result in severe leukopenia, agranulocytosis, thrombocytopenia and aplastic anemia in certain individuals, although toxic effects have been sporadic and have occurred in only a small proportion of individuals receiving the antibiotic.

The potential toxicity of antibiotics toward the hematopoietic tissues is now well recognized, but the basic mechanism involved is still obscure. For this reason, studies were carried out to determine whether the compounds mentioned have any demonstrable effect on the respiratory metabolism of separated leukocytes. The action of two other known bone marrow depressants, atabrine and urethane, was also observed for comparison. The following report summarizes the results obtained.

METHODS

The method for separating leukocytes from whole blood has been previously described. Fibrinogen was used as the sedimenting agent, and was diluted with isotonic 1.15 per cent KCl pH 7.5 or with isotonic saline. The red cells were allowed to sediment for

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### Table 1

<table>
<thead>
<tr>
<th>Substance Used</th>
<th>Molarity of Stock Solution</th>
<th>Quantity of Stock Used (ml.)</th>
<th>Final Molarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>$6.0 \times 10^{-2}$</td>
<td>0.20</td>
<td>$4.3 \times 10^{-3}$</td>
</tr>
<tr>
<td>ATP-Na*</td>
<td>$1.5 \times 10^{-2}$</td>
<td>0.20</td>
<td>$1.1 \times 10^{-3}$</td>
</tr>
<tr>
<td>DPN*</td>
<td>$1.5 \times 10^{-2}$</td>
<td>0.20</td>
<td>$1.1 \times 10^{-3}$</td>
</tr>
<tr>
<td>HDP†</td>
<td>$1.0 \times 10^{-1}$</td>
<td>0.15</td>
<td>$3.7 \times 10^{-3}$</td>
</tr>
<tr>
<td>Cytochrome C*</td>
<td>$3.1 \times 10^{-4}$</td>
<td>0.20</td>
<td>$2.2 \times 10^{-5}$</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>$1.0 \times 10^{-1}$</td>
<td>0.10</td>
<td>$7.1 \times 10^{-3}$</td>
</tr>
<tr>
<td>Succinate-Na</td>
<td>$1.1 \times 10^{-1}$</td>
<td>0.20</td>
<td>$7.9 \times 10^{-3}$</td>
</tr>
<tr>
<td>Sucrose</td>
<td></td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>Phosphate buffer, pH 7.5</td>
<td>1.25</td>
<td>0.15</td>
<td>$6.7 \times 10^{-3}$</td>
</tr>
<tr>
<td>Tris buffer, pH 7.5</td>
<td>1.48</td>
<td>0.10</td>
<td>$3.3 \times 10^{-3}$</td>
</tr>
</tbody>
</table>

* Purchased from Sigma Laboratories.
† Purchased from Schwartz Laboratories.
‡ Sucrose 0.8434 Gm. was added directly to the flask.

Abbreviations used: ATP = adenosine triphosphate
DPN = diphosphopyridine nucleotide
HDP = hexose diphosphate or fructose 1,6 diphosphate
Tris = tris (hydroxymethyl) aminomethane.

twenty minutes at room temperature. Following this, the leukocyte-rich supernatant plasma was aspirated and transferred to a walk-in cold room where all subsequent handling of the cells was carried out at 4 C. The white cells were washed twice with alkaline isotonic KCl, centrifugations being carried out at 225 g for seven minutes to minimize platelet contamination. A small percentage of erythrocytes remained, but this was disregarded since no oxygen uptake could be demonstrated when pure red cell suspensions of comparable cell concentration were added to control Warburg flasks. After the washing procedure, the leukocytes were suspended in approximately 4.0 ml alkaline isotonic KCl and the cell concentration was adjusted to 40,000-80,000 per cu. mm. Cell counts were done on the suspension in quadruplicate, and successive 1.0 ml. aliquots were removed and homogenized for one minute in a lucite homogenizer. The homogenates were then recombined and were mixed well prior to use.

Warburg flasks containing a standard substrate cofactor system were prepared immediately before the experiment. The system used is listed in table 1.

Sucrose was added since Schneider has shown that mitochondria in rat liver homogenates maintain their morphologic structure more satisfactorily in a hypertonic medium. After a number of trials it was found that sucrose 0.88 Molar resulted in maximal oxygen uptake. This molarity was therefore employed in all subsequent experiments. Tris buffer was used in addition to phosphate buffer since the pH of the flask contents could not be maintained constant otherwise.

All drugs were added to the incubation medium in crystalline form. This was done to avoid increasing the fluid volume unduly. Potassium hydroxide 0.5 Molar and a filter paper wick were placed in the center wells. Cold homogenate 0.5 ml. was then added to the main portion of the flasks. Following this, the flasks were attached to the manometers and were allowed to equilibrate for ten minutes in the water bath at 38 C. The manometer stopcocks were then closed and readings were taken every fifteen minutes for three hours. Determinations were done in duplicate, using air as the gaseous medium.

Total oxygen uptake and the uptake for each fifteen minute period were calculated as outlined by Umbreit, Burris and Stauffer. All values were converted to micromoles of oxygen consumed per 10⁹ leukocytes. Representative curves were then graphed and the initial rate of oxygen consumption per hour was determined. This was calculated from the slope of a line constructed tangent to the portion of the curve included between the zero and fifteen minute points.
EFFECT OF ANTIBIOTICS ON LEUKOCYTE RESPIRATION

TABLE 2.—Leukocyte Oxygen Consumption in Controls

<table>
<thead>
<tr>
<th>System</th>
<th>No. of Experiments</th>
<th>Initial Rate O₂ Uptake per Hour*</th>
<th>Three Hour O₂ Uptake*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>80</td>
<td>505 ± 187</td>
<td>746 ± 235</td>
</tr>
</tbody>
</table>

* Oxygen uptake is expressed as micromoles per 10³ wbc ± standard deviation.

TABLE 3.—Effect of Chloramphenicol on Leukocyte Respiration

| Molar Concentration | No. of Experiments | Initial Rate O₂ Uptake per Hour (%) of Control | % Inhibition | % Inhibition
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1 × 10⁻²</td>
<td>3</td>
<td>9.3</td>
<td>90.7</td>
<td>17.3</td>
</tr>
<tr>
<td>5.1 × 10⁻²</td>
<td>5</td>
<td>29.0</td>
<td>71.0</td>
<td>39.0</td>
</tr>
<tr>
<td>2.5 × 10⁻³</td>
<td>18</td>
<td>52.8</td>
<td>47.2</td>
<td>69.6</td>
</tr>
<tr>
<td>1.1 × 10⁻³</td>
<td>10</td>
<td>69.5</td>
<td>30.5</td>
<td>70.0</td>
</tr>
<tr>
<td>5.5 × 10⁻⁴</td>
<td>5</td>
<td>86.0</td>
<td>14.0</td>
<td>91.0</td>
</tr>
</tbody>
</table>

* Expressed as the mean of all experiments performed.

RESULTS

Controls:

Leukocytes were obtained from normal subjects or from patients with leukocytosis of infection. All experiments were internally controlled so that flasks containing drugs could be compared with duplicate flasks to which no drugs had been added. Leukocyte oxygen consumption obtained in all controls is presented in table 2. It is apparent that leukocytes exhibit readily measurable respiratory activity under the conditions employed. This is reproducible in replicate flasks within 10 per cent, and appears to be relatively consistent in repeated determinations on the same individual. The following data serve to illustrate this:

<table>
<thead>
<tr>
<th>Patient</th>
<th>Time</th>
<th>Initial Rate O₂ Uptake/hr.</th>
<th>Three Hour O₂ Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.S.</td>
<td>Initial</td>
<td>573</td>
<td>858</td>
</tr>
<tr>
<td>A.S.</td>
<td>1 day</td>
<td>565</td>
<td>850</td>
</tr>
<tr>
<td>D.L.</td>
<td>Initial</td>
<td>713</td>
<td>848</td>
</tr>
<tr>
<td>D.L.</td>
<td>1 day</td>
<td>643</td>
<td>855</td>
</tr>
<tr>
<td>C.P.</td>
<td>Initial</td>
<td>475</td>
<td>568</td>
</tr>
<tr>
<td>C.P.</td>
<td>5 day</td>
<td>510</td>
<td>543</td>
</tr>
<tr>
<td>C.P.</td>
<td>6 day</td>
<td>495</td>
<td>460</td>
</tr>
</tbody>
</table>

Chloramphenicol:

Forty-one experiments were performed with chloramphenicol in concentrations varying from 5.5 × 10⁻⁴ Molar to 6.1 × 10⁻³ Molar. The results are listed in table 3. It can be seen that significant inhibition was obtained at concentrations of 1.1 × 10⁻³ Molar and above. With the highest concentration, 6.1 × 10⁻³ Molar, almost complete inhibition occurred. This is graphically illustrated in figure 1.
FIG. 1.—The Effect of Chloramphenicol on Leukocyte Respiration. Effect of chloramphenicol $6.1 \times 10^{-3}$ Molar on the oxygen uptake of leukocyte homogenate in a fortified system. All values are expressed in micromoles per $10^{10}$ leukocytes. Standard manometric technic was used in all experiments.

FIG. 2.—The Effect of Broad-Spectrum Antibiotics on Leukocyte Respiration. Comparison of the effect of chloramphenicol, oxytetracycline and chlortetracycline $2.5 \times 10^{-3}$ Molar on the oxygen uptake of leukocyte homogenates in a fortified system.

**Chloramphenicol and other antibiotics:**

The comparative effects of chloramphenicol, chlortetracycline, oxytetracycline, streptomycin and penicillin are presented in table 4. The first three of these are also graphically illustrated in figure 2. It is readily apparent that the only significant mean inhibition of oxygen uptake occurred with chloramphenicol. The drug concentration used in all comparison experiments was $2.5 \times 10^{-3}$


Table 4.—Comparative Effect of Various Antibiotics on Leukocyte Respiration

<table>
<thead>
<tr>
<th>Drug Used</th>
<th>Molar Concentration</th>
<th>No. of Experiments</th>
<th>Initial Rate O₂ Uptake (CP of Control)</th>
<th>% Inhibition</th>
<th>H₂ O₂ Uptake (CP of Control)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol</td>
<td>2.5 X 10⁻³</td>
<td>18</td>
<td>52.8</td>
<td>47.2</td>
<td>69.6</td>
<td>30.4</td>
</tr>
<tr>
<td>Chlortetracycline</td>
<td>2.5 X 10⁻³</td>
<td>8</td>
<td>104.0</td>
<td>0</td>
<td>99.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>2.5 X 10⁻³</td>
<td>6</td>
<td>113.0</td>
<td>0</td>
<td>120.5</td>
<td>0</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>2.5 X 10⁻³</td>
<td>10</td>
<td>99.8</td>
<td>0.2</td>
<td>104.4</td>
<td>0</td>
</tr>
<tr>
<td>Penicillin</td>
<td>2.5 X 10⁻³</td>
<td>10</td>
<td>101.2</td>
<td>0</td>
<td>100.3</td>
<td>0</td>
</tr>
</tbody>
</table>

* Expressed as the mean of all experiments performed.

Table 5.—Effect of Atabrine and Urethane on Leukocyte Respiration

<table>
<thead>
<tr>
<th>Drugs Used</th>
<th>Molar Concentration</th>
<th>No. of Experiments</th>
<th>Initial Rate O₂ Uptake (CP of Control)</th>
<th>% Inhibition</th>
<th>H₂ O₂ Uptake (CP of Control)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atabrine</td>
<td>1 X 10⁻³</td>
<td>2</td>
<td>19.0</td>
<td>81.0</td>
<td>33.0</td>
<td>67.0</td>
</tr>
<tr>
<td>Atabrine</td>
<td>5 X 10⁻⁴</td>
<td>6</td>
<td>33.0</td>
<td>67.0</td>
<td>30.2</td>
<td>69.8</td>
</tr>
<tr>
<td>Atabrine</td>
<td>1 X 10⁻³</td>
<td>1</td>
<td>59.1</td>
<td>40.8</td>
<td>83.9</td>
<td>16.1</td>
</tr>
<tr>
<td>Atabrine</td>
<td>1 X 10⁻⁵</td>
<td>1</td>
<td>100.4</td>
<td>0</td>
<td>104.6</td>
<td>0</td>
</tr>
<tr>
<td>Urethane</td>
<td>1 X 10⁻¹</td>
<td>2</td>
<td>27.0</td>
<td>73.0</td>
<td>41.8</td>
<td>58.2</td>
</tr>
<tr>
<td>Urethane</td>
<td>5 X 10⁻³</td>
<td>1</td>
<td>16.0</td>
<td>84.0</td>
<td>41.7</td>
<td>58.3</td>
</tr>
<tr>
<td>Urethane</td>
<td>1 X 10⁻³</td>
<td>1</td>
<td>92.6</td>
<td>7.4</td>
<td>95.7</td>
<td>4.3</td>
</tr>
</tbody>
</table>

* Expressed as the mean of all experiments performed.

Molar. At this drug level some variability in the amount of inhibition by chloramphenicol was noted, but 16 of the 18 determinations gave results below 69% of the control initial rate, the remaining two values being 72% and 86% respectively. The mean value was 52.8% and one low value of 17% was found. Total three hour oxygen uptakes showed a similar range of variability, the mean value being 69.6% of the control.

Chlortetracycline demonstrated little or no inhibition of the initial rate of oxygen uptake in five of the eight experiments performed. The other three showed values of 68%, 79% and 80% of control values. Total three hour oxygen uptake indicated even less inhibitory tendency. The three hour uptake value corresponding to the 79% initial uptake was found to be 94%, while the other two three hour oxygen uptakes were 82% and 68% respectively.

No significant inhibition of leukocyte respiration occurred with oxytetracycline. Instead, moderate stimulation of oxygen uptake was noted in almost all experiments. The mean initial rate for all flasks containing oxytetracycline was 113% and the three hour uptake was 120.5%. Values for the initial rate ranged from 91% to 134%, and three hour uptakes ranged from 99% to 134%.

Penicillin and streptomycin showed no inhibitory or stimulatory effect of consequence. All mean values were similar to those found in the controls.

Atabrine and urethane:

The former proved to be the most active respiratory depressant used. In six experiments atabrine 5 X 10⁻⁴ Molar demonstrated inhibitory activity similar
to that obtained with chloramphenicol $5 \times 10^{-3}$ Molar. On the other hand, urethane was much less active than either atabrine or chloramphenicol. In the few experiments performed a concentration of at least $5 \times 10^{-3}$ Molar was found necessary to obtain comparable inhibition of oxygen uptake. All results are listed in table 5.

**DISCUSSION**

The results indicate that chlortetracycline, oxytetracycline, penicillin and streptomycin have little or no effect on leukocyte respiratory activity under the experimental conditions employed. Chloramphenicol, on the other hand, produced significant inhibition of leukocyte respiration when used in the same concentration. Moderate inhibition of oxygen uptake occurs with chloramphenicol at concentrations of $1 \times 10^{-2}$ Molar, and this increases progressively until almost complete inhibition is noted at $6.1 \times 10^{-2}$ Molar. The inhibitory activity observed with chloramphenicol is intermediate between that observed with atabrine and with urethane. It is somewhat less active than the former, but more active than the latter.

The concentrations employed exceed those usually found in the serum of patients receiving these compounds by mouth, but are similar to those found to be effective in vitro with other commonly used biochemical or pharmacologic inhibitors such as malonate, azide or iodoacetate. It is not known whether hematopoietic tissues would be exposed to drug levels of equal magnitude in vivo. The possibility exists, however, that selective concentration may occur, since it has been shown that thiouracil, for example, may be found in the bone marrow at levels up to approximately 350 times those found in the serum. It should be pointed out, however, that the demonstrable activity of chloramphenicol in suppressing oxygen consumption of leukocyte homogenates in vitro cannot be assumed to represent a mechanism for hematopoietic depression in vivo. The present experiments delineate a pharmacologic activity. They do not permit interpretation of this activity as being an explanation for the bone marrow suppression occasionally observed after in vivo administration.

The toxic effect of chloramphenicol toward the hematopoietic tissues is most likely due to the nitrobenzene radical in its chemical structure. This was first suggested since naturally occurring compounds do not ordinarily contain this group. Nitrobenzene by itself is known to produce methemoglobinemia in susceptible individuals and may, paradoxically, result in polycythemia.

The site of action of chloramphenicol in bacterial enzyme systems and in animal tissue homogenates has been extensively studied by a number of investigators. Inhibition of an aerobic dehydrogenase, xanthine oxidase, by chloramphenicol has been observed in liver homogenates by Swenson, Swanson and Bethell. Since oxygen is the hydrogen acceptor of compounds activated by this flavin-containing enzyme, its activity contributes to cellular respiration. This fact suggested that a possible site of action in the leukocyte might include this class of enzymes. However, experiments now in progress in this laboratory have thus far failed to indicate a relationship between flavin-dependent enzymes and the observed inhibition of respiration by chloramphenicol. Riboflavin and flavin-adenine-dinucleotide (FAD), for example, do not reverse the inhibition of leukocyte respiration produced by this antibiotic.
Such reversal has been observed by Gots and Sevag\textsuperscript{41, 45} when these substances are added to bacterial suspensions which have been inhibited by sulfathiazole, atabrine or other compounds which appear to act primarily on flavin-linked enzyme systems.

Cytochrome oxidase likewise does not appear to be a site of action. This enzyme is probably present in the leukocyte since other workers\textsuperscript{36, 37} have shown that leukocyte respiration is partially inhibited by cyanide. Cytochrome oxidase is extremely sensitive to this inhibitor, but can be “by-passed” by an auto-oxidizable dye such as methylene blue, which acts as a hydrogen acceptor. When methylene blue is added to flasks containing chloramphenicol, the degree of inhibition of leukocyte respiration is not significantly affected.

Further studies will be necessary to elucidate whether or not the metabolism of Krebs cycle intermediates in the leukocyte is affected by chloramphenicol. This is of importance since another broad spectrum antibiotic, chlortetracycline, has been shown by Van Meter and Oleson\textsuperscript{38} to inhibit the respiration of liver homogenates by apparently blocking a part of the Krebs cycle. These authors used a fortified system similar to that used by Pardee and Potter,\textsuperscript{39} and found that the most marked effect was observed when citrate was omitted.

Additional work will also be required to determine if the entry of active metabolites such as pyruvate or “active acetate” into the tricarboxylic acid cycle is modified in any way by chloramphenicol. This is worthy of investigation in view of the studies by Umbreit\textsuperscript{40, 41} on the inhibition of respiration in certain bacteria by streptomycin. He has shown that the inhibitory effects observed are due to the action of streptomycin on either the so-called pyruvate-oxalacetate condensation mechanism or on the formation of a new intermediate, 2-phospho-4-hydroxy-4-carboxy-adipic acid.

It is hoped that biochemical studies of this nature will help to further elucidate basic toxic mechanisms that now tend to limit the use of many valuable therapeutic compounds.

**Summary**

1. Leukocyte homogenates show active, reproducible respiratory activity in a fortified system.
2. Chloramphenicol inhibits leukocyte respiration in vitro when used in concentrations similar to other commonly used inhibitors.
3. Chlortetracycline, oxytetracycline, penicillin and streptomycin have no significant effect on leukocyte respiration.
4. Urethane and atabrine produce inhibition of leukocyte respiration, but the latter is more active.
5. The possible sites of action of chloramphenicol in cellular metabolism are discussed.

**Summario in Interlingua**

1. Homogenatos leucocytic exhibit in un systema fortificate un positive e reproducibile activitate respiratorii.
2. Chloramphenicol inhibi le respiration leucocytic in vitro si illo es usate in concentrationes simile a illos de altere inhibitores de uso commun.
3. Chlorotetracyclina, oxytetracyclina, penicillina, e streptomycina ha nulle effetos significative super le respiration leucocyty.
4. Urethano e atabrinha produce un inhibition del respiration leucocyty, sed le secunde de iste agentes es plus active.
5. Es discutite le possibile locos del action de chloramphenicol in le metabolismo cellular.

REFERENCES
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242


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