Entry of Methotrexate into *Streptococcus pneumoniae*: a Study on a Wild-type Strain and a Methotrexate Resistant Mutant

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Entry of methotrexate (MTX) into the folate prototrophic bacterium *Streptococcus pneumoniae* was poorly inhibited by folate or its natural derivative folinic acid, suggesting that if MTX is transported via a folate transporter, the affinity of that transporter for MTX is higher than for folate. In the range of concentrations tested, MTX uptake was non-concentrative and decreased in ATP-depleted bacteria. When the external concentration of MTX was increased from $1 \times 10^{-7} \text{M}$ to $1 \times 10^{-6} \text{M}$, uptake became saturated and was insensitive to ionophores. However, when external MTX concentrations were increased to $1 \times 10^{-5} \text{M}$, uptake increased linearly, and was inhibited by the ionophores carbonyl cyanide m-chlorophenylhydrazone (CCCP) and valinomycin, suggesting that the process was energized by the proton motive force ($\Delta p$) at this concentration. A model for MTX entry in *S. pneumoniae* is proposed with respect to these results.

The high level of resistance to MTX of the nonsense mutant *amiA9* cannot be entirely explained by a decrease in MTX uptake.

INTRODUCTION

Interest in folate antimetabolites derives from their use as chemotherapeutics and as tools for studying reactions involved in cellular metabolism of folate and its derivatives. Inhibitory and toxic effects of these compounds, principally methotrexate (MTX) and aminopterin, are well documented in micro-organisms and mammalian cells in tissue culture. Resistance to folate analogues, which has been extensively described in bacterial and mammalian systems, is generally associated with mutations affecting the target enzyme dihydrofolate reductase, thymidilate synthetase or the transport of the antimetabolite (for review see Blakley, 1969). In L1210 leukaemia cells, substrate competition studies indicate that two separate transport systems are involved in the uptake of folate compounds (Huennekens *et al.*, 1978). The folate prototrophic bacterium *Lactobacillus casei* utilizes a single system for the transport of a variety of folate derivatives (Huennekens *et al.*, 1978) and it is likely that MTX is accumulated via the same system as folate (Cooper, 1970; Henderson & Huennekens, 1974). This could account for the high sensitivity of *L. casei* to the antifolates. The folate prototrophic bacterium *Streptococcus pneumoniae* is also highly sensitive to the antifolates MTX and aminopterin (Ephrussi-Taylor *et al.*, 1965). Resistant strains bearing mutations in the genes coding for dihydrofolate reductase (Sirotnak *et al.*, 1964) or thymidilate synthetase (Friedman & Ravin, 1972) have been isolated. Other resistant strains, like the *amiA* mutants (Ephrussi-Taylor *et al.*, 1965), have a lower transmembrane potential ($\Delta \psi$) (Trombe *et al.*, 1984), exhibit a wild-type like dihydrofolate reductase (Trombe & Sicard, 1975) and do not possess any detoxifying activity against the antifolates (Trombe, 1972). MTX inhibition is not overcome by related chemicals such as folate, folinic acid, thymine, thiamin, methylcytosine, methyladenine or a mixture of thymidine, glycine and methyladenine even when their concentrations in the growth medium are 20 times higher.

Abbreviations: MTX, methotrexate; CCCP, carbonyl cyanide m-chlorophenylhydrazone.
higher than the MTX concentration (Trombe, 1972). The amiA mutants are resistant to MTX concentrations 10- to 20-fold higher than the concentration toxic for the wild-type strain (Ephrussi-Taylor et al., 1965). Previous reports suggest that in S. pneumoniae, MTX shares the folate uptake system (Sirotnak et al., 1967a) and that the amiA mutants (Trombe & Sicard, 1973) are altered in the affinity of the folate transporter for MTX (Sirotnak et al., 1967b).

In order to understand the relationship between the decrease of Δψ and the loss of affinity of the folate transporter for MTX in the amiA mutants, we have analysed the uptake of MTX in a wild-type strain and in an isogenic strain bearing the nonsense amiA9 mutation lying at the beginning of the locus (Gasc et al., 1979). The results presented show that entry of MTX into S. pneumoniae is a complex process that is energy and temperature dependent. No evidence was obtained for a folate transporter-mediated uptake of MTX. Moreover, MTX resistance of the amiA9 strain cannot be entirely explained by a decrease in MTX uptake.

METHODS

Media and organisms. The MTX-sensitive strain of Streptococcus pneumoniae C13 was derived from the strain R36A (Tiraby et al., 1975). It is auxotrophic for valine, leucine, isoleucine, arginine, asparagine, histidine, glutamine and uracil but does not require folic acid for its growth in synthetic medium (Sicard, 1964). Bacterial growth is inhibited by 1 × 10−6 M-MTX in peptone/yeast extract medium and by 4 × 10−5 M-MTX in synthetic medium (Trombe, 1972); hence, resistant mutants were selected on plates containing 1 × 10−5 M-MTX (Sicard, 1964). The amiA9 mutant used in this study carries a nonsense mutation (Gasc et al., 1979).

Preparation of the bacteria for transport assays. Bacteria were grown to exponential phase (OD550 0.7) in a peptone/yeast extract medium (Trombe & Sicard, 1975). Cells were harvested by centrifugation at 4 °C, washed and resuspended to about 3 mg protein ml−1 in the buffer used in the uptake experiments (see below). The cell density was routinely measured at 550 nm; 1 ml culture at an OD550 of 1.0 contained 0.3 mg protein and the intracellular water space was estimated as 0.96 µl (Sirotnak et al., 1967a).

Transport assay. Unless otherwise mentioned, bacteria were diluted to 0.6 to 2 mg protein ml−1 in salt-buffered medium (Sicard, 1964) containing: 0.5% NaCl, 0.2% NH4Cl, 0.04% KCl, 12% Na2HPO4 and 0.48% Trizma base pH 7.55 (Sigma). They were allowed to glycolyse at 20 °C for 2 min before [3H]MTX was added to start the reaction. Uptake was stopped at the desired time by diluting 0.5 ml of the reaction mixture into 2 ml ice-cold salt-buffered medium. The samples were then centrifuged in the cold for 5 min at 30000 g. The cells were resuspended in 2 ml salt-buffered medium, filtered through 0.45 µm nitrocellulose filters (Millipore HA) and washed with 2 ml salt-buffered medium. Filters were then dried and the radioactivity determined (Trombe et al., 1984).

Zero time points were obtained by diluting the bacteria 100-fold in the salt-buffered medium at 4 °C. [3H]MTX was then added and the sample was treated as above. Centrifugation of the bacteria before filtration has the advantage of giving low blank values without any loss of the radioactivity retained by the cells. Results are expressed as pmoles (mg protein)−1.

ATP deprivation. Streptococcus pneumoniae relies upon glycolysis as a source of energy. Therefore sodium arsenate, an analogue of phosphate, was used to deplete the cells of ATP. The bacteria were suspended in salt-buffered medium in which phosphate was substituted by arsenate were allowed to glycolyze at 20 °C for 20 min. MTX uptake was then measured as above except that arsenate was present instead of phosphate in all the media.

The control was obtained with bacteria treated in parallel in phosphate-containing medium.

Treatment with ionophores. The protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) (1 × 10−3 M) or the K+ ionophore valinomycin (1 × 10−5 M) were added to the bacterial suspension. After 15 min, transport was measured as above. Inhibition was calculated by comparison with a control experiment using non-treated bacteria.

Chemicals. Aminopterin (4-aminofolic acid) was obtained from Sigma and folinic acid (5-formyl-5,6,7,8-tetrahydrofolic acid) from K & K Laboratories, Plainview, NY, USA. Methotrexate (4- amino-10-methylfolic acid) was kindly provided by Specia Laboratories, 16 rue Clisson, Paris Cedex 13, France. [3',5',7-3H]methotrexate sodium salt, 98% radiochemically pure, was purchased from Amersham. The other chemicals were of analytical grade.

RESULTS

Specificity of MTX uptake in S. pneumoniae

The specificity of the MTX uptake system was verified by competition tests where [3H]MTX uptake was measured in the presence of different chemicals. Among the chemicals tested, only aminopterin, another folate analogue, strongly inhibited MTX uptake (Table 1). The other related folates, folic acid and folinic acid, were as poor inhibitors as unrelated compounds such
MTX uptake by folate prototrophic bacteria

Fig. 1. [3H]MTX uptake in the wild-type and amiA9 mutant strains. [3H]MTX (1 μM, 1613 c.p.m. pmol⁻¹) uptake was measured at 20 °C (●) and at 4 °C (□) for the wild-type strain and at 20 °C for the mutant strain (○).

Table 1. Effect of some folate derivatives and amino acids on [3H]MTX uptake

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Percentage inhibition of MTX uptake</th>
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<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Aminopterin</td>
<td>92</td>
</tr>
<tr>
<td>Folic acid</td>
<td>19</td>
</tr>
<tr>
<td>Folinic acid</td>
<td>20</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>20</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>4</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>0</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>28</td>
</tr>
</tbody>
</table>

as leucine or isoleucine. This suggests that aminopterin and MTX share the same uptake route in S. pneumoniae. Moreover, if the antifolates were transported via a natural folate transporter, the affinity of this transporter for MTX was much higher than for the natural folates tested. It is possible that competition for MTX entry by folate, leucine or isoleucine occurred at the energetic level rather than as competition for a site on a transporter.

Time course of [3H]MTX uptake in the wild-type and the amiA9 strain

MTX uptake was strongly temperature dependent between 4 °C and 20 °C (Fig. 1). The initial rate of uptake at 20 °C was 1.18 pmol h⁻¹ (mg protein)⁻¹ for both wild-type and amiA9 strains. The plateau was reached after 1 h incubation. The amount of MTX retained by the bacteria was then equivalent to 0.9 pmol (mg protein)⁻¹ for the wild-type strain and 0.6 pmol (mg protein)⁻¹ for the mutant (Fig. 1). These values were equivalent to MTX intracellular concentrations of 3 × 10⁻⁷ M and 2 × 10⁻⁷ M respectively. Consequently, no accumulation of MTX inside the bacteria could be observed under our experimental conditions.

Variation of MTX uptake with the external concentration

MTX uptake as a function of the external concentration measured at 20 °C and 37 °C presented a biphasic profile (Fig. 2), suggesting that two types of mechanism were involved in MTX entry in S. pneumoniae, the first becoming saturated at a low external MTX concentration, and the second not saturated up to an external MTX concentration of 25 μM. Both processes were enhanced at 37 °C when compared to 20 °C (Fig. 2). Again, however, no accumulation against the concentration gradient could be measured in the range of concentrations studied.
Fig. 2. [$^3$H]MTX uptake at increasing external concentrations of MTX in the wild-type strain at 20 °C (●) and 37 °C (○). [$^3$H]MTX uptake was measured after 30 min incubation. The radioactivity content of the uptake media was adjusted to 1300 c.p.m. µl⁻¹.

Fig. 3. [$^3$H]MTX uptake at increasing external MTX concentrations in the wild-type strain (●) and the amiA9 mutant strain (○) at 37 °C. [$^3$H]MTX uptake was measured after 30 min incubation. The radioactivity content of the uptake media was adjusted to 1300 c.p.m. µl⁻¹.

Table 2. Effect of energetic inhibitors on MTX uptake in the wild-type and amiA9 mutant strains

Bacteria were incubated for 15 min at room temperature in uptake medium containing $2 \times 10^{-2}$ M-arsenate, $1 \times 10^{-6}$ M-valinomycin or $1 \times 10^{-3}$ M-CCCP. Uptake was initiated by addition of [$^3$H]MTX at the required concentration and was measured after 60 min. Inhibition was calculated by reference to control experiments performed in parallel but without the addition of the inhibitors. Uptake in control experiments with the wild-type strain was equivalent to 0.6 pmol µl⁻¹ when the external MTX concentration was $1 \times 10^{-6}$ M and 75 pmol µl⁻¹ when the external MTX concentration was $1 \times 10^{-4}$ M; for the mutant strain the values were 0.4 pmol µl⁻¹ and 50 pmol µl⁻¹ respectively.

<table>
<thead>
<tr>
<th>Percentage inhibition</th>
<th>1 $\times$ $10^{-6}$ M-MTX</th>
<th>1 $\times$ $10^{-4}$ M-MTX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>amiA9</td>
</tr>
<tr>
<td>Arsenate</td>
<td>35</td>
<td>25</td>
</tr>
<tr>
<td>Valinomycin</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>CCCP</td>
<td>0.1</td>
<td>0</td>
</tr>
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The response was similar for the mutant strain, but the level of MTX retained was slightly lower than for the wild-type strain (Fig. 3). Therefore, the nonsense amiA9 mutation reduced both steps of MTX uptake but did not lead to the loss of one of them. On the other hand, the small reduction of MTX uptake in the amiA9 strain cannot entirely account for its high level of resistance (10 to 20 times higher than the wild-type strain).

Energetics of [$^3$H]MTX uptake

Streptococci rely upon glycolysis as a source of energy. The protonmotive force derives mainly from the hydrolysis of ATP by the membrane F1, F0 ATPase (Harold, 1977). Consequently, depletion of the intracellular pool of ATP by the phosphate analogue arsenate results in a reduction of the primary and secondary uptake processes (Trombe et al., 1984). On the other hand, the K⁺ ionophore valinomycin and the protonophore CCCP will specifically inhibit those processes requiring the protonmotive force. When the ATP pool of the bacteria was decreased by arsenate treatment to 25% of its control value (see Methods), the amount of MTX retained by the wild-type and the mutant strains were lower than in the control, whatever the external concentration (i.e $1 \times 10^{-6}$ M or $1 \times 10^{-4}$ M) (Table 2). In contrast, valinomycin and CCCP
inhibited MTX uptake at an external concentration of $1 \times 10^{-4}$ M but were without any effect when the external MTX concentration was $1 \times 10^{-6}$ M (Table 2). The biphasic aspect of the plots in Fig. 2 suggests the coexistence of two energetically distinct mechanisms involved in MTX uptake in *S. pneumoniae*; if this is so, neither of them disappears in the nonsense *amiA9* mutant.

**DISCUSSION**

In *S. pneumoniae*, the uptake of the toxic antifolate MTX is poorly inhibited by folate and the natural derivative, folic acid. Therefore, if MTX uptake occurs via a natural folate transporter, it is likely that the affinity of this transporter for MTX is greater than for folate. These results disagree with earlier reports by Sirotnak et al. (1967a, b), which suggested that there is an active, specific and saturable transporter for folate that is shared by MTX in *S. pneumoniae*. Sirotnak et al. (1967b) thought that some *amiA* mutations (Trombe & Sicard, 1973) led to a decreased affinity of the transporter for MTX. Wild-type strain differences might account for these discrepancies. Indeed Sirotnak's wild-type strain was tenfold more sensitive to MTX than the one used in the present study. Therefore, a folate transporter responsible for MTX uptake might exist in the former strains. Such differences might exhibit side effects on the expression of the *amiA* mutation (Trombe et al., 1984). In any case, with the strains used in this study, the decrease in MTX entry in the *amiA9* mutant cannot account entirely for its level of MTX resistance.

On the other hand, the duality of the energetic dependence of MTX entry (Table 2) and the biphasic profile of the curves (Figs 2, 3) could be explained if two mechanisms with different energy requirements and characteristics were involved in MTX uptake, the first relying upon ATP alone, and the second relying upon the protonmotive force ($\Delta p$). One possibility is that both mechanisms exist and function in parallel at high extracellular concentrations of MTX. Another possibility is that the arsenate-sensitive step is necessary and precedes the step that is dependent on the protonmotive force (sensitive to ionophores). It is possible that the MTX molecules are first bound to specific sites in the membrane at low extracellular MTX concentration by an ATP-dependent mechanism; this would correspond to the saturable part of the plot in Fig. 2. At this stage the MTX molecules may be protonated if the local pH is low enough (Goldman et al., 1968). Indeed the membrane of *S. pneumoniae* contains acidic phospholipids (Trombe et al., 1979). Once protonated, the MTX molecules might cross the membrane in response to $\Delta p$; this step would correspond to the linear part of the plot in Fig. 2. In *amiA9* both processes were reduced somewhat but neither disappeared. Since *amiA9* bears a nonsense mutation lying at the beginning of the gene (Gasc et al., 1979), it is unlikely that the *amiA* gene is the structural gene of a MTX transporter. One possibility is that *amiA9* defines a new target for MTX in *S. pneumoniae*. The function of the *amiA* gene is, in addition, involved in the establishment of the transmembrane potential ($\Delta\psi$) (Trombe et al., 1984).

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**REFERENCES**


