We characterized a highly purified preparation of the chromosomally encoded dihydrofolate reductase (DHFR) from a trimethoprim-susceptible (Tmp\(^s\); strain MAP) and two trimethoprim-resistant (Tmp\(^r\)) strains (MAP/47 and MAP/42) of *Haemophilus influenzae*. The enzymes were purified between 650- and 3000-fold by gel-filtration and dye-ligand chromatography. The apparent molecular mass of the three proteins was 18400 Da by PAGE under denaturing and non-denaturing conditions. Total enzyme activity was greater in all fractions from the Tmp\(^r\) strains compared with the Tmp\(^s\) isolate. The three enzymes had a similar \(K_m\) for dihydrofolate (7, 9 and 5 \(\mu\)M) and NADPH (2, 5 and 6 \(\mu\)M). However, the Tmp IC\(_{50}\) (the concentration necessary for 50\% inhibition of DHFR activity) for the Tmp\(^s\) strain MAP was 0.001 \(\mu\)M, whereas DHFR from the Tmp\(^r\) strains MAP/47 and MAP/42 had values of 0.1 \(\mu\)M and 0.3 \(\mu\)M respectively. The methotrexate IC\(_{50}\) of the MAP/42 DHFR was 0.06 \(\mu\)M in comparison with the enzyme from MAP (0.008 \(\mu\)M) and MAP/47 (0.007 \(\mu\)M). Isoelectric focusing indicated that the DHFR from MAP/42 had a different isoelectric point (pI 7.6) compared with the enzymes from MAP and MAP/47 (pI 7.3). Peptide mapping after digestion with trypsin revealed one major peptide fragment (7.9 kDa) in the DHFR of MAP and MAP/47 and three major tryptic fragments (7.9, 9.6 and 12.5 kDa) in DHFR from MAP/42. We conclude that trimethoprim resistance in *H. influenzae* results from overproduction of structurally altered DHFR(s).

**INTRODUCTION**

Bacterial resistance to trimethoprim (Tmp) is commonly due to resistance plasmids which encode (a) dihydrofolate reductase(s) (DHFR(s)) with lower affinity for Tmp. There are six classes of R-plasmid-encoded DHFRs in Gram-negative micro-organisms (Sköld & Widh, 1974; Pattishall et al., 1976; Fling et al., 1982; Young & Amyes, 1986; Sundstrom et al., 1987; Wylie et al., 1988) and one class in Gram-positive bacteria (Archer et al., 1986). Additional mechanisms of resistance include chromosomal mutation leading to overproduction of DHFR (Smith & Calvo, 1982; Flensburg & Sköld, 1987), a decreased outer-membrane permeability to Tmp (Werner & Goeth, 1984; Gutmann et al., 1985) or thymine auxotrophy (Maskell et al., 1977).

DHFRs from several bacteria, e.g. *Neisseria gonorrhoeae*, *Streptococcus faecium*, *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus pneumoniae*, have been partially purified and characterized (Nixon & Blakley, 1968; Sirotnak et al., 1969; Novak et al., 1983; Baccanari et al., 1984; Young & Amyes, 1986). The amino acid sequence of these enzymes shows considerable inter- and intra-species differences (Smith & Calvo, 1980; Fling & Richards, 1983; Baccanari et al., 1984), even though they may possess common kinetic properties.

We previously reported that Tmp resistance in *Haemophilus influenzae* was chromosomally mediated and associated with increased DHFR activity in sonicated cells (de Groot et al., 1988). We describe here the partial purification and properties of DHFR from three strains, two of which are trimethoprim-resistant. All are isogenic, except for the Tmp\(^r\) determinant. All three enzymes have a molecular mass of approx. 18400 Da, but have structural and functional differences.

**MATERIALS AND METHODS**

**Bacterial strains**

Strain MAP (Catlin et al., 1972) [previously described as strain R906 (de Groot et al., 1988)] is Tmp\(^s\) [minimal inhibitory concentration (MIC) 0.5 \(\mu\)g/ml]. It was used as a recipient for transformation with DNA derived from Tmp\(^r\) strains R1047 (MIC 30 \(\mu\)g/ml) and R1042 (MIC 100 \(\mu\)g/ml) (de Groot et al., 1988). In the present study, two trimethoprim- and streptomycin-resistant transformants shown to be erythromycin-resistant were identified as MAP/1047-1 and MAP/1042-1 respectively (de Groot et al., 1988). These previously described isogenic *Haemophilus influenzae* strains were used in the present study: a Tmp\(^s\) strain [MAP (MIC 0.5 \(\mu\)g/ml)], a low-level Tmp\(^r\) strain [MAP/1047-1 (MIC 30 \(\mu\)g/ml; called MAP/47 here)] and a high-level Tmp\(^r\) strain [MAP/1042-1 (MIC 100 \(\mu\)g/ml, called MAP/42 here)].

**Media**

*Haemophilus influenzae* cells were grown in brain-heart-infusion agar or broth (Difco) supplemented with 10 \(\mu\)g of haemin chloride/ml, 10 \(\mu\)g of histidine/ml and 2 \(\mu\)g of \(\beta\)-NAD\(^+/\)ml (sBH1) (de Groot et al., 1988). Solid media were incubated overnight at 36.5 °C in 5% \(CO_2\), whereas liquid cultures were incubated at 37 °C in air and shaken at 150 rev./min.

**Materials**

Dihydrofolic acid (DHF), \(\beta\)-NADPH, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phenylmethanesulphonyl fluoride, dithiothreitol, Tmp and methotrexate were used: DHFR(R), dihydrofolate (reductase); Tmp, trimethoprin; \(s\), -susceptible; \(r\), -resistant; IC\(_{50}\), concentration necessary for 50\% inhibition of DHFR activity; MIC, minimal inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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Abbreviations used: DHF(R), dihydrofolate (reductase); Tmp, trimethoprin; \(s\), -susceptible; \(r\), -resistant; IC\(_{50}\), concentration necessary for 50\% inhibition of DHFR activity; MIC, minimal inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The antibiotic stock solution of Tmp used for determination of the 50 % inhibitory constant (IC50) was 5 mg/ml (17.24 mm) in 50 mM-HCl. The stock solution of methotrexate was 4.55 mg/10 ml of distilled water (1 mm). They were stored at 4 °C. Sephadex G-75 was supplied by Pharmacia, Uppsala, Sweden. Matrix Gel Green A, ultrafiltration cell and YM 10 membranes were purchased from Amicon Corporation (Lexington, MA, U.S.A.). Ampholines (pH 5–8) were obtained from LKB (Bromma, Sweden). Sequencing-grade trypsin was obtained from Boehringer-Mannheim (Indianapolis, IN, U.S.A.). Protein molecular-mass standards, ultrapure glycerol and (NH4)2SO4 were supplied by BRL (Gaithersburg, MD, U.S.A.). Bradford protein assay mixture was purchased from Bio-Rad (Richmond, CA, U.S.A.). All other chemicals were of reagent grade.

Enzyme purification

Cultures (4 litres) of each strain were grown aerobically overnight to about 10⁶ cells/ml in SBHI. Cells were pelleted at 10000 g and frozen at −20 °C until the subsequent steps. All subsequent steps were performed at 4 °C. Cell pellets from a total of 12 litres of culture (approx. 12 g of cells) were resuspended in 120 ml of 50 mM-sodium phosphate buffer, pH 7.4, and pelleted at 10000 g. The pellets were resuspended in 120 ml of phosphate buffer, pH 7.4, containing 1 mM-dithiothreitol, 1 mM-sodium EDTA, 50 mM-KCl and 0.1 mM-phenylmethanesulphonyl fluoride and sonicated at 20 W at 0–4 °C for 30 s ten times (5 min total) in a W370 cell disintegrator (Heat Systems Ultrasonics). After centrifugation of the sonicated cells at 10000 g for 1 h, the supernatant was collected and DNAase was added to yield a final concentration of 50 μg/ml. The solution was incubated at room temperature for 15 min and centrifuged at 10000 g for 30 min. Remaining nucleic acids were precipitated by the addition of 0.1 vol. of 5 % (w/v) streptomycin sulphate solution in 50 mM-phosphate buffer, pH 7.4.

After centrifugation the supernatant was used for (NH4)2SO4 fractionation. The fraction precipitating between 45 and 80 % saturation contained more than 90 % of the DHFR activity. The (NH4)2SO4 pellet was dissolved in 20 ml of 50 mM-phosphate buffer, pH 7.4, dialysed overnight against the same buffer and applied to a Sephadex G-75 column (75 cm × 1.25 cm). The sample was eluted with the same buffer containing 20 % (v/v) glycerol at 10 ml/h. The eluate was monitored at 280 nm, and 2 ml fractions were collected. All fractions were assayed for DHFR activity. Those with DHFR activity were pooled and applied to a Matrix Gel Green A column (2.6 cm × 40 cm) equilibrated with 50 mM-phosphate buffer. The column was washed for a minimum of 24 h with 540 ml of the same buffer: this effluent did not contain detectable DHFR activity. The samples were then eluted at 10 ml/h with the same buffer containing 20 % (v/v) ultrapure glycerol and a linear salt gradient (0–2 M-KCl). Fractions with DHFR activity were pooled and concentrated at 345 kPa (50 lb/in²) in an Amicon ultrafiltration cell with a YM-10 membrane and stored at 4 °C.

Enzyme assay

DHFR activity was measured spectrophotometrically with a Beckman model DU 6 spectrophotometer as described previously (Osborn & Huennekens, 1958). Enzyme activity was detected by measuring the DHF-dependent decrease in the absorbance at 340 nm with time at 30 °C. The standard enzyme assay contained 0.1 mmol of NADPH, 50 mM-sodium phosphate buffer, pH 7.4, and 10 μM-mercaptoethanol with or without DHF (0.1 mmol) in a final volume of 1 ml. The linear decrease in absorbance was measured during the first 30–60 s to estimate initial velocity. The decrease in absorbance was corrected for that occurring in the absence of DHF and enzyme source. One enzyme unit was defined as the amount required to reduce 1 μmol of DHF/min on the basis of a molar absorption coefficient of 12.3 × 10³ litre·mol⁻¹·cm⁻¹ (Hilcoat et al., 1967). Specific activity was expressed by enzyme units/mg of protein. Studies of enzyme kinetics were performed with DHFR eluted from the Green A matrix gel. Kinetic parameters at different concentrations of substrate, cofactor or inhibitors were obtained by three to five replicate measurements. The Kₘ for DHF and NADPH was estimated from the equation:

\[ \frac{v}{IC_{50} + [I]} = \frac{V_{max}}{[S]} \]

in which v, [S], Vmax and Kₘ are (respectively) initial velocity, substrate concentration, maximum reaction velocity and Michaelis–Menten constant. The means ± S.D. for the replicates and the P values were calculated by using the methods of Bevington (1969) and Choi (1978). The Michaelis–Menten kinetic analyses were performed by preincubating DHF and NADPH for 3 min before starting the reaction with enzyme source. IC₅₀ was determined for all three preparations using different concentrations of Tmp or methotrexate with DHF and NADPH concentration constant at 0.1 mm.

Tmp and methotrexate IC₅₀ values were calculated by using the equation:

\[ v = V_{max} \cdot \frac{[I]}{IC_{50} + [I]} \]

in which v, Vmax and [I] are (respectively) observed velocity, maximum reaction velocity and concentration of the inhibitor. IC₅₀ values were obtained after preincubation of NADPH, enzyme source and inhibitor for 3 min. The reaction was then started by adding DHF. A non-linear least-squares fit was used to determine the values of Vmax and IC₅₀.

Protein determination

Protein concentration of samples was measured as described by Bradford (1976), with BSA as the standard.

PAGE

SDS/PAGE was performed on 10–12 % acrylamide separating gels with 4 % acrylamide stacking gels, using the method of Laemmli (1970). Electrophoresis was continued at 20 °C and 50 V until the Bromophenol Blue marker migrated to the bottom of the gel. Proteins were detected by using the silver-staining technique of Wray et al. (1981). Non-denaturing continuous gel electrophoresis was performed on 10 % acrylamide slab gels described by Hames (1981). Enzyme preparations were suspended in 10 % (w/v) sucrose/0.2 % Bromophenol Blue/10 mM-Tris/HCl, pH 8.0. Samples were electrophoresed for 16 h at 140 V. DHFR activity in the gel was detected by using the system described by Hiebert et al. (1972). NADPH (8.3 mg), DHF (2.2 mg), MTT (16.5 mg) and 0.5 M-Tris/HCl, pH 7.4 (5 ml), were mixed in a total volume of 50 ml. The gels were incubated in this solution for 15 min at 37 °C, at which time blue bands were evident. These bands were cut from the gel and minced into small pieces. The minced fragments were resuspended in running buffer, subjected to SDS/12 % PAGE and silver-stained as described above to estimate molecular mass.

Isoelectric focusing

Isoelectric focusing was performed under non-denaturing conditions in a tube-gel electrophoresis system (Bio-Rad model 150A). Gels were prepared as described by Eder (1972). The
lower reservoir was filled with 0.05 M-H₄SO₄ as anode solution and the upper chamber was filled with 0.03 M-NaOH as the cathode solution. The gels were prerun at 1 mA/gel for 15 min. Enzyme preparations in 30 mM-phosphate buffer, pH 7.4, containing 10% sucrose, and 6% Ampholines (pH 5–8) were loaded, focused for 7 h and DHFR activity detected by the method of Hiebert et al. (1972) described above. The pH gradient was measured by slicing an unstained gel into 0.5 cm sections, suspending each section in 1 ml of deionized water, equilibrating at 21 °C for 1 h and measuring the pH. Bands with DHFR activity were excised from the gel, minced into small pieces, and subjected to SDS/12%-PAGE; proteins were detected by silver stain as described above.

Peptide mapping

Peptide mapping was performed as described by Cleveland et al. (1977). After Green A matrix gel chromatography, fractions with DHFR activity derived from strains MAP, MAP/47 and MAP/42 were electrophoresed on SDS/15%-PAGE minigels. The predominant 18.4 kDa band was excised from the gel after revealing protein bands with Ponceau S stain (Salinovich & Montelaro, 1986). The gel fragments were placed in 0.125 M-Tris/HCl (pH 8.0)/0.1% SDS/1 mM-EDTA and electroeluted in a Bio-Rad model-422 electroeluter using 50 mM-NH₄HCO₃ (pH 8.0)/0.1% SDS. Elution was performed at 10 mA/sample for 4 h. Each sample was freeze-dried in a Speed-Vac (Savant Instruments), and 100 mg of trypsin in 1 ml of 1 mM-HCl was added to each sample. A gel fragment not containing detectable protein was processed as a control. Digestion was continued for 18 h at 38 °C. Samples were then dried and stored at 4 °C. The tryptic digests were resuspended in 50 μl of 0.1 M-sodium phosphate, pH 7.4, and radiiodinated with Na125I by the chloramine-T procedure (McConahey & Dixon, 1980). 125I-labelled samples and molecular-mass standards were electrophoresed overnight on a SDS/15%-PAGE gel. The gel was dried after fixing in methanol/acetic acid/water (9 : 2 : 9, by vol.) for 1 h. Autoradiographs were made after 20 min, 30 min, 1 h, 4.5 h and 18 h of exposure, with a screen between gel and film to intensify the bands.

![Molecular mass (kDa)](image)

**Table 1. Purification of DHFR from isogenic H. influenzae strains**

Fractions (column 2) are identified as follows: 1, cell lysate; 2, after streptomycin sulphate treatment; 3, after (NH₄)₂SO₄ fractionation; 4, after Sephadex G-75; 5, after Green A Matrix chromatography.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fraction</th>
<th>Total enzyme activity (units *)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP</td>
<td>1</td>
<td>6016</td>
<td>2693</td>
<td>2.2</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5691</td>
<td>2537</td>
<td>2.2</td>
<td>1</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5285</td>
<td>1441</td>
<td>3.7</td>
<td>1.3</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2195</td>
<td>9</td>
<td>243.9</td>
<td>103</td>
<td>36</td>
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<tr>
<td></td>
<td>5</td>
<td>1220</td>
<td>0.2</td>
<td>697.5</td>
<td>3193</td>
<td>20</td>
</tr>
<tr>
<td>MAP/47</td>
<td>1</td>
<td>49024</td>
<td>3174</td>
<td>15.5</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>49024</td>
<td>3050</td>
<td>16.1</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>48862</td>
<td>834</td>
<td>58.6</td>
<td>3.8</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>43252</td>
<td>23</td>
<td>1880.5</td>
<td>120</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>13984</td>
<td>0.8</td>
<td>17479.6</td>
<td>1132</td>
<td>29</td>
</tr>
<tr>
<td>MAP/42</td>
<td>1</td>
<td>47642</td>
<td>2856</td>
<td>16.7</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>57480</td>
<td>2641</td>
<td>21.8</td>
<td>1.3</td>
<td>121</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>56179</td>
<td>1045</td>
<td>53.8</td>
<td>3.1</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>18211</td>
<td>40</td>
<td>455.3</td>
<td>27</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7724</td>
<td>0.9</td>
<td>8581.8</td>
<td>648</td>
<td>16</td>
</tr>
</tbody>
</table>

* μmol of tetrahydrofolate produced/min from DHF and NADPH (Hilcoat et al., 1967).

**Fig. 1. SDS/PAGE analysis of purified MAP, MAP/47 and MAP/42 DHFR**

Molecular-size standards are shown in the extreme left and right lanes. Lane A, 2.8 μg of protein from MAP/42; lane B, 2.8 μg of protein from MAP/47; and lane C, 2.8 μg of protein of partially purified DHFR from strain MAP.

**RESULTS**

**Enzyme purification**

Initial attempts at purification of DHFR were associated with loss of enzyme activity in the steps after (NH₄)₂SO₄ fractionation.
This problem was solved by the addition of 20% (v/v) glycerol during gel-filtration steps. The purification scheme for DHFR from the three strains is summarized in Table 1. These data are the average of three replicate purifications using frozen cells from a total of 12 l-litre cultures of each strain. Total enzyme activity was calculated after correction for DHF-independent NADPH oxidase activity. This activity was considerable in the first two steps of the purification procedure, but present in minute quantities after (NH₄)₂SO₄ precipitation, and not detectable after the gel-filtration steps. Total enzyme activity of the final electrophoretically pure preparations from MAP/47 and MAP/42 was 6–10-fold higher than those derived from MAP. The specific activity of DHFR derived from these strains was 8-fold the specific activity of DHFR from MAP in the initial fractions, but became similar as the enzyme was purified. Purification for the three enzymes varied between 650- to more than 3000-fold, with recovery of total activity between 16 and 29%.

Molecular mass

The molecular masses of the native enzymes were estimated by gel filtration on Sephadex G-75 in comparison with reference proteins. DHFR activity was eluted as a single activity peak corresponding to a molecular mass of approx. 18000 Da. SDS/PAGE of fractions with high specific activity indicated a protein with a relative mobility equivalent to 18.4 kDa (Fig. 1). When the same enzyme fractions were analysed by non-denaturing gel electrophoresis, we detected DHFR activity in the lanes containing extract of MAP and MAP/47, but not from MAP/42. Bands with DHFR activity were electroeluted from the gel and analysed by SDS/PAGE; each contained a single protein with a mobility equivalent to 18.4 kDa.

Isoelectric focusing

Fraction 5 derived from the strains was subjected to isoelectric focusing and DHFR activity was detected in the gel as described
DISCUSSION

above. Fraction 5 from MAP and MAP/47 had a different pI compared with MAP/42 (Fig. 2). A major band corresponding with a pI of 7.6 was seen with MAP/42, whereas a pI of 7.3 was found with the fractions from MAP and MAP/47. When fraction 3 of each strain was subjected to isoelectric focusing, a faint band with a pI of 7.1 could be seen in fractions from MAP and MAP/47. The apparent molecular mass of the fraction containing most of the DHFR activity was confirmed by elution from the isoelectric-focusing gel followed by SDS/PAGE; the protein had a relative mobility equivalent to 18.4 kDa.

Enzyme kinetics

The kinetics of fraction 5 of each DHFR were studied to determine whether functional differences existed. Kinetic parameters, including $K_m$ for DHF, $K_a$ for NADPH and $IC_{50}$ for Tmp and methotrexate are summarized in Table 2 and depicted in Fig. 3. Statistical analysis using the Aspen–Welch test (Choi, 1978) showed significant differences in $K_m$ for DHF between MAP and MAP/42 ($P < 0.05$), whereas those between MAP and MAP/42 were not ($P > 0.1$). In addition, significant differences in $K_m$ for NADPH were found between MAP and MAP/47 ($P < 0.05$) and MAP and MAP/42 ($P < 0.025$). However, $K_m$ values for DHF and NADPH showed less than a 3-fold difference between the three strains. By contrast, approx. 100- and 200-fold differences were measured between the Tmp $IC_{50}$ values of MAP and MAP/47 ($P < 0.0005$), and MAP and MAP/42 ($P < 0.005$) respectively. In addition, a 9-fold difference was found between the methotrexate $IC_{50}$ values of MAP and MAP/47 ($P < 0.0005$), whereas no significant difference was observed between the methotrexate $IC_{50}$ values of MAP and MAP/47.

Peptide mapping

Proteolytic digestion of fraction 5 from the three strains resulted in the generation of one major peptide of 7.9 kDa in DHFR from strains MAP and MAP/47 and three major peptides (7.9, 9.6 and 12.5 kDa) from strain MAP/42. In addition, an 18.4 kDa band and a 22 kDa trypsin band were seen on the autoradiographs.

Table 2. Comparison of kinetic properties of various H. influenzae DHFRs

<table>
<thead>
<tr>
<th>Strain</th>
<th>$K_m$ ($\mu M$) for:</th>
<th>$IC_{50}$ ($\mu M$) for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DHF</td>
<td>NADPH</td>
</tr>
<tr>
<td>MAP</td>
<td>6.71 ± 1.14</td>
<td>2.01 ± 0.19</td>
</tr>
<tr>
<td>MAP/47</td>
<td>8.60 ± 0.64</td>
<td>5.05 ± 0.85</td>
</tr>
<tr>
<td>MAP/42</td>
<td>4.75 ± 0.81</td>
<td>6.00 ± 0.68</td>
</tr>
</tbody>
</table>

The $K_m$ and $V_{max}$ for DHF and NADPH of the three DHFRs varied by 2–3-fold, a difference we considered to be physiologically unimportant. In contrast, the Tmp $IC_{50}$ for the enzymes from MAP/47 and MAP/42 Tmp$^a$ strains was 100- and 200-fold higher than the Tmp $IC_{50}$ for the DHFR from Tmp$^a$ MAP. The methotrexate $IC_{50}$ for MAP/42 was approx. 10-fold higher than the Tmp $IC_{50}$ values for MAP and MAP/47. These data suggest that DHFR resistance in H. influenzae is caused not only by overproduction of DHFR, but also by changes in enzyme structure, resulting in a lower affinity of DHFR for antifolates. Comparison of the kinetic parameters from a variety of chromosomally and plasmid-encoded DHFRs (Table 3) shows that the values of $H. influenzae$ DHFR $K_m$ for DHF and NADPH are similar to those found with chromosomally and plasmid-mediated DHFR in E. coli and N. gonorrhoeae. Similarly, the Tmp $IC_{50}$ of $H. influenzae$ is in the same range as Tmp$^a$ E. coli. The Tmp $IC_{50}$ values for DHFR derived from Tmp$^a$ H. influenzae are intermediate between those from Tmp$^a$ E. coli DHFR and that of the plasmid-encoded DHFR from Tmp$^a$ E. coli. This is not surprising in view of the relatively low Tmp MICs of Tmp$^a$ H. influenzae strains (30 µg/ml and 100 µg/ml) compared with the higher MICs (>1000 µg/ml) of certain plasmid-encoded enzymes from Tmp$^a$ E. coli.

The apparent molecular mass of all these $H. influenzae$ enzymes was 18000 Da. This is similar to the molecular mass of chromosomally and plasmid-mediated DHFR derived from other Gram-positive and Gram-negative micro-organisms (Pattishall et al., 1976; Tennhammar-Ekman & Sköld, 1979; Baccanari et al., 1984; Joyner et al., 1984). Isoelectric focusing confirmed the presence of two DHFR bands, one corresponding to the pI of fraction 2 (MAP/42), which is the same as those seen in other Gram-positive and Gram-negative micro-organisms. Alternatively, the endogenous DHFR might be detected. The presence of structural differences between DHFRs from MAP/42 and the other strains was supported by the peptide maps. Only one major tryptic peptide fragment was seen in DHFRs

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Table 3. Michaelis–Menten and inhibition constants of DHFRs in various micro-organisms

<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>Strain</th>
<th>Source</th>
<th>MIC (µg/ml)</th>
<th>Kₘ (µM) for:</th>
<th>IC₅₀ (µM) for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>DHF</td>
<td>NADPH</td>
<td>Tmp</td>
</tr>
<tr>
<td><em>H. influenzae</em></td>
<td>MAP</td>
<td>Chromosome</td>
<td>0.5</td>
<td>6.7</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>MAP/47</td>
<td>Chromosome</td>
<td>30</td>
<td>8.6</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>MAP/42</td>
<td>Chromosome</td>
<td>100</td>
<td>4.8</td>
<td>6.1</td>
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<td><em>E. coli</em></td>
<td>J5</td>
<td>Chromosome</td>
<td>0.2</td>
<td>1.2</td>
<td>7.0</td>
</tr>
<tr>
<td>R67</td>
<td>Plasmid</td>
<td>&gt;1000</td>
<td>5.6</td>
<td>12.3-17.3</td>
<td>3.8-57</td>
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<tr>
<td>pAZ1</td>
<td>Plasmid</td>
<td>64</td>
<td>0.4</td>
<td>N.D.</td>
<td>2.1</td>
</tr>
<tr>
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<td>Plasmid</td>
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<td>37</td>
<td>N.D.</td>
<td>0.2</td>
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<tr>
<td><em>N. gonorrhoeae</em></td>
<td>F62</td>
<td>Chromosome</td>
<td>48</td>
<td>2.1</td>
<td>13</td>
</tr>
<tr>
<td>T47</td>
<td>Chromosome</td>
<td>1200</td>
<td>26</td>
<td>10</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

from MAP and MAP/47, whereas three major fragments were observed in DHFR from MAP/42.

Using DHFR preparations purified 600-3000-fold, we found structural and functional differences between the DHFR of the three strains. We suggest that the genetic basis for these changes may be similar to those encountered in regulatory mutants of DHFR in *E. coli* K12 (Sheldon, 1977). Studies by Smith & Calvo (1980) revealed that the genetic basis of Tmp⁺ induced by mutagenesis was a mutation in the fol gene, resulting in a DHFR with lower affinity for Tmp, and a second mutation in the promoter, resulting in an increased synthesis of DHFR-specific mRNA.

REFERENCES