Mechanism of R Factor-mediated Chloramphenicol Resistance

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The results of early studies on the mode of expression of the resistance transfer factor (RTF) in enteric bacteria were summarized by T. Watanabe (Bacteriol. Rev. 27:87, 1963). The biochemical mechanism of R factor-mediated resistance to chloramphenicol was attributed to a decreased permeability of resistant cells to the antibiotic in view of (i) inconclusive evidence of antibacterial inactivation, and (ii) the experiments of S. Okamoto and D. Mizuno (J. Gen. Microbiol. 35:125, 1964), which suggested that the cell-free synthesis of polypeptide in R factor strains was fully sensitive to chloramphenicol. The first direct evidence for an apparent permeability barrier to chloramphenicol was the observation (J. Unowsky and M. Rachmeler, J. Bacteriol. 92:358, 1966) that a three- to fourfold greater uptake of 14C-chloramphenicol occurred with chloramphenicol-sensitive (R−) Escherichia coli as compared with resistant (R+) cells. Similar findings were obtained (W. V. Shaw, unpublished data) by similar techniques.

S. Okamoto and Y. Suzuki (Nature 208:1301, 1965) demonstrated, however, that R+ strains of E. coli with the chloramphenicol resistance determinant were able to inactivate the antibiotic in the presence of acetyl-coenzyme A (acytly-CoA). Subsequently, it has been shown conclusively that chloramphenicol-resistant R factor strains of E. coli inactivate the antibiotic by enzymatic acetylation to form the 3-acetyl and 1,3-diacyt derivatives (W. V. Shaw, J. Biol. Chem. 242:687, 1967; Y. Suzuki and S. Okamoto, J. Biol. Chem. 242:4722, 1967). More recent studies (W. V. Shaw and R. F. Brodsky, Antimicrobial Agents and Chemotherapy—1967, in press) have shown that 19 consecutive clinical isolates of enteric bacteria with an R factor for chloramphenicol resistance contain the enzyme chloramphenicol acetyltransferase.

The present collaborative studies were undertaken to determine whether the acetylation mechanism of resistance might account for the apparent permeability defect. For this work, the original cultures of R+ and R− E. coli K-12 employed in the permeability studies were used, namely, CSH-2 (F−) and the same strain harboring the R factor 222 (R+) with resistance determinants for chloramphenicol, tetracycline, streptomycin, and sulfonamides. Figure 1 shows the results of thin-layer chromatography of the products resulting from incubation of 14C-chloramphenicol with acetyl-CoA and crude cell extracts prepared from both strains as previously described (W. V. Shaw, J. Biol. Chem. 244:687, 1967). Both the mono- and diacytlated forms of chloramphenicol resulted from incubation of the extract from the R+ chloramphenicol-resistant strain, but not with that from the R− organism. Using a sensitive spectrophotometric assay (W. V. Shaw and R. F. Brodsky, J. Bacteriol. 95:28, 1968), we found that the R+ extract contained 0.9 unit per mg (protein) of chloramphenicol acetyltransferase, a specific activity comparable to that seen with other R+ cultures. It seems clear, therefore, that bacteria with the apparent permeability defect are capable of acetylating chloramphenicol.

A striking positive correlation has been demonstrated between the antibacterial activity of the resistant bacterial culture and the extent to which they are accumulated by bacterial cell suspensions (D. Vazquez, Biochim. Biophys. Acta 114:277, 1966). Further studies by D. Vazquez (Biochim. Biophys. Acta 114:289, 1966) have shown that inactive analogues show little affinity for the bacterial ribosome and are ineffective as inhibitors of protein synthesis. On the basis of the finding of chloramphenicol acetyltransferase in 222/CSH-2 and the work of Vazquez, it was postulated that the reported impermeability of strain 222/CSH-2 is due to failure of chloramphenicol-3-acetate to bind to the bacterial ribosomes, resulting in no net accumulation of radioactivity within resistant cells, as compared with the sensitive cells which lack the acetylating enzyme. To test this hypothesis, ribosomes from R− E. coli were prepared and incubated with
radioactive chloramphenicol or chloramphenicol-3-acetate. The latter was prepared by incubating chloramphenicol-3-14C (Nuclear-Chicago) with acetyl-CoA (P.L. Laboratories) and crude cell-free extract containing chloramphenicol acetyltransferase, followed by ethyl acetate extraction and purification of the product by thin-layer chromatography as described (W. V. Shaw, J. Biol. Chem. 242:687, 1967). The results (Table 1) show a 20-fold decrease in the affinity of *E. coli* ribosomes for the acetylated product, a finding compatible with the latter's ineffectiveness as an antibiotic and with the permeability data cited above.

When considered in the light of information currently available, the enzymatic data for strain 222/CSH-2 and the ribosomal binding studies support the hypothesis that the primary biochemical event related to the R factor type of chloramphenicol resistance is acetylation of the antibiotic. The evidence suggests that the apparent perme-

**Table 1. Binding of chloramphenicol and chloramphenicol-3-acetate to Escherichia coli ribosomes**

<table>
<thead>
<tr>
<th>Radioactive compound</th>
<th>14C in ribosomal pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>9,500</td>
</tr>
<tr>
<td>Chloramphenicol-3-acetate</td>
<td>460</td>
</tr>
</tbody>
</table>

*Ribosomes from R* E. coli* were prepared by sonic disruption of 25 g of bacterial cells in 100 ml of buffer containing 0.05 M Tris (pH 7.8), 0.02 M magnesium acetate, and 0.06 M KCl. The supernatant fluid was centrifuged at 30,000 × g for 30 min, and the pellet was discarded. The ribosomal pellet obtained after centrifugation at 100,000 × g for 2 hr was washed twice by resuspension in 6 ml of buffer followed by repeated 100,000 × g centrifugation. The binding experiments were conducted by adding 14C-chloramphenicol or 14C-chloramphenicol-3-acetate (5 μM; specific activity, 5 μCi/μmole) to 6.2 ml of the ribosomal buffer solution (65 optical density units at 260 μm). After incubation for 10 min at 4°C, the samples were centrifuged at 100,000 × g for 2 hr. The ribosomal pellet obtained in each instance was resuspended in 1.0 ml of buffer, and a sample was taken for scintillation counting in Bray's solution. A correction for radioactivity trapped in the pellet was made by subtracting the pellet radioactivity in control tubes to which nonradioactive chloramphenicol or chloramphenicol-3-acetate was added at a concentration of 0.3 mM (100-fold excess). The radioactivity attributed to trapping was 700 and 420 counts/min for chloramphenicol and the 3-acetate derivative, respectively. The radioactivity extracted from ribosomal pellet after incubation with chloramphenicol-3-14C was chromatographically indistinguishable from authentic chloramphenicol as determined by the techniques described in Fig. 1.

**Fig. 1. Chloramphenicol acetylation by a cell extract of chloramphenicol-resistant Escherichia coli (222/CSH-2).** Each incubation mixture contained 0.02 mM chloramphenicol-3-14C (5 μCi/μmole), 0.1 mM acetyl-CoA, and cell-free extract (0.2 mg of protein) in the presence of 100 mM Tris(hydroxymethyl)aminomethane (Tris)-chloride (pH 7.8) in a final volume of 1.0 ml. After 10 min of incubation, the reaction mixtures were extracted with ethyl acetate and chromatographed in the chloroform-methanol system described previously (W. V. Shaw, J. Biol. Chem. 242:687, 1967). The radioautograph depicts the formation of the 1-acetyl (A), 3-acetyl (B), and 1,3-diacyl (C) derivatives of chloramphenicol (CM) in the incubation containing extract from R* E. coli*. The identity of the radioactive products observed with the R* extract was confirmed by the coincidence of each of the radioactive areas with the spots seen when the thin-layer sheet was observed under ultraviolet light. Authentic nonradioactive reference compounds (0.1 μmole each of the acetyl derivatives) were added to a 0.1 volume of the radioactive extract prior to chromatography.
ability defect observed may be a secondary phenomenon due to a failure of the acetylated antibiotic to bind to bacterial ribosomes.

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