Characterization of Chloramphenicol Acetyltransferase from Chloramphenicol-resistant Staphylococcus aureus

W. V. SHAW AND R. F. BRODSKY

Department of Medicine, Columbia University College of Physicians and Surgeons, New York, New York 10032

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Chloramphenicol-resistant strains of Staphylococcus aureus contain an inducible enzyme which inactivates chloramphenicol by acetylation in the presence of acetyl coenzyme A. The products of acetylation are chromatographically indistinguishable from those obtained with chloramphenicol-resistant Escherichia coli carrying an R factor. The kinetics of induction of chloramphenicol acetyltransferase are complicated by the inducer's effect on protein biosynthesis and its fate as chloramphenicol 3-acetate, which is not an inducer of the enzyme. The E. coli and S. aureus enzymes have been compared, with the conclusion that they are identical with respect to molecular weight (approximately 78,000) and pH optimum (7.8), but differ with respect to heat stability, substrate affinity, electrophoretic mobility, and immunological reactivity. Antiserum prepared against enzyme from E. coli contains precipitating antibody, which inactivates the E. coli enzyme, but neither precipitates nor neutralizes the activity of S. aureus enzyme.

Evidence accumulated in recent years suggests that strains of Staphylococcus aureus which are resistant to chloramphenicol (chl-r) owe their resistance to inactivation of the antibiotic (5, 13). More recent studies by Suzuki, Okamoto, and Kono (20) indicate that acetyl coenzyme A (CoA) is required for inactivation of chloramphenicol by extracts of resistant S. aureus, and that the primary product is likely to be a monoacetyl derivative. Their experiments were prompted by the earlier observations of Okamoto and Suzuki (14) that RTF (R factor) containing strains of Escherichia coli carrying the chl-r determinant contain a chloramphenicol-inactivating enzyme. More recent studies (17, 18) have identified the major products of chloramphenicol acetylation as the 3-acetoxy and 1,3-diace- toxy derivatives of chloramphenicol, and have characterized the enzyme responsible for acetylation. In view of the likely similarities between the mechanisms of inactivation by S. aureus and R+ enteric bacteria, and in view of the recent reports of S. Mitsuhashi (12) that all chl-r strains of S. aureus isolated in that laboratory inactivated chloramphenicol, it seemed of interest to compare the acetylaing enzyme prepared from chl-r S. aureus and from an R+ strain of E. coli with the chl-r determinant.

Prior to the studies reported here, it was noted that, whereas the presence of acetylating enzyme (chloramphenicol acetyltransferase) was a constitutive property of chl-r E. coli harboring an R factor (14, 17), the enzyme from S. aureus appeared to be present only after growth in the presence of chloramphenicol (13, 20). An unexpected analogy between the chl-r genome in S. aureus and episomal chloramphenicol resistance in E. coli was pointed out by Chabbert et al. (2) and by Mitsuhashi (12), who reported that the chloramphenicol resistance in S. aureus was lost spontaneously and after treatment with acridines, suggesting an extrachromosomal (plasmid) location for the chl-r genome.

The present studies were aimed at comparing chloramphenicol acetyltransferase from S. aureus with that from E. coli in regard to: control of synthesis of the enzyme, substrate specificity, pH optima, heat stability, electrophoretic mobility, molecular weight, and cross reactivity with antisera prepared against the purified E. coli enzyme.

MATERIALS AND METHODS

Bacterial strains. Numerous clinical isolates of chloramphenicol-resistant S. aureus have been found capable of acetyllating chloramphenicol (W. Shaw, unpublished data). The strain used for the present study was obtained from R. P. Novick. S. aureus
strain C 22.1 is a chloramphenicol-resistant trans-
ductant of *S. aureus* strain 8325. The origin of the
*chl-r* determinant was a clinical isolate of *S. aureus,
provided by Y. Chabbert. The isolate had been shown to
undergo spontaneous loss of chloramphenicol resis-
tance at a high frequency which was increased by
treatment with acriflavine (2). Similar results, sug-
gesting that the *chl-r* genome is extrachromosomal,
have been reported for chloramphenicol-resistant
strains of *S. aureus* isolated in Japan (12). The mini-
mal inhibitory concentration of chloramphenicol for
*S. aureus* C 22.1 was 50 μg per ml. The chlorampheni-
col-susceptible control for the present studies was *S.
aureus* 8325, the propagating strain for phase 47 of
the International Typing Series, which was also ob-
tained from R. P. Novick.

*E. coli* K-10 (Hfr) and *E. coli* R$_s$/K-10 designate
the *chl-s* and *chl-r* organisms, respectively, and were
described previously (17). The R$_s$ episome is a stable
segregant of a more complex R factor and contains
resistance determinants to only chloramphenicol and
tetracycline. The presence of chloramphenicol acetyl-
transferase is a constitutive property of *E. coli* R$_s$
K-10.

**Culture methods.** All growth studies were carried
out in Penassay broth (Difco) without additional
supplements. *S. aureus* was generally grown at 37 C
on a rotary shaker in 250 ml of medium, unless other-
wise specified. Chloramphenicol was added in mid-
logarithmic phase to induce such cultures, and the
bacteria were harvested in early stationary phase. *E.
coli* was grown in similar fashion, except that aerated
20-liter carboys were used to obtain sufficient bac-
terial cells for enzyme purification, and chlorampheni-
col was added at the time of inoculation to insure the
growth of a chloramphenicol-resistant population of
cells. *E. coli* was harvested in stationary phase, at
which time maximal enzyme-specific activities were
obtained.

Preparation of cell-free extracts. *E. coli* extracts
were prepared by sonic disruption and centrifugation
as described previously (17). *S. aureus* extracts were
prepared by lysis of washed cell suspensions with
lysothaphin (16). Cultures (250 ml) were harvested by
centrifugation, the pellet was washed with 50 ml of
0.05 M tris(hydroxymethyl)aminomethane (Tris)
buffer, pH 7.5, in 0.145 M NaCl and resuspended in 10
ml of the same buffer. After the addition of
lysothaphin to a final concentration of 10 units per
ml, the suspension was incubated for 15 min at 37 C.
The lysate was further solubilized by the addition of
deoxyribonuclease (final concentration 50 μg per ml)
and incubated for an additional 15 min. The clear
supernatant fluid obtained after centrifugation
(10,000 × g for 20 min) was used without further
purification.

Induction of chloramphenicol acetyltransferase in *S.
aureus*. In contrast to *E. coli* and other enteric orga-
nisms harboring R factors (18), the chloramphenicol
acyetylating enzyme is found in *S. aureus* only after the
addition of antibiotic to the medium. Chlorampheni-
col, or its congeners, were added in the mid-logarith-
mic phase at a final concentration of 0.5 mM. After
approximately 90 to 120 min, enzyme specific activity
reached a plateau and the cells were harvested. For
the experiments described in Table 2, cells were
arbitrarily harvested 90 min after the addition of
chloramphenicol and were lysed as described above.

**Spectrophotometric assay of chloramphenicol acetyl-
transferase.** Previous studies with enteric bacteria
have shown that the chloramphenicol acetylating
enzyme may be conveniently assayed by following the
chloramphenicol-dependent disappearance of acetyl
CoA in the presence of suitable enzyme (17, 18). The
original assay has been modified by analogy with sim-
ilar assays for serine acetylase (8) and thioaceta-
loside transacetylase (1) utilizing 5,5'-dithiobis-2-
nitrobenzoic acid (DTN). The reaction is followed by
observing the increase in absorbance at 412 μm,
coincident with the formation of free CoA and its re-
action with DTN to form the mixed disulfide. A molar
extinction coefficient of 13,600 for the liberated
thionitrobenzoic acid was assumed for the pur-
pes of this study (8). The reaction was carried out at 37 C
in a Gilford recording spectrophotometer with
thermostatic control. Each cuvette contained: Tris
chloride, pH 7.8 (0.1 M); acetyl CoA (0.1 mM); and
enzyme in a final volume of 1.0 ml. The reaction
was initiated by the addition of chloramphenicol (0.1 mM)
to the reference cuvette. One unit of enzyme was
taken to be the amount sufficient to acetate 1 μmole of
chloramphenicol per minute under the standard
conditions specified.

Preparation of purified chloramphenicol acetyltrans-
ferase from *E. coli* R$_s$/K-10. Bacterial cells (25 g, wet
weight) were suspended in a final volume of 100 ml of
Tris chloride, pH 7.8 (10 mM), containing β-merca-
ptoethanol (0.01 mM). After sonic disruption and cen-
trifugation as described (17), the following purification
steps were undertaken at 0 to 3 C on 100 ml of crude
extract. Streptomycin sulfate was added in a final con-
fcentration of 1%, and the precipitate was discarded
after centrifugation. Finely powdered ammonium
sulfate was added to 50% saturation. The precipitate
obtained after centrifugation was dissolved to 60 ml in
standard buffer consisting of Tris chloride, pH 7.8
(10 mM); β-mercaptoethanol (0.5 mM); and chloram-
phenicol (0.2 mM). The buffered extract was heated at
70 C for 10 min, cooled immediately, and centrifuged.
The resulting supernatant solution was brought to 50%
saturation with ammonium sulfate; the precipitate
was then dissolved in 10 ml of Tris chloride, pH 7.8
(10 mM). The latter was exchanged with pH 7.0
sodium phosphate (10 mM) by passing the extract
over a Sephadex G-25 column (2.5 × 30 cm) pre-
equilibrated with phosphate buffer. Alumina C$_y$ gel
[0.7 mg (wt weight) per mg of protein] was added to
adsorb the enzyme. After centrifugation, the gel was
washed once with 0.01 M phosphate buffer and cen-
trifuged. The adsorbed enzyme was eluted by suspend-
ing the pellet in 0.5 M sodium phosphate buffer, pH 7.0.
The supernatant fluid obtained after centrifugation
was exchanged with the standard buffer by using a
Sephadex G-25 column as noted above. The resulting
pale yellow solution was applied to a diethylamino-
etyl (cellulose) column (2.5 × 20 cm) equilibrated
with standard buffer. The enzyme was eluted at 0.19
M NaCl by using a linear sodium chloride gradient.
(from 0 to 0.4 m) prepared in standard buffer. The peak tubes were pooled and concentrated by vacuum ultrafiltration; the resultant colorless fluid was applied to a Sephadex G-100 column (2.5 × 90 cm). Gel filtration was accomplished by using standard buffer containing 0.2 m NaCl. The tubes showing peak activity were pooled and concentrated as described above. The resulting enzyme (204 units and 1.33 mg of protein) revealed a single band on disc gel electrophoresis at pH 8.8, and represented a 200-fold purification with a 10% overall yield. Throughout the purification, enzyme activity was quantitated by the spectrophotometric assay noted above. Protein was determined by the method of Lowry (10).

Preparation of antiserum to chloramphenicol acetyltransferase. The purified enzyme from E. coli Rf/K-10 was mixed with an equal volume of complete Freund’s adjuvant, and a total of 1 mg of enzyme protein was injected into the hind toe pads of a 2-kg male albino rabbit. The rabbit was bled from the ear artery 4 weeks after immunization. The resulting antiserum was found to neutralize enzyme activity when mixed with chloramphenicol acetyltransferase from E. coli Rf/K-10 (see Fig. 8). Furthermore, a single major precipitin line was obtained when antiserum was reacted with the E. coli enzyme by the agar double-diffusion technique (15). Two additional faint lines were also noted when the crude antiserum was used for such studies, but the latter were not seen when antiserum had been previously absorbed with an equal volume of crude E. coli K-10 (Rf) extract (5 mg of protein per ml) overnight at 3 C. The absorbed antiserum was centrifuged, and the supernatant fluid was used for the studies described in Fig. 7 and 8. The unabsorbed antiserum failed to react with S. aureus enzyme, as judged by the absence of precipitin lines or neutralization of enzyme activity. Normal serum from a nonimmunized rabbit failed to yield precipitin lines and did not neutralize the E. coli enzyme. All double-diffusion studies were performed with 0.7% Noble Agar (Difco) containing 0.85% sodium chloride, 2.5% glycine, and 0.85% sodium barbiturate. The pH was adjusted to 7.6 with HCl, and merthiolate (0.01%) was added to prevent bacterial growth.

Disc gel electrophoresis. Electrophoretic analysis was carried out with the Buchler apparatus using the anionic (Tris-glycine; running pH 9.3) technique described by Davis (4). Purified enzyme or crude extracts were applied to the stacking gel in a volume of 0.05 ml or less and allowed to migrate until the tracking dye reached the bottom of each tube. The gels were removed and stained for protein with 0.25% Amido Black, or for chloramphenicol acetyltransferase. The latter method was adapted from the Kredich and Tompkins (8) histochemical technique for detecting serine transacylase. Each gel was immersed in a small test tube which contained the following in a final volume of 1.0 ml: Tris chloride, pH 7.8 (100 mM); chloramphenicol (0.25 mM); acetyl CoA (0.25 mM); 0.1 mg of phenazine methosulfate (Sigma) and 1.0 mg of nitro blue tetrazolium (Sigma). Chloramphenicol was omitted from the control tubes which showed a diffuse faint background of the formazan resulting from the nonspecific cleavage of acetyl CoA. A single sharp band, which corresponded to the location of enzyme activity, appeared in tubes containing chloramphenicol (Fig. 6). The location of enzyme activity was determined independently by slicing the gel into 1-mm segments and assaying for enzyme extracted by freezing and thawing in Tris chloride, pH 7.8 (10 mM).

Sucrose gradient centrifugation. The sedimentation constants of the purified E. coli enzyme and of S. aureus strain C 22.1 were determined by the sucrose density technique of Martin and Ames (11). Linear gradients of 5 to 20% sucrose were prepared in Tris chloride, pH 7.8 (10 mM) containing 50 mM sodium chloride. The reference standards used were two times crystallized human hemoglobin (Mann), crystalline horse heart myoglobin (Mann), and yeast alcohol dehydrogenase (Worthington). Gradients of 5.0 ml were run for 18 hr at 37,000 rev/min in an SW 39 Spinco rotor. Tubes were punctured, and 10-drop fractions were collected (approximately 300 ± 5 drops).

Chemicals. Chloramphenicol and its congeners were obtained from Mildred Rebstock of Parkes, Davis and Co., Detroit, Mich.; 3-14C-chloramphenicol was from Nuclear Chicago Corp., Des Plaines, Ill.; DTN (5,5′-dithiobis-2-nitrobenzoic acid) was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis.; and CoA was from P-L Laboratories, Milwaukee, Wis. Acetyl CoA was synthesized from acetic anhydride and CoA by the method of Simon and Shemin (19). Whatman diethylaminoethyl cellulose (microgranular) was purchased from H. Reeve-Angel Co., Clifton, N. J. Alumina C gel was the product of Calbiochem, Los Angeles, Calif. Lysostaphin was obtained from the Mead Johnson Research Institute, Evansville, Ind.

RESULTS

Products of chloramphenicol acetylation. When cell-free extracts of chloramphenicol-resistant E. coli (Rf) were incubated with 14C-chloramphenicol and an excess of acetyl CoA, the major products were chloramphenicol 3-acetate and chloramphenicol 1,3-diacetate (17). The products formed from 14C-chloramphenicol and acetyl CoA in the presence of S. aureus extract were identical with those obtained with the E. coli enzyme (Fig. 1). As has been reported previously for the E. coli enzyme, small amounts of the 1-acetate derivative were found as well, and may be the product of a side reaction rather than an intermediate in the formation of the diacetate (17). Control experiments (not shown) demonstrated that the 1-acetate is not a substrate for the addition of a second acetyl substituent in the 3-position, whereas, the 3-acetate derivative serves as a substrate for the formation of chloramphenicol diacetate. No acetylated products were noted when extracts from the chloramphenicol-susceptible strain (8325) of S. aureus was incubated under similar conditions (Fig. 1, strip 1).
absence of products was also noted with extract from an uninduced culture of resistant strain C 22.1 (not shown).

Kinetics of enzyme induction and chloramphenicol acetylation by cell suspensions of *S. aureus* C 22.1. Figure 2 depicts the sequence of events which ensued when chloramphenicol was added to a culture of resistant *S. aureus* C 22.1. The addition of antibiotic was followed promptly by a modest decrease in the exponential growth rate as estimated by the change in turbidity of the cul-

**FIG. 1.** Formation of monoacetyl and diacetyl derivatives of chloramphenicol by cell-free preparations of resistant *Staphylococcus aureus* and *Escherichia coli*. Crude-cell extracts were prepared from an induced culture of the resistant strain C 22.1 (Strip 2) and from the uninduced sensitive strain 8325 (Strip 1) as described in Materials and Methods. A crude extract of *E. coli* R*ppK-10* was prepared for comparison (Strip 3) by methods previously described (17). Approximately 0.1 unit of enzyme from the resistant strains was incubated with acetyl coenzyme A (0.1 mM) and 14C-chloramphenicol (18 μC per μmole; 0.05 mM) in tris(hydroxymethyl)aminomethane chloride, pH 7.8 (0.1 M), for 10 min at 37°C. The radioactive material was extracted and chromatographed as described (17) with the silica gel/chloroform-methanol system. The radioautographs demonstrate the presence of unaltered chloramphenicol (Rf = 0.19) in Strip 1 and the formation of the 1-acetoxy (Rf = 0.36), 3-acetoxy (Rf = 0.49), and 1,3-diacetoxy (Rf = 0.79) derivatives in Strips 2 and 3.

**FIG. 2.** Induction of chloramphenicol acetyltransferase in *Staphylococcus aureus* C 22.1. Growth was initiated by the addition of 5 ml of an overnight culture (Penassay broth) to 50 ml of fresh medium. The latter was placed on a rotary shaker at 37°C, and the growth of the induced (A) and uninduced (control) culture (O) was estimated by following the increase in turbidity with a Klett photoelectric colorimeter with a no. 66 filter. Klett units are proportional to optical density (OD) at 660 μm. A reading of 150 Klett units is equivalent to 1.0 OD and represents 4.8 x 10^-1 bacterial cells per ml. The growth ordinate (Klett units) was plotted linearly to stress the absence of logarithmic growth during the induction phase, as well as the slight decrease in growth rate of the induced culture after the addition of chloramphenicol. The latter was induced at the end of the logarithmic phase of growth (163 min) by the addition of 14C-chloramphenicol (1 μC per μm) at a concentration of 0.1 mM. The disappearance of chloramphenicol (▼), and the appearance of acetylated products were followed by extracting 1-ml portions with ethyl acetate followed by thin-layer chromatography and autoradiography (see Fig. 1). The radioactive areas were cut out and counted in a scintillation spectrometer. Because less than 10% of the total counts added were accounted for by the sum of chloramphenicol 1-acetate and the 1,3 diacetate derivative, they were not plotted. As indicated (●), the bulk of product was in the form of chloramphenicol-3-acetate. Portions of culture taken for enzymatic activity (●) were chilled, centrifuged, washed, and lysed as described in Materials and Methods. The supernatant fluid was then assayed for chloramphenicol acetyltransferase activity by the standard spectrophotometric assay method. The specific activity is plotted as millimicromoles of chloramphenicol acetylated per minute per milligram of protein.

Chloramphenicol disappeared from the medium over a 60-min period; during this time, chloramphenicol 3-acetate accumulated and small amounts of the 1-acetate and the 1,3-diacetate derivatives were detected (not shown). Synthesis of chloramphenicol acetyltransferase began within 10 min of induction, but showed a more rapid and linear rate of increase as chloramphenicol disappeared from the medium. The activity of the enzyme was plotted on a specific activity basis, to stress the preferential synthesis of chlorampheni-
col acetyltransferase following addition of the antibiotic.

Properties of chloramphenicol acetyltransferase from S. aureus. Figure 3 depicts the effect of pH on enzyme activity as measured in Tris-maleate buffer over the range of 6.0 to 8.8. An optimal pH of 7.8 was estimated from the data of Fig. 3. Identical results were obtained with E. coli R4/K-10 (17).

Striking differences in heat stability existed between the S. aureus and E. coli enzymes (Fig. 4). Rapid inactivation of the E. coli R4/K-10 preparation was observed at 75°C, whereas the S. aureus extract was remarkably resistant to heat denaturation at that temperature. A moderate degree of protection of the E. coli enzyme was observed when the heating was carried out in the presence of chloramphenicol and mercaptoethanol (not shown). The latter technique was useful for the purification of E. coli enzyme, in which a heat step at 70°C was used (see Materials and Methods). The stability of S. aureus enzyme was not judged to be due to stabilizing proteins in the cell extract, since the addition of extract from strain 8325 (chl-s) or uninduced C 22.1 (chl-r) failed to stabilize the E. coli R4/K-10 enzyme to heating at 75°C.

Chloramphenicol acetyltransferase from S. aureus C 22.1 was also compared with its counterpart in E. coli R4/K-10, as regards specificity towards the acetyl acceptor. A number of isomers and analogues of chloramphenicol were compared by following the rate of acetylation of each compound (at 0.1 mm) in the presence of enzyme and acetyl CoA. As has been reported for the E. coli enzyme (17), crude chloramphenicol acetyltransferase from S. aureus acetylated only the biologically active D-threo stereoisomer. Similarly, the two enzyme preparations showed diminished acetylating activity against analogues with alterations of the propanediol side chain. Substitution of the 1-phenyl moiety and the nature of the acyl group at the 2-amino position produced variable effects on the rate of acetylation. Two examples of the divergent specificity of the E. coli and S. aureus enzymes toward such substrates were chosen for the determination of apparent affinity (K_m).

Figure 5 illustrates the reciprocal plot of the kinetic data obtained when S. aureus enzyme was incubated with varying concentrations of chloramphenicol. Table 1 summarizes the results of K_m determinations on chloramphenicol and two analogues of the same stereochemical conformation. The S. aureus and E. coli enzymes showed marked differences in their affinities for the same substrate. Especially striking in this regard was the difference in K_m for the dibromoacetyl
analogue of chloramphenicol. The affinity of each enzyme for chloramphenicol was such that antibiotic acetylation was assured at low concentrations (5 μM) of chloramphenicol, where antibiotic activity was readily seen.

Specificity of inducer for chloramphenicol acetyltransferase in S. aureus C 22.1. As suggested by the data of Fig. 2, it might be possible to define the structural requirements of the inducer by measuring the amount of enzyme synthesized in the presence of a number of chloramphenicol congeners under defined conditions. The results summarized in Table 2 represent the synthesis of chloramphenicol acetyltransferase which occurred in the 90 min following the addition of inducer. It is noteworthy that induction was stereospecific in that the d-erythro isomer was essentially devoid of activity as an inducer. The products of acetylation, chloramphenicol-3-acetate, and chloramphenicol-1,3-diacetate were similarly ineffective. The presence and nature of a substituent at the 2-amino position affected induction, but was less striking. The data of Table 2 reflect the effectiveness of each compound as an inducer, and also its effect as an inhibitor of protein synthesis, the presumed explanation for the initially slow rate of synthesis of enzyme during the early phase of induction in Fig. 2, when chloramphenicol was still present at inhibitory levels. A time-course similar to Fig. 2 was performed with the 1-phenyl analogue, which is inactive as an antibiotic. Such an experiment (not shown) demonstrated a linear increase in enzyme from the time of addition of the inducer, a result expected for an inducer which is not also an inhibitor of protein synthesis.

Physical and immunological characterization of chloramphenicol acetyltransferase from S. aureus and E. coli. When analyzed by the sucrose density gradient method of Martin and Ames (11), the enzymes of S. aureus C 22.1 and E. coli R6/K-10 were found to have identical molecular weights in that: (i) their sedimentation profiles were superimposable; and (ii) when the S. aureus and E. coli enzymes were mixed, there was no broadening or skewing of the sedimentation pattern. On the basis of sucrose gradients run in the presence of human hemoglobin (68,000) and yeast alcohol dehydrogenase (125,000) as standards, an approximate molecular weight of 78,000 was obtained.

Figure 6 illustrates the results of polyacrylamide gel electrophoresis of the S. aureus and E. coli enzymes. The control tubes were incubated in the presence of all reagents except chloramphenicol. The chloramphenicol-dependent bands noted (relative mobility approximately 0.5) of S. aureus and E. coli were superficially very similar, but, in the tube containing both enzymes, the band is

TABLE 1. Comparison of crude Staphylococcus aureus and Escherichia coli enzymes with respect to affinities (Km) for chloramphenicol and selected analogues

<table>
<thead>
<tr>
<th></th>
<th>S. aureus C 22.1</th>
<th>E. coli R6/K-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol</td>
<td>2.7 μM</td>
<td>6.1 μM</td>
</tr>
<tr>
<td>Dibromoacetyl analogue</td>
<td>22 μM</td>
<td>3.7 μM</td>
</tr>
<tr>
<td>1-Phenyl analogue</td>
<td>68 μM</td>
<td>21 μM</td>
</tr>
</tbody>
</table>

* Concentration of chloramphenicol and analogues required for half-maximal reaction velocity (Km) was calculated from data similar to that of Fig. 5.

b An earlier value of 15 μM may have been in error (17). The current 5,5'-dithiobis-2-nitrobenzoic acid (DTN) spectrophotometric assay of acetyl CoA cleavage is more sensitive than the previous measurement of the decrease in absorption at 232 mμ, which occurs on hydrolysis of acetyl CoA. All Km determinations were performed with crude cell-free extracts of E. coli and S. aureus. Determination of the Km for chloramphenicol with purified E. coli enzyme gave an identical value.

d D - Threo - 1 - p - nitrophenyl - 2 - dibromoacetamido-1,3-propanediol.

e D - Threo - 1 - phenyl - 2 - dichloroacetamido-1,3-propanediol.
TABLE 2. Inducer specificity of chloramphenicol analogues

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chloramphenicol acetyltransferase (per cent of control)</th>
<th>Antibiotic activity of analogue (per cent, of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Threo chloramphenicol</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>D-Erythro chloramphenicol</td>
<td>4</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Chloramphenicol-3-acetate</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Chloramphenicol-1,3-diaceate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chloramphenicol base (free amine)</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>3-Propanol analogue</td>
<td>4</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Monochloro analogue</td>
<td>16</td>
<td>41</td>
</tr>
<tr>
<td>Azidoacetyl analogue</td>
<td>32</td>
<td>20</td>
</tr>
<tr>
<td>Cyanoacetyl analogue</td>
<td>42</td>
<td>36</td>
</tr>
<tr>
<td>1-Phenyl analogue</td>
<td>70</td>
<td>&lt;2</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*a* D - Threo - 1 - p - nitrophenyl - 2 - amino - 1,3 - propanediol.

*b* DL - 1, p - Nitrophenyl - 2 - dichloroacetamido - 3 - propanol.

*c* D - Threo - 1 - p - nitrophenyl - 2 - monochloroacetamido-1,3-propanediol.

*d* D - Threo - 1 - p - nitrophenyl - 2 - azidoacetamido-1,3-propanediol.

*e* D - Threo - 1 - p - nitrophenyl - 2 - cyanoacetamido-1,3-propanediol.

*f* D - Threo - 1 - phenyl - 2 - dichloroacetamido - 1,3-propanediol.

*Bacteria were grown in 50 ml of Pen assay broth, induced in mid-logarithmic phase and harvested 90 min after the addition of chloramphenicol or related compounds (see Materials and Methods). Enzyme in the control culture induced by chloramphenicol had a specific activity of 0.3 units per mg of protein.*

Antibiotic activity data provided by Mildred Rebstock (personal communication) based on a turbidimetric assay with *Shigella sonnei*. It is assumed, but not proven, that such activity is an expression of the inhibition of protein synthesis by the analogue in question.

somewhat broader than with either enzyme alone, suggesting heterogeneity.

The availability of electrophoretically pure chloramphenicol acetyltransferase from *E. coli R6/K-10* permitted a comparison of possible cross-reactivity between the *S. aureus* extract and the antiserum to the *E. coli* enzyme. Figure 7 illustrates the precipitin line obtained by the double-diffusion technique, when antiserum to *E. coli* enzyme is reacted with crude extract from strain R6/K-10. No such line is seen in well 1, which contained the same amount of enzyme (*S. aureus C 22.1*) as that of well 6 (*E. coli R6/K-10*).

![FIG. 6. Polycrystalline electrophoresis of crude Staphylococcus aureus C 22.1 enzyme and purified chloramphenicol acetyltransferase from Escherichia coli R6/K-10. Approximately 0.1 units of enzyme in a volume of 0.05 ml was applied to the top of each gel column and subjected to electrophoresis as detailed in Materials and Methods. After electrophoresis, the gels were incubated at 42 °C with the tetrazolium reagents and acetyl coenzyme A. Tubes 1 and 2 contained enzyme from *S. aureus*; tubes 3 and 4 contained *E. coli* enzyme. Tubes 5 and 6 contained equivalent amounts of both enzyme preparations. Gels 2, 4, and 6 were incubated in the presence of chloramphenicol (0.25 μM); the controls, gels 1, 3, and 5, were not incubated with chloramphenicol.](http://jb.asm.org/)
have merely lost conjugal fertility determinants (6). Conversely, no isolates of R+ (chl-r) enteric bacteria have been described which do not owe their resistance to the chloramphenicol-acetylating enzyme. Mitsuhashi (12) has recently summarized the genetic and epidemiologic aspects of antibiotic resistance in S. aureus with the conclusion that: (i) all resistant strains of S. aureus inactivate chloramphenicol; and (ii) the chl-r determinant is extrachromosomal, but does not cotransduce with other antibiotic-resistance (penicillin, erythromycin) determinants carried on plasmids. The most striking difference between gene expression in R+ enteric bacteria and in chloramphenicol-resistant S. aureus is that chloramphenicol acetyltransferase is constitutive in the former, but is inducible in S. aureus. The interpretation of the induction data of Fig. 2 must be tentative, but it is clear that the ability of chloramphenicol to inhibit protein synthesis must exert a negative effect during the early phase, after addition of antibiotic. Furthermore, the major product of the acetylating enzyme, chloramphenicol 3-acetate, is not an inducer (Table 2), so that chloramphenicol has none of the properties expected of a “gratuitous” inducer (7), a compound which is not a substrate and does not influence the metabolism of the induced cells. A search is currently underway for an analogue of chloramphenicol which is a potent inducer, is inactive as an antibiotic, and is not a substrate.

Although the products of chloramphenicol acetylation by S. aureus have not been as thoroughly characterized as have those of the R+ enteric system (17), it is clear from Fig. 1 that they are chromatographically indistinguishable. Fig. 1 gives the misleading impression that the 1,3-diaceylate derivative is a major product of acetylation, whereas Fig. 2 indicates that the formation of the diaceylate is very slow. As with the E. coli enzyme, the spectrophotometric assay of acetylation by S. aureus extracts measures the formation of the monoaceyl compound. Only with prolonged incubation in the presence of an excess of aceyl CoA and enzyme (Fig. 1) does the diaceylate appear in significant amounts.

Although both the S. aureus and E. coli en-
zymes have the same molecular weight (approximately 78,000) by sucrose density gradient centrifugation and the same pH optimum (7 to 8), it is clear from the remainder of the comparative data that they show striking differences in heat stability (Fig. 4), substrate affinities (Table 1), and immunological cross-reactivity (Fig. 7 and 8). Less impressive is the suggestion that they differ slightly in electrophoretic mobility (Fig. 6) on polyacrylamide gel. Although not shown in Fig. 7, the antiserum recalls the Ag. coli R₈/K-10 enzyme shows remarkable resolving power in that agar double-diffusion studies have revealed lines of identity with E. coli enzyme and with those from strains of Shigella sonnei, Salmonella typhimurium and Proteus mirabilis that harbor the R₈ episome (W. Shaw, unpublished data). Cross-reacting material has been identified with certain chl-s point mutants of R⁺ (chl-r) cultures of E. coli, but not with mutants having a deletion of the chl-r genome.

The remarkable efficiency of neutralization of E. coli chloramphenicol acetyltransferase by the antiserum recalls earlier studies on enzyme inactivation by specific antibody (3). No information is available as to whether the E. coli antibody inactivates by combining at the active site, inhibits by steric hindrance, or alters activity by evoking critical changes in tertiary structure which affect the active site indirectly.

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LITERATURE CITED