JOURNAL OF BACTERIOLOGY, Jan. 1968, p. 28-36
Vol. 95, No. 1

Printed in U.S.A.

# Characterization of Chloramphenicol Acetyltransferase from Chloramphenicol-resistant *Staphylococcus aureus*

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Received for publication 7 August 1967

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* harboring 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 *p*H 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-diacetoxy 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 acetylating 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, *p*H 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 acetylating 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 transductant 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 resistance at a high frequency which was increased by treatment with acriflavine (2). Similar results, suggesting that the *chl-r* genome is extrachromosomal, have been reported for chloramphenicol-resistant strains of *S. aureus* isolated in Japan (12). The minimal inhibitory concentration of chloramphenicol for *S. aureus* C 22.1 was 50 μg per ml. The chloramphenicol-susceptible control for the present studies was *S. aureus* 8325, the propagating strain for phage 47 of the International Typing Series, which was also obtained from R. P. Novick.

*E. coli* K-10 (Hfr) and *E. coli* $R_6$/K-10 designate the *chl-s* and *chl-r* organisms, respectively, and were described previously (17). The $R_6$ episome is a stable segregant of a more complex R factor and contains resistance determinants to only chloramphenicol and tetracycline. The presence of chloramphenicol acetyltransferase is a constitutive property of *E. coli* $R_6$/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 otherwise 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 bacterial cells for enzyme purification, and chloramphenicol 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 lysostaphin (16). Cultures (250 ml) were harvested by centrifugation, the pellet was washed with 50 ml of 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 7.5, in 0.145 M NaCl and resuspended in 10 ml of the same buffer. After the addition of lysostaphin 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 organisms harboring R factors (18), the chloramphenicol acetylating enzyme is found in *S. aureus* only after the addition of antibiotic to the medium. Chloramphenicol, or its congeners, were added in the mid-logarithmic 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 acetyltransferase.* 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 similar assays for serine acetylase (8) and thiogalactoside transacetylase (1) utilizing 5,5′-dithiobis-2-nitrobenzoic acid (DTN). The reaction is followed by observing the increase in absorbance at 412 mμ, coincident with the formation of free CoA and its reaction with DTN to form the mixed disulfide. A molar extinction coefficient of 13,600 for the liberated thionitrobenzoic acid was assumed for the purposes 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, *p*H 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 acetylate 1 μmole of chloramphenicol per minute under the standard conditions specified.

*Preparation of purified chloramphenicol acetyltransferase from E. coli $R_6$/K-10.* Bacterial cells (25 g, wet weight) were suspended in a final volume of 100 ml of Tris chloride, *p*H 7.8 (10 mM), containing β-mercaptoethanol (0.01 mM). After sonic disruption and centrifugation 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 concentration 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, *p*H 7.8 (10 mM); β-mercaptoethanol (0.5 mM); and chloramphenicol (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, *p*H 7.8 (10 mM). The latter was exchanged with *p*H 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 $C_\gamma$ gel [0.7 mg (wet 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 centrifuged. The adsorbed enzyme was eluted by suspending the pellet in 0.5 M sodium phosphate buffer, *p*H 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 diethylaminoethyl (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 *p*H 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* $R_6$/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* $R_6$/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 ($R^-$) 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 0.83% sodium barbiturate. The *p*H was adjusted to 7.6 with HCl, and merthiolate (0.01%) was added to prevent bacterial growth.

*Disc gel electrophorsis.* Electrophoretic analysis was carried out with the Buchler apparatus using the anionic (Tris-glycine; running *p*H 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 transacetylase. Each gel was immersed in a small test tube which contained the following in a final volume of 1.0 ml: Tris chloride, *p*H 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, *p*H 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, *p*H 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 Parke, Davis and Co., Detroit, Mich.; 3-$^{14}$C-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* ($R^+$) were incubated with $^{14}$C-chloramphenicol and an excess of acetyl CoA, the major products were chloramphenicol 3-acetate and chloramphenicol 1,3-diacetate (17). The products formed from $^{14}$C-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 extract from the chloramphenicol-susceptible strain (8325) of *S. aureus* was incubated under similar conditions (Fig. 1, strip 1). The

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 culture. 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 chloramphenicol

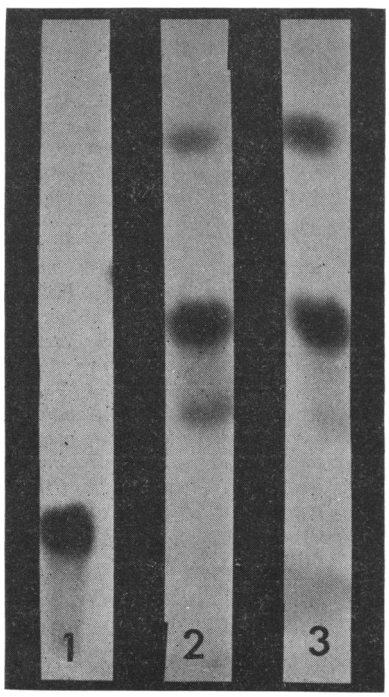


FIG. 1. *Formation of monoacetyl and diacetyl derivatives of chloramphenicol by cell-free preparations of resistant Staphylococcus aureus and Escherichia coli. Crude-cell extracts were prepared from an induced culture of the resistant strain C 22.1 (Strip 2) and from the uninduced sensitive strain 8325 (Strip 1) as described in Materials and Methods. A crude extract of E. coli $R_6$/K-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 $^{14}$C-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 ($R_f$ = .19) in Strip 1 and the formation of the 1-acetoxy ($R_f$ = .36), 3-acetoxy ($R_f$ = .49), and 1,3-diacetoxy ($R_f$ = .79) derivatives in Strips 2 and 3.*

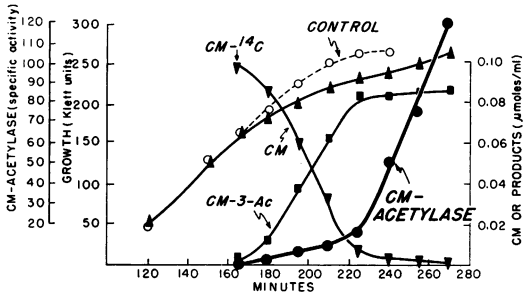


FIG. 2. *Induction of chloramphenicol acetyltransferase in Staphylococcus aureus C 22.1. Growth was initiated by the addition of 5 ml of an overnight culture (Penassay broth) to 50 ml of fresh medium. The latter was placed on a rotary shaker at 37 C, and the growth of the induced (▲) and uninduced (control) culture (○) 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 mμ. A reading of 150 Klett units is equivalent to 1.0 OD and represents 4.8 × $10^{-7}$ 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 (165 min) by the addition of $^{14}$C-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.*

col acetyltransferase following addition of the antibiotic.

*Properties of chloramphenicol acetyltransferase from S. aureus.* Figure 3 depicts the effect of *p*H on enzyme activity as measured in Tris-maleate buffer over the range of 6.0 to 8.8. An optimal *p*H of 7.8 was estimated from the data of Fig. 3. Identical results were obtained with *E. coli* $R_6$/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* $R_6$/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* $R_6$/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* $R_6$/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

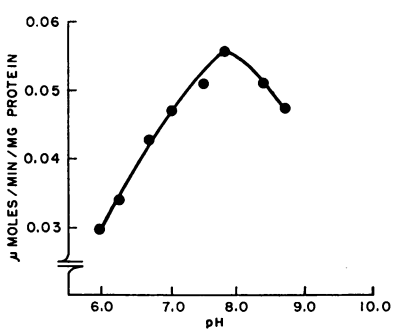


FIG. 3. *The pH optimum of chloramphenicol acetyltransferase from Staphylococcus aureus C 22.1. Enzyme was prepared from an induced culture as described in Materials and Methods and assayed in 0.1 M tris(hydroxymethyl)aminomethane-maleate buffer at the pH specified. Spectrophotometric measurements of enzyme activity were performed by the standard 5,5'-dithiobis-2-nitrobenzoic acid assay of chloramphenicol-dependent acetyl coenzyme A disappearance. Each determination was carried out at 37 C with 0.1 unit of the acetylating enzyme.*

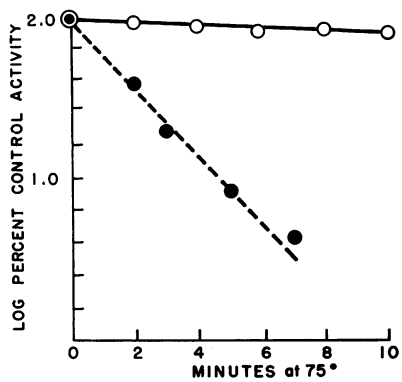


FIG. 4. *Heat stability of chloramphenicol acetyltransferase derived from Staphylococcus aureus C 22.1 and from Escherichia coli $R_6$/K-10. Crude cell extracts were adjusted to a protein concentration of 5 mg per ml in pH 7.8 tris(hydroxymethyl)aminomethane chloride (0.1 M), heated at 75 C for the time indicated, and immediately cooled in an ice-water bath prior to assay with the standard 5,5'-dithiobis-2-nitrobenzoic acid method. The preparation from S. aureus contained 0.4 units per ml before heating, whereas the E. coli extract assayed at 8 units per ml. The results of heat denaturation are expressed as the per cent of the activity present in the unheated extract. Not shown are the results of reciprocal mixing experiments designed to rule out the possibility that S. aureus or E. coli might contain factors tending to stabilize or accelerate inactivation at 75 C. When S. aureus C 22.1 enzyme was mixed with E. coli K-10 extract (chl-s), and E. coli $R_6$/K-10 was mixed with S. aureus 8325 (chl-s), identical inactivation profiles were obtained.*

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

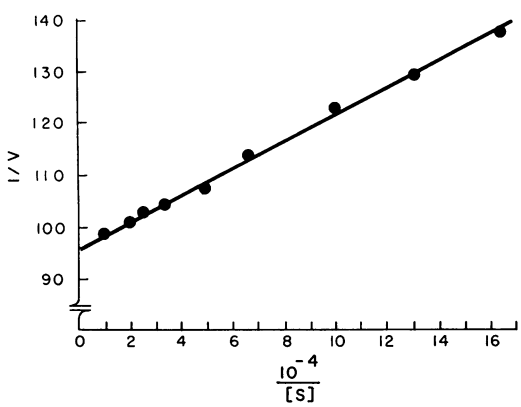


FIG. 5. *Reciprocal (Lineweaver-Burk) plot of the initial reaction velocity with respect to chloramphenicol concentration. The source of chloramphenicol acetyltransferase was an induced culture of Staphylococcus aureus C 22.1, which yielded 0.4 units per ml at a protein concentration of 4.3 mg per ml. The standard spectrophotometric 5,5'-dithiobis-2-nitrobenzoic acid assay of chloramphenicol-dependent acetyl coenzyme A disappearance was used to measure the initial velocities. The $K_m$ calculated for the slope was 2.7 μM (9). The initial reaction velocity (V) is expressed in μmoles of chloramphenicol acetylated per minute.*

analogue of chloramphenicol. The affinity of each enzyme for chloramphenicol was such that antibiotic acetylation was assured at the 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 chloramphanicol-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* $R_6$/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 ($K_m$) for chloramphenicol and selected analogues*[a]

| | *S. aureus* C 22.1 | *E. coli* $R_6$/K-10 |
|---|---|---|
| Chloramphenicol | 2.7 μM | 6.1 μM[b] |
| Dibromoacetyl analogue[c] | 22 μM | 3.7 μM |
| 1-Phenyl analogue[d] | 68 μM | 21 μM |

[a] Concentration of chloramphenicol and analogues required for half-maximal reaction velocity ($K_m$) 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 $K_m$ determinations were performed with crude cell-free extracts of *E. coli* and *S. aureus*. Determination of the $K_m$ for chloramphenicol with purified *E. coli* enzyme gave an identical value.

[c] D - Threo - 1 - *p* - nitrophenyl - 2 - dibromoacetamido-1,3-propanediol.

[d] D - Threo - 1 - phenyl - 2 - dichloroacetamido-1,3-propanediol.

TABLE 2. *Inducer specificity of chloramphenicol analogues*

| Compound | Chloramphenicol acetyltransferase (per cent of control)[g] | Antibiotic activity of analogue (per cent of control)[h] |
|---|---|---|
| D-Threo chloramphenicol | 100 | 100 |
| D-Erythro chloramphenicol | 4 | <1 |
| Chloramphenicol-3-acetate | 7 | 0 |
| Chloramphenicol-1,3-diacetate | 0 | 0 |
| Chloramphenicol base (free amine)[a] | 16 | 0 |
| 3-Propanol analogue[b] | 4 | <1 |
| Monochloro analogue[c] | 16 | 41 |
| Azidoacetyl analogue[d] | 32 | 20 |
| Cyanoacetyl analogue[e] | 42 | 36 |
| 1-Phenyl analogue[f] | 70 | <2 |
| None | 0 | |

[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.

[g] Bacteria were grown in 50 ml of Penassay 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.

[h] 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* $R_6$/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 $R_6$/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* $R_6$/K-10).

The *E. coli* antiserum not only contains precipitating antibody, but also neutralizes enzyme activity, when reacted against crude *E. coli* $R_6$/K-10 extract (Fig. 8). Its inability to neutralize the *S. aureus* enzyme parallels the absence of precipitating antibody noted in Fig. 7.

## DISCUSSION

A substantial amount of information is now available concerning chloramphenicol acetyltransferase from R-factor resistant enteric bacteria and from resistant *S. aureus*. Although some comparisons may be premature, the most striking similarities and differences should be summarized for the purpose of planning and interpreting future experiments. Although trace levels of the acetylating enzyme may be found in a variety of enteric bacteria (18), there is good reason to believe that the high levels which confer chloramphenicol resistance are found only in $R^+$ cultures of enteric bacteria in which the *chl-r* determinant is episomal. An effort has been made in this laboratory to ascertain the existence of chloramphenicol-resistant enteric bacteria which carry the *chl-r* on the chromosome. Thus far, six isolates have been obtained, which fail to transfer the *chl-r* character on mixed cultivation with a suitable recipient, but it is not clear whether such strains carry a chromosomal *chl-r* genome or

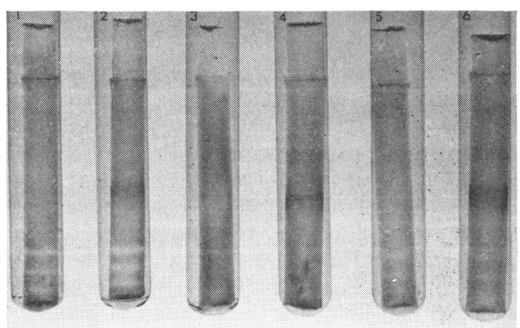


FIG. 6. *Polyacrylamide electrophoresis of crude Staphylococcus aureus C 22.1 enzyme and purified chloramphenicol acetyltransferase from Escherichia coli $R_6$/K-10. Approximately 0.1 units of enzyme in a volume of 0.05 ml was applied to the top of each gel column and subjected to electrophoresis as described 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 mM); the controls, gels 1, 3, and 5, were not incubated with chloramphenicol.*

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.

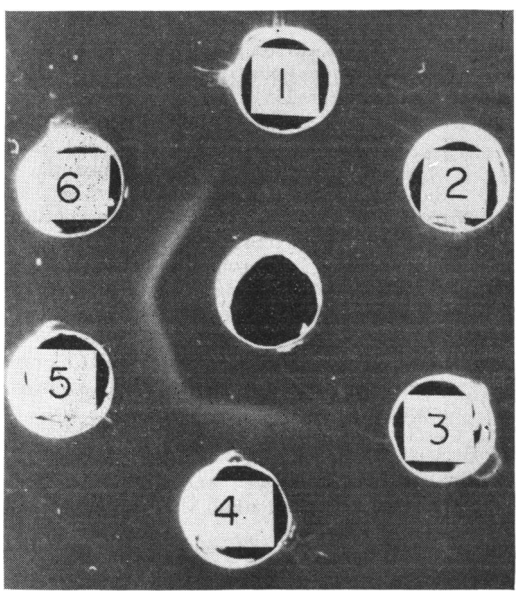


FIG. 7. *Double-diffusion study demonstrating the reaction of antiserum to purified chloramphenicol acetyltransferase of Escherichia coli origin with enzyme present in crude extracts of induced Staphylococcus aureus C 22.1 and E. coli $R_6$/K-10. The center well contained rabbit antiserum to the purified E. coli enzyme, prepared as described in Materials and Methods. Outer wells 2 and 3 contained crude extract from the chl-s strains of S. aureus 8325 and E. coli K-10, respectively. Well 1 contained extract from induced S. aureus C 22.1, whereas the remaining wells were filled with dilutions of E. coli $R_6$/K-10 extract. The wells with enzyme contained the following amounts of chloramphenicol acetyltransferase: well 1, 0.075 units; wells 4, 5, and 6 contained 0.30, 0.15, and 0.075 units, respectively. Precipitation lines were visible after 5 hr and were photographed after 18 hr at room temperature.*

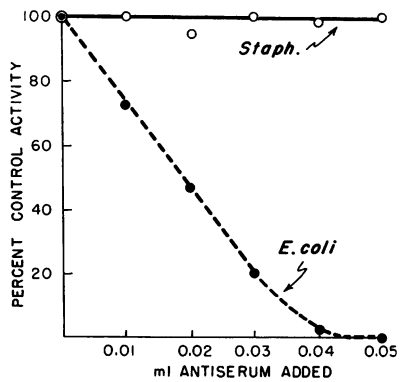


FIG. 8. *Neutralization of chloramphenicol acetyltransferase activity by antiserum to Escherichia coli enzyme. Increasing amounts of antiserum were added, as indicated, to 0.05 ml of crude enzyme prepared from induced S. aureus C 22.1 and E. coli $R_6$/K-10 in a final volume of 0.30 ml containing 0.5% sodium chloride. The control activities of the Staphylococcus aureus and E. coli enzymes were 1.5 and 8.0 units per ml, respectively. After incubation at 3 C for 18 hr, the tubes were thoroughly agitated before samples were taken for the spectrophotometric enzyme assay. When treated in similar fashion with normal rabbit serum, neither the S. aureus nor the E. coli enzyme showed any detectable loss of activity.*

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-diacetate derivative is a major product of acetylation, whereas Fig. 2 indicates that the formation of the diacetate is very slow. As with the *E. coli* enzyme, the spectrophotometric assay of acetylation by *S. aureus* extracts measures the formation of the monoacetyl compound. Only with prolonged incubation in the presence of an excess of acetyl CoA and enzyme (Fig. 1) does the diacetate 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 *p*H 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 prepared to *E. coli* $R_6$/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 $R_6$ 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.

## ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI07344 from the National Institute for Allergy and Infectious Diseases. W. V. Shaw is a Career Scientist of the Health Research Council of the City of New York.

The authors wish to thank Richard P. Novick for the culture of *S. aureus* and for stimulating discussions of plasmid genetics. We are also indebted to Mildred Rebstock of Parke, Davis and Co. for a generous supply of chloramphenicol analogues and to Herbert Rosenkrantz for suggestions regarding enzyme characterization.

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