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Mechanism of Chloramphenicol Resistance in *Staphylococcus epidermidis*

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The mechanism of chloramphenicol resistance in several multiple-resistant *Staphylococcus epidermidis* strains has been studied and shown to be due to the presence of the enzyme, chloramphenicol acetyltransferase. As with *S. aureus*, the inactivating enzyme in *S. epidermidis* appears to be the product of a structural gene on the chloramphenicol plasmid because resistance and enzyme activity are concurcurrently lost after growth in acridine orange or at elevated temperatures. The synthesis of chloramphenicol acetyltransferase in *S. epidermidis* has been compared with the function of a similar enzyme in chloramphenicol-resistant *S. aureus* with the conclusion that the kinetics of induction, products of the reaction, and general properties of the enzymes are identical. The chloramphenicol acetylating enzyme from *S. epidermidis* has been purified to a state of homogeneity and compared with the analogous purified *S. aureus* enzyme. Both purified preparations consist of native enzymes with molecular weights of 80,000, and evidence is presented that is consistent with their being made up of four identical subunits of 20,000 each. The two staphylococcal enzymes are identical with respect to *p*H optimum, apparent affinity (K_m) for chloramphenicol, heat denaturation, and immunological reactivity, but they differ in electrophoretic mobility, chromatographic behavior, substrate specificity, and sensitivity to inhibition by mercuric ion.

The genetic and biochemical mechanisms of antibiotic resistance in staphylococci have received increased emphasis since earlier studies from several laboratories characterized the extrachromosomal nature of the penicillinase gene and stressed its complexity (15, 16, 21). In addition to the well-defined penicillinase "plasmid," a number of other resistance determinants in staphylococci possess characteristics which suggest an extrachromosomal location. Erythromycin, tetracycline, and chloramphenicol resistance have all exhibited properties consistent with those expected for plasmid-linked loci (4, 11, 16, 22). Chloramphenicol resistance in *Staphylococcus aureus* has received special emphasis because of the demonstration that resistance is due to an inducible enzyme which acetylates and thereby inactivates the antibiotic (14, 25, 26). The inactivating enzyme, chloramphenicol acetyltransferase, appears to be the product of a structural gene on the chloramphenicol plasmid since resistance and enzyme activity are concurrently lost by growth at elevated temperatures or in the presence of acridine dyes (4, 22). The ease with which generalized transduction can be effected in *S. aureus* (5, 8, 15) and the precision with which the chloramphenicol acetylating enzyme can be characterized suggest that chloramphenicol resistance may be as useful a determinant as penicillin resistance in analyzing the functions and general properties of staphylococcal plasmids.

Although most studies to date have examined *S. aureus*, recent reports suggest that in *S. epidermidis* the determinant for penicillinase production (2) as well as the determinants for tetracycline resistance and chloramphenicol resistance are located at extrachromosomal (plasmid) sites (D. W. Bentley and M. H. Lepper, Bacteriol. Proc., p. 93, 1969). The chloramphenicol resistance determinant has been shown to be irreversibly lost at a high (0.1%) frequency and in a segregated manner when chloramphenicol-resistant *S. epidermidis* is grown in acridine orange or at elevated temperatures (Bentley and Lepper, Bacteriol. Proc., p. 93, 1969). The obvious similarity between these findings and those for the chloramphenicol resistance determinant in *S. aureus* (4, 8, 12, 13, 22) prompted the present

study which examines the mechanism of chloramphenicol resistance in *S. epidermidis* and compares it with that previously described for *S. aureus* (25, 29).

MATERIALS AND METHODS

Bacterial strains and media. All 157 chloramphenicol-resistant *S. epidermidis* isolates from the epidemiological survey (Bentley and Lepper, Bacteriol. Proc., p. 93, 1969) inactivated chloramphenicol when tested by the method of Sabath (22). Several chloramphenicol-resistant strains and their corresponding chloramphenicol-sensitive clones (in which chloramphenicol resistance had been eliminated after growth in acridine orange) were examined for the presence of the enzyme, chloramphenicol acetyltransferase. Further studies were performed with a single strain, 39 NC, which in addition to being chloramphenicol-resistant was also resistant to tetracycline and penicillin G as well as to cadmium and mercury ions when tested for heavy metal resistance by the disc method of Novick and Roth (17). This strain represented a biotype 6 *S. epidermidis* according to the biotyping method previously reported (3).

The strain of *S. aureus* used was C22.1, a chloramphenicol-resistant transductant of propagating strain 47 of the International Typing Series. Its origin and characteristics were previously described (25). Both strains were maintained on Penassay Agar (Difco) containing 50 μg of chloramphenicol per ml to insure against loss of the resistance determinant. Chloramphenicol acetyltransferase was induced by the growth of the resistant strains of *S. epidermidis* and *S. aureus* in the presence of 3-deoxychloramphenicol (29). Penassay Broth (Difco) was used routinely for growth in liquid media.

Assay of chloramphenicol acetyltransferase. The enzymatic acetylation of chloramphenicol was measured by a coupled spectrophotometric assay which measures the chloramphenicol-dependent cleavage of acetyl-coenzyme A (acetyl-CoA; 25). Reaction velocities were measured at 37 C with a Gilford recording spectrophotometer. The reduction of 5,5-dithio-bis-2-nitrobenzoic acid (Ellman's reagent, or DTN) by reduced CoA was followed at 412 nm. One unit of enzyme is that activity which acetylates 1 μmole of chloramphenicol per min under standard conditions (25). Crude cell lysates were prepared by using lysostaphin-deoxyribonuclease treatment and centrifugation as previously described (25).

Purification of chloramphenicol acetyltransferase from S. epidermidis. The protocol described previously (29) for similar studies with *S. aureus* was followed with minor modifications. A 1-liter overnight culture of *S. epidermidis* grown in Penassay broth and chloramphenicol (final concentration of 0.1 mM) was used to inoculate 29 liters of the same media. To insure maximal induction, 3-deoxychloramphenicol, a "gratuitous inducer" of chloramphenicol acetyltransferase in *S. aureus*, was added in the mid-exponential phase of growth at a final concentration of 5 μM. Bacteria were harvested by centrifugation in early stationary phase with a yield of 103 g (wet weight) of cell paste. The cells were washed once with 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.5) containing 145 mM sodium chloride (Tris-saline buffer) and resuspended to a total volume of 500 ml for cell lysis. A crude cell-free extract was prepared by using lysostaphin and deoxyribonuclease and incubating at 37 C for 2 hr as previously described. After clarification by centrifugation at 10,000 rev/min (Sorvall RC-2B centrifuge and GSA rotor operating at 4 C) for 30 min, the pale amber fluid was made 0.1 M with Tris-hydrochloride (*p*H 7.8) buffer before the addition of finely ground ammonium sulfate to 75% saturation. The resulting precipitate was dissolved in 10 mM Tris-hydrochloride (*p*H 7.8) containing 0.2 mM chloramphenicol and 0.5 mM 2-mercaptoethanol (TCM buffer), and dialyzed overnight against 40 volumes of the same buffer. The resulting extract was applied to a diethylaminoethyl (DEAE) cellulose column (2.5 by 18.5 cm) and eluted with 1 liter of TCM buffer containing a linear gradient from zero to 0.4 M sodium chloride. Fractions (10 ml) were collected and chloramphenicol acetyltransferase was eluted in tubes 25 through 31 which were pooled and concentrated (by Diaflo ultrafiltration) to 7.5 ml under nitrogen. The pooled and concentrated DEAE effluent was applied to Sephadex G-100 (regular bead) column (2.5 by 90 cm) which was eluted with TCM buffer containing 0.2 M sodium chloride, and 5-ml fractions were collected. The peak tubes were then pooled, concentrated, and dialyzed overnight against TCM buffer without sodium chloride. The final DEAE cellulose column was identical in all respects with that used after the ammonium sulfate step except that the *p*H of the TCM buffer was 7.0. The peak tubes were eluted at the same location in the gradient, pooled, and concentrated to a final volume of 6 ml yielding 246 units with a specific activity of 6.8 units per mg of protein (*see* Table 5).

The protein concentration of column effluents was estimated by ultraviolet absorption at 280 nm, but all measurements for calculations of purification or studies with purified material were made by the method of Lowry et al. (20).

Disc gel electrophoresis. Polyacrylamide disc gel electrophoresis was performed with the Buchler system by using the modifications previously described (25). Estimates of molecular weight of the proposed subunits were made by the sodium dodecyl sulfate modification described by Weber and Osborn (28) by using commercially available proteins which were homogeneous by conventional disc gel electrophoresis. The localization of chloramphenicol acetyltransferase by a coupled histochemical stain by using tetrazolium blue was previously reported (25).

Immunological methods. Rabbit antibody to the purified enzyme from *S. epidermidis* was raised in rabbits by the toe pad method with a total of 1 mg of protein mixed with Freund's Incomplete Adjuvant (Difco). Animals were bled for control sera before immunization and again 2 and 4 weeks after the toe pad injections. The non-immune control sera failed to react with purified enzyme as judged by the absence of a precipitin reaction in agar double diffusion and the failure of such sera to neutralize enzyme activity.

Chemicals. D-Threo chloramphenicol and its related

isomers and analogues were obtained from M. Rebstock of Parke, Davis & Co., Ann Arbor, Mich.; chloramphenicol-3-^{14}C was the product of Nuclear-Chicago Corp., Des Plaines, Ill.; DTN was purchased from Aldrich Chemical Co., Milwaukee, Wis.; and CoA was prepared by P-L Laboratories, Milwaukee, Wis. Acyl-CoA compounds were synthesized as previously described (25). Whatman DEAE (microgranular) was purchased from H. Reeve Angel Co., Clifton, N.J., and Sephadex G-100 was purchased from Pharmacia Fine Chemicals, Piscataway, N.J. Lysostaphin was the gift of the Mead Johnson Research Institute, Evansville, Ind. Agarose (6%) was the product of Bio-Rad Laboratories, Richmond, Calif.; the reagents required for gel electrophoresis were purchased from Eastman Chemicals, Rochester, N.Y.

RESULTS

Chloramphenicol acetyltransferase in extracts of resistant S. epidermidis. Chloramphenicol acetyltransferase was present in the crude cell lysostaphin lysates from all the chloramphenicol-resistant *S. epidermidis* strains examined; there was no evidence of enzyme activity in any of the corresponding chloramphenicol-sensitive clones. Figure 1 demonstrates the chromatographic identity of the products obtained after incubation of the staphylococcal cell extracts with ^{14}C-chloramphenicol and acetyl-CoA. With more prolonged incubation times (*not shown*) and a large excess of enzyme and acetyl-CoA, more complete conversion of the monoacetate to the diacetoxy derivative has been demonstrated.

The availability of a "gratuitous inducer" of chloramphenicol acetyltransferase in *S. aureus* (29) suggested that a similar induction system might be observed in *S. epidermidis*. Figure 2 illustrates the linear kinetics of induction after the addition of 3-deoxychloramphenicol to cultures of both *S. aureus* and *S. epidermidis*. Comparable rates of induction and specific activities were obtained for both species. Induction by the addition of chloramphenicol (*not shown*) revealed the sigmoid profile of induction noted previously for *S. aureus* (25, 29). Such kinetics have been attributed to an initial phase of inhibition of protein synthesis followed by induction and the ultimate cessation of net synthesis resulting from acetylation and concomitant inactivation of the inducer. To avoid the uncertainties of induction by the parent compound, the analogue was routinely used for enzyme induction in both *S. aureus* and *S. epidermidis*.

Comparative enzymology of chloramphenicol acetyltransferase in staphylococci. Before the purification of the acetylating enzyme from *S. epidermidis*, a number of comparative studies were performed with crude cell-free extracts of both strains of staphylococci. Figure 3 describes

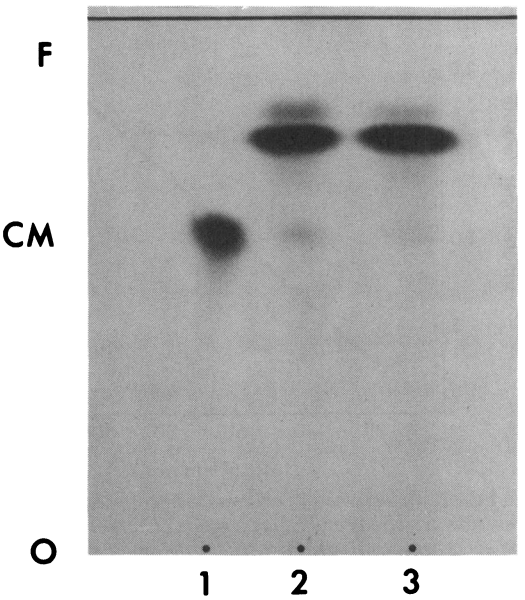

FIG. 1. *Radioautographic demonstration of products of inactivation in S. aureus and S. epidermidis. Crude cell-free extracts were prepared with lysostaphin from exponential-phase cultures of each organism grown in the presence of 50 μg of chloramphenicol per ml. Lysates containing approximately 0.2 mg of protein were incubated in the presence of 10 mM Tris-hydrochloride (pH 7.8), 0.2 mM chloramphenicol-3-^{14}C (2 μCi per μmole), and 0.5 mM acetyl-CoA at 37 C for 15 min in a final volume of 1 ml. The reaction mixture was extracted with ethyl acetate, and the extract was chromatographed on alumina thin-layer sheets as previously described (23). The designations O and F refer to the origin and solvent front, whereas CM indicates the mobility of radioactive chloramphenicol. Reaction 1 contained the complete mixture except for enzyme. Reactions 2 and 3 contained approximately 0.2 units of chloramphenicol acetyltransferase from S. aureus and S. epidermidis, respectively. The enzyme-containing reactions (2 and 3) show essentially complete conversion of chloramphenicol (CM) to a major faster moving compound (chloramphenicol-3-acetate) and a less distinct derivative of still faster mobility (chloramphenicol-1,3-diacetate). The identity of the radioactive acetylated products was confirmed by co-chromatography with authentic unlabeled compounds (23).*

the *p*H dependence of the monoacetylation reaction as determined spectrophotometrically for *S. aureus* and *S. epidermidis*. The *p*H optima (7.8) are comparable and identical to that noted for the R-factor mediated enzyme of *Escherichia coli* (23).

Measurements of the apparent affinity of chloramphenicol acetyltransferase for chloramphenicol were previously made by using the spectrophotometric technique and conventional

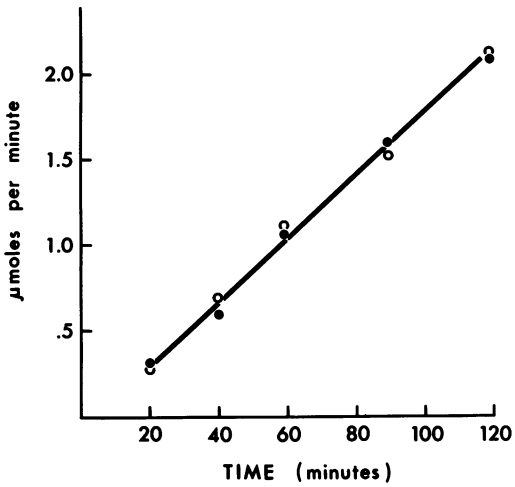

FIG. 2. *Induction of chloramphenicol acetyltransferase in S. aureus and S. epidermidis. Cultures (50 ml) of each organism were induced in the mid-exponential phase of growth by the addition of 3-deoxychloramphenicol at a final concentration of 0.02 mM (29). Samples (5 ml) withdrawn at the times indicated were centrifuged, and the pellets were washed with standard buffer before lysis and enzyme assay. Chloramphenicol acetyltransferase in S. aureus (●) and S. epidermidis (○) is plotted as micromoles of chloramphenicol acetylated per minute by the total enzyme recovered in each 5-ml aliquot.*

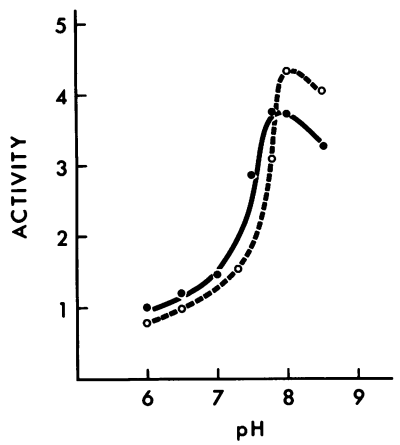

FIG. 3. *Effect of pH on enzyme activity for chloramphenicol acetyltransferase from S. aureus (○) and S. epidermidis (●). The initial reaction rate was determined spectrophotometrically in the presence of 0.05 M Tris-maleate buffer at the specified pH.*

kinetic analysis (9). Since such measurements revealed significant differences between the acetylating enzyme from *E. coli* and *S. aureus* (25, 29), it appeared likely that useful information might be obtained by a similar comparison of the staphylococcal enzymes. Kinetic data (*not shown*) gathered over a concentration range of 3 to 100 μM gave K_m values for chloramphenicol of 3.2 μM (*S. epidermidis*) and 3.5 μM (*S. aureus*). Since the *p*H and affinity studies revealed no significant differences in catalytic properties between the crude staphylococcal enzymes, several other properties were compared. From past experience it appeared that the kinetics of heat denaturation might be a useful means of detecting differences between two types of chloramphenicol acetyltransferase (25); such was not the case, however, since the per cent loss in activity after heating at 70 C for various periods of time was virtually the same for both enzymes (Table 1).

Previous studies have led to the conclusion that measurement of the rate of acetylation of various analogues and isomers of chloramphenicol is a useful means of detecting subtle differences in closely related enzymes (24, 25). The results of such a comparison between *S. aureus* and *S. epidermidis* are summarized in Table 2. The *N*-dibromoacetyl analogue of chloramphenicol showed acetyl acceptor activity greater than that observed for the parent antibiotic, and essentially no activity was observed for the free amine of chloramphenicol. A stereochemical preference for the D-threo configuration was observed for the *S. aureus* enzyme, as was previously seen (24, 25), but it is noteworthy that the D-erythro stereoisomer was a better acetyl acceptor for the *S. epidermidis* enzyme than for that of *S. aureus*.

TABLE 1. *Heat stability of chloramphenicol acetyltransferase from S. epidermidis and S. aureus*

Min at 70 C	Activity remaining[a]	
	S. epidermidis	*S. aureus*
0[b]	100	100
3	87	86
5	78	78
9	65	63
15	48	50

[a] Values expressed as per cent of unheated control. Chloramphenicol acetyltransferase was induced in *S. aureus* and *S. epidermidis* as described in Fig. 1. Crude lysates of equivalent enzyme activity (0.5 units per ml) and protein concentration (2 mg per ml) were prepared, and 0.1-ml portions were heated in small stoppered test tubes for various periods of time by immersion into a water bath at 70 C. The samples were removed at the specified times and placed in an ice-water bath until the spectrophotometric assays were performed.

[b] Control.

An analogous divergence in specificity was also seen with the *N*-glycolyl analogue of chloramphenicol which was acetylated by the *S. epidermidis* enzyme at a rate almost twice that observed for *S. aureus*. In keeping with earlier observations (24, 25), alterations of the 1,3-propanediol side chain of chloramphenicol markedly reduced acetyl acceptor activity for both enzymes (1,3-butanediol and 3-dimethyl-1,3-propanediol analogues). Experiments were also performed to test the specificity of the enzymes in question with regard to the nature of the acyl donor, since the R-factor-mediated chloramphenicol acetyltransferase of *E. coli* has been shown to be capable of synthesizing the propionyl and butyryl esters of chloramphenicol (23). Table 3 summarizes the results of such experiments in which the acetyl, propionyl, butyryl, and palmityl thio-esters of CoA were tested by using the spectrophotometric assay of chloramphenicol-dependent thio-ester cleavage in the presence of the acetylating enzyme. The *E. coli* and staphylococcal enzymes share a similar preference for the short-chain fatty acyl thio-esters, but the relative rates of reaction were different in each case. The overall differences in specificity of the staphylococcal enzymes for the acyl donors and acceptors were small but were sufficiently reproducible to suggest that different techniques might reveal other differences.

Previous studies with chloramphenicol acetyltransferase from enteric bacteria revealed a protective effect of reduced mercaptans on enzyme activity that suggested the possible involvement of free sulfhydryl groups in enzyme activity (23, 27). Accordingly, a survey was made of the effects of several commonly used sulfhydryl inhibitors on the staphylococcal enzymes. The results are described in Table 4. Although *p*-mercuribenzoate (0.5 mM) and *N*-ethyl maleimide (2 mM) gave comparable degrees of inhibition for both enzymes, a striking dissociation was noted with mercuric chloride (0.5 mM). The greater sensitivity of the *S. epidermidis* enzyme to inhibition by the latter reagent was confirmed in a subsequent experiment (Fig. 4) wherein the effect of mercuric ion was examined over a broad concentration range. The inhibition was irreversible, as judged by the absence of reactivation after prolonged dialysis in the presence of mercaptans.

Purification of chloramphenicol acetyltransferase from S. epidermidis. Conventional techniques of salt precipitation, ion exchange chromatography, and gel filtration have provided highly purified preparations of chloramphenicol acetyltransferase from *S. aureus* and *E. coli* (25, 29). A similar purification scheme was adopted

TABLE 2. *Substrate specificity of chloramphenicol acetyltransferase from S. epidermidis and S. aureus*

Compound	Acetyl acceptor activity[a]	
	S. epidermidis	*S. aureus*
Isomers		
D-Threo chloramphenicol (CM)	100	100
D-Erythro chloramphenicol	32	23
L-Threo chloramphenicol	4	6
L-Erythro chloramphenicol	0	6
N-acyl substituent[b]		
Dibromoacetyl	152	122
Formyl	23	22
Glycolyl	55	23
None (free amine of CM)	2	0
C_1-C_3 analogues[c]		
3-Dimethyl-1,3-propanediol	0	0
1,3-Butanediol	4	6
3-Propanol	46	64
1-Hydroxypropane	0	0

[a] Relative ability of each compound to act as an acetyl acceptor in the presence of *S. aureus* enzyme and acetyl-CoA under the standard conditions described for the spectrophotometric assay. Values are expressed as per cent of the initial velocity observed with CM when each compound was tested at 0.1 mM.

[b] For CM, the C_2 substituent is *N*-dichloroacetyl.

[c] For CM, the side-chain carbon skeleton is 1,3-propanediol. All compounds in this group contained the 1-*p*-nitrophenyl and 2-dichloroacetamido structure of the parent compound (CM).

TABLE 3. *Species specificity of chloramphenicol acetyltransferase for various acyl donors*[a]

Acyl-coenzyme A	Species		
	S. aureus	*S. epidermidis*	*E. coli*
	%	%	%
Acetyl	100	100	100
Propionyl	64	74	94
Butyryl	23	44	64
Palmityl	0	0	0

[a] Assay conditions as in Table 1 except that the acyl acceptor in each case was D-threo chloramphenicol. An equimolar amount (0.2 mM final concentration) of the appropriate acyl donor was added to start the reaction. Comparable amounts of each enzyme were used to give initial control (100%) rates of acylation of approximately 0.02 μmoles per min.

TABLE 4. *Inhibition of chloramphenicol acetyltransferase by sulfhydryl reagents*

Treatment	Residual activity[a]	
	S. aureus	*S. epidermidis*
None (control)	100	100
N-ethyl maleimide (2 mM)	55	35
p-Mercuribenzoate (0.5 mM)	34	34
Mercuric chloride (0.5 mM)	55	6

[a] Values expressed as per cent of control. The experiment was carried out as follows. One milliliter of the appropriate enzyme containing approximately 5 units of activity was incubated in the presence of 10 mM Tris-hydrochloride (*p*H 7.8), sodium chloride (200 mM), and the inhibitor at the specified final concentration for 10 min at 37 C. Each sample was then dialyzed for 18 hr against 500 ml of the Tris-sodium chloride buffer at 4 C. Residual chloramphenicol acetyltransferase activity was then assayed spectrophotometrically and related to the control sample preincubated in the absence of inhibitors.

for *S. epidermidis* with the results summarized in Table 5. The overall yield and degree of purification compare favorably with that for *S. aureus* reported previously. The sharp and congruent elution profiles of enzyme activity and protein on DEAE cellulose (Fig. 5) and Sephadex G-100 (Fig. 6) were accompanied by an impressive purification, whether judged by increases in specific activity (Table 5) or analysis by disc gel electrophoresis (Fig. 7). The latter illustration shows the faster moving faint bands of contaminating material in sample 2 after Sephadex gel filtration. Accordingly, a final purification step with a second DEAE cellulose column at a lower *p*H was used to achieve the homogeneous material seen in sample 3. When unstained gels similar to those of Fig. 7 were examined for chloramphenicol acetyltransferase activity by a previously described histochemical technique (25), the purified protein and enzyme activity bands showed identical mobilities (*not shown*).

Comparative studies of purified chloramphenicol acetyltransferase from S. epidermidis and S. aureus. The preliminary experiments noted above indicated that the staphylococcal enzymes were similar as regards *p*H optima, apparent affinity for chloramphenicol, and heat stability when compared in the crude state. Although differences in substrate specificity and sensitivity to mercuric ion were noted, no data were presented which might firmly establish a clear-cut difference between the two closely related enzymes. The first suggestion of such evidence came from comparisons of elution profiles of the staphylococcal enzymes from DEAE cellulose under conditions similar to those of Fig 5. Whereas the *S. epidermidis* enzyme was eluted at 71 mM sodium chloride, the *S. aureus* activity appeared to migrate more rapidly. Figure 8 confirms the earlier impression of nonidentity by showing that the relative mobilities of the two purified staphylococcal enzymes are indeed different. That the observed absolute differences in mobility are real is apparent from the middle gel in Fig. 8, wherein the two preparations were mixed before electrophoresis and two clearly separable bands may be seen.

Immunological comparison of the purified staphylococcal enzymes. Previous reports stressed the lack of immunological cross-reactivity of chloramphenicol acetyltransferase from enteric bacteria (R-factor-containing *E. coli*) with that of *S. aureus* (23, 29). The rabbit antiserum prepared against the purified *E. coli* enzyme failed to precipitate or neutralize the *S. aureus* prepa-

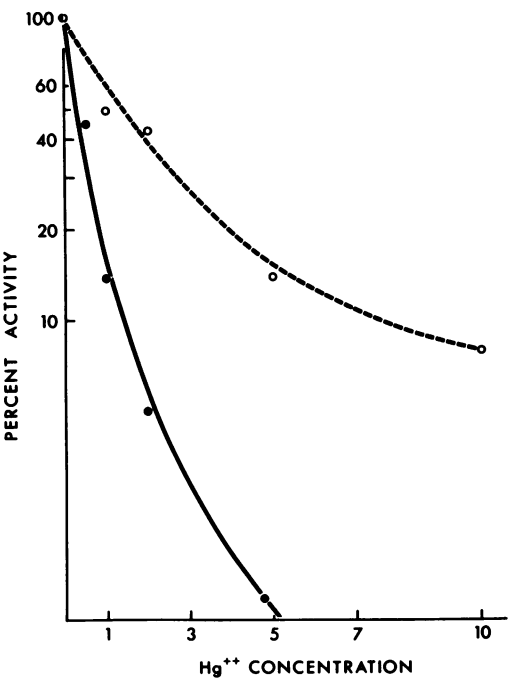

FIG. 4. *Concentration dependence of mercuric chloride inhibition of chloramphenicol acetyltransferase. Each point represents a single determination of residual activity after inhibition by mercuric chloride under the conditions described in Table 3. Enzyme activity from S. epidermidis (●) and S. aureus (○) has been plotted as the per cent remaining as compared with the untreated control. The abscissa is plotted as the molar concentration of mercuric ion × 10⁴.*

TABLE 5. *Purification of chloramphenicol acetyltransferase S. epidermidis*[a]

Step	Preparation	Vol	Total enzyme	Total protein	Specific activity	Recovery	Purification
			units	*mg*	*units/mg*	%	
1	Crude lysate	500	1810	4200	0.4	100	1.0
2	Ammonium sulfate precipitate	104	1456	676	2.2	81	5.5
3	DEAE peak (*p*H 7.8)	64	768	70	11	42	28
4	Sephadex G-100 peak	17	510	10.2	50	28	125
5	DEAE peak (*p*H 7.0)	6	246	3.6	68.5	14	171

[a] From 103 g (net weight) of cells.

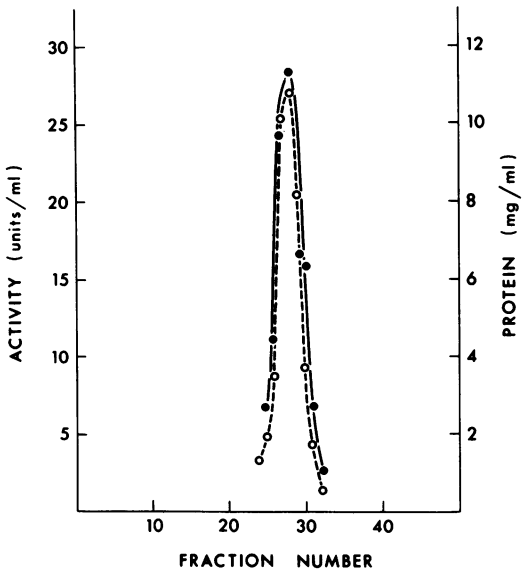

FIG. 5. *DEAE cellulose chromatography of chloramphenicol acetyltransferase from S. epidermidis. Each 10-ml fraction was assayed for enzyme activity* (○) *and protein* (●). *Tubes 25 through 31 yielded a 53% recovery of the total activity applied to the column (see Table 5). The concentration of sodium ion in the peak tube (no. 28) was 71 mM as determined by flame photometry. Conductivity measurements (not graphed) were consistent with the beginning of the effluent salt gradient at tube 9 and confirmed the linearity of the sodium chloride gradient.*

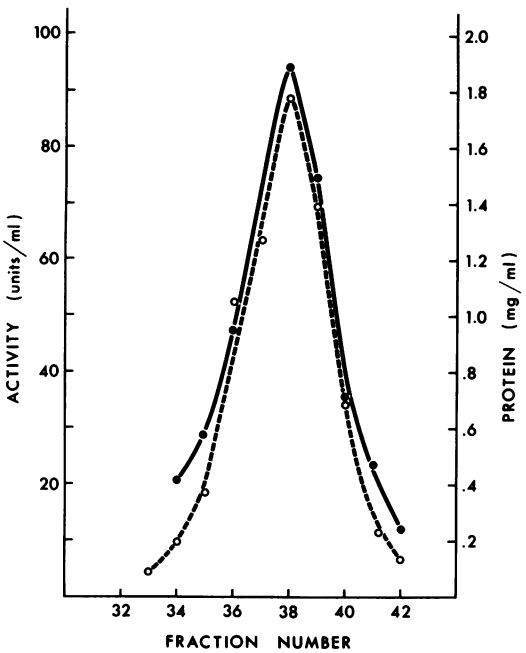

FIG. 6. *Gel filtration of chloramphenicol acetyltransferase from S. epidermidis on Sephadex G-100. The peak tubes (fractions 35 through 40) were pooled and yielded 510 units of enzyme activity, representing 67% of the applied sample. Each 5-ml fraction was assayed for enzyme activity* (○) *and protein* (●). *The void volume of the column was 120 ml as determined with Dextran Blue.*

ration in contrast to its reactivity with the homologous antigen. When also tested against the purified *S. epidermidis* enzyme there was also no evidence of (i) an immune precipitate by double diffusion in agar or (ii) neutralization of enzyme activity (*not shown*). However, when the purified *S. epidermidis* enzyme was used as an antigen, the rabbit antiserum was highly effective against both the homologous (*S. epidermidis*) and closely related (*S. aureus*) enzymes, but failed to react with that of *E. coli*. Figure 9 depicts the parallel neutralization of both enzymes when activity is titrated by increasing amounts of antiserum. Similarly, a single line of identity was observed by agar double diffusion when the staphylococcal enzymes were run in adjacent wells against the anti-*S. epidermidis* antibody (*not shown*).

Molecular weight and subunit structure of staphylococcal chloramphenicol acetyltransferase. Calibration of the preparative Sephadex G-100 column (Fig. 6) with appropriate purified pro-

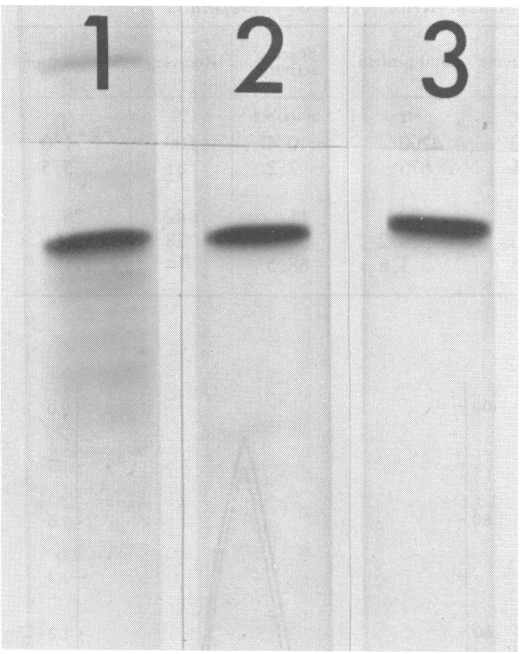

FIG. 7. *Disc gel electrophoretic evidence of purification of chloramphenicol acetyltransferase from S. epidermidis. Polyacrylamide gel electrophoresis was carried out at pH 8 as previously described (25). The lower pole corresponds to the anode in each case. The stain was Amido Black. Gels 1, 2 and 3 contained enzymes from S. epidermidis as follows: (1) 50 μg of pooled peak from the first (pH 7.8) DEAE column; (2) 36 μg from the Sephadex G-100 peak, and (3) 32 μg from the second (pH 7.0) DEAE peak.*

tein markers (apoferritin, bovine serum albumin, hemoglobin, myoglobin, and cytochrome *c*) as described by Andrews (1) gave a preliminary value of approximately 80,000 for the molecular weight of the native enzyme from *S. epidermidis*. The determination was repeated with a calibrated analytical gel filtration column (1.5 by 80 cm) containing 6% agarose. Chloramphenicol acetyltransferase for both *S. aureus* and *S. epidermidis* gave identical elution profiles and relative mobilities consistent with a molecular weight of 80,000 (Fig. 10). Figure 10 compares favorably with the previously reported estimate of 78,000 for the crude *S. aureus* enzyme as determined by gel filtration and sucrose density centrifugation (25).

Because of the relatively large size of chloramphenicol acetyltransferase, it seemed likely that the native enzyme might consist of a complex of two or more subunits. This possibility was investigated by the technique of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (28). Figure 11 summarizes the results obtained by that technique wherein the purified staphylococcal enzymes gave single polypeptide bands of approximately 20,000 for *S. aureus* and 20,500 for *S. epidermidis*. Taking into account an expected precision of the method, the results are compatible with the hypothesis that the staphylococcal enzymes are made up of polypeptide subunits of the same molecular weight and that the native enzyme most likely exists as a tetramer of four such subunits. The failure to detect enzyme activity (*not shown*) in the material eluted from

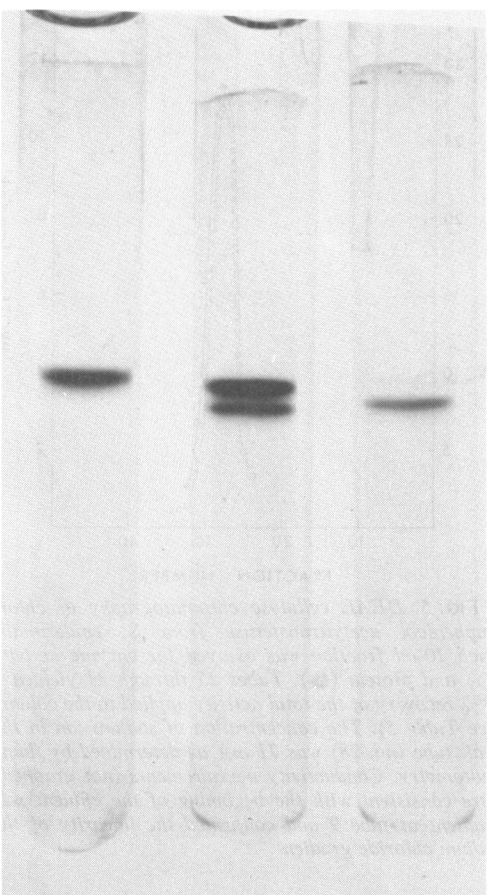

FIG. 8. *Chloramphenicol acetyltransferase from S. aureus and S. epidermidis: comparison of the purified enzymes by disc gel electrophoresis. The enzyme preparations in each case were from pooled eluates of the second (pH 7.0) DEAE column. The gel on the left contained 55 μg of S. epidermidis enzyme, and that on the far right approximately 20 μg of purified S. aureus material. The center gel contained a mixture of the two specimens in the same quantities used for the samples run individually. The conditions were in all respects similar to those of Fig. 7.*

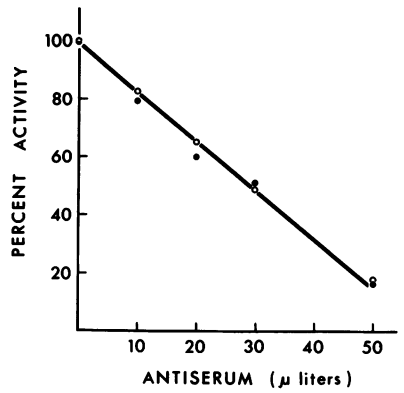

FIG. 9. *Neutralization of chloramphenicol acetyltransferase by antiserum to purified S. epidermidis enzyme. Increasing amounts of rabbit antiserum were added as indicated to 0.2 units of enzyme from S. aureus (●) and S. epidermidis (○) in a final volume of 0.2 ml and in the presence of 0.9% sodium chloride. After incubation for 2 hr at 0 C, the samples were centrifuged, and enzyme activity was determined in the supernatant fluid by the spectrophotometric assay. Preimmunization sera showed no neutralization of the activity in either enzyme preparation.*

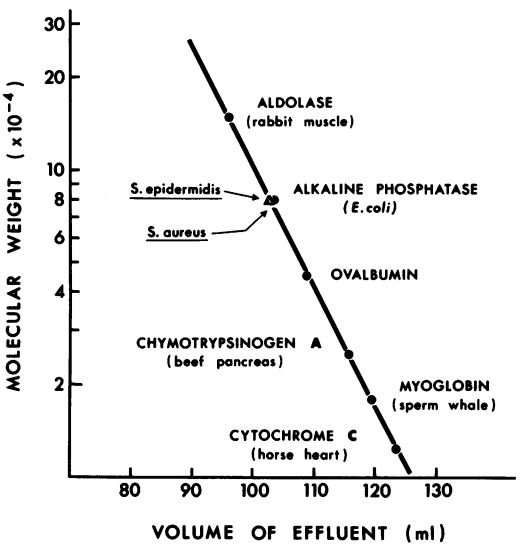

FIG. 10. *Agarose gel filtration behavior of native chloramphenicol acetyltransferase. A column (1.5 by 80 cm) of 6% agarose in 0.05 M sodium phosphate (pH 7.0) with 0.1 M sodium chloride was calibrated with purified commercially available proteins of known molecular weight. The marker elution profiles were determined by absorbance at 280 nm or by enzymatic assay (1). Interpolation of the experimental points for S. aureus and S. epidermidis gave an estimate of 80,000 for the molecular weight of native chloramphenicol acetyl-transferase from both species.*

appropriate unstained sections of the sodium dodecyl sulfate-polyacrylamide gels suggests that the monomeric material is catalytically inactive. Studies are in progress to ascertain whether the proposed subunits are identical and whether they can be shown to reassociate to form the active native enzyme.

DISCUSSION

Whereas earlier reports of the enzymatic inactivation of chloramphenicol stressed the characteristics of the acetyltransferase enzyme from R-factor-containing enteric bacteria (23, 27), it has become clear that chloramphenicol-resistant strains of *S. aureus* also contain an acetylating enzyme which has similar properties (25, 26, 29). At the genetic level it is of interest that chloramphenicol resistance in *S. aureus* is apparently also mediated by an extrachromosomal element ("plasmid") as judged by the high rates of appearance of sensitive cells in cultures grown at elevated temperatures or treated with acridine dyes (4, 13, 22).

Similar findings have now been demonstrated for chloramphenicol-resistant *S. epidermidis*. The strains examined were obtained during an epidemiological study in which certain evidence suggested that the chloramphenicol resistance

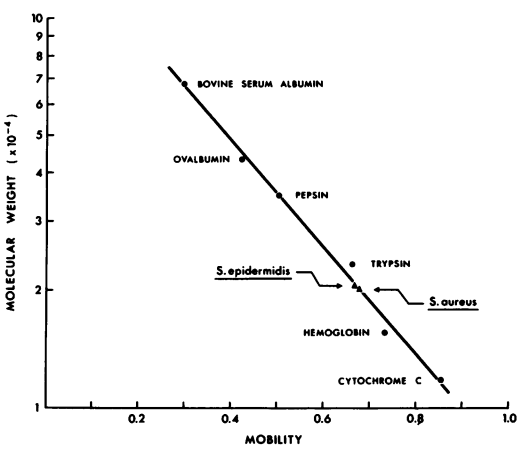

FIG. 11. *Electrophoretic mobility of subunits of chloramphenicol acetyltransferase on sodium dodecyl sulfate-polyacrylamide gel columns. Twenty microgram samples of the purified staphylococcal enzymes and commercially available proteins were treated with sodium dodecyl sulfate and mercaptoethanol and subjected to electrophoresis by the method of Weber and Osborn (28). The normal degree of cross-linking was achieved by the use of 0.6 g of methylene bis-acrylamide per 100 ml of gel solution. The subunit sizes were estimated to be 20,000 for S. aureus (▲) and 20,500 for S. epidermidis (△).*

determinant might be located at an extrachromosomal (plasmid) site (Bentley and Lepper, Bacteriol. Proc., p. 93, 1969). The findings, that the chloramphenicol-resistant *S. epidermidis* strains studied contain chloramphenicol acetyltransferase which inactivates chloramphenicol and that the chloramphenicol-eliminated clones from these strains no longer have this enzyme, are additional evidence that the determinant responsible for chloramphenicol resistance may be located on a plasmid.

There is now little doubt that plasmid-mediated chloramphenicol resistance in staphylococci is due to the same enzymatic mechanism of inactivation as has been described in enteric bacteria harboring R factors (23, 27) or possessing a chromosomal resistance determinant (H. W. Jacobsen, Jr., and W. V. Shaw, Bacteriol. Proc., p. 60, 1970). Unlike the constitutive synthesis of chloramphenicol acetyltransferase in the gram-negative species, the enzyme is inducible in both *S. epidermidis* and *S. aureus*. Although the genetic and molecular aspects of induction have yet to be examined in similar detail, it seems likely that future studies may reveal features analogous to those described for the penicillinase plasmids of staphylococci (16, 21).

The results of the present study indicate that the chloramphenicol acetylating enzyme found in *S. epidermidis* is similar, if not identical, to that of *S. aureus* as far as *p*H optima (7.8), apparent affinity for chloramphenicol, kinetics of heat denaturation, and immunological reactivity. The enzymes from the two staphylococcal species appear to differ only with respect to electrophoretic behavior and sensitivity to inhibition by inorganic mercuric ion. The differential reactivity with respect to substrates is reproducible but probably trivial compared with the above differences. In view of the evidence that the native enzymes from both species of staphylococci have the same molecular weight (Fig. 10), it is apparent that the different mobilities on disc gel electrophoresis (Fig. 8) are due to differences in charge rather than size. The same may be said for the *E. coli* enzyme which shows identical behavior on gel filtration (W. V. Shaw, *unpublished data*), but more rapid migration than the *S. aureus* enzyme in electrophoresis at alkaline *p*H (25, 29). Although amino acid composition studies of the purified enzymes are in progress, it is apparent that subtle differences may not be detected readily without resorting to analysis of peptides split by enzymatic or chemical methods.

The high degree of inhibition of both staphylococcal enzymes noted for sulfhydryl reagents and especially by inorganic mercuric ion may prove to be a useful observation for future comparative studies. The observation that the latter type of inhibition is not reversed by dialysis against mercaptoethanol suggests that mercaptide formation may lead to irreversible changes in tertiary structure. The enhanced sensitivity of the *S. epidermidis* enzyme to mercuric ion is reproducible but thus far unexplained. The availability of milligram amounts of the purified protein will permit sulfhydryl titrations and conformational studies that may help to explain the differential reactivity.

A most gratifying observation during the present study was the ease with which subunits of chloramphenicol acetyltransferase could be demonstrated by the recently described method of Weber and Osborn (28). Taken together, the data of Fig. 10 and 11 are consistent with earlier estimates of the size of the similar *E. coli* enzyme (25) and compatible with a tetrameric structure of subunits of 20,000 molecular weight. More recent preliminary experiments using the technique of polyacrylamide gel electrophoresis in 8 M urea have revealed single bands for both staphylococcal enzymes, suggesting that they probably consist of four identical subunits. *N*-terminal amino acid studies currently in progress should provide stronger evidence for the proposed structure.

In the event that such a formulation proves to be correct, a novel experimental situation can be envisioned if chloramphenicol-resistant strains of either *S. aureus* or *S. epidermidis* can be transduced by phage from the complementary resistant species to yield progeny bearing two functionally identical enzymes with different electrophoretic mobilities. Although similar in a formal genetic sense to Richmond's observations of the enzymes dictated by compatible penicillinase plasmids (19), such diploids might well be expected to have a *minimum* of two isoenzymes and, theoretically, as many as five. This assumption presupposes the possibility of random association of the subunit polypeptide chains of each type to form native enzyme. If, for illustrative purposes, the chloramphenicol acetyltransferase polypeptide found in *S. aureus* is designated α and that of *S. epidermidis* β, the diploid should yield not only the expected α_4 and β_4 enzymes but the intermediate $\alpha_1\beta_3$, $\alpha_2\beta_2$, and $\alpha_3\beta_1$ forms as well. The occasional instances of interspecies phage-mediated transfer of antibiotic resistance in staphylococci suggest that construction of the desired genotype may be feasible (7). Success in isolating the desired dual-plasmid strain of staphylococcus would also provide another system for confirming Richmond's observation of a novel type of control of total enzyme synthesis in *S. aureus* diploid for the plasmid-linked

penicillinase gene (20). In any event, the demonstration of electrophoretically different types of chloramphenicol acetyltransferase among resistant staphylococci may prove to be a useful observation for more general epidemiologic and genetic studies (6, 18).

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