JOURNAL OF BACTERIOLOGY, June 1969, p. 1248-1257
Vol. 98, No. 3

Kinetics of Induction and Purification of Chloramphenicol Acetyltransferase from Chloramphenicol-resistant *Staphylococcus aureus*

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Received for publication 20 February 1969

Plasmid-mediated chloramphenicol resistance in *Staphylococcus aureus* has been shown to involve acetylation of chloramphenicol by an enzyme induced by growth in the presence of the antibiotic and certain analogues. Analysis of the kinetics of induction has been complicated by (i) the intrinsic inhibitory effects of chloramphenicol on induced enzyme synthesis and (ii) the rapid disappearance of inducer after synthesis of the acetylating enzyme. The compound related to D-threo chloramphenicol which lacks a C_3 hydroxyl substituent (3-deoxychloramphenicol) is a potent inducer of chloramphenicol acetyltransferase but is ineffective as an antibiotic and is not a substrate for the enzyme. The availability of such a "gratuitous" inducer has simplified an analysis of the kinetics of induction of chloramphenicol acetyltransferase. The enzyme from induced bacteria has been purified to homogeneity and has been compared with the analogous enzyme present in *E. coli* which harbors a resistance transfer factor with the chloramphenicol resistance determinant.

Prior studies of certain chloramphenicol-resistant (Chl-R) strains of *Staphylococcus aureus* suggested that antibiotic inactivation was the mechanism of resistance (6, 11). Subsequent workers confirmed these observations and demonstrated that inactivation is the result of acetylation of chloramphenicol by acetyl coenzyme A (acetyl CoA) in the presence of the inducible enzyme, chloramphenicol acetyltransferase (8, 20, 23). The latter experiments were prompted by the parallel finding that extracts of enteric bacteria carrying an episomal *chl* locus (R factor) were capable of catalyzing the acetylation of chloramphenicol to 3-acetoxy and 1,3-diacetoxy derivatives (17, 18, 22). In addition to the similarities in the biochemical mechanism of resistance to chloramphenicol, it was also apparent that analogies existed between the episomal determinant for resistance in enteric bacteria and a probable extrachromosomal (plasmid) *chl* locus in *S. aureus* (3, 8). More recent experiments have shown that the *chl* determinant is lost spontaneously at a high frequency which is increased by growth at elevated temperatures and by serial subculture in the absence of chloramphenicol (16).

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In view of the probable similarities between *S. aureus* and *Escherichia coli* with respect to (i) the extrachromosomal location of the *chl* locus and (ii) the enzymatic basis of resistance, a comparative study of chloramphenicol acetyltransferase was undertaken. A previous report indicated that the products of the enzyme reaction were identical and that the *p*H optima (7.8) and molecular weights (78,000) for each enzyme were similar (20). The *S. aureus* enzyme was found to be more resistant to heat inactivation and failed to react with antiserum prepared against enzyme from Chl-R *E. coli* (R+). The enzymes also differed in apparent affinity (K_m) for chloramphenicol and in electrophoretic mobility by the polyacrylamide disc gel technique (20). Earlier experiments were performed with crude cell extracts. The availability of highly purified enzyme has permitted a more rigorous analysis of certain selected properties.

In the course of the comparative studies of the gene product of the *chl* determinant, it was observed that the synthesis of chloramphenicol acetyltransferase in *S. aureus* is induced by the presence of chloramphenicol as well as by certain analogues of the antibiotic, whereas the presence of enzyme is a constitutive property of Chl-R

E. coli (R+). Of special interest was the observation which prompted the present study, namely, that the kinetics of enzyme synthesis followed a sigmoid time course. Rapid synthesis of the acetylating enzyme occurred only after a long lag period (40 to 60 min) following the addition of chloramphenicol, and the resulting linear rate of synthesis ceased abruptly after the resulting acetylation of the inducer (20).

The experiments to be reported deal with the kinetics of chloramphenicol acetyltransferase induction under "gratuitous" conditions (7) by use of an inducer that (i) is relatively ineffective as an inhibitor of protein synthesis and (ii) is not a substrate for the enzyme. It was hoped that the availability of such a compound might yield induction kinetics which failed to show the prolonged lag phase and the postinduction plateau often noted with induction under nongratuitous conditions. It was also hoped that the reproducible and sustained synthesis of enzyme observed with a gratuitous inducer would be an asset in obtaining maximal enzyme yields for purposes of purification. An unrealized ancillary hope was the consideration that the screening of analogues of chloramphenicol might yield a compound which was not an inducer but which was a potent antibiotic. However, no such analogue has been noted, nor have compounds been found which are effective antibiotics but which lack affinity for the acetylating enzyme (19).

MATERIALS AND METHODS

Bacterial strains. All studies were performed with *S. aureus* C22.1, which is resistant to chloramphenicol but sensitive to penicillin G, tetracycline, streptomycin, erythromycin, and lincomycin. This strain, which was kindly provided by R. P. Novick, was obtained by transduction of the *chl* determinant from a clinical strain of *S. aureus*, isolated by Y. Chabbert, to *S. aureus* 8325. The origins and characteristics of these strains were summarized in a previous report (20).

Culture methods. Bacteria were grown in Penassay Broth (Difco) at 37 C in a rotary shaker water bath. In typical experiments, the sterile medium (125 ml) was inoculated with 1 ml of an overnight (16 hr) culture of *S. aureus* C22.1 grown in the absence of chloramphenicol. In view of the high rate of loss of plasmid-linked chloramphenicol resistance (16), strain C22.1 was frequently subcultured on Penassay Agar containing 50 μg of chloramphenicol per ml to assure the maintenance of plasmid-rich stock cultures for the preliminary overnight incubation. Induction of chloramphenicol acetyltransferase was generally initiated in the midexponential phase of growth by the addition of chloramphenicol or a related compound at the final concentrations indicated. Cell density was estimated with a Klett photoelectric colorimeter and a no. 66 filter (660 nm) which gave typical midexponential values of 100 Klett units [0.65 optical density (OD) unit and approximately 8×10^7 colony-forming units per ml].

Samples were taken after induction, as indicated for the determination of enzyme activity and total protein. Portions (5 ml) of each culture were filtered by vacuum on membranes (0.45 μm pore size; Millipore Corp., Bedford, Mass.), after which the cells were washed with 5 ml of ice-cold 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 7.5, containing 145 mM sodium chloride. The filters were placed in tubes containing 0.5 ml of the above (Tris-saline) buffer and were agitated on a vortex mixer to resuspend the bacteria. Lysis was accomplished enzymatically by the addition of lysostaphin as previously reported (20).

Assay of chloramphenicol acetyltransferase. The rate of chloramphenicol acetylation was determined for cell extracts, prepared as described above, by the spectrophotometric method and the reaction conditions described in earlier studies (20). The method is based upon the stoichiometric liberation of 1 mole of reduced CoA per mole of chloramphenicol acetylated by acetyl CoA and by the coupled reaction of reduced CoA with 5,5'-dithio-bis-2-nitrobenzoic acid (DTN), which is determined at 412 nm. Appropriate controls were run to correct for thioester cleavage unrelated to chloramphenicol acetylation by observing the rate of DTN reduction in the absence of chloramphenicol. One unit of enzyme is considered to be that amount of enzyme which catalyzes the acetylation of 1 μmole of chloramphenicol under specified conditions (20).

Protein determination. All protein analyses were performed on samples of the culture which had not been subjected to enzymatic lysis. A five-ml amount of the culture was centrifuged rapidly; then the cell pellet was washed with cold Tris-saline buffer and was resuspended in 5% trichloroacetic acid for 30 min at 4 C. The resulting precipitate was collected by centrifugation and heated with 1 N NaOH for 16 hr at 37 C. The solubilized protein was determined by the method of Lowry et al. (9).

Purification of chloramphenicol acetyltransferase from S. aureus C22.1. The induction of the chloramphenicol acetylating enzyme was accomplished on a preparative scale by taking advantage of the conditions of gratuitous induction observed with 3-deoxychloramphenicol (D-threo-1-*p*-nitrophenyl 2-dichloroacetamido-1-hydroxy propane), an analogue which lacks the C_3 substituent of the parent antibiotic. An 18-liter amount of Penassay Broth was inoculated with 1 liter of an overnight culture grown in the presence of chloramphenicol (20 μg per ml). The bacteria were grown on a rotary shaker at 37 C in 1 liter batches and were induced early in the exponential phase of growth by the addition of 3-deoxychloramphenicol (0.005 mM). The cells were harvested in early stationary phase by centrifugation at 10,000 rev/min for 20 min with the GSA rotor of a Sorvall RC-2B centrifuge operating at 4 C. The culture flasks were kept in an ice bath until centrifugation, and all subsequent steps were performed at 4 to 6 C unless otherwise noted. The cell paste (44.2 g, wet weight) was washed with 1 liter of Tris-saline buffer

(see above), and was resuspended in 900 ml of the same solution for cell disruption at 37 C with lysostaphin and deoxyribonuclease as described previously (20). Lysis was complete in approximately 1 hr, as judged by the change in OD of the suspension at 660 nm. The crude lysate was clarified by centrifugation and yielded 780 ml of pale amber fluid containing 1,037 units of enzyme activity. The crude preparation was taken to 100 mM Tris (*p*H 7.8) and was made 0.1 mM with respect to 2-mercaptoethanol and chloramphenicol before precipitation with ammonium sulfate. The latter was accomplished by the addition of 392 g of finely ground ammonium sulfate to 900 ml of the buffered extract (70% saturation). The precipitate resulting after 2 hr was removed by centrifugation as described above, and the supernatant fluid (900 ml) was taken to 90% saturation by the addition of 121 g of ammonium sulfate. The final precipitate was collected by centrifugation and resuspended to a final volume of 65 ml in 10 mM Tris (*p*H 7.8) containing 0.2 mM chloramphenicol and 0.1 mM 2-mercaptoethanol (TCM buffer). The resulting solution, which contained 436 units of enzyme, was dialyzed against 50 volumes of TCM buffer for 16 hr without loss of activity. The desalted ammonium sulfate fraction was then applied to a column (2.5 by 20 cm) of diethylaminoethyl (DEAE) cellulose and was eluted with 2 liters of TCM buffer containing sodium chloride in a linear gradient from 0 to 400 mM. Enzyme activity was eluted at approximately 190 mM sodium chloride. The fractions with maximum specific activity were pooled to a final volume of 135 ml, which contained 244 units of enzyme. After concentration to 8 ml by ultrafiltration under nitrogen, the pooled DEAE eluate was applied to a Sephadex G-100 column (2.5 by 90 cm) for gel filtration in the presence of TCM buffer containing 200 mM sodium chloride. The peak fractions were combined to yield a final volume of 43 ml containing 174 units of enzyme. The resulting G-100 pooled eluate was then concentrated as before to a volume of 5 ml. The concentrated purified material in TCM-sodium chloride was found to be stable to freezing and thawing but was divided into small samples for storage at −20 C before further study.

Immunochemical analysis. The purified enzyme preparation from *S. aureus* C22.1 was examined by the agar double-diffusion technique, as described previously, by use of a rabbit antiserum to purified chloramphenicol acetyltransferase of the R-factor type prepared from *E. coli* (20). A single precipitation line was noted when the antiserum was reacted with the enteric enzyme, but none was observed when the purified *S. aureus* was tested as the antigen.

Disc gel electrophoresis. Purification of the staphylococcal acetylating enzyme was monitored by the technique of polyacrylamide disc gel electrophoresis at *p*H 9.3, with the Buchler apparatus and conditions described by Davis (5). Enzyme activity was localized by the histochemical stain described previously (20), and protein was detected by staining with amido black. The mobility of purified (G-100) enzyme was found to coincide precisely with the protein band revealed by amido black.

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. Acetyl-CoA was synthesized by the method of Simon and Shemin (21). 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.

RESULTS

Induction of chloramphenicol acetyltransferase by chloramphenicol. As described previously (20), the addition of D-threo chloramphenicol (0.1 mM) to a logarithmic-phase culture of *S. aureus* C22.1 resulted in a nearly linear increase in acetylating activity after a 60-min lag when the enzyme was measured in lysates of whole cells prepared with lysostaphin. Figure 1 demonstrates that, in addition to the duration of the lag, both the ultimate rate of synthesis and the peak level achieved are a function of the concentration of chloramphenicol present. At the lowest concentration tested (0.005 mM), an immediate but unsustained increase in enzyme activity was noted, whereas the addition of 0.1 mM chloramphenicol led to a prolonged lag followed by a maximal rate of

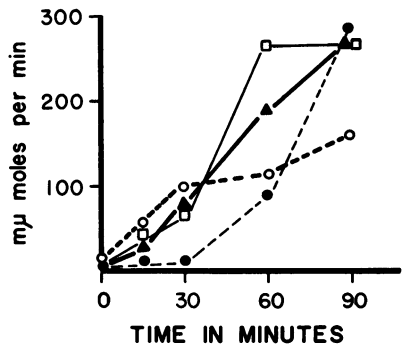

FIG. 1. *Induction of chloramphenicol acetyltransferase by chloramphenicol; concentration dependence. S. aureus C22.1 was grown to the midlogarithmic phase of growth in the absence of chloramphenicol. The culture was then divided into four portions and was induced at zero-time by the addition of chloramphenicol at a final concentration of 0.005 mM (○), 0.03 mM (▲), 0.05 mM (□), and 0.1 mM (●). Samples (5 ml) of each culture were taken at each point indicated. The bacterial cells were collected and washed by membrane filtration (Millipore Corp.), lysed, and assayed for enzyme. Enzyme activity is expressed as the rate of chloramphenicol acetylation observed in cell lysates prepared from 5-ml samples of each culture.*

synthesis and a plateau, usually observed only after approximately 120 min (not shown). Previous studies suggested that the cessation of enzyme induction is due to conversion of chloramphenicol to the acetoxy derivatives which are inactive as inducers (19, 20). Intermediate concentrations of chloramphenicol (0.05 and 0.03 mM) gave proportionately shorter lags before induction, suggesting that the inhibitory effect of chloramphenicol on induced enzyme synthesis might be responsible. Since the complex effects of variations in chloramphenicol concentration appeared to be a major impediment to an analysis of the induction process, we attempted to find a compound related to chloramphenicol which might be a potent inducer but which would not (i) act as an inhibitor of protein synthesis or (ii) serve as a substrate.

Induction of acetylating enzyme by isomers and analogues of chloramphenicol. Table 1 is a summary of selected examples from experiments with more than 30 compounds related to chloramphenicol. Data are presented for the ability of each analogue or isomer to serve as an inducer when compared with the parent compound at the same concentration. The data from earlier work (19) relating to each compound's ability to serve as an acetyl acceptor have also been tabulated for comparative purposes. A suitable gratuitous inducer (7) would be expected to show inducer activity comparable to chloramphenicol but should be inactive both as a substrate and as an antibiotic. Only one analogue of chloramphenicol (D-threo-1-*p*-nitrophenyl-2-dichloroacetamido-1-hydroxy propane) showed promise as such an inducer (Table 1). For convenience, we have chosen to describe this compound as 3-deoxychloramphenicol, a trivial name which stresses both its steric and structural identity with the parent compound as well as the absence of a C_3 hydroxyl group, which has been shown to be essential for significant antibacterial activity (2) and acetyl acceptor activity (19).

The remaining data in Table 1 indicate that the D,threo conformation is essential for induction and that an appropriate *N*-acyl substituent is necessary. The structure of the carbon skeleton is also critical in that (i) two asymmetric carbon atoms are required for the preferred stereochemistry, (ii) introduction of methyl groups at C_1 and C_3 abolishes activity, and (iii) methylation of 3-hydroxyl prevents induction and masks the acetylation site. Although not shown in Table 1, an extensive survey of *para*-substituted 1-phenyl-analogues has led to the conclusion that this moiety is unimportant as a determinant of inducer activity (W. V. Shaw and E. Winshell, *unpublished data*).

TABLE 1. *Induction of chloramphenicol acetyltransferase by analogues and isomers of chloramphenicol*

Compound	Induction rate (per cent of control)[a]	Acetyl acceptor activity (per cent of control)[b]
Isomers		
D-Threo chloramphenicol (CM)	100	100
D-Erythro chloramphenicol	3	20
L-Threo chloramphenicol	19	0
L-Erythro chloramphenicol	21	0
N-acyl analogues[c]		
N-dibromoacetyl	115	94
N-monochloroacetyl	37	79
N-acetyl	100	75
None (free amine)	<5	0
$C_1 - C_3$ *analogues*[d]		
1-Methyl-1,3-propanediol	<5	25
1,3-Butanediol	10	0
3-Methyl-1,3-butanediol	19	0
1-Hydroxy ethane	7	0
1-Hydroxy propane	60	0

[a] Each compound was compared as an inducer with the parent antibiotic (CM) at a concentration of 0.05 mM under conditions similar to those of Fig. 1, except that enzyme activity was assayed in each case 90 min after induction. The control induction value for CM was 0.35 μmoles acetylated per min. Enzyme activity was determined in lysates prepared from 5-ml samples of each culture.

[b] 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 (19).

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

[d] For CM, the side chain carbon skeleton is 1,3-propanediol. All compounds tested contained the 1-*p*-nitrophenyl and 2-dichloroacetamido substituents of the parent compound (CM).

Chloramphenicol acetyltransferase induction with 3-deoxychloramphenicol. The optimal conditions for enzyme induction with 3-deoxychloramphenicol were determined by varying the concentration of inducer over a broad range (Table 2). The stability of the analogue toward the acetylating enzyme permitted measurable induction at a concentration as low as 0.0002 mM, whereas similar experiments with chloramphenicol did not lead to detectable increases in enzyme at concentrations below 0.001 mM. No further increase

TABLE 2. *Concentration dependence of induction with 3-deoxychloramphenicol*

Inducer concn (μM)	Increase in chloramphenicol acetyltransferase (units per 5 ml of culture)[a]	Rate of growth (per cent of control)[b]
0	0	100
0.2	0.041	100
0.5	0.087	100
1.0	0.107	98
2.0	0.142	96
5.0	0.161	95
10	0.162	87
20	0.073	77
50	0.032	64
100	0	55

[a] Enzyme activity is expressed as the increase in chloramphenicol acetyltransferase observed 30 min after induction with the analogue. The uninduced control value was 0.012 unit per 5 ml at zero-time.

[b] Growth was estimated by the turbidity change at 660 nm. The control showed an increase of 0.40 OD unit, whereas cultures induced in the presence of the analogue showed smaller increments in turbidity, expressed as a per cent of the increase noted with the uninduced culture.

in the rate of induction was observed with concentrations of 3-deoxychloramphenicol in excess of 0.0005 mM, and a decrease was noted at 0.02 mM. This inhibition may relate to the observation that the minimal inhibitory concentration (MIC) of 3-deoxychloramphenicol is 0.03 mM for both *S. aureus* C22.1 (Chl-R) and for the Chl-S strain (*S. aureus* 8325) which lacks the resistance plasmid. By way of contrast, the MIC for chloramphenicol under similar conditions (Penassay Broth; 18 hr at 37 C) is 0.4 mM for *S. aureus* C22.1 and 0.005 mM for the sensitive strain which lacks the acetylation enzyme. Taken together the above observations suggest that 3-deoxychloramphenicol possesses sufficient antibiotic activity to limit its value for induction studies at concentrations greater than 0.01 mM and that the similar MIC values of both the sensitive and resistant strains are a reflection of the inability of either strain to inactivate the analogue by acetylation.

A more detailed comparison of chloramphenicol and its 3-deoxy analogue was carried out with the combined enzymatic and radioautographic techniques described in Fig. 2. Both compounds were added to logarithmic-phase cultures of *S. aureus* C22.1, but the parent compound was labelled with ^{14}C so that its disappearance and the coincident production of the 3-acetoxy derivative could be quantitated. The data clearly indicate that the rate of synthesis of the acetylating enzyme declines abruptly when the concentration of ^{14}C-chloramphenicol is less than 0.002 mM, whereas the initial rate of induction observed with the 3-deoxy analogue is sustained. Figure 2 also indicates that the lag before enzyme synthesis is similar for both compounds when they are tested as inducers at subinhibitory concentrations.

A test of the overall performance of 3-deoxychloramphenicol as a gratuitous inducer is summarized in Fig. 3, wherein chloramphenicol acetyltransferase activity is plotted as a function of the total protein present in an equal sample of the culture at a number of points in time throughout the logarithmic phase of growth. The slope of the line is a measure of the proportionality of enzyme induction to total protein synthesis occur-

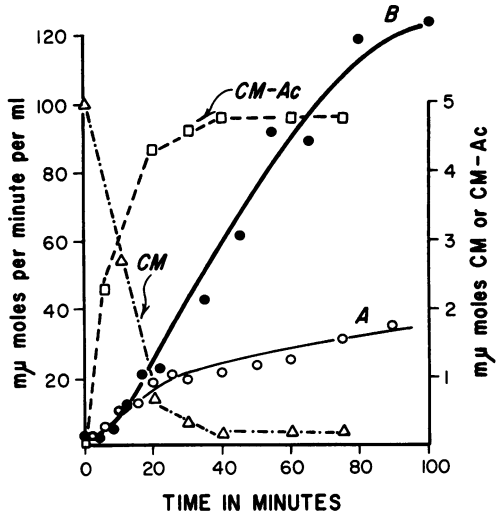

FIG. 2. *Comparison of chloramphenicol and the 3-deoxy analogue as inducers of chloramphenicol acetyltransferase. Curves A (○) and B (●) describe the appearance of enzyme activity after induction with chloramphenicol and the 3-deoxy analogue, respectively. Enzyme was assayed in lysostaphin lysates prepared from 5-ml samples. S. aureus C22.1 was grown to the midlogarithmic phase of growth, at which point the culture was divided so that A was induced with 0.005 mM ^{14}C-chloramphenicol (5 μc per μmole) and B with the same concentration of the nonradioactive 3-deoxy analogue. The disappearance of chloramphenicol (CM) and the appearance of the corresponding monoacetate (Cm-Ac) were determined by thin-layer chromatography and radioautography as described previously (20). Ethyl acetate extraction and thin-layer chromatography were also performed in identical fashion on the culture induced by the 3-deoxy analogue. When viewed under short-wave ultraviolet light, single spots of identical mobility were observed for samples taken at the onset of induction and after 100 min. The rate of chloramphenicol acetylation refers to the enzyme obtained from cell lysates prepared from 1 ml of culture medium.*

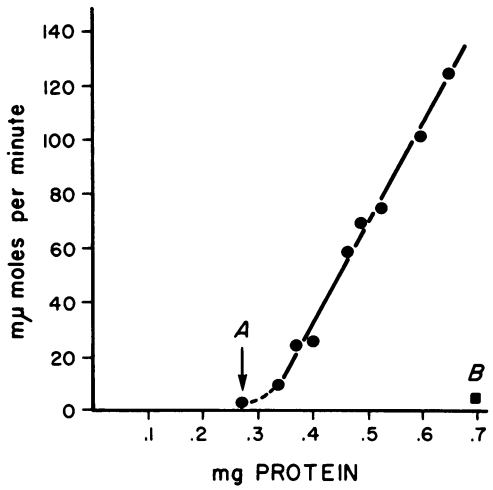

FIG. 3. *Induction of chloramphenicol acetyltransferase as a function of total protein synthesis. After induction of an early logarithmic-phase culture of S. aureus C22.1 at point A with 0.1 mM 3-deoxychloramphenicol, samples were taken over a 60-min period for the determination of enzyme activity and total protein. The abscissa and ordinate represent the protein and enzyme recovered, respectively per 5-ml sample of the induced culture. Point B indicates the enzyme detected in a control culture after 60 min of incubation in the absence of inducer.*

ring during growth. A crude estimate of the fraction of total protein synthesis accounted for by chloramphenicol acetyltransferase may be made by reference to Table 4 (see below), wherein the purified enzyme is noted to have a specific activity of 17.4 units per mg of protein. The differential rate of synthesis of enzyme (slope in Fig. 3) can be calculated and expressed as 0.37 unit per mg of protein synthesized, or, by use of the specific activity conversion factor, approximately 0.02 mg of enzyme per mg of total protein is synthesized after induction. Although the estimate of 2% is, at best, a first approximation, it is of the same order of magnitude observed with β-galactosidase in *E. coli* (7) and is consistent with the ease with which homogeneous acetylating enzyme was isolated after a modest degree of purification (Table 4).

Characterization of the induction process. Whereas an interpretation of the events occurring after the addition of inducer was complicated in the case of chloramphenicol, the desoxy analogue proved to be a more suitable inducer for such studies when tested at subinhibitory concentrations. Figure 4 depicts the time course of induction after the addition of 3-deoxychloramphenicol to an exponential-phase culture of *S. aureus* C22.1 grown at 37 C. Under the conditions specified, the lag before detectable enzyme synthesis began was approximately 3 min, an observation which is consistent with the total time required for transcription and translation of the structural gene for β-galactosidase in *E. coli* (7).

It is also apparent that removal of the inducer after induction results in a prompt cessation of enzyme synthesis. Figure 5 indicates the course of such an experiment, in which a culture was divided 30 min after induction with 3-deoxychloramphenicol. The cells from each half of the culture were collected and washed in similar fashion and were resuspended in fresh medium either with or without the inducer. In the latter case, a decrease in the rate of enzyme synthesis was detected as early as 7 min after filtration. Such a figure may not represent the minimum time required for deadaptation, since the technical aspects of the procedure precluded measurements at earlier times.

An inducible system of the type proposed on the basis of the foregoing observations might be expected to be sensitive to puromycin, which is a potent inhibitor of protein synthesis at the translational level (12). The experiment described in Fig. 6 indicates that the synthesis of chloramphenicol acetyltransferase ceases after the addition of puromycin to a culture in which the inducer is still present.

A novel effect of temperature on an inducible system has been described for penicillinase pro-

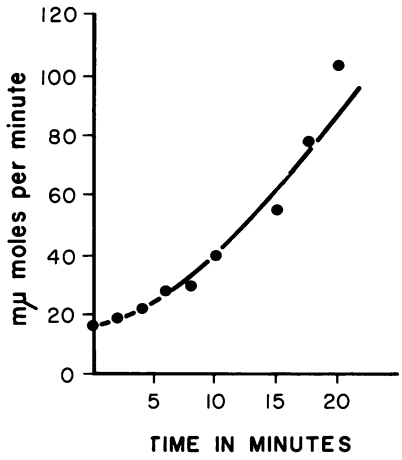

FIG. 4. *Time course of induction of chloramphenicol acetyltransferase. At zero-time, a midlogarithmic-phase culture of S. aureus C22.1 was induced with 3-deoxychloramphenicol at a final concentration of 0.005 mM. Samples were taken for rapid membrane filtration (Millipore Corp.) and subsequent lysis of the collected bacterial cells. Enzyme activity is expressed as chloramphenicol acetyltransferase present in each 5-ml sample.*

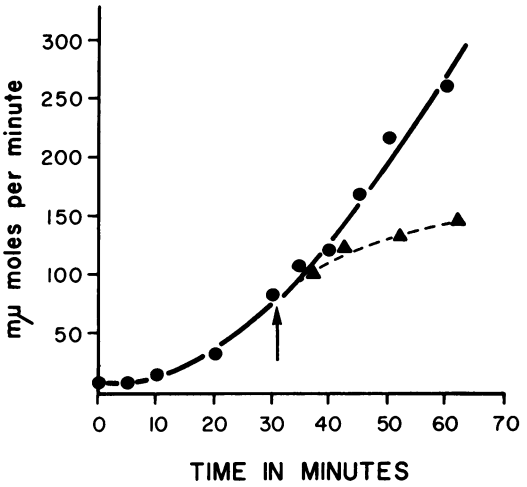

FIG. 5. *Dependence of enzyme synthesis on the presence of inducer. A midlogarithmic-phase culture of S. aureus C22.1 was induced at zero-time with 3-deoxychloramphenicol (0.005 mM). After 30 min of incubation, the culture was divided and each half of the cell mass was collected by filtration (see text), washed with fresh culture medium, and resuspended in medium without inducer (▲) or in the presence of 3-deoxychloramphenicol at the same concentration employed for induction. The solid circles (●) represent the results of enzyme assays in cells collected before washing and also after resuspension in the presence of inducer. The ordinate expresses enzyme activity per 5-ml sample of the cell suspension.*

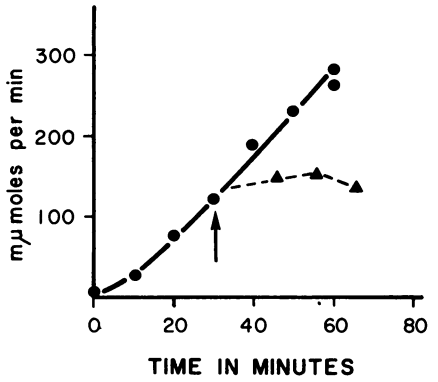

FIG. 6. *Inhibition of chloramphenicol acetyltransferase synthesis by puromycin. The induction conditions were identical to those of Fig. 5. The culture was divided 30 min after induction, and puromycin (final concentration, 50 μg per ml) was added to one portion (▲), whereas the other half was allowed to incubate in the absence of the inhibitor (●). Enzyme activity was determined and expressed as described in Fig. 5.*

duction in *S. aureus*, in that growth at 42 C results in derepression that is not dependent upon the presence of inducer and is presumed to be due to the presence of a heat-labile repressor (4).

The temperature dependence of induction for chloramphenicol acetyltransferase was examined in similar fashion over the range from 32 to 42 C (Table 3). Uninduced control cultures showed no significant increase in the basal level of enzyme when induction was carried out at elevated temperatures. The temperature dependence noted for the analogue-related induction process may well reflect the general effects of temperature on cell metabolism rather than a characteristic of the inducible system in question.

Induction was observed to be rather insensitive to *p*H changes over a range from 6.0 to 7.0 when phosphate-buffered Penassay Broth was used for the induction medium. A 25% decrease in rate of synthesis was observed at *p*H 5.5, whereas only a 10% reduction was noted at *p*H 7.5 (data not shown).

Purification of chloramphenicol acetyltransferase from S. aureus C22.1. When the optimal conditions for enzyme induction were defined for 3-desoxy-chloramphenicol, a large-scale growth experiment was undertaken to provide enzyme for purposes of purification. Table 4 summarizes the results of a three-step procedure which resulted in greater than 100-fold purification and the isolation of enzyme which appeared to be a homogeneous material, as judged by the absence of contaminating protein bands after disc gel electrophoresis (Fig. 7). Studies are currently in progress to determine whether higher yields of purified enzyme can be achieved by taking advantage of the relative heat stability of the *S. aureus* enzyme (20).

TABLE 3. *Effect of temperature on induction of chloramphenicol acetyltransferase*[a]

Temp	Enzyme specific activity (units per mg of protein)				
	Zero-time	20 min		40 min	
		Control	Inducer	Control	Inducer
C					
32	0.016	0.014	0.049	0.018	0.143
37	0.012	0.014	0.074	0.016	0.173
42	0.022	0.020	0.170	0.024	0.369

[a] A culture of uninduced *S. aureus* C22.1 was grown at 37 C to the early logarithmic phase of growth, at which time it was divided into portions for further growth at the temperatures indicated. After 20 min of equilibration, each culture was induced with 3-deoxychloramphenicol (0.01 mM). The control cultures represent samples treated in similar fashion, save for the absence of induction with the analogue. Samples of 5 ml were taken before induction (zero-time) and at 20-min intervals for assays of enzyme activity and total protein.

TABLE 4. *Purification of chloramphenicol acetyltransferase from S. aureus C22.1*[a]

Step	Prepn	Vol (ml)	Total enzyme (units)	Total protein (mg)	Specific activity (units per mg)	Per cent recovery	Purification
1	Crude lysate	780	1,037	6,326	0.16	100	1.0
2	Ammonium sulfate precipitate	65	436	618	0.70	42	4.4
3	DEAE peak	135	244	32	7.6	24	48
4	G-100 peak	43	174	10	17.4	17	109

[a] From 44 g (wet weight) of cells.

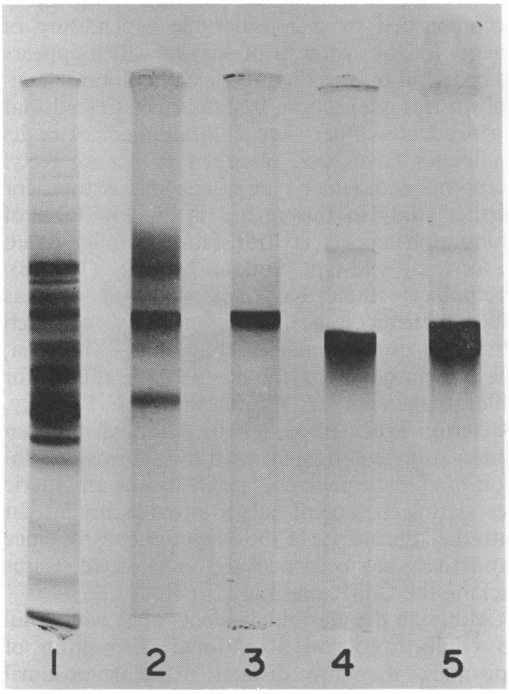

FIG. 7. *Disc gel electrophoresis of purified chloramphenicol acetyltransferase from S. aureus C22.1 and R+ E. coli (Chl-R). Disc gel electrophoresis was performed at pH 8, and protein was detected by staining with amido black. The lower pole of each gel corresponds to the anode. Gels 1, 2, and 3 contained enzyme from S. aureus C22.1 as follows: (1) 95 μg of ammonium sulfate-precipitated protein, (2) 72 μg of protein from DEAE peak, and (3) 40 μg of protein Sephadex G-100 peak. Gel 4 contained 40 μg of highly purified chloramphenicol acetyltransferase from E. coli (20). Gel 5 was run with 40 μg each of the purified S. aureus and E. coli enzymes. In each instance, the major protein bands were found to correspond with enzyme activity by use of the histochemical stain described previously (20) or by elution of enzyme activity from serial slices of unstained gels.*

Comparison of purified chloramphenicol acetyltransferase from S. aureus with the R factor-mediated E. coli enzyme. Earlier experiments with crude lysates of *S. aureus* C22.1 (20) demonstrated that the chloramphenicol acetyltransferase of micrococcal origin was similar to that found in R+ strains of Chl-R *E. coli* with regard to *p*H optimum (7.8) and approximate molecular weight (78,000). The preparations were found to differ, however, with respect to (i) the apparent affinity of each enzyme for chloramphenicol and selected analogues, (ii) electrophoretic mobility on polyacrylamide gel, and (iii) reactivity with antiserum prepared against purified enzyme from *E. coli* (20).

The differences in electrophoretic behavior and immunological reactivity have been confirmed and extended with the highly purified *S. aureus* C22.1 enzyme prepared as described in Table 4. In Fig. 7, a disc gel electrophoretic analysis of the purification of chloramphenicol acetyltransferase from *S. aureus* (gels 1, 2, and 3) is compared with a sample of the previously described (20) enzyme from R+ *E. coli* (gel 4). The possibility that the slight differences in mobility between the *S. aureus* (gel 3) and *E. coli* (gel 4) enzymes might represent an artifact is rendered unlikely by the results of gel 5; this gel contained equal amounts of both preparations and showed a broadening of the stained protein band consistent with the pattern expected for two proteins of slightly different mobilities.

The technique of double diffusion in agar gel was used to compare the reactivity of the purified *S. aureus* and *E. coli* enzymes with antiserum prepared against the enteric enzyme. Figure 8 demonstrates that, regardless of whether the enzymes are compared at equivalent amounts of purified protein (A) or identical levels of enzyme activity (B), there is no evidence of a reaction with the *S. aureus* enzyme, whereas there is a clearly discernible precipitin band formed with the *E. coli* preparation. Absorption of the antiserum with the *S. aureus* protein failed to diminish its ability to precipitate the *E. coli* enzyme. Furthermore, as was noted previously with crude extracts (20), the addition of antiserum to chloramphenicol acetyltransferase from *S. aureus* showed no effect on enzyme activity, whereas the purified *E. coli* enzyme was irreversibly inactivated under similar conditions (data not shown).

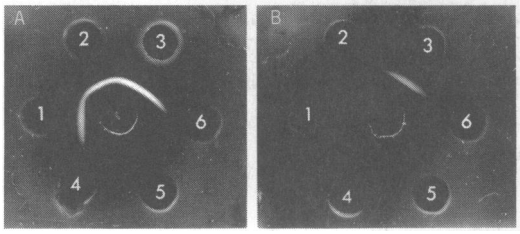

FIG. 8. *Immunological analysis of purified chloramphenicol acetyltransferase by double diffusion in agar. Center wells contained undiluted rabbit antiserum to E. coli enzyme (20). In both A and B, the numbers 1, 2, and 3 refer to E. coli enzyme and 4, 5, and 6 refer to enzyme from S. aureus. In experiment A, the enzymes were compared by use of the same quantities of enzyme as follows: wells 1 and 4 (0.05 unit), wells 2 and 5 (0.15 unit), and wells 3 and 6 (0.5 unit). In experiment B, the corresponding pairs contained equal amounts of protein; wells 1 and 4 (10 μg), wells 2 and 5 (30 μg), and wells 3 and 6 (100 μg). The photograph was made after 24 hr of incubation at 25 C.*

DISCUSSION

The growing body of information which relates to chloramphenicol resistance of *S. aureus* suggests that the present observations might best be discussed in the context of (i) mechanisms of chloramphenicol inactivation among other clinically important resistant bacteria, (ii) the general topic of antibiotic resistance mediated by extrachromosomal genes, and (iii) the possible therapeutic insights which might be gained from the present study.

An earlier report (20) stressed the similar aspects of enzymatic acetylation, as observed in Chl-R *S. aureus* and in enteric bacteria harboring a resistance transfer factor episome with the chloramphenicol determinant. The identity of the main products, chloramphenicol 3-acetate and chloramphenicol 1,3-diacetate, was established in both instances (17, 20, 22). Further studies, which stressed the superficial similarities (*p*H optima, molecular weight, and specificity for compounds related to chloramphenicol), suggested a greater degree of homology between the two types of enzyme than has been apparent from subsequent experiments. On the basis of the evidence presented here and the data reported previously (19, 20), there is ample justification for the view that the chloramphenicol acetyltransferase of *S. aureus* is quite distinct from that found in *E. coli* and related enteric bacteria. It should be emphasized, however, that the differences noted (substrate affinity, heat stability, electrophoretic mobility, and immunological reactivity) could result from quite minor substitutions in amino acid sequence. In spite of the availability of highly purified enzymes of both types, the delineation of such differences will be a formidable task in view of the molecular weight of the enzymes in question and their apparent lack of subunit structure (W. V. Shaw, *unpublished data*). In any event, the most striking difference from a biological point of view is the inducibility of the *S. aureus* enzyme in contrast to the constitutive synthesis of the homologous enzyme in the enteric species. The resulting high-level resistance of the enteric bacteria (MIC > 200 μg per ml), in contrast to Chl-R *S. aureus* (MIC = 50 μg per ml), is obviously accomplished by a considerable expenditure of energy for the synthesis of enzyme which appears to be useful only in the presence of chloramphenicol. In this connection, it should be stressed that no substrates other than chloramphenicol or its analogues have been observed to act as acetyl acceptors with either type of enzyme. A topic for further study in this regard is the low level of chloramphenicol acetyltransferase activity noted in certain wild-type enteric bacteria (15, 18). Preliminary studies have suggested that, whereas chloramphenicol acetylating activity of such strains is quite similar to that of R+ bacteria, the nonepisomal enzyme has a lower affinity for chloramphenicol (W. V. Shaw and R. F. Brodsky, Bacteriol. Proc., p. 32, 1968). Although such an observation is consistent with the stepwise evolution of chloramphenicol specificity for an enteric acetylating enzyme, it fails to provide any insight into the appearance of the *S. aureus* enzyme since no activity has been detected in sensitive strains lacking the Chl-R plasmid (20).

Although the plasmid elements of *S. aureus* fail to conform to the operational description of episomes, they are distinct extrachromosomal genetic elements containing a wide variety of resistance determinants (13). In contrast to the rigorous demonstration of an extrachromosomal locus for penicillinase in *S. aureus* (13, 14), only circumstantial evidence has been presented for plasmid-linked chloramphenicol resistance (3, 8, 10, 16). In addition, although a detailed analysis is now available for the penicillinase plasmid, little is known about the genetic control of chloramphenicol acetyltransferase in *S. aureus*. The isolation of constitutive (i^-) as well as temperature-sensitive and superinducible mutants of chloramphenicol acetyltransferase would afford meaningful comparisons with the penicillinase plasmid.

In contrast to the striking effectiveness of the semisynthetic penicillins which are resistant to hydrolysis by the β-lactamase of *S. aureus*, there is little to suggest that medically useful analogues of chloramphenicol will be found which are (i) not substrates or (ii) ineffective as inducers of the

acetylating enzyme. An extensive survey of analogues and isomers of chloramphenicol has revealed that compounds which are not acetylated are essentially devoid of antibacterial activity (19) and that a similar correlation exists for analogues which are ineffective inducers of chloramphenicol acetyltransferase (20). An unexpected result of the search for clinically useful compounds has been the finding that the 3-deoxy analogue of chloramphenicol possesses favorable characteristics for enzyme induction under gratuitous conditions. By avoiding the complications resulting from the use of the parent antibiotic which is an inhibitor and a substrate as well as an inducer, it has been possible to analyze this system in some detail and to compare it with other well-defined inducible enzymes.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant A1-03744 from the National Institute of Allergy and Infectious Diseases and by a Career Scientist award to W. V. S. by the Health Research Council of the City of New York.

The authors thank Mildred Rebstock, Parke, Davis & Co., for her continuing interest in these investigations and for generous gifts of chloramphenicol analogues.

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