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Purification and Properties of Gentamicin Acetyltransferase I†

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ABSTRACT: Gentamicin acetyltransferase I is induced 13-fold in R factor resistant *Escherichia coli* by high concentrations (1 mg/ml) of gentamicin in the growth medium. The enzyme is maximally released from bacteria by osmotic shock in late-log phase, unlike previously studied periplasmic enzymes. Streptomycin sulfate and ammonium sulfate precipitations of shockate followed by affinity and ion-exchange chromatography recover 51% of the induced enzyme with a 360-fold increase in purity (12% of 4400-fold, uninduced). The purified enzyme appears homogeneous by six criteria, the first aminoglycoside inactivating enzyme so purified. Sodium dodecyl sulfate electrophoresis, amino acid analysis, and sedimentation analyses indicate a tetrameric protein of 63000 molecular weight. The protein does not contain tryptophan. Kinetic analyses yield apparent values of: V_{max} = 3.4 ± 0.2 μmol per min mg at pH 8 (optimum), K_m (acetyl-CoA) = 3.9 ± 0.2 μ*M*, K_m (gentamicin C_{1a}) = 0.3 ± 0.08 μ*M*, K_I (gentamicin substrate inhibition) = 160 ± 29 μ*M*. The activity of the enzyme is stable to a variety of conditions, including lyophilization and prolonged storage, and can be monitored by two convenient spectrophotometric assays.

Resistance to aminoglycoside antibiotics in many species of bacteria is determined by the presence of R factors which direct the synthesis of a new family of enzymes (Benveniste and Davies, 1973). These enzymes inactivate aminoglycoside antibiotics by three separate mechanisms: acetylation, adenylylation and phosphorylation. A thorough understanding of the mechanisms of resistance is dependent upon characterization of these unusual enzymes, which in turn is dependent upon substantial purification of the enzymes in a reasonable quantity. In addition, considerable interest exists in preparing stable forms of the antibiotic inactivating enzymes in quantity, for the purpose of developing rapid and specific clinical assays of antibiotics in body fluids (Williams et al., 1975; Smith and Smith, 1974; Holmes and Sanford, 1974; Haas and Davies, 1973). Despite differences between inactivating mechanisms, the enzymes show a number of similarities. Notable among these are extremely low yields of enzyme from cellular extracts and a limited stability, particularly upon exposure to cellulose and Sephadex column resins, properties which have greatly hindered purification (D. B. Northrop, J. Davies, and co-workers, unpublished results; Smith and Smith, 1974). This paper describes the purification of gentamicin acetyltransferase I to

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homogeneity, the first aminoglycoside inactivating enzyme so purified, using methods which specifically overcome these problems. This enzyme was first identified and partially purified from R^+ *Pseudomonas aeruginosa*, and catalyzes the transfer of an acetyl group from acetyl-CoA to the 3-amino group of the gentamicin C antibiotics and sisomicin (Brzezinska et al., 1972). In addition, the methods described have been found applicable to the purification of gentamicin adenylyltransferase and neomycin phosphotransferase II, and may find utility in the purification of other inactivating enzymes.

Materials and Methods

Chemicals. Samples of veterinary grade gentamicin sulfate (potency, 559 μg/mg as free base) and purified gentamicin C_{1a} (purity, 93–95%) were provided by Dr. Gerald Wagman of Schering Corp. Standard proteins used for sodium dodecyl sulfate gel electrophoresis were: bovine serum albumin (Sigma), chymotrypsinogen A (bovine pancreas, Sigma), myoglobin (horse heart, Mann), and cytochrome *c* (horse heart, Sigma). The materials for disc gel and dodecyl sulfate gel electrophoresis, Affi-Gel 10, DEAE Bio-Gel A, and Bio-Gel P-2 were from Bio-Rad. Streptomycin sulfate, Nbs_2[1], Tris, and EDTA were from Sigma. P-81 phosphocellulose ion-exchange paper was from Whatman, acetyl-CoA from P-L Biochemicals, enzyme grade ammonium sulfate from Schwarz/Mann, and [1-^{14}C]acetyl-CoA was from ICN.

Growth of Bacteria. Escherichia coli C-600 containing R factor JR88 were obtained from Dr. Julian Davies. Stock cultures were maintained in medium containing 8 g/l. of peptone, 5 g/l. of yeast extract, and 5 g/l. of glucose. To prevent R factor segregation, veterinary grade gentamicin (15 μg/ml) was initially added to all media. Enzyme was isolated from *E. coli* grown in 15-l. Model FS314 New Brunswick fermentators with constant stirring and aeration (10 l./min), containing 10 l. of the above medium inoculated with 1 l. of an overnight culture. The fermentor was submerged in a water bath maintained at 37°C. Later it was found that the enzyme production was increased during growth if higher concentrations of gentamicin were used in the inoculum (see below).

Protein Determinations. Protein concentrations during purification were determined by measuring absorbance at 280 nm and assuming 1 OD_{280} = 1 mg/ml of protein. Values determined by this method agree closely with results obtained by the colorimetric method of Lowry et al. (1951). Following purification, ultraviolet spectra of GAcT I[1] were determined in Tris-HCl (pH 7.8) and in 0.1 *N* NaOH. A value for $E_{293}(1\%)$ of 134 was obtained as described by Bencze and Schmid (1957). From the spectral data, $E_{293}(1\%)$, and assuming 24 tyrosines per 63000 daltons of enzyme, a value of 1.40 mg/ml per OD_{280} at pH 7.8 was calculated for pure GAcT I.

Amino Acid Analysis. Samples of purified GAcT I were hydrolyzed in 6 *N* HCl at 110°C in evacuated glass tubes, one for 24 hr, and one for 48 hr. A sample was also alkylated with iodoacetate according to the procedure of Crestfield et al. (1963), evacuated, and hydrolyzed for 24 hr at 115°C in 4 *N* methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole for the determination of cysteine and tryptophan. Approximately 15 μg of protein hydrolysate was chromatographed in each analysis, using a Durrum 500 amino acid analyzer. The ratio of tyrosine to tryptophan content was determined according to the spectrophotometric method of Goodwin and Morton (1946) assuming six tyrosines per subunit.

Electrophoresis. Disc gel electrophoresis was conducted in a Bio-Rad Model 300A cell driven by a Model 400 power supply. Electrophoresis of native enzyme followed the procedure described by Gabriel (1971). Molecular weight determinations by dodecyl sulfate gel electrophoresis were performed at room temperature as described by Bio-Rad Laboratories (1974).

Enzyme Assays. The procedure for a radioactive assay (assay 1), which follows the formation of [^{14}C]acetylgentamicin, is described by Benveniste and Davies (1971). This assay was used during the initial studies because of the low enzyme levels and the presence of material that interfered with the spectrophotometric assays. Enzyme activity during purification and the initial kinetic studies was followed by monitoring the disappearance of the thioester bond of acetyl-CoA, ϵ 4500 (Stadtman, 1955), at 232 nm (assay 2), using a Gilford Model 240 spectrometer and a Leeds and Northrop Speedomax XL-610 recorder. Full scale optical densities from 0.04 to 0.1 were used. The temperature of the cuvette compartment was maintained at 25°C. All reactions were carried out in a total volume of 0.45 ml in self-masking micro-cuvettes with a 10-mm light path purchased from Hellma Cells, Inc. Assays of enzyme activity during purification were carried out with reaction mixtures containing 133.3 μM acetyl-CoA, 24.7 μM gentamicin C_{1a}, 10 m*M* Tris-HCl (pH 7.8), 0.15 m*M* EDTA, and 1 m*M* magnesium acetate. Kinetic measurements were carried out with reaction mixtures that contained 8.88 m*M* Tris-HCl (pH 7.8), 0.133 m*M* EDTA, 8.88 m*M* $(NH_4)_2SO_4$, and varied concentrations of acetyl-CoA and gentamicin C_{1a}. Reactions were started by addition of enzyme kept at 0°C at the appropriate dilution. The high background absorbance limits the usefulness of this assay at very high acetyl-CoA or protein concentration. A second spectrophotometric assay (assay 3) was employed in later kinetic measurements to gain sufficient sensitivity necessary to assay GAcT I at very low substrate concentrations. The production of the free sulfhydryl of CoASH was linked to a chemical reaction with Nbs_2 (Benveniste and Davies, 1971). Absorbance at 412 nm (ϵ 15700) in 0.1 *M* Tris at pH7.8 (Alpers et al., 1965) was monitored using the same instrument and cuvettes as above. Kinetic measurements were done according to the procedure described for assay 2 except that 0.555 m*M* Nbs_2 was included in the reaction mixture.[2] Unless otherwise specified, international units are used and velocities are units per mg of protein.

Data Processing. Velocities were first plotted graphically as double reciprocal plots, and the data were then fitted to appropriate rate equations by the least-squares method, assuming equal variance for the velocities (Wilkinson, 1961). The fits were performed by a digital computer with the Fortran programs of Cleland (1967). Reciprocal plots were fitted to eq 1, if linear, or to eq 2, if substrate inhibition was observed. The regression lines for dodecyl sulfate electrophoresis and equilibrium sedimentation molecular weight

[1] Abbreviations used are: GAcT I, gentamicin acetyltransferase I; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).

[2] It was later found that an initial 5–10 sec lag in the rates in this assay could be eliminated by increasing the Nbs_2 concentration to 5.5 m*M* without inhibition of the enzyme.

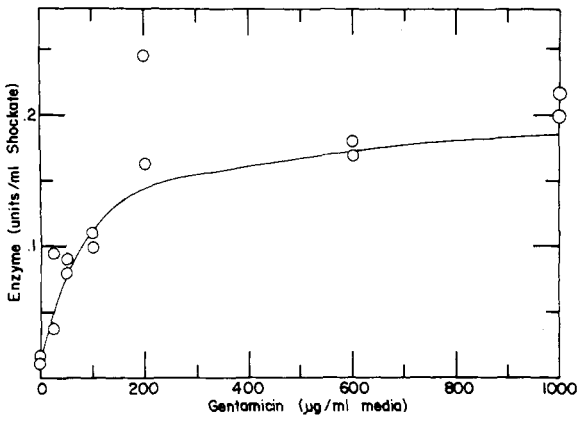

FIGURE 1: Induction of GAcT I with gentamicin. *E. coli* were grown stepwise in slowly increasing crude gentamicin concentrations to a final concentration in the medium of 1 mg/ml; 0.5 ml of culture from this high antibiotic concentration was then used to inoculate flasks containing 50 ml of medium at the following crude gentamicin concentrations: 0, 10, 25, 50, 100, 200, 600, and 1000 μg/ml. Each culture was transferred four times to a new medium containing antibiotic and grown overnight. The fifth transfer was a 5-ml inoculum to each of duplicate flasks containing the respective antibiotic concentrations. These cultures were then grown to an OD_{550} = 0.8, harvested, and osmotically shocked (see text). The GAcT I activity in the shockate was determined by assay 2.

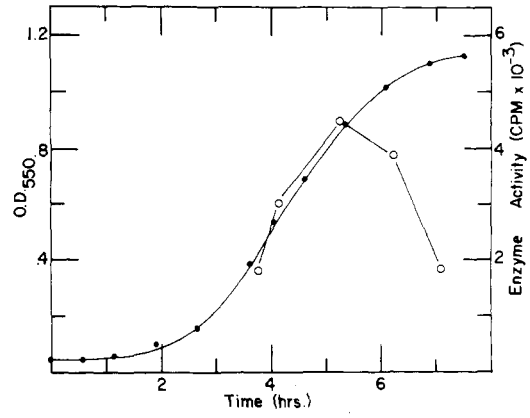

FIGURE 2: Extraction of GAcT I as a function of bacterial growth. Medium containing 15 μg/ml of crude gentamicin (500 ml) was inoculated with 5 ml from an overnight culture. Growth was followed by optical density at 550 nm and GAcT I activity was determined from 45 ml of culture harvested at various stages of growth and stored at 0°. The pellets were all osmotically shocked identically and the GAcT I activity in the shockate was determined by assay 3.

determinations were calculated by the Madison Academic Computing Center Program REGAN 2.

$$v = VA/(K + A) \quad (1)$$

$$v = VA/(K + A + A^2/K_I) \quad (2)$$

Results

Induction of GAcT I. The specific activity of GAcT I in shockate obtained at peak enzyme production (see below) was increased 13.5-fold by increasing the concentration of veterinary grade gentamicin in the inoculum. *E. coli* were gradually adapted to growth in medium containing 1 mg/ml of veterinary grade gentamicin by successive transfers into medium containing increasing concentrations of antibiotic. Attempts to achieve adaptation by a single transfer were unsuccessful. Once adapted, the bacteria were then transferred back to medium containing various lower antibiotic concentrations and grown for 25–30 generations in order to compare GAcT I production and antibiotic concentrations in cells with common history. The activity of GAcT I in shockate vs. the veterinary grade gentamicin concentration is shown in Figure 1 and found to follow a saturation phenomena. The enzyme was half-maximally induced at a veterinary grade gentamicin concentration of 78 μg/ml. These results also show that the induction is reversible, with the activity of GAcT I returning to normal levels after 25–30 generations in medium containing no added antibiotic. However, reversibility is gradual since high enzyme levels were present in the cells even after 5–6 generations. This latter finding was exploited to prepare sufficient enzyme for study but avoid the impracticalities of either growing large batches of bacteria or consuming an excessive amount of antibiotic. By growing the inoculum in high antibiotic concentrations (>150 μg/ml) and then transferring to medium with no antibiotic added, induced levels of GAcT I could still be attained. To demonstrate that the induced and uninduced GAcT I are in fact the same enzyme, samples of both were purified and partially characterized.

Harvesting of Cells. The yield of enzyme obtained by osmotic shock varies considerably as a function of the growth curve of *E. coli*, and reaches a peak in late-log phase as shown in Figure 2. The peak represents more than a tenfold increase over the yield obtained from overnight cultures (presumably well into stationary phase). To maximize the yield of enzyme, it is necessary to harvest the cells within the narrow time period of peak activity. For small volumes of media (<5 l.), cells were rapidly harvested by centrifugation at 5000*g* for 10 min in a Beckman J21-B preparative centrifuge, as soon as an OD_{550} of 0.8 was reached. Once obtained, the pellet of cells was stored at 0°C for upwards to 24 hr with no significant loss of activity, and could therefore be combined with other preparations. Alternatively, with large volumes of media (33 l.), the fermentor was chilled by the addition of ice to the water bath to slow the growth of cells and allow sufficient time for harvesting. Chilling of medium as soon as the OD_{550} reached 0.75 increased the optimum time of harvesting to approximately 2 hr.

Preparation of Osmotic Shockate. Harvested cells were osmotically shocked according to the method of Nossal and Heppel (1966). The procedure followed was a modification of this method by J. Davies (personal communication), described here for a 1-l. culture of bacteria. Harvested cells were washed twice at room temperature with 200 ml of 30 m*M* Tris-HCl at pH 7.3, and mixed well for 15 min at room temperature. The washed pellet was resuspended in 100 ml of 20% sucrose containing 3 m*M* EDTA in 33 m*M* Tris-HCl at pH 7.3, and mixed well for 15 min at room temperature. All subsequent purification steps were carried out at 4°C. The pellet was collected by centrifugation at 10000*g* for 10 min, decanted, thoroughly drained, and finally swabbed with cotton-tipped applicator sticks to minimize the presence of residual sucrose. The bacterial cells were osmotically shocked by resuspending the pellet in 16 ml of 0.5 m*M* $MgCl_2$, using a Vortex mixer. After stirring or shaking the suspension for 15 min, the supernatant was collected by centrifugation at 20000*g* for 10 min and adjusted to 0.15 m*M* EDTA. This supernatant is the "osmotic shockate" and can be stored at −20°C for several days without substantial loss of activity. All waste products preceding the preparation of the shockate were autoclaved to minimize the risk of spreading R-factor resistance.

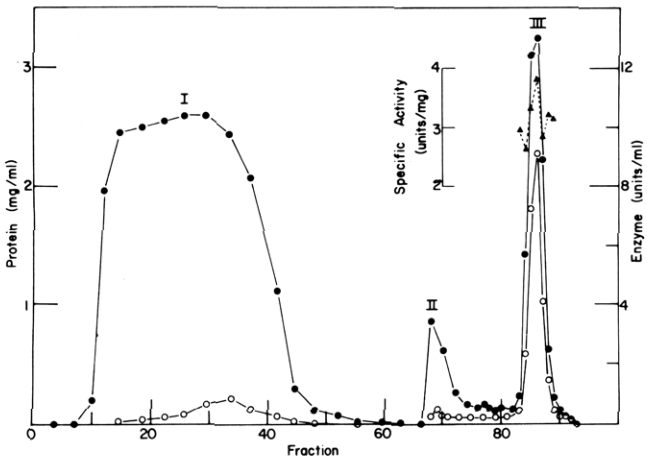

FIGURE 3: Affinity chromatography of induced GAcT I on gentamicin C_{1a}–Affi-Gel 10. The plots represent: protein (●), GAcT I activity (○), and specific activity (△). Fraction I was eluted with buffer A, fraction II with buffer A containing 20 m*M* ammonium sulfate, and fraction III with 0.1 *M* potassium acetate buffer (pH 4.5) containing 0.15 m*M* EDTA and 0.1 *M* ammonium sulfate. The column was 1 × 30 cm and eluted at 0.5 ml/min into 2.3-ml fractions. Peak fractions were brought to pH 7.4–8.0 with 2 *M* Tris-HCl (pH 8.2) and assayed for GAcT I activity. The elution profile of uninduced GAcT I was similar to the above except that no enzyme activity was detected prior to fraction II (see text).

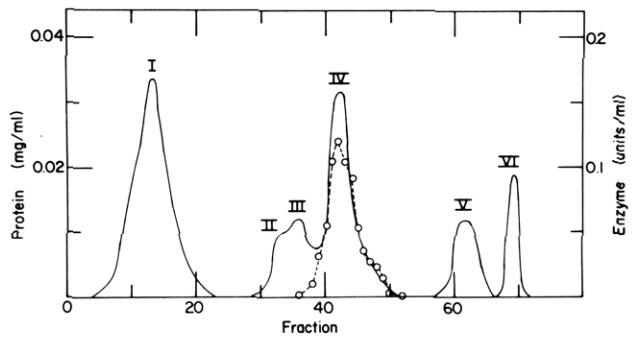

FIGURE 4: Chromatography of uninduced GAcT I from DEAE-agarose. The plots represent: protein, from an Isco Type IV UV column monitor (—), and GAcT I activity (○). A linear gradient of ammonium sulfate (0–0.08 *M* in buffer A) was run from fraction 26 to fraction 67. Fractions 68–74 were eluted with 0.3 *M* ammonium sulfate in buffer A. The column was 19 cm and was eluted at a flow rate of 0.5 ml/min. Fraction volumes were 1 ml.

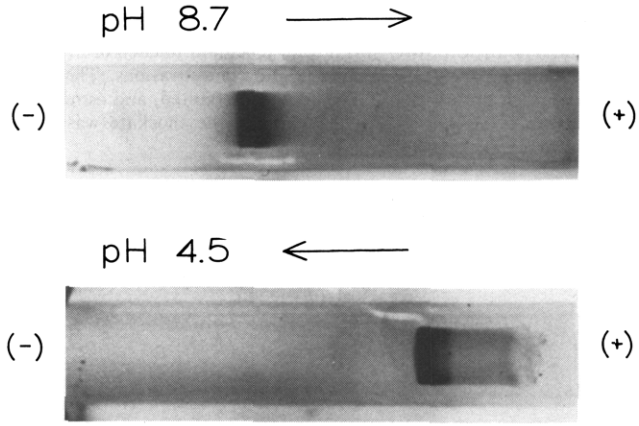

FIGURE 5: Disc gel electrophoresis of induced GAcT I. Samples containing 70 μg of protein were placed on 7.5% polyacrylamide gels and run at 3 mA/tube. Gels were stained for protein in a solution containing 0.1 g of Coomassie Blue, 45 ml of methanol, and 45 ml of H_2O, and diluted threefold with water just before use. Destaining was carried out in 10% Cl_3CCOOH for 12 hr.

Streptomycin Sulfate Precipitation. The osmotic shockate was brought to 1.5% w/w in streptomycin sulfate to precipitate nucleic acids. The white, stringy, precipitate was centrifuged at 15000*g* for 15 min and the pellet discarded. The supernatant displayed artificially high activity with the radioactive assay which was not seen with the spectrophotometric assays.

Ammonium Sulfate Precipitation. The streptomycin sulfate supernatant was brought to 65% saturation by gradual addition of solid ammonium sulfate. After the mixture was stirred for 1 hr, the precipitate was collected by centrifugation at 30000*g* for 15 min and redissolved in a minimum volume of 0.15 m*M* EDTA and 10 m*M* Tris-HCl buffer (pH 7.8) (buffer A). This redissolved ammonium sulfate precipitate can be frozen and stored for at least a month with only a slight loss in enzyme activity. Intermediate ammonium sulfate fractionations yielded only slight increases in purity and were not routinely employed.

Affinity Chromatography. The affinity resin was prepared by coupling 300 mg of gentamicin C_{1a} to 3 g of activated agarose (Affi-Gel 10) according to the procedure described by Bio-Rad Laboratories (1973). The coupling reaction is a displacement of *N*-hydroxysuccinimide by unidentified primary amine groups of gentamicin C_{1a} at a neutral pH. The redissolved ammonium sulfate precipitate was first desalted on Bio-Gel P-2 equilibrated with buffer A and then applied to the affinity column, also equilibrated with buffer A. The enzyme was eluted from the affinity column in a single sharp peak by lowering the pH of the eluent to 4.5, shown in Figure 3. Attempts to elute the enzyme by high ionic strength produced a broad dilute peak. The results obtained by affinity chromatography varied as a function of the amount and possibly the specific activity of enzyme added to the column. When a high amount of GAcT I was applied to the affinity column (>5 units/ml of column resin) the fractions containing enzyme were approximately 80% pure, whereas the application of low amounts of GAcT I (<0.3 unit/ml of column resin, uninduced enzyme, not shown) produced enzyme of only 35% purity. Apparently, the affinity column yields a more effective purification when more fully loaded.

DEAE-Agarose Chromatography. Neutralized fractions from the gentamicin affinity column were desalted on Bio-Gel P-2 equilibrated with buffer A and then applied to a DEAE Bio-Gel A column also equilibrated with buffer A. Six protein fractions were separated on this column during the purification of the uninduced enzyme, shown in Figure 4. One fraction passed through the column (I), four fractions eluted with a linear gradient (II–V), and a final fraction eluted at high ionic strength (VI). GAcT I activity was associated with only one of the fractions (IV). In contrast, during the purification of the induced enzyme, only two protein fractions eluted from the DEAE-agarose column. Virtually all of the enzyme activity applied to the DEAE column eluted in the gradient as a single protein peak with constant specific activity. The other protein fraction eluted corresponds to peak VI of Figure 4. The results of the purification of both the uninduced and induced GAcT I are summarized in Table I.

Disc Gel Electrophoresis. A sample of induced GAcT I from the final step of purification was subjected to disk gel electrophoresis at pH values of both 4.5 and 8.7. The results in Figure 5 show a single protein band at both pH values, with a slight nonbanded smearing of the low pH band. Sep-

Table I: Purification of Gentamicin Acetyltransferase I.[a]

Purification Step	Protein (mg/ml)	Enzyme Activity (units/ml)	Specific Activity (units/mg)	Total Volume (ml)	Total Units	Recovery (%)	Purification
1. Shockate	0.894	0.0042	0.0047	780	3.27	100	1
	(3.04)	(0.192)	(0.063)	(2160)	(415)	(100)	(1)
2. 1.5% streptomycin sulfate	0.677	0.0036	0.0053	780	2.8	86	1
	(2.30)	(0.154)	(0.067)	(2160)	(333)	(80)	(1)
3. 65% ammonium sulfate	6.51	0.240	0.037	8	1.9	59	8
	(11.10)	(4.24)	(0.380)	(60)	(354)	(61)	(6)
4. Gentamicin agarose	0.148	0.198	1.34	6.7	1.33	41	285
	(1.35)	(4.20)	(3.10)	(54)	(227)	(55)	(49)
5. DEAE-agarose	0.031	0.107	3.44	4	0.43	13	732
	(1.97)	(7.49)	(3.80)	(30)	(225)	(54)	(60)

[a] Results from the preparation of induced enzyme from 132 l. of bacterial culture are shown in parentheses. Uninduced enzyme was prepared from 33 l. of culture.

Table II: Amino Acid Composition of Gentamicin Acetyltransferase I.[a]

Amino Acid	Residue/His Found	Residue/ Subunit Nearest Integer	Weight[e] Residue per Subunit
Aspartic acid	6.3	13	1495
Threonine[b]	2.8	6	606
Serine[b]	4.3	9	783
Glutamic acid	6.8	14	1806
Proline	4.3	9	873
Glycine	5.1	10	570
Alanine	8.0	16	1136
Valine	3.0	6	594
Methionine[b]	1.0	2	262
Isoleucine	2.6	5	565
Leucine	6.4	13	1469
Tyrosine	3.1	6	978
Phenylalanine	2.0	4	588
Histidine	1.0	2	274
Lysine	2.4	5	640
Arginine	3.3	7	1092
Cysteine[c]	3.8	8	824
Tryptophan[d]	0.1	0	0
Total	66.3	135	14,555

[a] Assuming a subunit molecular weight of 16000. [b] Extrapolated to zero time of hydrolysis. [c] Determined as carboxymethylcysteine. [d] Determined spectrophotometrically. [e] Values are corrected for a loss of H_2O in protein.

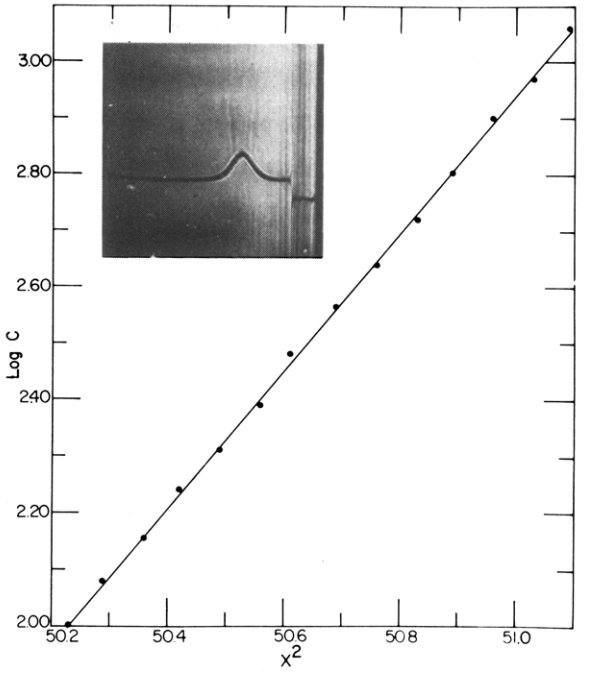

FIGURE 6: Molecular weight determination of GAcT I by equilibrium sedimentation. The rotor speed was 25980 rpm, protein concentration was 0.4 mg/ml, rotor temperature was 5°C, and the solvent was 10 m*M* Tris-HCl (pH 7.8), 10 m*M* $(NH_4)_2SO_4$, and 0.15 m*M* EDTA. Insert: Sedimentation pattern of GAcT I. The rotor speed was 59780 rpm, protein concentration was 5.2 mg/ml, rotor temperature was 20°C, and the solvent was same as above. The frame shown was taken 40 min after the attainment of maximal rotor speed.

arate gels were run simultaneously with the gels stained for protein. These gels were sliced (3 mm/slice), incubated with Tris buffer at 4°C for 4 hr, and assayed for GAcT I activity. Activity was only associated with the regions corresponding to the stained areas at both pH values. Disc gel electrophoresis of 5 μg of uninduced GAcT I obtained from fraction IV of the DEAE-agarose column also gave one band of the same mobility. Dodecyl sulfate gel electrophoresis of GACT I on 12% polyacrylamide gels also gave a single band. Comparison of the mobility of GAcT I in dodecyl sulfate with standard proteins indicates a minimum apparent molecular weight of 17000 ± 2000 for the enzyme protein.

Analytical Ultracentrifugation. The sedimentation pattern of a purified sample of induced GAcT I is shown in Figure 6. Only a single component is apparent having an s_{obsd} = 4.2. Results of equilibrium sedimentation centrifugation are also shown in Figure 6. The plot of the log of concentration vs. radius squared is linear with a slope of 1.222, which corresponds to a molecular weight of 63000, using a partial specific volume of 0.72 ml per g calculated from the amino acid composition (Yphantis, 1964).

Amino Acid Composition. The results of amino acid analysis are listed in Table II. Tryptophan was not detected in hydrolysates of alkylated enzyme and its absence was confirmed by spectral methods. The ultraviolet spectrum of GAcT I in 0.1 *N* NaOH has a λ_{max} at 293 nm with no detectable shoulder at 280 nm, with a 280/294 ratio corresponding to a 26.4 ratio of tyrosine to tryptophan.

pH Dependence of Enzymatic Activity. The activity of purified GAcT I shows a broad pH optimum from pH 5.5 to pH 8.5, with a peak in 0.1 *M* Tris-HCl buffer at pH 7.7–8.0. GAcT I has no activity at pH 4.5, the pH at which the enzyme elutes from the gentamicin-agarose affinity col-

Table III: Michaelis and Inhibition Constants.[a]

Enzyme	Substrate	K_m (μM)	K_I (μM)
Uninduced[b]	Acetyl-CoA	4.8 ± 1.5	
	Gent. C_{1a}	0.5 ± 0.3	382 ± 130
Induced[c]	Acetyl-CoA	3.9 ± 0.2	
	Gent. C_{1a}	0.3 ± 0.08	160 ± 29

[a] Concentrations of fixed substrates were: acetyl-CoA, 133 μM; gentamicin C_{1a}, 25 μM. [b] Determined by assay 2. [c] Determined by assay 3.

umn, but activity is fully restored upon neutralization of the enzyme, even after incubation at pH 4.5 overnight at 4°C. However, at pH 4.0, enzymatic activity is irreversibly lost.

Kinetic Constants. The apparent kinetic constants were determined from initial velocity measurements on both the induced and uninduced GAcT I and are given in Table III. Data for the uninduced enzyme were obtained using assay 2, and the induced using assay 3. Within the limits of the errors obtained, the two sets of data agree, indicating an equivalence of the two assay methods and the two forms of the enzyme. Double reciprocal plots of initial velocity data obtained with induced GAcT I reveal significant substrate inhibition by gentamicin C_{1a}. Preliminary studies suggest that gentamicin C_{1a} substrate inhibition is uncompetitive vs. acetyl-CoA concentrations. Using the calculated $E_{280}(1\%)$ for pure GAcT I and correcting for substrate inhibition, the maximal velocity is 3.4 ± 0.2 units/mg, corresponding to a turnover number of 214 mol of product per mol of enzyme per min, assuming a molecular weight of 63000.

Stability Characteristics. The activity of purified GAcT I is stable to a variety of conditions. Treatment with 2 m*M* *N*-methylmaleimide, addition of 0.1 m*M* dithiothreitol, dialysis against 0.1 m*M* EDTA, or addition of magnesium to dialyzed enzyme had no effect on enzymatic activity. Lyophilization of purified enzyme initially caused a loss of 10% of the enzymatic activity, but no additional loss of activity upon storage for periods up to 1 month at either refrigerator or room temperature. Control samples of purified enzyme stored under refrigeration in solutions of buffer A containing 20 m*M* ammonium sulfate lost 35% of the enzymatic activity in 1 month.

The enzyme also binds tightly to a CoA-Agarose affinity column, but no active enzyme could be recovered. Similarly, gel-filtration chromatography was not a successful means of purification as the enzyme lost substantial activity on Sephadex columns and eluted in more than a column volume upon passage over Bio-Gel P-100, the degree of spreading being a function of the ionic strength of the eluent. Nevertheless, the effect was negligible with crude protein fractions, and Bio-Gel P-2 was routinely used for desalting purposes since dialysis of crude fractions also caused excessive losses of activity which could not be traced to a dialyzable cofactor.

Discussion

Gentamicin acetyltransferase I was purified to homogeneity as evidenced by six criteria: migration of the protein as a single band under acidic (a) and alkaline (b) conditions, and in the presence of dodecyl sulfate (c); sedimentation as a single component in the ultracentrifuge (d); linearity of sedimentation equilibrium data (e); and constancy of specific activity by gradient elution during ion-exchange chromatography in the final stage of purification (f). Of equal importance is the present ability to prepare the enzyme in quantity with a high yield. Most of the increase in recovered enzyme occurred prior to the formal purification steps, namely by induction (13-fold, Figure 1) and by careful timing of cell harvesting (>10-fold, Figure 2). The term "induction" is probably a misnomer, as the increased cellular level of enzyme is more likely a result of gene amplification rather than regulation at the operon level. Rownd et al. (1973) have observed an increase in chloramphenicol transacetylase in response to increased concentrations of chloramphenicol, during plasmid studies on *P. mirabilis* containing R factor NR1. The increase in enzyme was accompanied by a proportionate increase in R factor DNA. Furthermore, the bacteria required multiple generations to either maximize or minimize cellular levels of R factor DNA. In the present study, changes in GAcT I of *E. coli*, responding to either an increase or decrease in concentrations of gentamicin, did require multiple generations for full expression, although no direct evidence of gene amplification was obtained.

The effect of the timing of cell harvesting on the amount of enzyme released by the osmotic shocking procedure has not been previously described, although we have also seen the same effect during the preparation of gentamicin adenylyltransferase and neomycin phosphotransferase II (Goldman and Northrop, 1975). The sharp drop in recovered enzyme as the bacteria enter the stationary phase of the growth cycle does not appear to reflect a change in the cellular level of enzyme but rather a change in the cell membranes or cell wall such that the bacteria are less susceptible to osmotic shock. A comparison between the amount of GAcT I released by sonication vs. osmotic shocking supports this view, as do the results of Rownd et al. (1973) who observed an increase in the cellular levels of both R factor NR1 and chloramphenicol transacetylase after *P. mirabilis* entered stationary phase.

In contrast, Nossal and Heppel (1966) did not observe a decrease in the chromosomal enzymes cyclic phosphodiesterase, 5′-nucleotidase, or acid phosphatase, released by osmotic shock from *E. coli* in stationary phase vs. logarithmic phase, except under conditions incorporating 5×10^{-4} *M* Mg^{2+} in the shock medium. Nevertheless, the reported decreases were less than we have observed with the antibiotic inactivating enzymes and we observe no significant difference in the amount of these enzymes released as a function of Mg^{2+} concentrations. Since the osmotic shock method has frequently been used as a means for identifying the intracellular location of a variety of proteins, i.e., cytoplasmic vs. periplasmic, an intriguing hypothesis is that the differential of the amount of shocked enzyme obtained in stationary vs. logarithmic cells varies as a function of more subtle differences of the periplasmic location. If the plasma membrane or cellular components comprising the periplasmic space change upon entry into the stationary phase, then "intracellular-like" periplasmic proteins (the antibiotic inactivating enzymes which utilize intracellular substrates ATP and coenzyme A) may not be as readily released by osmotic shock if they lie within the inner portion of the periplasmic space; whereas "extracellular-like" periplasmic proteins (cyclic phosphodiesterase, 5′-nucleosidase, and acid phosphotase which catalyze hydrolytic reactions in common with extracellular enzymes) may be released equally well from stationary or logarithmic cells if they lie within the external portion of the periplasmic space.

Alternatively, the differential may be dependent upon the molecular weight of periplasmic proteins. Smith and Wyatt (1974) suggested that cell walls have a molecular sieving property after noting that both R factor and chromosomal β-lactamases with a molecular weight of about 20000 are released by osmotic shock, but those with molecular weights of 30000 or more are retained by a variety of gram-negative bacteria in stationary phase. With an average molecular weight of 63000 GAcT I is one of the larger proteins to be released by osmotic shock, and this may have a bearing on the differential release. The molecular sieving argument is weak, however, since 5′-nucleotidase does not display the differential release yet it has a molecular weight of 59000 (Neu, 1967). The presence and location of the single band observed on dodecyl sulfate electrophoresis show GAcT I consists of four subunits of equal size. This is the first aminoglycoside inactivating enzyme to be identified as having a subunit structure.

Attempts to quantitate differences between sonication and osmotic shocking were complicated by a variation in the susceptibility of *E. coli* to sonication as a function of the growth cycle together with inactivation of GAcT I as a function of the time of sonication. However, an estimate of the amount of enzyme in intact cells at the time of maximum susceptibility of release by osmotic shock was obtained by varying the time of sonication and comparing GAcT I activities in the sonicates. Inactivation was found to be first order with a rate constant of 0.0506 sec^{-1} ($t_{1/2}$ = 13.7 sec). A comparison of the activity at zero time of sonication, extrapolated from the inactivation curve, and results obtained by the osmotic shocking procedure indicate that osmotic shock releases 92% of GAcT I in late logarithmic phase and accomplished a sixfold purification of the enzyme from the total, soluble protein. The overall purification represented by Table I is therefore 360-fold for the induced enzyme, and 4400 for the uninduced enzyme.

The low level of enzymatic activity extracted from resistant bacteria early in the study of aminoglycoside resistance prompted a question as to whether or not the enzymes were present in sufficient quantity to satisfy their proposed role as the biochemical mediators of resistant characteristics. The number of molecules of GAcT I per cell in uninduced R factor *E. coli* was therefore estimated as follows, using measurements of the number of viable cells per ml of bacterial culture obtained by a plate count at the time of cell harvesting, the enzymatic activity per ml of culture following osmotic shocking, and the molecular weight and specific activity of purified enzyme: (0.00032 unit/ml of culture)(6.02 $\times 10^{23}$ molecules/mol)/[(5.1 $\times 10^{8}$ cells/ml of culture)(3.4 units/mg)(10^{3} mg/g)(63000 g/mol)] = 1763 molecules/cell. The uninduced R factor *E. coli* cell therefore contains roughly two or more times the number of GAcT I molecules as the average number of molecules of other proteins estimated in *E. coli* (Watson, 1970). Since this number will increase up to 13-fold upon exposure of the bacteria to antibiotic, the proposed function of the enzyme is not inconsistent with its concentration.

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