Aminoglycoside Resistance in Bacteria Mediated by Gentamicin Acetyltransferase II, an Enzyme Modifying the 2′-Amino Group of Aminoglycoside Antibiotics†

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ABSTRACT: Certain bacterial strains resistant to aminoglycoside antibiotics have been shown to contain an enzyme which acetylates the 2′-amino group of the aminohexose ring of gentamicin, tobramycin, kanamycins, and related antibiotics. This enzyme, gentamicin acetyltransferase II, is the first enzyme shown to modify this class of antibiotics at the 2′ position.

Studies on the mechanism of resistance to antibiotics in clinical isolates of resistant bacteria have revealed a variety of mechanisms by which resistance is determined (Benveniste and Davies, 1973a). In the case of the aminoglycoside antibiotics, resistance is due to the presence of enzymes which inactivate the antibiotic by O-phosphorylation, O-adenylylation, or N-acetylation (see Table I). Because of the structural similarities between aminoglycosides various cross-resistance patterns are possible. For example, the phosphotransferases (Yagisawa *et al.*, 1972; Brzezinska and Davies, 1973; Umezawa *et al.*, 1973) are responsible for resistance to antibiotics such as neomycin, kanamycin, and other compounds containing a 3′-hydroxyl group (see Figure 1); gentamicin adenylylate synthetase mediates resistance to gentamicin, kanamycin, and tobramycin, but not to the neomycin-type antibiotics (Benveniste and Davies, 1971a). Recently we described gentamicin acetyltransferase I (GAT_I[1]) which acetylates the 3-NH_2 group of deoxystreptamine in gentamicin (Brzezinska *et al.*, 1972) and is associated with a phenotype of gentamicin resistance and kanamycin sensitivity in *Pseudomonas aeruginosa*. Subsequently, this mechanism of resistance has been found in clinical isolates of *Escherichia coli* (S. Biddlecome, P. J. L. Daniels, and J. Davies, unpublished observations). We now report the characterization of a new gentamicin acetyltransferase which is present in other isolates of gentamicin-resistant, kanamycin-sensitive strains of bacteria. This enzyme, gentamicin acetyltransferase II, catalyzes the acetylation of the 2′-amino group of a number of aminoglycoside antibiotics and represents the first report of enzymatic inactivation at this position of the amino sugar ring, in resistant bacteria.

Materials and Methods

Bacterial Strains. Providencia 164 was kindly provided by Dr. J. A. Waitz. This strain was isolated from a patient by Dr. Morwan Uwaydah of the School of Medicine, American University of Beirut, Republic of Lebanon. The strain is resistant to most aminoglycosides; the exceptions are gentamicin B, kanamycin A, and BB-K8, a derivative of kanamycin A (Kawaguchi *et al.*, 1972).

Enzymatic Assays. The phosphocellulose paper binding assay, as described previously for the N-acetylation of kanamycin and gentamicin, using [^{14}C]acetyl coenzyme A as substrate, was employed in all assays during the enzyme preparation and substrate studies (Benveniste and Davies, 1971a).

Enzymatic Inactivation. The preparation of N-acetylated antibiotics for structural studies was carried out as previously described (Benveniste and Davies, 1971a; Brzezinska *et al.*, 1972). *N*-Acetylsisomicin was purified by ion exchange chromatography and its purity checked by thin layer chromatography on silica gel.

Enzyme Preparation. Providencia 164 was grown in rich medium (yeast extract–tryptone–glucose); the cells were harvested by centrifugation and washed with 10 mM Tris–50 mM NH_4Cl (pH 7.8) at 4°. The pellet was resuspended in 20 mM Tris, 10 mM $Mg(OAc)_2$, 25 mM NH_4Cl, 10 mM KCl, and 2 mM dithiothreitol (pH 7.5). The cells were disrupted by passage through a French pressure cell or by sonication, and the cell debris and ribosomes were removed by centrifugation. This supernatant was used as a source of enzyme for most purposes; it could be concentrated conveniently by ammonium sulfate precipitation (40–70%). The enzyme has been purified by affinity chromatography using a gentamicin C_1–indubiose column (M. Chevereau and F. LeGoffic, unpublished observations).

Chemicals. [^{14}C]Acetyl coenzyme A was obtained from New England Nuclear, and acetyl coenzyme A from Calbiochem and Sigma. The gentamicins, sisomicin, and gentamines were provided by the Schering Corporation, tobramycin by Dr. Kay Koch of Lilly Research Laboratories, kanamycins A, B, and BB-K8, and butirosins A and B by Dr. K. E. Price of Bristol Laboratories, kanamycin C by Dr. H. Umezawa, neomycins by Dr. George Whitfield, Jr., the Upjohn Co., and paromomycin and butirosin by Dr. T. H. Haskell of Parke, Davis and Co.

Results

Providencia 164, a clinical isolate from Lebanon, was found to be resistant (to varying degrees) to most aminoglycosides, including gentamicin, tobramycin, neomycin, and butirosin, but it was found to be sensitive to gentamicin B, kanamycin A, and its derivative BB-K8. This resistance pattern was unusual and different from that associated with any of the previously

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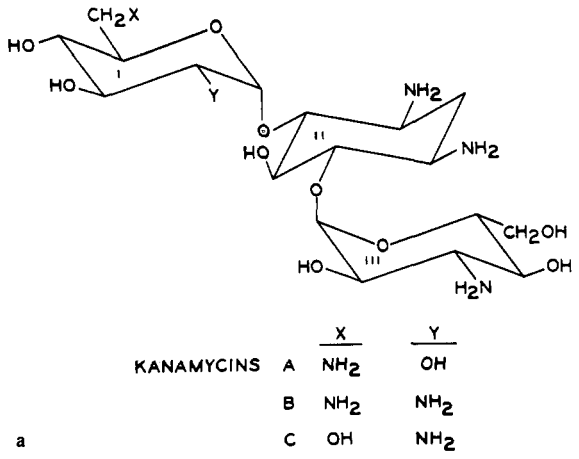
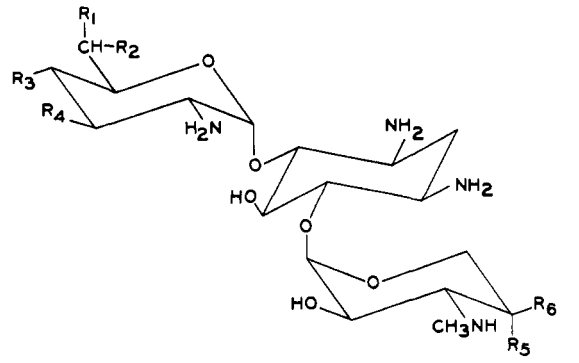
[1] Abbreviation used is: GAT, gentamicin acetyltransferase.

known mechanisms of aminoglycoside resistance (Table I). Extracts of the resistant strain were prepared by sonication and assayed for their ability to catalyze modification of the gentamicins; it was found that acetyltransferase activity was present in the extracts. No phosphotransferase or other known drug modifying activity was present. As shown in Table II, the acetyltransferase from *Providencia* 164 has broad specificity; as will be shown later, the substrate activities vary greatly with pH. We propose to call this activity gentamicin acetyltransferase II (GAT_{II}). The crude enzyme preparation was found to be stable on repeated freezing and thawing and was stored routinely at −20°.



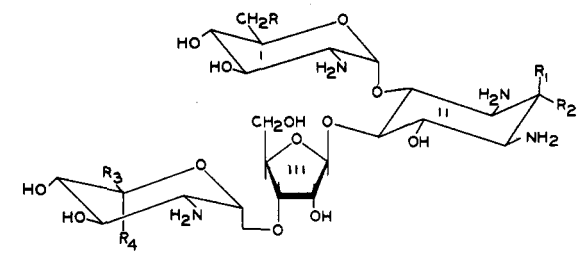
		X	Y
KANAMYCINS	A	NH_2	OH
	B	NH_2	NH_2
	C	OH	NH_2

a



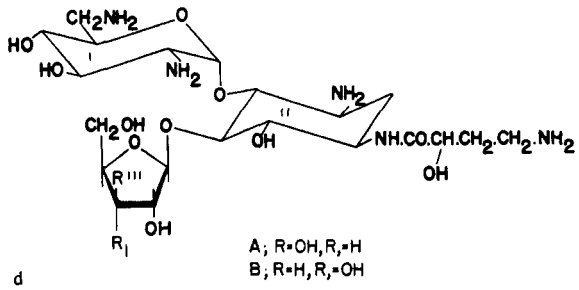
		R_1	R_2	R_3	R_4	R_5	R_6
GENTAMICINS	A	H	OH	OH	OH	H	OH
	C_{1a}	H	NH_2	H	H	OH	CH_3
	C_2	CH_3	NH_2	H	H	OH	CH_3
	C_1	CH_3	$NHCH_3$	H	H	OH	CH_3

b



	R	R_1	R_2	R_3	R_4
NEOMYCIN B	NH_2	H	H	H	CH_2NH_2
NEOMYCIN C	NH_2	H	H	CH_2NH_2	H
PAROMOMYCIN	OH	H	H	CH_2NH_2 or H	H or CH_2NH_2

c



A; R=OH, R_1=H
B; R=H, R_1=OH

d

Preparation and Purification of Acetylated Sisomicin. The substrate activities (Table II) of gentamicin II suggested that the enzyme modified the 2′-amino group, since the only compounds which were not substrates were kanamycin A, BB-K8, and gentamicin B, all of which have a 2′-hydroxyl group. To confirm this supposition it was decided to inactivate a quantity of sisomicin (Cooper *et al.*, 1971; Reimann *et al.*, 1971) with the *Providencia* enzyme; the 4,5 double bond in the 2,6-diaminosugar ring of this antibiotic facilitates the recognition of fragments in subsequent mass spectral analyses. Sisomicin (8 mg) was inactivated by incubation of the drug, acetyl coenzyme A, and enzyme in a total volume of 35 ml at 35° for 6 hr. The product was purified by ion exchange chromatography as previously described, and the acetylated sisomicin eluted from BioRex 70 with 2.0 M NaCl. After purification on a second BioRex column and elution with ammonium hydroxide, the product was collected and shown to be homogeneous by thin layer chromatography on silica gel.

Properties and Structure Determination of Acetylsisomicin. Thin layer chromatography on silica gel (methanol–ammonia, 3:1) showed that *N*-[^{14}C]acetylsisomicin produced by GAT_{II} was a single component (R_F 0.6), clearly distinct from sisomicin (R_F 0.5). Acetylsisomicin showed very weak antibacterial activity against wild-type strains of *E. coli* but this activity was not found with any gentamicin-resistant strains.

The proton magnetic resonance (pmr) spectrum of the inactivated sisomicin was determined in D_2O solution at 100 MHz using a Varian XL-100 instrument. In view of the small sample available (0.6 mg) 25 scans were taken and accumulated with a CAT. Three singlet methyl resonances of approximately equal intensity were observed at δ 1.24 (CH_3C), 2.01 (CH_3CON), and 2.61 (CH_3N) ppm relative to an external sodium 2,2-dimethyl-2-silapentane-5-sulfonate reference. The remainder of the spectrum was consistent with the structure proposed but was not well resolved due to the small sample used. The inactivated product was confirmed to be 2′-*N*-acetylsisomicin by its mass spectrum (Figure 2A) which is shown together with that of sisomicin (Figure 2B). The molecular ion at *m/e* 489 is consistent with a mono-*N*-acetylsisomicin and the peak at *m/e* 169 (ion a) locates the acetate group on the unsaturated sugar ring. Peaks characteristic of garosamine (*m/e* 160, 118) and 2-deoxysteptamine (*m/e* 191, 173, 163, 145) (Daniels *et al.*, 1971) are unshifted in the spectrum of the acetate. Attachment of the acetate group to the 2′-nitrogen atom is demonstrated by the relatively intense peak at *m/e* 404

FIGURE 1: (a) The structures of the kanamycins. Positions in ring I are numbered 1′–6′, in ring II 1–6, and in ring III 1″–6″. Position Y is the 2′ position. Tobramycin is 3′-deoxykanamycin B. BB-K8 is 1-HABA-kanamycin A (see Figure 1d). (b) The structures of the gentamicins. Numbering is as indicated for the kanamycins. Sisomicin is 4′,5′-dehydrogentamicin C_{1a}. (c) The structures of the neomycins. Positions are numbered as in the kanamycins. Lividomycin B is 3′-deoxyparomomycin. Ribostamycin has rings I, II, and III as in neomycin B. (d) The structure of the butirosins. The hydroxyaminobutyric side chain at position 1 of the deoxystreptamine ring (II) is referred to as HABA.

TABLE I: Enzymes Modifying the Deoxystreptamine–Aminoglycoside Antibiotics.[a]

Enzyme	Modification	Substrates[b]	References
Neomycin phosphotransferase I	Phosphorylation of 3′-OH and 5′′-OH	Neomycin, kanamycin, paromomycin, lividomycin, ribostamycin	Yagisawa *et al.*, 1972; Brzezinska and Davies, 1973; Umezawa *et al.*, 1973
Neomycin phosphotransferase II	Phosphorylation of 3′-OH	Neomycin, kanamycin, paromomycin, butirosin, ribostamycin	Yagisawa *et al.*, 1972; Brzezinska and Davies, 1973
Gentamicin adenylylate synthetase	Adenylylation of 2′′-OH	Gentamicin, kanamycin, tobramycin, sisomicin	Martin *et al.*, 1971; Witchitz and Chabbert, 1971; Benveniste and Davies, 1971b; Kabins *et al.*, 1971; Yagisawa *et al.*, 1971; Kobayashi *et al.*, 1971.
Kanamycin acetyltransferase	Acetylation of 6′-NH_2	Neomycin, kanamycin (A and B), tobramycin, butirosin, ribostamycin, gentamicin, BB-K8, sisomicin	Okanishi *et al.*, 1967; Benveniste and Davies, 1971a
Gentamicin acetyltransferase I	Acetylation of 3-NH_2	Gentamicin, sisomicin	Brzezinska *et al.*, 1972; Kobayashi *et al.*, 1972
Gentamicin acetyltransferase II	Acetylation of 2′-NH_2	Gentamicin, tobramycin, butirosin, sisomicin, kanamycin B and C	This paper

[a] As of Sept 1973. [b] Most efficient substrates.

corresponding to ion b, formed by the retro-diene cleavage indicated. The corresponding cleavage of sisomicin and 6′-*N*-acetylsisomicin gives an ion at *m/e* 362 which is absent from the spectrum of the *Providencia* inactivated product. High resolution measurement of the peak at *m/e* 404 (found, 404.224; $C_{17}H_{32}N_4O_7$ requires 404.227) confirmed the composition of ion b.

Thus the acetyltransferase from *Providencia* 164 catalyzes acetylation of the 2′-NH_2 group of a number of aminoglycoside antibiotics; this is a new form of enzymatic inactivation–modification occurring in clinical isolates of resistant bacteria.

Characteristics of the Enzymatic Acetylation of Aminoglycoside Antibiotics. A number of aminoglycosides with a

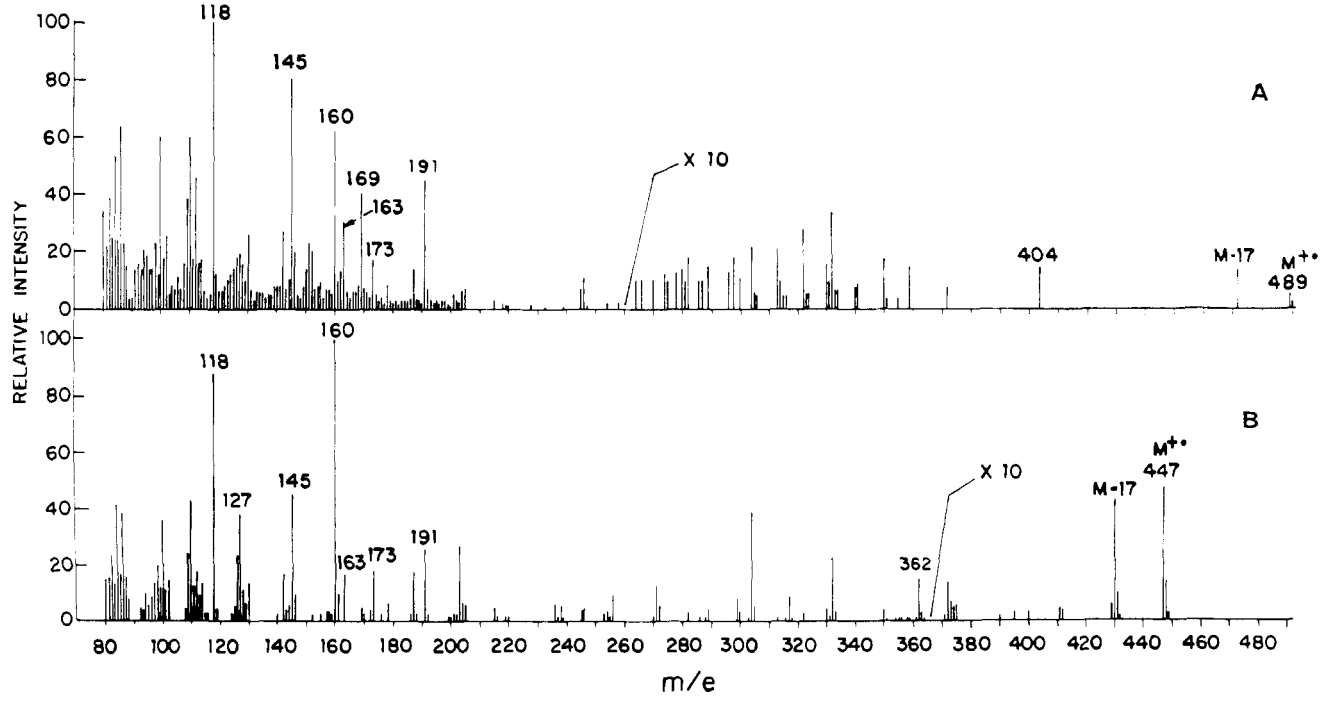


FIGURE 2: The mass spectra of 2-*N*-acetylsisomicin (A) and sisomicin (B).

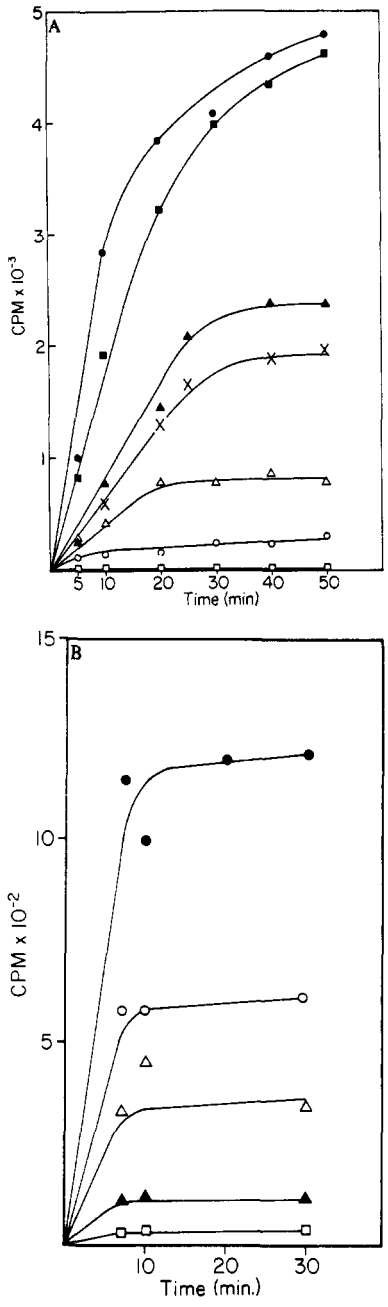


FIGURE 3: Kinetics of acetylation of various aminoglycoside substrates. Reaction mixtures were prepared as described under Materials and Methods. At the times indicated, samples were withdrawn from the reaction mixture and pipetted onto 1-cm squares of phosphocellulose paper which were washed with water, dried, and counted in a toluene-based scintillation fluid: (A) (●) gentamicin C_1; (■) dideoxykanamycin B; (▲) tobramycin and kanamycin C; (×) kanamycin B; (△) gentamicin B; (□) neomycin B; (□) kanamycin A, or no drug; (B) (●) gentamicin C_1; (○) butirosin A; (△) butirosin B; (▲) ribostamycin; (□) neomycin B.

2′-amino group were tested as substrates and were found to vary in their susceptibility to the enzyme. Compounds with a 2′-hydroxyl group were, of course, not substrates (Table II). When the kinetics of the acetylation reaction were examined, it was found that many of the aminoglycosides were acetylated at approximately the same rate, but not to the same extent (Figure 3). A possible explanation for this behavior would be that the 2′-*N*-acetylated products differ in their abilities to act as inhibitors of the enzyme. However, such product inhibition cannot be a factor in determining levels of resistance

TABLE II: Efficiency of Different Aminoglycosides as Substrates for Gentamicin Acetyltransferase II.[a]

Antibiotic	%
Gentamicin C_1	100
Gentamicin C_{1a}	86
Dideoxykanamycin	92
Sisomicin	71
Tobramycin	58
Kanamycin C	40
Butirosin (complex)	30
Ribostamycin	15
Kanamycin B	14
Neomycin B	10
Gentamicin B	7
Kanamycin A	0
BB-K8	0

[a] Determined at pH 6.6, 30-min incubation at 37°.

in intact bacteria, since *Providencia* 164 did not show wide variations in levels of resistance to antibiotics which varied markedly in the extent of reaction with the crude enzyme.

All reactions were routinely supplemented with $Mg(OAc)_2$ and dithiothreitol and the requirement for these additions has not been examined. However, the acetyltransferase from *Providencia* 164 demonstrates a wide variety of pH optima for different substrates (Figure 4). Such effects have also been found for kanamycin acetyltransferase, although the pH effects are more drastic with the *Providencia* enzyme (GAT_{II}) described in this communication. The enzyme is also subject to inhibition by related nonsubstrates or poor substrates (Table III). Kanamycin B is a strong inhibitor of this reaction although this effect varies somewhat depending on the pH of the reaction mixture. Greater levels of inhibition were shown

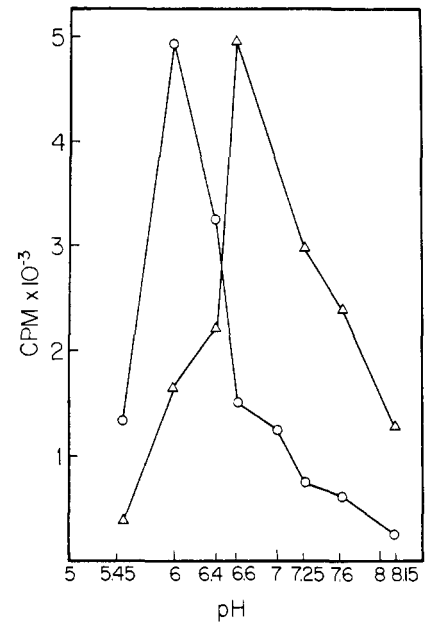


FIGURE 4: pH dependence of acetylation reactions with gentamicin acetyltransferase II. Reaction mixtures were prepared at the indicated pH, using appropriate buffers: (○) kanamycin C; (△) gentamicin C.

TABLE III: Inhibition of Gentamicin Acetyltransferase II by Aminoglycosides.[a]

Aminoglycosides Present	Extent of Reaction (%)
Gentamicin C_{1a}	100
Gentamicin C_{1a} + kanamycin A	50
Gentamicin C_{1a} + kanamycin B	15

[a] Reactions were carried out at pH 6.6.

in the case of kanamycin acetyltransferase (Benveniste and Davies, 1971a).

Discussion

We have described the characterization of gentamicin acetyltransferase II, an enzyme which acetylates the 2′-amino group of gentamicin and other aminoglycoside antibiotics, using acetyl coenzyme A as acetyl donor. This enzyme was first found in a clinical isolate of *Providencia* and subsequently in gentamicin-resistant, kanamycin-sensitive strains of *Proteus* isolated from urinary tract infections by Dr. S. Cohen (unpublished observations). It is probable that GAT_{II} is also responsible for the gentamicin resistance of a number of clinical isolates of *Providencia* strains reported in a hospital in Caen, France (Morel and Freymuth, 1973; M. Chevereau and F. LeGoffic, unpublished observations). There are now four mechanisms by which gentamicin resistance is determined in clinical isolates of bacteria (see Table I). These resistance mechanisms differ in antibiotic resistance spectrum and GAT_{II} is the widest in this respect, since it confers resistance to gentamicin and to a number of structurally unrelated antibiotics, such as butirosin and lividomycin. It is perhaps of interest that GAT_I and GAT_{II} both confer resistance to gentamicin but not to kanamycin A; gentamicin is replacing kanamycin A as the antibiotic of choice for clinical treatment of serious Gram-negative infections.

Both gentamicin adenylylate synthetase (Martin *et al.*, 1971; Benveniste and Davies, 1971b; Kabins *et al.*, 1971; Witchitz and Chabbert, 1971) and gentamicin acetyltransferase I (S. Biddlecome, unpublished observations) have been shown to be R-factor determined and thus transferable between various Gram-negative species. In *Providencia* 164, at least, the gene for GAT_{II} is not demonstrably transferable. The fact that this character has since been found in strains of *Proteus* would suggest that R-factor mediated transfer of this activity does exist.

The behavior of GAT_{II} is worthy of continued investigation; it is not clear what the requirements for substrate activity are, and the reasons for the different pH optima for (apparently) closely related substrates should be examined. Since *Providencia* 164 is resistant to a variety of aminoglycosides, GAT_{II} is either not subject to such pH dependence in the cell, or the pH of the enzyme environment in the cell is compatible with a range of inactivation reactions. The 3′-deoxyaminoglycosides (gentamicin, tobramycin, and dideoxykanamycin B) are the most effective substrates, but other compounds such as butirosin, gentamicin B, and kanamycin C are also substrates for GAT_{II}. It is of interest to note that chemical conversion of kanamycin B (an inhibitor of GAT_{II} to dideoxykanamycin B (Umezawa *et al.*, 1972) in order to produce an antimicrobial agent resistant to phosphorylative inactivation has at the same time produced a compound which is more susceptible to inactivation by GAT_{II}. In fact, dideoxykanamycin, at pH 6.6, is a much better substrate than tobramycin or kanamycin B. The loss of the 3′- and 4′-hydroxyl groups increases substrate activity of the molecule in a manner which is presently unknown. In addition, 2′,6′-diamino compounds are generally poorer substrates than 2′-amino compounds (*e.g.*, kanamycin B is less effective than kanamycin C and neomycin is less effective than paromomycin). Removal of the 2′-amino group is the only modification which blocks GAT_{II} action, but it will be interesting to see whether the substitution of a hydroxyaminobutyric acid side chain at the 1 position of the kanamycin–gentamicin type antibiotics will protect the antibiotics from acetylation. Such substitution in the neomycin series, *e.g.*, ribostamycin *vs.* butirosin, does not result in substantial reduction of substrate activity. An enzyme (or enzymes) with activity similar to GAT_{II} has been found in extracts of *Streptomyces spectabilis* (spectinomycin-producing) and *Streptomyces tenebrairius* (tobramycin-producing) (Benveniste and Davies, 1973b; J. Dowding, unpublished results). The function of the enzyme in antibiotic-producing strains is not known, but it has been suggested that this source may represent the origin of the inactiviting enzymes.

Acknowledgments

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A Simple and Precise Assay of the Enzymatic Conversion of Cholesterol into Pregnenolone†

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ABSTRACT: A simple and precise assay is described for measurement of the enzymatic conversion of cholesterol into the C_{21} steroid, pregnenolone. This procedure determines quantitatively the amount of the isotopically labeled C_6 fragment (isocaproic acid) formed from cholesterol, when the sterol substrate bears a radioisotope, ^{14}C or ^{3}H, in the side chain. The method is based upon the fact that the substrate is separated from the C_6 product by percolation through a column of alumina. The unreacted sterol is adsorbed out of the aqueous incubation medium by the adsorbant, whereas the small molecular weight fragment filters through. The radioactivity in the eluate serves as a direct measure of the cleavage enzyme activity and thereby provides the basis for a rapid assay which is convenient for the study of many aspects of this important enzyme.

The biosynthetic processes, by which steroid hormones are made from cholesterol, all begin with cleavage of a C_6 fragment from the side chain of the sterol. Most of the other enzymes involved in steroid hormone formation, *e.g.*, hydroxylases and Δ^5-3β-hydroxysteroid dehydrogenase–isomerase etc., proceed at rates far faster than that characterizing the side-chain cleavage of cholesterol (Koritz and Kumar, 1970). It is natural, therefore, that this rate-limiting scission is generally considered to be the principal step at which the control of the rate of hormone synthesis is exerted. The locus of action of the various trophic hormones (ACTH, LH,[1] etc.) is usually thought to be at the reaction, cholesterol → pregnenolone + isocaproic acid (Garren, 1968).

Consequently, it is evident that a rapid, easy, and sensitive assay is of great value for a study of the detailed nature of the reaction and of the factors which influence it. Although many assays have been described, none combines adequate sensitivity with ease of manipulation. In those assays which use nonradioactive cholesterol (either of endogenous or exogenous origin) as a substrate, the pregnenolone[1] formed was measured colorimetrically (Koritz, 1962), by gas–liquid chromatography (Young and Hall, 1969), by radioimmunoassay (Bermudez *et al.*, 1970), or by the appearance of DPNH[1] that occurs when pregnenolone (isolated by chromatography) is oxidized enzymatically to progesterone (Simpson *et al.*, 1972). In those determinations in which radioactive cholesterol was used as a substrate, measurement of the isotope associated with more polar products (Halkerston *et al.*, 1961), with specific C_{21} steroids after chromatography (Simpson and Boyd, 1967), or with the labeled C_6 side-chain fragment [determined by gas–liquid chromatography (Burstein *et al.*, 1971), by extraction (Raggatt and Whitehouse, 1966), by stream distillation (Constantopoulos and Tchen, 1961), or by difference following the removal of the volatile fragment by distillation (Kimura *et al.*, 1966; Doering and Clayton, 1969)] served as the end point in the analysis. Most of these methods are insensitive, and almost all are time consuming.

The method presented in this paper measures directly the radioactive C_6 fragment formed from the cleavage of [26-^{14}C]cholesterol, by a procedure that is both rapid and simple. After the enzyme reaction is arrested by dilution with an alkaline buffer containing $HgCl_2$, the entire mixture is poured onto a small column of aluminum oxide. The small molecular weight product percolates through with the buffer while the unchanged radioactive cholesterol is adsorbed out of the solution, and is retained on the column. The ^{14}C in the eluate was found to be associated only with isocaproic acid, which is produced in stoichiometric quantities with pregnenolone when cholesterol is used as a substrate or with pregnenolone sulfate when cholesterol sulfate is the precursor. The ^{14}C in the eluate is thus a quantitative measure of the extent of the reaction and may be used as the basis of an assay. The method is simple and so may readily monitor kinetic experiments in which consecutive samples can be taken only seconds apart.

Experimental Procedure

Materials and Methods

Labeled Substrates. [26-^{14}C]Cholesterol (45 Ci/mol) and [1,2-^{3}H]cholesterol (45 Ci/mmol) (purchased from New En-

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[1] Abbreviations used are: ACTH, adrenocorticotrophic hormone; LH, luteinizing hormone; Et_3N, triethylammonium; pregnenolone, 3β-hydroxy-5-pregnen-20-one; DPNH, reduced diphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide.