There are two main similarities in the metabolism of progesterone and 15α-hydroxyprogesterone in rabbit liver, in that the C-20 reduction is toward 20α and that this reduction in both is not very extensive.

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Gentamicin Resistance in Strains of *Pseudomonas aeruginosa* Mediated by Enzymatic N-Acetylation of the Deoxystreptamine Moiety*

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ABSTRACT: Strains of *Pseudomonas aeruginosa* which are gentamicin resistant but tobramycin sensitive have been found to inactivate gentamicin by enzymatic acetylation. Physical and chemical characterization of the purified acetylgentamicin has revealed that the 3-amino group of the 2-deoxystreptamine moiety is acetylated in the antibiotic. *In vitro* studies with the partially purified enzyme have shown that the gentamicin C antibiotics and sisomicin are all excellent substrates, whereas closely related antibiotics such as tobramycin, kanamycins A, B, and C, and gentamicin A are either poor substrates or not acetylated.

The amino glycoside antibiotics, which are effective agents against gram-negative infections, can be enzymatically inactivated by many resistant strains isolated in clinical situations. A number of different enzymes have been identified in R-factor-containing strains which can modify these antibiotics by phosphorylation, acetylation, or adenylylation (Davies *et al.*, 1971). In Pseudomonads, neomycin–kanamycin resistance *via* phosphorylation has been studied by Umezawa *et al.* (1968) but other amino glycoside resistance mechanisms have not been identified. The antibiotic resistance patterns of bacterial strains provide a convenient way of recognizing the mechanism of resistance, since different enzymes have different amino glycoside substrate specificities. We recently obtained several clinical isolates of *Pseudomonas aeruginosa* which showed a resistance pattern different from those already known, since they were gentamicin resistant but tobramycin (nebramycin factor 6) sensitive. All other *Pseudomonas* strains which we have studied are sensitive to both antibiotics. Several strains of *Klebsiella* and *Escherichia coli* have been shown to be resistant to both antibiotics as a result of enzymatic adenylylation (Benveniste and Davies, 1971b). Analysis of extracts of the resistant *P. aeruginosa* strains has revealed a new acetylating enzyme with high specificity for the gentamicin C antibiotics.

Physical and chemical studies of the inactivated antibiotic have shown that acetylation occurred on the 3-amino group of the 2-deoxystreptamine ring. This is the first report of enzymatic modification of this cyclitol moiety in amino glycoside antibiotics.

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During the course of this work, Mitsuhashi *et al.* (1971) reported inactivation of gentamicin by a cell-free extract of a Pseudomonas strain in the presence of acetyl coenzyme A; the specificity of the enzyme and structure of the inactivated product was not determined.

Materials and Methods

Strains. P. aeruginosa strains 130 and 209, as well as other clinical isolates, were provided by Dr. J. A. Waitz. These strains were resistant to neomycin, kanamycin, streptomycin, and gentamicin, but sensitive to tobramycin.

Preparation of Cell-Free Extracts. P. aeruginosa 130 was grown to late-log phase in yeast extract–tryptone medium (6 l.). The cells were harvested by centrifugation and washed with 10 mM Tris–50 mM NH_4Cl, pH 7.8 (at 4°). The pellet (25 g) was resuspended in 130 ml of the same buffer at 4° (from this point all manipulations were carried out at 4–10°) and disrupted by sonication. The cell debris was removed by centrifugation at 30,000*g* for 30 min. The supernatant was centrifuged at 105,000*g* to remove ribosomes. Nucleic acids were precipitated by the addition of streptomycin sulfate to a final concentration of 1.5%. The resulting supernatant was adjusted to a protein concentration of 10 mg/ml and made 35% in ammonium sulfate; the precipitate was removed by centrifugation and the supernatant adjusted to 50% ammonium sulfate. The precipitate (35–50%) was collected by centrifugation, dissolved in 3 ml of 10 mM Tris (pH 7.8), and dialyzed exhaustively against the same buffer.

Partial Purification of the Acetylating Enzyme. The dialyzed 35–50% ammonium sulfate precipitate was applied to a 1.5 × 17.5 cm column of DEAE-cellulose (Whatman DE52) which had previously been equilibrated with 10 mM Tris (pH 7.8). A linear gradient (400 ml) from 10 mM Tris (pH 7.8) to 10 mM Tris–0.3 M NH_4Cl (pH 7.8) was applied at a flow rate of 20 ml/hr. Fractions were assayed for gentamicin-acetylating activity and the enzyme was found to elute at 0.22 M NH_4Cl. The fractions containing the enzyme activity were combined, concentrated by ultrafiltration, and dialyzed *vs.* 10 mM Tris (pH 7.8). This partially purified enzyme (*ca.* 15-fold purification) was used in substrate studies.

Radioactive Assay for Acetylating Activity. The enzyme assay, using [^{14}C]CoASAc,[1] was performed as previously described for the N-acetylation of kanamycin (Benveniste and Davies, 1971a). All substrates were assayed at pH 7.8 at 30°. This enzyme does not exhibit a substrate-dependent pH optimum as previously described for the 6′-N-acetylating enzyme (Benveniste and Davies, 1971a).

Preparation and Isolation of Acetylated Gentamicin C_{1a}. The reaction mixture for large-scale acetylation of gentamicin C_{1a} contained, in a total volume of 150 ml, 90 ml of a cell extract obtained by disrupting 30 g of *P. aeruginosa* 130 in a French press; an ATP-generating system consisting of 0.3 mmole of phosphoenolpyruvate and 5 mg of pyruvate kinase, 5 mmoles of ATP (adjusted to pH 6 with KOH), 20 μmoles of yeast coenzyme A, 0.23 mmole of gentamicin C_{1a}, 15 mmoles of Tris (pH 7.6 at 30°), 1.5 mmoles of magnesium acetate, 6 mmoles of ammonium chloride, 6 mmoles of potassium chloride, and 0.75 mmole of dithiothreitol. The incubation was carried out at 33° for 6 hr with gentle agitation. Progress of the inactivation was monitored by testing portions of the reaction mixture for antibiotic activity—60% of the gentamicin C_{1a} was acetylated after 6 hr. The acetylated product was purified by cation-exchange chromatography as described previously for the purification of 6-*N*-acetylgentamicin C_{1a} (Benveniste and Davies, 1971a).

Physical and Chemical Properties of Acetylated Gentamicin C_{1a}. Nuclear magnetic resonance (nmr) spectra were determined on a Varian A60A spectrometer at 60 MHz in D_2O solutions. Mass spectra were determined on an Atlas CH5 spectrometer using a direct inlet system. Melting points were taken on a Reichert thermopan apparatus and are uncorrected. Thin-layer chromatography was performed on silica gel plates and compounds were visualized with iodine vapor and by sulfuric acid charring. Paper chromatography was carried out with Whatman No. 1 paper and developed by the descending technique; the compounds were visualized by spraying with ninhydrin.

Hydrolysis of Acetylated Gentamicin C_{1a}. 3-*N*-Acetylgentamicin C_{1a} (1.98 mg) was heated under reflux with 2 N NaOH (2 ml) for 48 hr in a nitrogen atmosphere. The solution was acidified to pH 5.0 with sulfuric acid and separated from a small amount of precipitate (silica) by centrifugation. The filtrate was lyophilized to give a white solid (264.4 mg). An aliquot, assayed against *Staphylococcus aureus* showed an activity of 15 μg/mg *vs.* a gentamicin C standard (1000 μg/mg), representing 22% regeneration of gentamicin C_{1a} activity.

Another aliquot (1 mg, containing *ca.* 6.8 μg of product) was subjected to bioautogram using system A on Whatman No. 1 paper. The product migrated identically with gentamicin C_{1a} and showed a zone of inhibition approximately the same as a 5-μg spot of gentamicin C_{1a}.

Methanolysis of Acetylated Gentamicin C_{1a}. 3-*N*-Acetylgentamicin C_{1a} (33.4 mg) was heated at 83° with 6 N HCl in methanol (1.8 ml) for 2 hr. After this time thin-layer chromatography (system A) showed the complete disappearance of starting material. The solution was evaporated to a syrup which was chromatographed over silica gel (3 g) using the lower phase of chloroform–methanol–concentrated ammonia–water (2:1:0.6:0.4, v/v) system as eluent. Fractions 5–26 afforded a syrupy anomeric mixture of methyl garosaminides (Cooper *et al.*, 1971a,b) identified by thin-layer chromatography and mass spectrometry. Fractions 76–128 gave 3-*N*-acetylgentamine C_{1a} (11.7 mg) as an amorphous solid.

In Vitro Polypeptide Synthesis. Reaction mixtures (100 μl) contained 66 mM Tris·HCl (pH 7.8 at 5°), 9 mM magnesium acetate, 50 mM NH_4Cl, 2.6 mM ATP, 60 μM GTP, 10 mM phosphoenolpyruvate, 2 μg of pyruvate kinase, 10 mM 2-mercaptoethanol, 3.3 mM dithiothreitol, 0.5 mM of each of the 20 amino acids (excluding valine), 50 μg of tRNA, 4 nmoles of [^{14}C]valine (25 μCi/μmole), and 1.6 OD units of a preincubated S-30 extract of *E. coli* (MRE 600). Incorporation was started by the addition of 1 OD_{260} unit of R 17 phage RNA. Incubation was carried out for 40 min at 37°, then 1.5 ml of 10% trichloroacetic acid was added, the tubes were heated at 90° for 20 min, cooled, filtered on glass fiber disks, and counted.

Chemicals. [^{14}C]CoASAc was obtained from New England Nuclear, CoASAc, and CoASH from Calbiochem. The gentamicins, gentamines, and sisomicin were obtained from the Schering Corp., tobramycin (nebramycin factor 6) from Dr. Kay Koch of the Lilly Research Laboratories; kanamycins A and B from the late Dr. A. Gourevitch of Bristol Laboratories, kanamycin C from Dr. H. Umezawa, neomycin B from Dr. G. B. Whitfield, Jr., of the Upjohn Co., and paromomycin from Dr. T. H. Haskell of Parke Davis and Co.

[1] Abbreviations used are: [^{14}C]CoASAc, [1-^{14}C]acetyl coenzyme A; CoASH, coenzyme A.

TABLE I: Efficiency of Different Antibiotics as Substrates for Gentamicin Acetyltransferase.[a]

	%
Gentamicin C_1	100
Gentamicin C_{1a}	107
Gentamicin C_2	110
6-*N*-Acetylgentamicin C_{1a}	76
Sisomicin	128
Gentamicin A	9
Gentamine C_1	25
Gentamine C_{1a}	4
Gentamine C_2	25
Kanamycin A	0
Kanamycin B	18
Kanamycin C	0
Neomycin B	0
Paromomycin	0
Tobramycin	14

[a] Each compound was incubated with a partially purified enzyme preparation from *P. aeruginosa* 130 and [^{14}C]COASAc under conditions previously described (Benveniste and Davies, 1971). Samples were removed at 10 min. Results are expressed relative to the acetylation of gentamicin C_1 (100%).

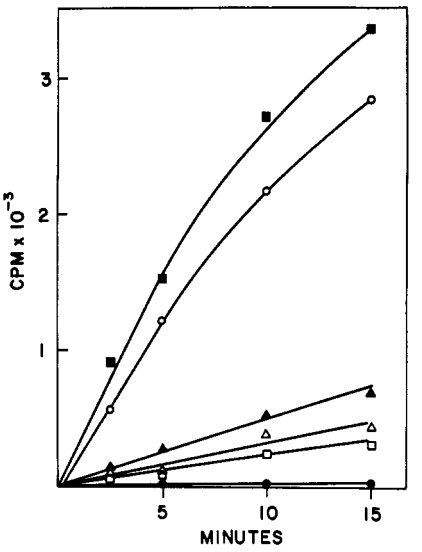

FIGURE 1: Acetylation of amino glycoside antibiotics by a partially purified enzyme fraction from *P. aeruginosa* 130. At the times indicated, samples were withdrawn from the reaction mixture (see Materials and Methods), pipetted onto 1-cm squares of phosphocellulose paper which were washed, dried, and counted in a toluene-based scintillation fluid. (■) Sisomicin, (○) gentamicin C_1 or C_{1a}, (▲) gentamine C_1, (△) kanamycin B, (□) tobramycin, (●) kanamycin A, kanamycin C, neomycin B, or no drug.

Results

P. aeruginosa 130 and 209, both clinical isolates from burn clinics, were found to be resistant to neomycin B, kanamycins A, B, and C, paromomycin, and gentamicin, but sensitive to tobramycin (20-μg disks). Assays of cell-free extracts of these strains, using the cation-exchange paper-binding assay (Ozanne *et al.*, 1969) indicated that neomycin, kanamycin, and paromomycin were inactivated by phosphorylation (unpublished observations). Phosphorylative inactivation of these aminoglycosides in Pseudomonads has been well documented (Umezawa *et al.*, 1968; Davies *et al.*, 1971).

When extracts of *P. aeruginosa* 130 or 209 were assayed for acetylating activity using [^{14}C]CoASAc, it was found that the gentamicin C antibiotics and sisomicin were good acceptors for the acetate moiety (Table I). This result indicated the presence of an acetyltransferase in these extracts, which was different from the enzyme found in several R-factor-carrying strains of *Escherichia coli* (Benveniste and Davies, 1971a). The latter enzyme has broad specificity and catalyzes the N acetylation of gentamicin C_{1a}, neomycin, kanamycins A and B, and tobramycin.

Partial Purification of Gentamicin-Acetylating Enzyme. Gentamicin acetyltransferase has been purified approximately 15-fold from a cell-free extract of *P. aeruginosa* 130 by precipitation of nucleic acids, ammonium sulfate fractionation, and DEAE-cellulose chromatography. The enzyme is stable on repeated freezing and thawing and can be stored at −20°.

Substrates for Gentamicin Acetyltransferase. Table I shows a comparison of the rates of acetylation of various substrates and Figure 1 gives some typical reaction kinetics. It can be seen that the most effective substrates are those antibiotics which contain a purpurosamine ring (as in gentamicin C_{1a}, see Figure 2A). None of the other amino glycoside antibiotics, including such closely related compounds as the kanamycins (Figure 2C) or tobramycin (Figure 2D), are good substrates for the transacetylase. However, kanamycin B and tobramycin did show some evidence of reaction compared to a nonsubstrate such as kanamycin A. The gentamines (degradation products of the gentamicins containing the purpurosamine and 2-deoxystreptamine moieties) were also poor substrates. None of the antibiotic nonsubstrates were inhibitors of the acetylation of gentamicin C_{1a}. The fact that 6-*N*-acetylgentamicin C_{1a} (Benveniste and Davies, 1971a) is a substrate for the enzyme shows that the 6-amino group of the purpurosamine ring is not acetylated by the *Pseudomonas* acetyltransferase. Apart from this however, no clear indication of the site of acetylation could be derived from these experiments.

Preparation and Purification of Acetylated Gentamicin C_{1a}. Since the substrate studies did not suggest the site of inactivation of the antibiotic, gentamicin C_{1a} (100 mg) was acetylated in a large-scale reaction as described in Materials and Methods. After ion-exchange chromatography of the product, the yield of pure acetylgentamicin C_{1a} was close to 100%. Acetylgentamicin C_{1a} was an amorphous solid, mp 130–145°, $[\alpha]_D^{26}$ +126° (*c* 0.37, H_2O); it was homogeneous by thin-layer chromatography and paper chromatography in four solvent systems (see Table II). This material was used for biological studies and for structure determination, as described below.

Identification and Structure of Acetylgentamicin C_{1a}. Hydrolysis with 2 N NaOH regenerated substantial gentamicin C_{1a} activity as indicated by bioassay and bioautogram. The compound showed infrared absorptions (Nujol) at 1640 and 1565 cm^{-1} consistent with a secondary amide group. The nmr spectrum (60 MHz, D_2O) showed absorptions as follows: δ 5.43, 5.10 (1 H each, broad bands, two anomeric protons), 1.96 (3 H, singlet, CH_3CO), 1.24 (*ca.* 3 H, singlet, CH_3C); *m/e* 492 (0.17%, $[M + 1]^+$), 491 (0.12%, M^+), 392 (3.8%), 364 (14%), 346 (5%), 233 (37%), 205 (21%), 187 (16%), 160 (88%, garosamine), and 129 (100%, purpurosamine C).

The mass spectrum exhibited a molecular ion at *m/e* 491

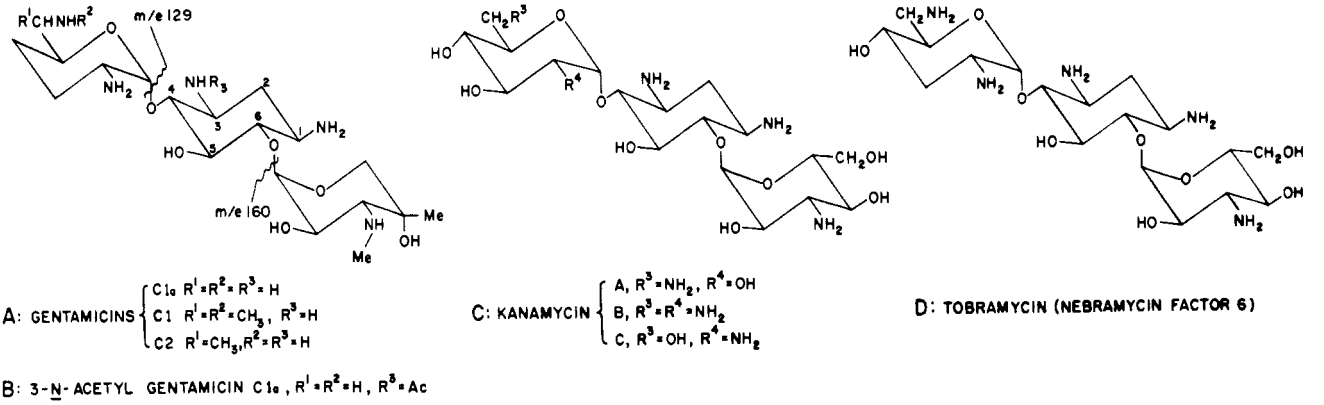

FIGURE 2: Structures of the gentamicins, kanamycins, and tobramycin.

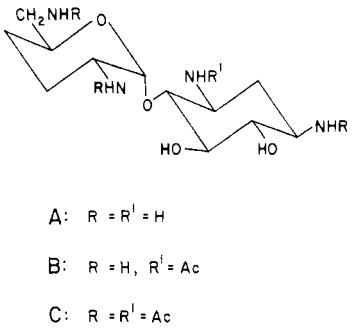

FIGURE 3: Structures of gentamine C_{1a} (A), 3-*N*-acetylgentamine C_{1a} (B), and tetra-*N*-acetylgentamine C_{1a} (C).

and a somewhat more intense $(M + 1)^+$ peak at *m/e* 492 consistent with a mono-N-acetylated gentamicin C_{1a}. Intense peaks at *m/e* 160 and 129 could be attributed to the garosamine and purpurosamine C ions (Cooper *et al.*, 1971b,c) (Figure 2A), indicating that the acetyl group was not attached to either of these parts of the molecule. A prominent series of peaks observed in gentamicin C_{1a} at *m/e* 191, 163, and 145 associated with the 2-deoxystreptamine unit (Daniels *et al.*, 1972), was absent from the spectrum of the acetylated compound; however, a series of peaks 42 mass units higher at *m/e* 233, 205, and 187 was present indicating that the extra *N*-acetyl group was attached to one of the 2-deoxystreptamine nitrogen atoms.

Methanolysis of the N-acetylated gentamicin C_{1a} afforded the corresponding *N*-acetylgentamine C_{1a} (Figure 3). This provided further support for the presence of an *N*-acetyl group since an *O*-acetyl group would not have survived the methanolysis conditions. *N*-acetylgentamine C_{1a} was an amorphous solid: $[\alpha]_D^{26}$ +50° (*c* 0.18, H_2O); nmr δ 5.50 (1 H, doublet J = 3.5 Hz, anomeric proton), 2.01 ppm (3 H, singlet, acetyl methyl group); *m/e* 332 $(M + 1)^+$, 332 $(M^{+}\cdot)$, 233, 205, 187, 129 (purpurosamine C ion). The chromatographic behavior of 3-*N*-acetylgentamine C_{1a} is shown in Table II.

The circular dichroism spectra of *N*-acetylgentamine C_{1a} were taken in cupra A (Bukhari *et al.*, 1970) and the tetramine copper sulfate (TACu) (Umezawa *et al.*, 1966) solutions. These spectra were essentially identical with those of gentamine C_{1a} but of opposite sign to those of tetra-*N*-acetylgentamine C_{1a} (Cooper *et al.*, 1971a). The results are shown in Table III. Since the circular dichroism bands of gentamine

TABLE II: Chromatographic Behavior of Gentamicin Derivatives.

Compound[a]	Chromatographic System[b]			
	A[c]	B[d]	C[c]	D[e]
Gentamicin C_{1a}	0.38	8.5	0.53	21
3-*N*-Acetylgentamicin C_{1a}	0.49	10.5	0.61	21.7
Gentamine C_{1a}	0.22	3.5	0.62	20.5
3-*N*-Acetylgentamine C_{1a}	0.26	3.6	0.66	20.7

[a] See Figures 3 and 4. [b] Solvent systems used were as follows: (A) lower phase of chloroform–methanol–28% ammonium hydroxide (1:1:1, v/v); (B) lower phase of chloroform–methanol–28% ammonium hydroxide (2:1:1, v/v); (C) ethyl acetate–ethanol–water–glacial acetic acid–pyridine (50:75:75:16:84, v/v); (D) 1-propanol–pyridine–glacial acetic acid–water (15:10:3:12, v/v). [c] Thin-layer chromatography on silica gel. [d] Paper chromatography; run for 3.5 hr, numbers represent distance of migration in millimeters. [e] Paper chromatography; run for 22 hr, numbers represent distance of migration in millimeters.

C_{1a} arise from the cuprammonium complex with its vicinal amino alcohol group,[2] a similar amino alcohol group is available for complexing in *N*-acetylgentamine C_{1a} which must therefore be the 3-*N*-acetyl compound shown. We conclude that the structure of the product of acetylation of gentamicin C_{1a} with the *Pseudomonas* acetyltransferase is 3-*N*-acetylgentamicin C_{1a} (Figure 2B).

Properties of Acetylgentamicin C_{1a}. Acetylation of gentamicin C_{1a} by an extract of *P. aeruginosa* 130 almost completely eliminates antibiotic activity; the compound is about 500 times less active than gentamicin C_{1a} in bacterial growth inhibition tests. On the other hand, the enzyme which acetylates the 6-amino group of the purpurosamine moiety in gentamicin C_{1a} only reduces the antibacterial activity some 10- to 20-fold (Benveniste and Davies, 1971a).

[2] TACu has been shown to complex exclusively with a vicinal amino alcohol group in compounds having both this group and a vicinal diol group (Umezawa *et al.*, 1966). The sign of the circular dichroism (Bukhari *et al.*, 1970) shows that this must also be the case for the complex with cupra A.

TABLE III: Circular Dichroism Spectra of Cuprammonium Complexes.

Compound[a]	Cupra A λ, nm ([θ])	TACu λ, nm ([θ])
Gentamine C_{1a}	*Ca.* 600 (−300)	582 (−440)
	Ca. 290 (+2400)	290 (+3100)[b]
3-*N*-Acetylgentamine C_{1a}	*Ca.* 590 (−290)	*Ca.* 580 (−350)
	290 (+2150)[b]	290 (+3400)[b]
Tetra-*N*-acetylgentamine C_{1a}	*Ca.* 600 (+200)	*Ca.* 580 (+87)
	300 (−1300)	*Ca.* 300 (−425)

[a] See Figure 4. [b] [θ] values at denoted wavelengths. Maxima for these complexes were at shorter wavelength and were not determined.

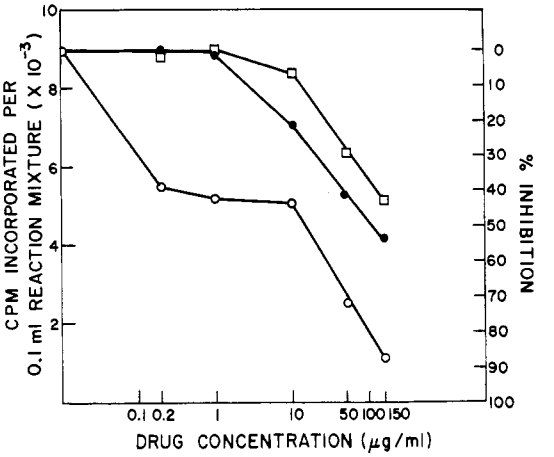

FIGURE 4: Inhibition of R 17 phage RNA-directed polypeptide synthesis *in vitro* by gentamicin C_{1a} and its acetylated derivatives. See Materials and Methods. (○) Gentamicin C_{1a}, (●) 6-*N*-acetylgentamicin C_{1a} (Benveniste and Davies, 1971a,b), (□) gentamicin C_{1a} acetylated on the 3-amino group of 2-deoxystreptamine.

The biological properties of the two acetylated derivatives were compared by testing their effectiveness as inhibitors of polypeptide synthesis in cell-free extracts of a sensitive strain of *E. coli*. As can be seen in Figure 4, gentamicin C_{1a} and 6′-*N*-acetylgentamicin C_{1a} (acetylated on the 6-amino group of the purpurosamine ring) are more effective inhibitors than 3-*N*-gentamicin C_{1a} (acetylated on the 3-amino group of the 2-deoxystreptamine ring).

Discussion

A gentamicin-resistant, tobramycin-sensitive strain of *P. aeruginosa* has been shown to produce an enzyme which, in the presence of [^{14}C]acetyl coenzyme A, transfers labeled acetate to antibiotics of the gentamicin C complex. Acetylgentamicin C_{1a} has been isolated and its structure determined by chemical and physical methods; the acetate residue is attached to the 3-amino group of the 2-deoxystreptamine ring of the antibiotic. This is the first example of specific modification of the 2-deoxystreptamine ring in an amino glycoside antibiotic and represents a new and specific form of enzymatic inactivation.

The gentamines, which lack the garosamine substituent on the 2-deoxystreptamine ring (Figure 3) are poor substrates for the enzyme. This indicates that the complete trisaccharide is required for optimal recognition by the enzyme. In addition, since kanamycin A is not a substrate for the enzyme and both kanamycin B and tobramycin are poor substrates, the number of amino groups and the degree of hydroxylation on the sugar moiety glycosidically linked to the 4 position of deoxystreptamine have a marked effect on the ability of the amino glycoside to act as a substrate. The failure of neomycin B to act as substrate is less surprising, since this antibiotic has glycosidic linkages on adjacent positions on the 2-deoxystreptamine ring and is considered to represent a separate subclass of the amino glycosides (Rinehart, 1969).

A number of clinical isolates of gentamicin-resistant, tobramycin-sensitive *P. aeruginosa* strains from various parts of the world have been examined and shown to contain gentamicin acetyltransferase (unpublished observations). The strain studied by Mitsuhashi *et al.* (1971) is presumably another example of this resistance mechanism.

Attempts to determine if gentamicin acetyltransferase is determined by a chromosomal or episomal gene have so far been inconclusive. No genetic transfer or "curing" of this characteristic has been found by the usual methods. There are now three different ways (adenylylation (Benveniste and Davies, 1971b), acetylation of the purpurosamine ring of gentamicin C_{1a} (Benveniste and Davies, 1971a), and acetylation of the deoxystreptamine ring (this paper)) by which the gentamicin antibiotics can be inactivated (wholly or partially) by resistant bacterial strains. The selectivity and specificity of these modifying enzymes may provide useful approaches to the preparation of semisynthetic amino glycosides with altered toxicity or resistance characteristics.

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Affinity Labeling of a Phosphorylcholine Binding Mouse Myeloma Protein†

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ABSTRACT: The phosphorylcholine binding γA myeloma protein of the Balb/c plasmacytoma TEPC 15 was immunospecifically purified. A phosphorylcholine analog, *p*-diazonium phenylphosphorylcholine, was synthesized and used an an affinity-labeling reagent. More than 90% of the specific modification occurred on the light chains. The most substantially labeled residue was a light-chain tyrosine. This residue is in a homologous position to the tyrosine which became labeled in similar studies performed on a nitrophenyl binding mouse myeloma protein. This finding suggests: (1) that all immunoglobulin "antigen" binding sites are located in the same topological sector of the Fab region, (2) that the tyrosine which becomes labeled may not be chemically neutral, and (3) that when present, this residue plays a similar role in antibodies of diverse specificities.

A considerable number of human and mouse myeloma proteins have been discovered which have definable binding activities. Because such immunoglobulins are homogeneous and usually available in substantial amounts they are potentially useful for helping to define the structural basis of antibody specificity. One set of such proteins—those that bind nitrophenyl ligands—is being explored in several laboratories. Particularly by the technique of affinity labeling, it has already been possible to directly implicate certain sections of the light- and heavy-chain variable regions as playing a critical role in the structure of the combining sites.

It would be advantageous to explore in similar detail a set of immunoglobulins which bind very different ligands than those referred to above. Such studies would be helpful in delineating those features of immunoglobulin combining sites which may be common to many or all immunoglobulins and those which are unique to sites reacting with a particular determinant or set of determinants. The group of Balb/c mouse γA myelomas which share the property of binding phosphorylcholine should be useful in this regard. The studies of Potter and Leon (1968), Potter and Lieberman (1970), and particularly Leon and Young (1971) have shown that several of these proteins, while having related binding activities, are clearly distinguishable on the basis of their binding constants for phosphorylcholine and its analogs.

The study reported here is the first of a series in which we have used the technique of affinity labeling to explore the combining sites of these proteins. A phosphorylcholine analog, *p*-diazonium phenylphosphorylcholine (DPPC),[1] was used to specifically modify the combining sites of phosphorylcholine binding protein from the tumor, TEPC 15. A highly labeled peptide was isolated and sequenced, and this has permitted us to define its location in the primary structure of the protein.

Materials and Methods

Tumor Lines. The mouse plasmacytoma TEPC 15 was obtained from Dr. Michael M. Potter and was maintained by subcutaneous passage in Balb/c mice. Ascites was collected by paracentesis of Balb/c or (Balb/c × C57BL/) F_1 mice which had been inoculated intraperitoneally with 0.5 ml of a tumor cell suspension 1 month earlier. In general 3–6 ml of ascites could be obtained per mouse.

p-Diazonium Phenylphosphorylcholine (DPPC). Methyl iodide (2 mmoles) was added to 20 mmoles of dimethylaminoethanol in 10 ml ether at 4° with stirring. The mixture was then stirred at room temperature for 18 hr. The white precipitate of choline iodide was washed with ether and dried. The yield was approximately 100%. The choline iodide, *p*-nitrophenyl phosphorodichloridate (2 mmoles) (Aldrich), and dry quinoline (2 mmoles) were each dissolved in 0.5 ml of dry acetonitrile, mixed, and stirred at 0° in the dark for 4–8 hr (Bird, 1967). Then 1.0 ml of pyridine and 0.2 ml of H_2O were added, and the solution was incubated at room temperature for 30 min. The solvents were flash evaporated, the residue was dissolved in H_2O, and the solution passed through a 40-ml Amberlite MB-3 column equilibrated with H_2O. The effluent was lyophilized. The product was analyzed by thin-layer chromatography on cellulose and silica gel (Eastman Chromogram) using either isopropyl alcohol–NH_4OH–H_2O (7:2:1, v/v) or 1-butanol–acetic acid–H_2O (5:2:4, v/v). In either case only a single component was observed. It was positive for phosphate (Hanes and Isherwood, 1949) and absorbed ultraviolet light. *Anal.* Calcd for $C_{11}H_{17}N_2O_6P_1$: C, 43.4; H, 5.7; N, 9.2; P, 10.2. Found: C, 43.5; H, 5.9; N, 9.4; P, 10.0. It had a melting point (cor) of 243°. The uv spectrum had a minimum at 237 nm and a maximum at 287 nm (Figure 1). The extinction coefficient, $\epsilon_{287.1\text{ cm}}$, was 1.05×10^4 at pH 7. This compound, *p*-nitrophenylphosphorylcholine, was stored

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[1] Abbreviations used are: DPPC, *p*-diazonium phenylphosphorylcholine; BBS, 0.2 M sodium borate buffer with 0.16 M NaCl; SDS, sodium dodecyl sulfate.