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Enzymatic Acetylation of Aminoglycoside Antibiotics by *Escherichia coli* Carrying an R Factor*

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ABSTRACT: Strains of *Escherichia coli* carrying an R factor which inactivate the aminoglycoside antibiotic kanamycin A by N acetylation have been found to acetylate a wide variety of other aminoglycosides. These include kanamycin B, neomycins B and C, some of the components of the gentamicin and nebramycin complexes, and the hybrimycins. The smallest antibiotic moiety required for recognition as a substrate by the acetylating enzyme is a 6-amino-6-deoxyhexose glycosidically linked to a streptamine or deoxystreptamine ring. Isolation of the purified acetylated antibiotics has revealed that acetylation does not necessarily result in inactivation of the drug. *N*-Acetylkanamycin A is not an antibiotic, whereas *N*-acetylkanamycin B, *N*-acetylneomycin B, and *N*-acetylgentamicin C_{1a} retain substantial antibiotic activity—although they are not as potent as the unacetylated parent compounds.

There are four enzymes found in strains carrying resistance (R) factors which can inactivate many of the aminoglycoside antibiotics. One enzyme adenylylates streptomycin and spectinomycin on the D-*threo*-methylamino alcohol moiety of their amino sugar and aminocyclitol rings, respectively (Yamada *et al.*, 1968; Benveniste *et al.*, 1970; Smith *et al.*, 1970). Streptomycin, but not spectinomycin, can also be phosphorylated at that same hydroxyl group by a phosphorylating enzyme (Ozanne *et al.*, 1969). Another enzyme phosphorylates neomycin, kanamycin, paromomycin, and some of the components of the gentamicin and nebramycin complexes on a hydroxyl group of the amino sugar moiety which is linked to 2-deoxystreptamine (Kondo *et al.*, 1968; Ozanne *et al.*, 1969).[1]

A fourth enzyme, first reported by Okamoto and Suzuki (1965), inactivates kanamycin by acetylation, since a crude extract of an *Escherichia coli* strain carrying R factor R-5 required acetyl coenzyme A to inactivate the drug. Subsequently, Umezawa *et al.* (1967) isolated the product of the enzymatic acetylation of kanamycin A and showed that the 6-amino group of its 6-amino-6-deoxy-D-glucose moiety was acetylated. Okanishi *et al.* (1967) compared several structurally related antibiotics as substrates for the acetylation reaction and concluded that whereas kanamycin A was inactivated, kanamycin C, paromomycin, and neomycin were not inactivated. Their results were obtained by use of a microbiological assay which measures the residual potency of these drugs after incubation with a crude cell extract and acetyl coenzyme A.

A more detailed study of this acetylating activity is the subject of this communication. We have modified our cation-exchange paper binding assay (Benveniste *et al.*, 1970) to provide a simple method of testing for the enzymatic acetylation of the aminoglycosides. In the presence of a partially purified acetylating enzyme obtained from a strain of *E. coli* carrying either R factor R-5 or NR79, we have found that, in addition to kanamycin A, the antibiotics kanamycin B,

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[1] M. Brzezinska, unpublished results.

neomycins B and C, the hybrimycins, and some of the components of the gentamicin and nebramycin complexes are also acetylated. A 6-amino group of a hexose, which the above compounds possess in common, is the moiety that is presumed to be acetylated. This enzyme is absent in *E. coli* strains which do not carry an R factor (*i.e.*, W677), and is not found in kanamycin-sensitive mutants of the R-factor-carrying strains. Structurally related antibiotics that do not contain this 6-amino group (such as paromomycin and gentamicin A) are potent inhibitors of the acetylation reaction.

The isolation and purification of several of the acetylated aminoglycosides has revealed that, although *N*-acetylkanamycin A is no longer an antibiotic, *N*-acetylkanamycin B, *N*-acetylneomycin B, and *N*-acetylgentamicin C_{1a} still retain considerable residual antibiotic activity.

Materials and Methods

Strains. E. coli W677 (*F*$^-$*thr*$^-$*leu*$^-$*thi*$^-$*lac*$_1^-$*mal*$_1^-$*gal*$_a^-$*gal*$_e^-$*xyl*$_1^-$*ara*$_1^-$*mtl*$_1^-$*tonA*$^-$) carrying either R factor R5, NR79, or NR79-5.

Preparation of Cell-Free Extracts. The cells were grown at 37° in a medium containing the following (per liter): dextrose, 5 g; peptone, 8 g, and yeast extract, 5 g; harvested in late logarithmic stage of growth and osmotically shocked using a modification of the procedure described by Nossal and Heppel (1966) (Benveniste *et al.*, 1970). The supernatant fluid obtained after this procedure is referred to as the "osmotic shockate" and is the source of enzyme for further purification.

The enzyme used to acetylate large quantities of the antibiotics was a cell extract of R5/W677. This strain (6 l.) was grown in medium containing 10 μg/ml of kanamycin—the presence of the drug was necessary to prevent segregation of the kanamycin-resistance character from R5/W677 during growth. This segregation did not occur with NR79/W677. The cells were washed twice with 10 mM sodium phosphate (pH 7) and suspended in 30–40 ml of 20 mM Tris (pH 7.5 at 30°), 10 mM magnesium acetate, 25 mM NH_4Cl, 10 mM KCl, and 2 mM dithiothreitol (buffer A). The cells were disrupted by passage through a French pressure cell, and the resulting suspension centrifuged at 30,000*g* for 30 min. Pancreatic deoxyribonuclease (4 μg/ml) (Worthington) was added and the suspension centrifuged at 100,000*g* for 2 hr. The supernatant was dialyzed *vs.* buffer A, and stored frozen until use.

Radioactive Assay. A typical reaction mixture contained osmotic shockate or partially purified acetylating enzyme, 5×10^{-9} mole of [^{14}C]CoASAc[2] (specific activity, 4 μCi/μmole), 5×10^{-9} mole of antibiotic, 3 μmoles of Tris-maleate buffer at pH's to be specified, 0.3 μmole of $MgCl_2$, and 6 μmoles of β-mercaptoethanol in a total volume of 30 μl. Incubation was at 30°, and at various times 10 μl was pipetted onto a 0.75 cm^2 of phosphocellulose paper (Whatman P-81). The squares were then immersed in hot distilled water (70–80°) for 2 min to stop the reaction, washed several times with large volumes of distilled water, dried, and counted in a Packard Tri-Carb scintillation spectrometer using a toluene-based fluid. Control reactions for nonspecific binding of [^{14}C]CoASH to the paper were run in the absence of either enzyme or antibiotic.

One unit of enzymatic activity is defined as that amount of enzyme producing 10^{-9} mole of acetylated antibiotic per min at 30°, under the assay conditions described. The amount of radioactivity as [^{14}C]acetate bound to the antibiotic, which is adsorbed on the phosphocellulose paper, is proportional to the amount of enzyme added to the assay mixture within the range of 0.2–7.0 units/ml. Protein was determined by the method of Lowry *et al.* (1951).

Colorimetric Assay. The reaction mixture contained, in a volume of 1 ml, an enzyme solution that had been partially purified by passage through a DEAE-cellulose column, 1 μmole of DTNB,[2] 0.18 μmole of [^{14}C]CoASAc, 0.1 mmole of Tris-maleate, 10 μmoles of $MgCl_2$, and 0.08 μmole of antibiotic. The increase in absorbance at 412 mμ was followed in a Coleman-Hitachi double-beam spectrophotometer. Control reactions were run in the absence of either enzyme or antibiotic. The enzyme solutions were dialyzed against buffers containing 10 mM Tris–10 mM $MgCl_2$ to remove β-mercaptoethanol which interferes with the assay by reacting with DTNB. The assay was linear to approximately 0.5 OD_{412}.

Partial Purification of Acetylating Enzyme. Streptomycin sulfate was added to a final concentration of 1.5% to an osmotic shockate containing 10 mM Tris-Cl (pH 7.4) (at 5°), 10 mM $MgCl_2$, and 0.6 mM β-mercaptoethanol. The suspension was centrifuged at 20,000*g* for 20 min and the pellet of nucleic acid discarded. The supernatant fluid was fractionated with solid ammonium sulfate, and the precipitate obtained from a 210–385-g/l. fraction was resuspended in 10 mM Tris-Cl (pH 7.4), 10 mM $MgCl_2$, and 0.6 mM β-mercaptoethanol (buffer B), and dialyzed against this same buffer.

The dialyzed ammonium sulfate fraction was applied to a column of DEAE-32-cellulose (Whatman, 1 mequiv/g) previously equilibrated with buffer B containing 0.05 M NaCl, and eluted with a linear gradient of NaCl from 0.05 to 0.15 M. The enzyme eluted at 0.09 M NaCl. The peak fractions were collected, concentrated by ultrafiltration (Diaflo, Amicon Corp.), and dialyzed *vs.* buffer B. This enzyme solution can be stored at −20° for several months with no detectable loss of activity.

Preparation and Isolation of N-Acetylated Antibiotics. The reaction mixture for the large-scale acetylation of gentamicin C_{1a} contained, in a total volume of 50–75 ml, 30 ml of a cell extract that had a protein concentration in the range of 10–15 mg/ml; an ATP-generating system consisting of 0.15 mmole of phosphoenolpyruvate and 3 mg of pyruvate kinase; 0.7 mmole of ATP (adjusted to pH 6 with KOH); 3 μmoles of yeast coenzyme A; 0.2 mmole of gentamicin C_{1a}, added gradually to avoid possible substrate inhibition, and buffer A at seven times its normal concentration. The incubation was carried out at 30° for 6–12 hr with gentle agitation.

Acetylation of kanamycins A and B was carried out in the same way, except that buffer A contained potassium Tris-maleate, pH 6.6 at 30°. Neomycin B was acetylated as described for kanamycin A, except that two times as much enzyme was used. When [^{14}C]acetate-labeled antibiotics were prepared, the magnesium acetate in buffer A was replaced by $MgCl_2$.

The reaction mixtures were centrifuged to remove any precipitate, applied to a 2 × 10 cm column of the cation-exchange resin Bio-Rex 70 (Bio-Rad, 100–200 mesh) and washed with 1 M NaCl containing 5 mM sodium phosphate (pH 7) until all the material absorbing at 260 and 280 mμ

[2] Abbreviations used are: [^{14}C]CoASAc, [1-^{14}C]acetyl coenzyme A; CoASH, coenzyme A; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); kan, kanamycin; neo, neomycin; gen, gentamicin.

had been eluted. The column had previously been washed with 1 M NaCl–5 mM sodium phosphate until the pH of the effluent was 7. A linear gradient of NaCl (containing 5 mM sodium phosphate, pH 7) was run from 1 M NaCl to the concentration of NaCl required to elute the acetylated antibiotic from the column. *N*-Acetylkanamycin A eluted at 1.4 M, kanamycin A at 3.0 M, *N*-acetylgentamicin C_{1a} at 1.7 M and gentamicin C_{1a} at 3.7 M, *N*-acetylkanamycin B at 2.6 M and kanamycin B at 5 M, and *N*-acetylneomycin B at 3.0 M and neomycin B at 5.1 M NaCl. The elution of the acetylated antibiotics was monitored by using the antibiotic acetylated with [^{14}C]acetate of high specific activity as marker.

The fractions containing acetylated antibiotic were then diluted to 0.7 M in NaCl, the sodium phosphate concentration adjusted to 5 mM and applied to a second column of Bio-Rex 70 previously equilibrated with 0.7 M NaCl and 5 mM sodium phosphate (pH 7). The column was washed successively with 0.5 l. of NH_4HCO_3 at concentrations of 0.025 and 0.05 M, and with 2 l. of 0.10 M NH_4HCO_3 to remove the majority of the NaCl. A linear gradient of NH_4HCO_3 was then run from 0.10 M to the concentration of NH_4HCO_3 at which the acetylated antibiotic eluted. *N*-Acetylkanamycin A eluted at 0.18 M NH_4HCO_3, *N*-acetylgentamicin C_{1a} at 0.20 M, *N*-acetylkanamycin B at 0.35 M, and *N*-acetylneomycin B eluted at 0.40 M. Although the unacetylated antibiotics are no longer present at this stage of the purification, they elute at approximately twice the NH_4HCO_3 concentrations required for the acetylated compounds. The fractions containing the acetylated antibiotics were then lyophilized to remove the NH_4HCO_3. The recovery of acetylated antibiotics from the columns was 95 to 100% for all four compounds. The final samples contained 2–5% NaCl. All operations were performed at room temperature, and column flow rates varied from 100 to 150 ml per hr, except for the equilibrations, which were done at flow rates of about 600 ml/hr.

Electrophoresis. Samples (2–8 μl) of reaction mixtures containing [^{14}C]CoASAc (54 μCi/μmole) or purified ^{14}C-acetylated antibiotics were spotted on Sepraphore III cellulose polyacetate electrophoresis strips (2.54 × 30.5 cm, Gelman Instrument Co.). The strips were developed in a water–pyridine–glacial acetic acid buffer (400:3:10, v/v, pH 3.9) for 2.5 hr at 1 mA/strip, and the radioactivity monitored with a Radiochromatogram scanner (Packard Instrument Co.).

In Vitro Polypeptide Synthesis. Tests of the effects of acetylated and unacetylated drugs on R17 RNA-directed polypeptide synthesis were carried out with minor modifications (Davies *et al.*, 1965) of the methods of Nirenberg (1964) using an S-30 extract obtained from a wild-type sensitive strain.

Chemicals. [^{14}C]CoASAc was obtained from New England Nuclear, CoASAc and CoASH from Calbiochem. The gentamicins were provided by Dr. David Cooper of Schering Corp., the nebramycins by Drs. Kay Koch and Sean O'Connor of Lilly Research Laboratories, the neomycins by Dr. G. B. Whitfield of Upjohn Co., the kanamycins by Dr. A. Gourevitch of Bristol Co., the hybrimycins and neobiosamine B by W. T. Shier and K. L. Rinehart, Jr., and paromomycin by Dr. T. H. Haskell of Parke-Davis Co.

Results

The resistance factors R5 and NR79 are known to confer resistance to high levels of streptomycin, chloramphenicol, tetracycline, sulfanilamide, and kanamycin A. We have also found them to confer resistance to spectinomycin (they therefore inactivate both streptomycin and spectinomycin by adenylylation (Benveniste *et al.*, 1970)), and to low levels of the nebramycin factors 4 and 6, to kanamycin B, neomycins B and C, gentamicin C_{1a}, and the hybrimycins. These R-factor strains are sensitive to paromomycin, gentamicin A, C_1, and C_2, and to nebramycin factor 2. Minimum inhibitory concentrations for some of these antibiotics are listed in Table I. R factor NR79-5 is a mutant which is completely sensitive to the kanamycins, neomycins, hybrimycins, nebramycins, and gentamicins, but resistant to streptomycin, spectinomycin, chloramphenicol, tetracycline, and sulfanilamide.

TABLE I: Resistance Levels of NR79/W667 and W677 to Various Aminoglycosides.

	MIC[a] (μg/ml)	
Aminoglycosides	W677	NR79/W677
Kanamycin A	6	30
Kanamycin B	3	9
Neomycin B	6	9
Neomycin C	8	13
Gentamicin C_{1a}	2	2.5
Gentamicin C_1	3	3
Gentamicin C_2	3	3
Nebramycin factor 2	4	3
Nebramycin factor 6	2	7
N-Acetylkanamycin A	>500	>500
N-Acetylkanamycin B	150	150
N-Acetylgentamicin C_{1a}	42	42
N-Acetylneomycin B	23	23

[a] 1×10^5 cells of a culture in logarithmic phase were added to 5 ml of ML broth containing varying concentrations of antibiotics and shaken at 37° for 8 hr. The minimum inhibitory concentration (MIC) is defined as the least amount of antibiotic required to prevent growth.

It has been previously shown that in several R-factor-carrying strains, neomycin, kanamycin, and paromomycin are inactivated by enzymatic phosphorylation (Kondo *et al.*, 1968; Okanishi *et al.*, 1968; Ozanne *et al.*, 1969). NR79 had not previously been examined, and since, like R5, it was resistant to kanamycin A but sensitive to paromomycin, we deduced that it was capable of acetylating kanamycin, as had already been shown for R5 by Okamoto and Suzuki (1965) and Umezawa *et al.* (1967).

A phosphocellulose paper binding assay that has been used to monitor the phosphorylation (Ozanne *et al.*, 1969) and adenylylation (Benveniste *et al.*, 1970) of antibiotics was used to detect the acetylation of any basic antibiotic that binds to the negatively charged phosphocellulose paper.

Enzymatic Assays. The acetylation of an aminoglycoside antibiotic can be monitored by measuring the transfer of radioactivity from [^{14}C]CoASAc to phosphocellulose paper in the presence of the antibiotic and the acetylating enzyme as described in Materials and Methods. Figure 1 shows the extent of acetylation of kanamycin A and neomycin B as determined by this assay.

For those compounds incapable of binding to phosphocellulose paper, a colorimetric assay with DTNB was used. Shaw and Brodsky (1968) used this assay successfully to

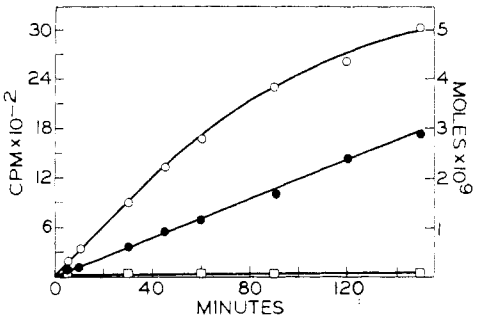


FIGURE 1: The enzymatic acetylation of kanamycin A and neomycin C by a partially purified enzyme preparation of NR79/W677. At the times indicated, 10 μl of a reaction mixture (containing 15×10^{-9} mole of antibiotic and 20×10^{-9} mole of [^{14}C]acetyl coenzyme A) was pipetted onto squares of phosphocellulose paper, which were washed, dried, and counted for radioactivity as described in Materials and Methods. (●) Moles $\times 10^9$ or counts per minute of *N*-acetylneomycin C; (○) moles $\times 10^9$ or counts per minute of *N*-acetylkanamycin A; (□) a reaction run in the absence of antibiotic, or an extract of strains NR79-5/W677 or W677 reacted in the presence of kanamycin A and [^{14}C]acetyl coenzyme A.

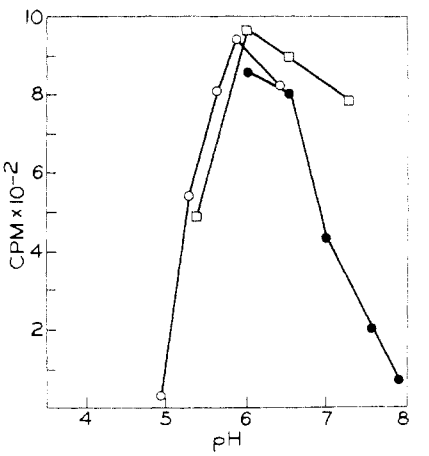


FIGURE 3: pH optimum for kanamycin acetylating activity. A reaction was run in the presence of kanamycin A, [^{14}C]CoASAc, and different buffers, and 10 μl spotted on phosphocellulose paper and counted. (○) Maleate buffer, (□) Tris-maleate buffer, (●) potassium phosphate buffer all at a final concentration of 0.1 M in the reaction mixture.

monitor the acetylation of chloramphenicol. The increase in absorbance at 412 mμ is coincident with the formation of free coenzyme A and its reaction with DTNB to form the mixed disulfide. DTNB was shown not to inhibit the acetylating enzyme. The endogenous reaction that can be measured by the increase in absorbance at 412 mμ when all the reactants except antibiotic are added in the cuvet is no longer present after the enzyme has been purified through DEAE-cellulose. Figure 2 shows how this assay system can be used to follow the acetylation of several aminoglycoside antibiotics.

Partial Purification and Properties of the Acetylating Enzyme. The acetylating enzyme has been purified approximately tenfold from an osmotic shockate of strain NR79/W677 by precipitation of nucleic acids, ammonium sulfate fractionation, and passage through a DEAE-cellulose column as described in Materials and Methods. The overall yield of activity at this stage is 70–80%. The enzyme eluted from DEAE-cellulose is free of any CoASAc hydrolase activity, which interferes with the DTNB assay by causing a very high blank value.

The acetylating enzyme is produced constitutively in cells grown in a rich medium. It requires Mg^{2+} both for activity and stability; dialysis in the absence of Mg^{2+} causes an irreversible loss of activity. The enzyme loses 35% of its activity when incubated for 10 min at 37° in the presence of buffer B; 70% of its activity is lost when incubated for the same period of time at 42°. Substrate amounts of acetyl coenzyme A completely protect the enzyme against this inactivation; kanamycin A alone affords only a very slight protection, and sodium acetate has no effect.

The pH optimum for the acetylation of kanamycin is approximately 5.8 (Figure 3). The neomycins, hybrimycins, and nebramycin factors 4 and 6 have a pH optimum near that value also. However, gentamicin C_{1a} and C_2 have an optimum near 7.6. These pH optimums do not reflect a pH-dependent binding of the aminoglycoside compounds to the phosphocellulose paper; purified ^{14}C-labeled N-acetylated antibiotics were shown to bind completely to the paper over a pH range of 5–8. The different pH optimums would there-

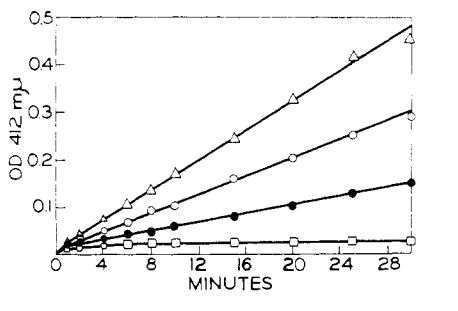
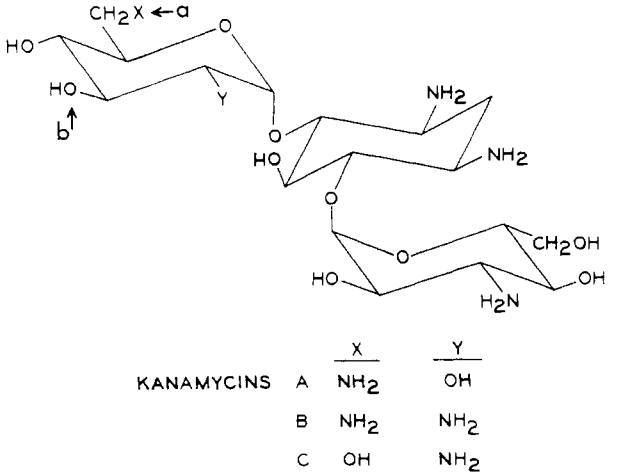


FIGURE 2: Acetylation of compounds monitored by the DTNB assay. The incubation mixture was reacted at room temperature in a 1-ml cuvet with a 1-cm path length as described in Materials and Methods. The increase in absorbance at 412 mμ was recorded as a function of time. (△) Acetylation of gentamicin C_{1a} at pH 6.7; (○) acetylation of nebramycin factor 6 at pH 5.8; (●) acetylation of neomycin B at pH 5.8; (□) a reaction mixture containing 6-amino-6-deoxy-D-glucose, and also a reaction run in the absence of antibiotic.



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

FIGURE 4: The structure of the kanamycins. The arrows indicate the sites where these antibiotics are enzymatically modified by N acetylation (a) and O phosphorylation (b).

TABLE II: Efficiency of Different Antibiotics and Related Compounds as Substrates for the Acetylating Enzyme at Various pH's.

	pH 5.8 N-Acetylated Product		pH 6.7 N-Acetylated Product		pH 7.6 N-Acetylated Product	
	moles × 10⁹[a]	%[b]	moles × 10⁹	%	moles × 10⁹	%
Kanamycin A	21.2	100	20.4	96	18.2	86
Kanamycin B	14.0	66	11.8	56	6.0	28
Neomycin B	13.0	61	8.5	40	5.4	25
Neomycin C	8.8	41	6.8	32	4.7	22
Neamine	21.2	100	10.4	49	6.6	31
Neobiosamine B	0	0	0	0	0	0
Paromomycin	0	0	0	0	0	0
Paromamine	0	0	0	0	0	0
Gentamicin C_{1a}	13.1	62	31.0	146	32.2	152
Gentamicin C_1	0	0	0	0	0	0
Gentamicin C_2	0.9	4	4.2	20	4.2	20
Gentamine C_{1a}	7.0	33			37.5	176
Gentamine C_1	0	0			0	0
Gentamine C_2	0	0			6.4	30
Gentamicin A	0	0	0	0	0	0
Gentamine A	0	0	0	0	0	0
6-Amino-D-glucose[c]	0	0	0	0	0	0
Hybrimycin A_1	29.2	138			26.8	126
Hybrimycin A_2	23.3	110			22.0	104
Hybrimycin A_3	38.5	182			45.0	213
Hybrimycin B_1	21.6	102			17.1	81
Hybrimycin B_2	19.5	92			19.0	90
Hybrimycin B_3	27.0	127			23.0	111
Nebramycin factor 2	0	0	0	0	0	0
Nebramycin factor 4	17.4	82	12.9	61	7.6	36
Nebramycin factor 6	28.6	135	26.0	123	19.4	92

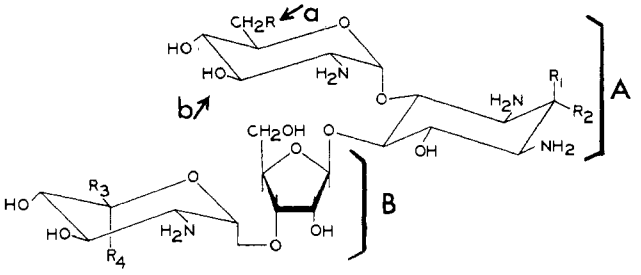
[a] Each compound listed was incubated with a partially purified enzyme preparation from NR79–W677 and [^{14}C]CoASAc at the pH's listed (Tris-maleate buffer). Aliquots were withdrawn at 2, 5, 15, 30, and 45 min to ensure that a linear assay was always occurring. The radioactivity observed was calculated as moles of acetylated product formed per milliliter of enzyme per minute and is listed above. The values represent the average of at least three different incubation mixtures. All the substrates listed were tested to ensure that they were not present in limiting or inhibiting amounts in the incubation mixture. [b] The per cent is relative to the rate of acetylation of kanamycin A at pH 5.8. [c] This compound was assayed by the colorimetric assay, using DTNB (see Materials and Methods, and Figure 2).

fore appear to reflect a property of the enzymatic reaction itself.

Substrates for the Acetylating Enzyme. Table II shows the rate of acetylation of a variety of antibiotics and related compounds at three different pH's by a partially purified enzyme preparation from NR79/W677. The structure of the kanamycins is shown in Figure 4, that of neomycin, paromomycin, and the hybrimycins in Figure 5 (Shier *et al.*, 1969), that of the gentamicins in Figure 6 (Cooper *et al.*, 1969), and the structure of nebramycin factor 6 in Figure 7. The structures of factors 2 and 4 are not known (Thompson and Presti, 1967; Koch and Rhoades, 1971). All of the compounds that are acetylated contain a 6-aminohexose which is linked to a streptamine, epistreptamine, or deoxystreptamine ring. It is unlikely that 6-aminoglucose would bind to phosphocellulose paper if it were N acetylated, so it was assayed for its activity as a substrate for acetylation by the DTNB assay (Figure 2). These data are further analyzed in the Discussion.

Since some of the compounds that were not substrates for the acetylating enzyme (*e.g.*, paromomycin and gentamicin A) resemble the substrates very closely, they were tested as possible inhibitors of the acetylation reaction. The results are listed in Table III. The antibiotics paromomycin and gentamicin A are the best inhibitors; paromamine, which is not an antibiotic, also inhibits effectively. The reactions in Table III were run at pH 6.7; at pH 7.6 paromomycin is less effective as an inhibitor of the N acetylation of kanamycin A and gentamicin C_{1a}. Figure 8 shows that the addition of the inhibitors while the reaction is in progress causes an immediate cessation of the acetylation.

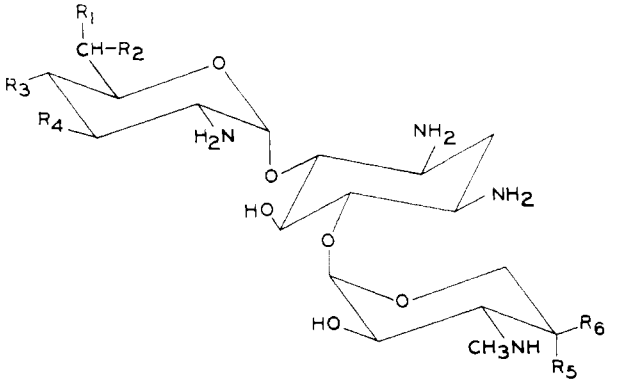
Preparation and Purification of N-Acetylated Antibiotics. As can be seen in Table II, gentamicin C_{1a} is one of the best substrates for the acetylating enzyme; nevertheless, strain W677, whether or not it contains the R factor, is quite sensitive to the drug. This might suggest that *N*-acetylgentamicin C_{1a} is an antibiotic, and this compound was purified in order that we might examine its properties more carefully. Gentamicin C_{1a} (100 mg) was acetylated in the presence of coenzyme A, sodium acetate, and ATP by a crude extract of R5/W677 as described in Materials and Methods. R5/W677 is preferable to NR79/W677 as a source of enzyme for these large-scale reactions because the acetylating activity is approximately threefold greater in this strain.



	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
HYBRIMYCIN A1	NH_2	OH	H	H	CH_2NH_2
HYBRIMYCIN A2	NH_2	OH	H	CH_2NH_2	H
HYBRIMYCIN B1	NH_2	H	OH	H	CH_2NH_2
HYBRIMYCIN B2	NH_2	H	OH	CH_2NH_2	H
PAROMOMYCIN	OH	H	H	CH_2NH_2 or H	H or CH_2NH_2

FIGURE 5: The structures of the neomycins, hybrimycins, and paromomycin. The arrows indicate the sites where these antibiotics are enzymatically modified by N acetylation (a) and O phosphorylation (b). Paromomycin is not acetylated. The part of the molecule indicated by bracket A is neamine, hybrimycin A_3, or B_3 (R = NH_2) or paromamine (R = OH). The part indicated by bracket B is neobiosamine.

The gentamicin C_{1a} and its *N*-acetyl derivative were separated by chromatography on a column of Bio-Rex 70, using a gradient of NaCl. The eluted acetylated antibiotic was reabsorbed onto another Bio-Rex 70 column, and the NaCl displaced with large volumes of NH_4HCO_3. The acetylated antibiotic was eluted with NH_4HCO_3, which was subsequently removed by lyophilization. The details of the purification are described in Materials and Methods. The use of ^{14}C-labeled acetylated gentamicin C_{1a} greatly simplified the identification of those fractions which contained the acetylated antibiotic. The yield of acetylated drug from the column was close to 100%, as judged by the recovery of ^{14}C-labeled material at



		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

FIGURE 6: The structure of the gentamicins.

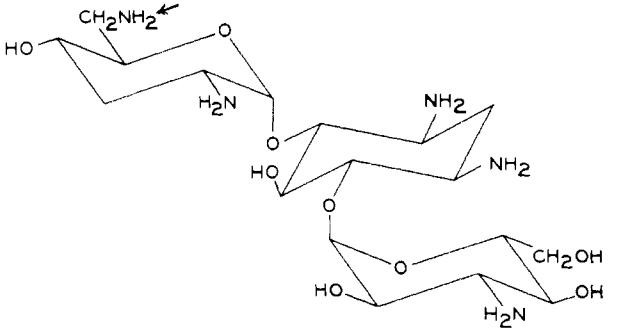


FIGURE 7: The structure of nebramycin factor 6. The arrow indicates the probable site of N acetylation.

the end of the purification. *N*-Acetylneomycin B, *N*-acetylkanamycin B, and *N*-acetylkanamycin A were also purified using the same procedure. The latter compound had already been isolated and characterized by Umezawa *et al.* (1967), using different techniques. The nuclear magnetic resonance spectra of these compounds was consistent with the presence of an *N*-acetyl group.

The extent of purity of the N-acetylated compounds was determined by thin-layer chromatography on silica gel; the R_F values in various solvents are listed in Table IV. By running several hundred micrograms of the acetylated antibiotics on the chromatograms, contamination with 0.5% of unacetylated antibiotic could have been detected. In all cases, the acetylated antibiotics migrated as one distinct spot.

A much more sensitive assay for the presence of unacetylated antibiotic is to test for acetylation in the presence of acetylating enzyme using the phosphocellulose paper binding assay. By using [^{14}C]CoASAc of high specific activity, a 0.05% contamination of an unacetylated antibiotic in each of the four acetylated antibiotics could have been detected (less than this level was hard to determine since some of the acetylated antibiotics inhibit the acetylation reaction). For the four N-

TABLE III: Inhibitors of the Acetylation Reaction.

Compounds Tested	% Inhibition[a] kan A	neo B	gen C_{1a}
Gentamicin A	64		81
Gentamicin C_1	10		9
Paromomycin	76	34	80
Paromamine (gentamine A)	41	6	43
Neobiosamine B	0	0	
Nebramycin 2	0		
6-Amino-D-glucose	0		
N-Acetyl-kan A	0		
N-Acetyl-neo B		20	
N-Acetyl-gen C_{1a}			30

[a] The per cent inhibition of the acetylation of kanamycin A, neomycin B, and gentamicin C_{1a} in the presence of equimolar amounts of the compounds tested. The incubations were carried out at pH 6.7 as described in Materials and Methods. The values shown represent the average of at least two experiments. The extent of inhibition does not change if the enzyme is preincubated in the presence of the inhibitors.

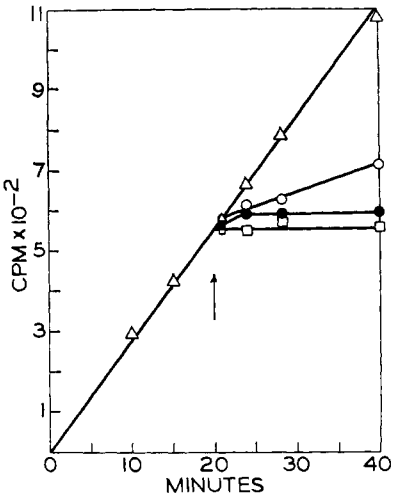


FIGURE 8: Inhibition of the acetylation of gentamicin C_{1a} by gentamine A and gentamicin A. Four reaction mixtures containing gentamicin C_{1a}, [^{14}C]acetyl coenzyme A, and partially purified enzyme in a total volume of 70 μl were incubated at 30°. At the times indicated, 10 μl was removed and counted as described in Materials and Methods. At 20 min, 1 μl of either water or an inhibitor was added. (△) The acetylation of 1×10^{-8} mole of gentamicin C_{1a}; (○) at 20 min, 1×10^{-8} mole of gentamine A was added; (□) at 20 min, 2×10^{-8} mole of gentamine A was added; (●) 1×10^{-8} mole of gentamicin A was added.

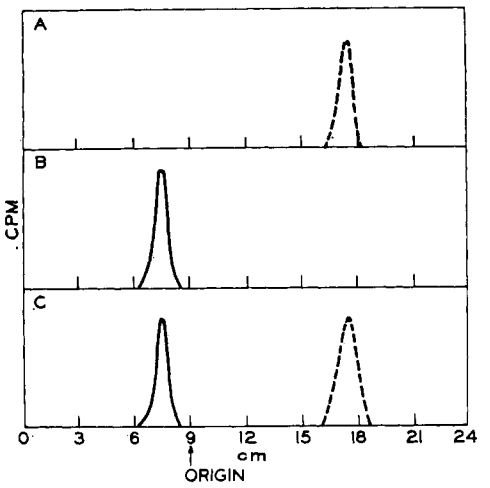


FIGURE 9: Electrophoresis of the products of the enzymatic acetylation of antibiotics. (A) Purified [^{14}C]*N*-acetylgentamicin C_{1a}; (B) [^{14}C]CoASAc or [^{14}C]sodium acetate; (C) a portion of a reaction mixture containing gentamicin C_{1a}, [^{14}C]CoASAc and acetylating enzyme. Electrophoresis was performed as described in Materials and Methods. The cathode lies to the right in the figures. The [^{14}C]*N*-acetylgentamicin A migrated to 17.6 cm; under the same conditions, [^{14}C]*N*-acetylneomycin B migrates to 18.2 cm, and [^{14}C]*N*-acetylkanamycin A to 16.7 cm.

acetylated antibiotics which we have isolated, there was less than 0.05% contamination with the parent compound, as judged by activity in the acetylation assay.

Electrophoresis on cellulose acetate strips showed that all of the radioactivity migrated to one distinct spot. When the products of an enzymatic reaction containing [^{14}C]CoASAc and antibiotic were run, two radioactive spots were obtained, one near the origin corresponding to [^{14}C]CoASAc or [^{14}C]-acetate, and the other which has migrated toward the cathode corresponding to the N-acetylated antibiotic. This is shown schematically for *N*-acetylgentamicin C_{1a} in Figure 9.

Properties of the Acetylated Antibiotics. As had previously been shown by Hori and Umezawa (1967), *N*-acetylkanamycin A is no longer an antibiotic. Table I shows that *N*-acetylkanamycin A is not able to inhibit the growth of *E. coli* W677 at concentrations up to 0.5 mg/ml. However, *N*-acetylneomycin B, *N*-acetylgentamicin C_{1a}, and *N*-acetylkanamycin B are still antibiotics; *N*-acetylneomycin B being approximately 4 times weaker, *N*-acetylgentamicin C_{1a} 10–20 times weaker, and *N*-acetylkanamycin B 50 times weaker than their respective nonacetylated parent compounds. If the antibiotic behavior of these three compounds were due to contamination with the unacetylated antibiotics, these contaminants would have to be present to an extent of about 25, 5–10, and 2%, respectively. Actually, less than 0.05% of the unacetylated antibiotics are present, as was previously mentioned.

In order to examine the biological properties of the acetylated antibiotics more carefully, they were compared to their parent compounds as inhibitors of *in vitro* polypeptide synthesis. The results are shown in Figure 10. As can be seen,

TABLE IV: R_F Values for Various Aminoglycosides.

	Solvent 1[a]	Solvent 2
Kanamycin A	0.61	0.56
N-Acetylkanamycin A	0.78	0.60
Kanamycin B	0.59	0.55
N-Acetylkanamycin B	0.80	0.61
Neomycin B	0.50	0.48
N-Acetylneomycin B	0.64	0.62
Gentamicin C_{1a}	0.78	0.31
N-Acetylgentamicin C_{1a}	0.95	0.47

[a] Solvent 1: upper phase of chloroform–methanol–28% ammonium hydroxide (2:1:1, v/v). Solvent 2: 1-propanol–pyridine–glacial acetic acid–water (16:10:3:10, v/v). The chromatograms were developed with either 0.1% ninhydrin in butanol or 50% sulfuric acid.

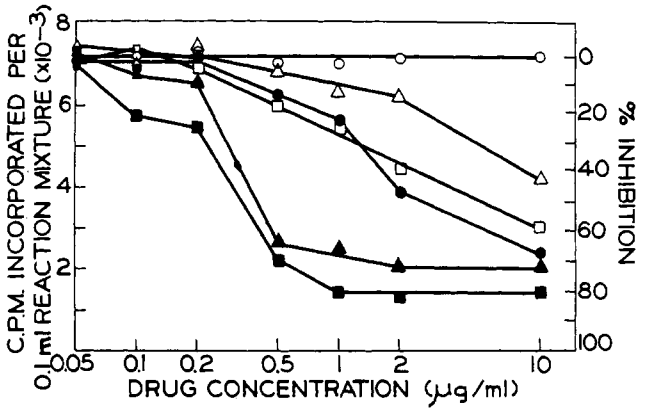


FIGURE 10: Inhibition of *in vitro* R17 phage RNA-directed polypeptide synthesis by acetylated and unacetylated antibiotics. The radioactivity in a 0.1-ml reaction mixture that had been incubated for 45 min at 37° with the concentration of antibiotic listed was filtered and counted in scintillation vials. [^{14}C]Valine (25 μCi/μmole) was used. All of the incubation tubes contained 3% polyethylene glycol. (○) *N*-Acetylkanamycin A, (●) kanamycin A, (△) *N*-acetylgentamicin C_{1a}, (▲) gentamicin C_{1a}, (□) *N*-acetylneomycin B, and (■) neomycin B.

N-acetylneomycin B and *N*-acetylgentamicin C_{1a} still inhibit polypeptide synthesis, whereas *N*-acetylkanamycin A does not, even at a concentration of 10 μg/ml. *N*-Acetylkanamycin B was not tested. The antibiotic properties of *N*-acetylneomycin B and *N*-acetylgentamicin C_{1a} could be due to a breakdown of these compounds during the ribosome incorporation to yield the unacetylated antibiotic. To eliminate this possibility an amino acid incorporation was carried out using valine and [^{14}C]*N*-acetylgentamicin C_{1a} or [^{14}C]*N*-acetylneomycin B, and portions of the mixture at time zero and after the 45-min incubation were spotted onto cellulose acetate strips. Electrophoresis of the strips revealed that no free [^{14}C]acetate was present at either time (less than 0.5% breakdown could have been detected).

The acetylated antibiotics were tested as inhibitors of the acetylation reaction. The results are listed as part of Table III. Equimolar concentrations of *N*-acetylneomycin B and *N*-acetylgentamicin C_{1a} inhibited the acetylation of neomycin B and gentamicin C_{1a} by 20 and 30%, respectively. *N*-Acetylkanamycin A did not inhibit the acetylation of kanamycin A even at a fivefold higher concentration than the substrate.

Kanamycin A and paromamine have both been shown to be phosphorylated on the 3-hydroxyl group of their amino sugar ring (see Figures 4 and 5) by an enzyme that is present in certain R-factor-carrying strains (Kondo *et al.*, 1968). Since neomycin B is also phosphorylated, the phosphorylation is presumed to occur on the same hydroxyl group (Ozanne *et al.*, 1969). Gentamicin C_{1a} and nebramycin factor 6, since they lack this hydroxyl group, are not phosphorylated (Ozanne *et al.*, 1969).[1] When *N*-acetylneomycin B and *N*-acetylkanamycin A were tested as possible substrates for the phosphorylation reaction, it was found that *N*-acetylneomycin B was phosphorylated as well as the unacetylated antibiotic, and *N*-acetylkanamycin A two-thirds as well as kanamycin A.

Discussion

An extract of a kanamycin-resistant strain of *E. coli* carrying either R-factor NR79 or R5 has been shown to contain an enzyme that, in the presence of [^{14}C]acetyl coenzyme A, transfers the labeled acetate to kanamycin A, kanamycin B, neomycins B and C, the hybrimycins, gentamicins C_{1a} and C_2, and nebramycin factors 4 and 6. Paromomycin, gentamicins A and C_1, and nebramycin factor 2 are not acetylated. The structural relationships between these antibiotics strongly suggest that in all cases it is a 6-amino group of a hexose ring that is acetylated.

N-Acetylkanamycin A is no longer capable of inhibiting either R17 RNA-directed polypeptide synthesis *in vitro*, or the growth of *E. coli*. However, *N*-acetylkanamycin B, *N*-acetylneomycin B, and *N*-acetylgentamicin C_{1a} are still antibiotics, although weaker than their unacetylated parent compounds.

The product of the enzymatic acetylation of kanamycin A by an extract of a strain carrying R factor R5 had previously been characterized by Umezawa *et al.* (1967). These authors showed that the antibiotic was acetylated on the 6-amino group of its 6-amino-6-deoxy-D-glucose moiety. Okanishi *et al.* (1967), after examining other aminoglycosides, concluded that neomycin, kanamycin C, and paromomycin were not acetylated. Their results were based on a microbiological assay that measured the residual potency of these antibiotics following incubation in the presence of a cell-free extract, coenzyme A, and ATP. Their failure to detect the acetylation of neomycin stems from the fact that *N*-acetylneomycin B is still an antibiotic. Also, the acetylation of neomycin proceeds very slowly, especially at a pH greater than 7.5, and 4 hr may have been insufficient time for a significant amount of neomycin to have been acetylated.

The enzyme that acetylates the aminoglycosides listed in Table II is a different enzyme from the one that has been shown to acetylate and inactivate chloramphenicol (Suzuki and Okamoto, 1967; Shaw, 1967). R factor NR79-5 is a segregant of NR79 which has lost kanamycin resistance but retains its chloramphenicol-resistance character; cell-free extracts of this strain can acetylate chloramphenicol but none of the aminoglycosides (see Figure 1).

Table II lists the relative efficiency of acetylation of various compounds compared to the acetylation of kanamycin A at pH 5.8. Neomycins B and C are acetylated, but paromomycin is not. Since the only difference between these antibiotics is the presence or absence of an amino group at the 6 position of the hexose unit attached to deoxystreptamine (Figure 5), the acetylation of neomycin must occur on the amino group at the 6 position. This is also clearly seen by comparing neamine with paromamine; the former is acetylated, while the latter is not.

It can be seen that kanamycin A is a better substrate for the acetylating enzyme than kanamycin B; the presence of a 2-amino group in the sugar ring therefore seems to depress the rate of acetylation. Kanamycin C was not tested, but since it does not contain a 6-amino group, it would not be expected to be acetylated.

The hybrimycins and neomycins are very closely related compounds (Figure 5); their only difference being the presence of a streptamine or epistreptamine ring in the former, and a 2-deoxystreptamine ring in the latter (Shier *et al.*, 1969). This difference affects the rate of acetylation, since the hybrimycins are better substrates than the analogous neomycins (*i.e.*, hybrimycins A_1 and B_1 are acetylated faster than neomycin B, and hybrimycins A_2 and B_2 better than neomycin C). Furthermore, the hybrimycins containing a streptamine ring are better substrates than those containing an epistreptamine ring (*i.e.*, hybrimycins A_1, A_2, and A_3 are better substrates than B_1, B_2, and B_3).

The structures of gentamicins C_{1a}, C_1, and C_2 are shown in Figure 6. Gentamicin C_{1a} is acetylated very well, C_2 poorly, and C_1 not at all. Therefore substitution on the 6-carbon (gentamicin C_2) or at the amino group on the 6-position (gentamicin C_1) effectively hinders or totally blocks the acetylation of that amino group by the enzyme. Gentamicin C_{1a}, C_1, and C_2 have similar biological activities in inhibiting the growth of various organisms (Waitz and Weinstein, 1969), or in inhibiting polypeptide synthesis (Davies *et al.*, 1969). Therefore, alkylation at the 6-carbon or at the amino group on the 6-carbon position of the hexose seems to be one method of preventing possible acetylation of an antibiotic by the acetylating enzyme without reducing its antibacterial efficiency. One might suppose that 6-*N*-methylkanamycin B or 6-*N*-methylneomycin would be resistant to enzymatic acetylation and retain biological activity.

Gentamine C_{1a} is acetylated at a faster rate than gentamicin C_{1a}, neamine faster than neomycin, and hybrimycin A_3 better than hybrimycin A_1 or A_2. The presence of additional substituents on the deoxystreptamine ring thus reduce the efficiency with which these compounds are acetylated.

The minimum structure required for enzymatic acetylation therefore seems to be a 6-aminohexose linked to a streptamine, epistreptamine, or deoxystreptamine ring (as in neamine, gentamine C_{1a}, or hybrimycins A_3 and B_3). 6-Amino-6-deoxy-

D-glucose is not acetylated (see Figure 2). Neobiosamine B is not acetylated, presumably because the sugar ring is attached to a ribose rather than a diaminocyclitol. Neobiosamine C was not tested.

It is assumed that one enzyme catalyzes all of these reactions because of the structural similarity in the substrates that are acetylated and because cell-free extracts of the mutant NR79-5/W677 are incapable of acetylating any of these antibiotics. Also, several mutants obtained from NR79/W677, which were selected for higher levels of resistance to kanamycin A, were found to yield higher levels of acetylation activity *in vitro* for all the aminoglycosides that are acetylated. Furthermore, a sample of DEAE-cellulose purified enzyme that was fractionated further by hydroxylapatite and Sephadex G-200 chromatography still acetylated all of these antibiotics.

The compounds listed in Table II that were not acetylated were tested as possible inhibitors of the acetylation reaction (Table III). Paromomycin inhibits the acetylation of neomycin, kanamycin, and gentamicin C_{1a}, whereas paromamine inhibits these reactions to a lesser extent. Thus, the intact antibiotic molecule is a better inhibitor than a portion of it; this is in contrast to the effectiveness of a compound as a substrate, where a part of an antibiotic, *i.e.*, neamine, is a more efficient substrate than the entire molecule, neomycin B. In clinical use the gentamicin complex would seem to have some advantage over the use of the isolated components of the mixture, since it contains inhibitors of the acetylation reaction; the complex also contains some inhibitors of the enzyme that catalyzes the phosphorylative inactivation of neomycin, kanamycin, and gentamicin A.[1]

Although gentamicin C_{1a} is one of the best substrates for the acetylation reaction, the R-factor-containing strain is almost completely sensitive to this antibiotic; this is apparently due to the fact that N acetylation of gentamicin C_{1a} does not completely eliminate the antibiotic activity of this compound. Consistent with this fact, we find that the N-acetylated drug is still an effective inhibitor of *in vitro* polypeptide synthesis. In addition, although the lack of resistance of the R-factor-containing strain to neomycin B might have been due to its low level of acetylation, especially if the enzymatic reaction occurs at pH 7 in the cell, it is most likely that sensitivity is due to the fact that *N*-acetylneomycin B is still an antibiotic. Although *N*-acetylkanamycin A is not an antibiotic, *N*-acetylkanamycin B retains a low level of antibiotic activity.

From these data we conclude that the presence of an amino group in either the 2 or the 6 position of the hexose moiety attached to deoxystreptamine of these aminoglycosides is an important structural element in determining antibiotic properties. When both of these groups are absent, or blocked by acetylation, the molecule is no longer an antibiotic. Thus, 6-*N*-acetylkanamycin A (which contains a 2-hydroxyl group on the same hexose that is acetylated) possesses no biological activity, whereas 6-*N*-acetylkanamycin B (with a 2-amino group) is still an antibiotic. Likewise, *N*-acetylneomycin B and *N*-acetylgentamicin C_{1a} which possess a 2-amino function still retain considerable biological activity. *N*-Acetylnebramycin factor 6, although not isolated and purified, would also be expected to retain biological activity. The details of these structure/activity relationships are currently under investigation.

The use of a compound that is resistant to all manner of R-factor mediated inactivation should be weighed against its effectiveness as an antibiotic. Gentamicin C_1 is completely resistant to inactivation by acetylation and phosphorylation (the heptose moiety is 3-deoxy). Likewise, nebramycin factor 2 is not acetylated or phosphorylated by any of the R-factor-containing strains that we have examined. For the kanamycin group of antibiotics, blocking the 6-amino group may be an effective way to prevent acetylation and concomitant inactivation, and the addition of a 3-amino group in the 6-amino-6-deoxy-D-glucose ring of kanamycin would prevent its inactivation by phosphorylation. Whether this compound would still be an antibiotic remains to be seen.

The neomycins, kanamycins, gentamicin A, and nebramycin factor 4 can be inactivated by phosphorylation by an enzyme extracted from a R-factor-carrying strain.[1] This phosphorylation has been shown to occur on the 3-hydroxyl group of the same sugar ring that is acetylated in kanamycin A (see Figures 4 and 5) (Kondo *et al.*, 1968). By structural analogy, this same hydroxyl group is believed to be phosphorylated in neomycin, gentamicin A, and nebramycin factor 4 (Ozanne *et al.*, 1969). One can distinguish *in vivo* between R-factor strains that phosphorylate or acetylate neomycin and kanamycin by the fact that if the acetylating enzyme is present, the strain will be sensitive to paromomycin and gentamicin A, whereas the strain that inactivates neomycin and kanamycin by phosphorylation will also be resistant to paromomycin and gentamicin A. In our experience, the number of R-factor containing strains that inactivate neomycin and kanamycin by phosphorylation are much more numerous than those that inactivate these drugs by acetylation. Also, since phosphorylation produces high levels of resistance in the strains carrying these R factors, it seems to be a much more effective way for the cell to protect itself against this group of aminoglycosides.

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Isolation and Properties of Subunits of Rat Pituitary Luteinizing Hormone*

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ABSTRACT: The isolation of subunits from rat pituitary luteinizing hormone (LH) is described. The subunits separate in the countercurrent distribution system: 40% (w/v) ammonium sulfate–0.2% dichloroacetic acid–1-propanol–ethanol (60:60:27:33). Two dissimilar subunits are obtained, that with a partition coefficient 0.1 is designated C-1, that with a partition coefficient of 10 is designated C-2. Similar subunits have previously been described for bovine and ovine luteinizing hormone. The C-1 subunit is characterized by a molar ratio 3:1 lysine:arginine, while C-2 has 9:1 arginine:lysine ratio. Although these ratios are not identical with those previously reported for LH subunits from other species they are similar. A higher content of tyrosine in C-1 than in C-2 also appears common, although the starting LH contained relatively little tyrosine compared to other proteins. The C-2 subunit was characterized by a very high proline content (the most abundant amino acid in this subunit and the starting LH) and a relatively high leucine content. Both subunits are glycopeptides containing glucosamine, galactosamine, mannose, galactose, and fucose; and each have a relatively high cystine content. Gel filtration studies and data on the Stokes radii are consistent with a molecular weight of approximately 15,500 for each subunit and 31,000 for the starting rat LH. The relative potency of the isolated rat LH was 1.55–1.72 units/mg measured by either the ovarian ascorbic acid depletion (OAAD) bioassay or radioimmunoassay (RIA), comparable to the potency of preparations from other species. The isolated subunits had 0.08 unit/mg by OAAD, but the RIA showed 0.07 unit/mg for C-1 and 1.1 units/mg for C-2. Thus a significant RIA reaction was retained for the C-2 subunit. Recombination of the two subunits in pH 7.0 buffer produced a 7-fold augmentation of the OAAD activity, which was essentially equipotent to the RIA response, both being about 35% of the starting LH potency.

Isolation of rat pituitary luteinizing hormone has been reported by three laboratories (Reichert and Midgley, 1968a; Ward *et al.* 1968; Fontaine and Burzawa-Gerard, 1968). Monroe *et al.* (1968) utilized purified rat LH[1] in the development of a radioimmunoassay for that hormone, but did not provide details of the preparative procedure. Ward *et al.* (1968) noted rather poor recoveries of rat LH and suggested this may be due to an unusually facile subunit dissociation of the hormone during its purification, although at that time subunits had not been demonstrated.

In this report, we wish to describe isolation of subunits of

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[1] Abbreviations used are: LH, luteinizing hormone; TSH, thyroid-stimulating hormone; RIA, radioimmunoassay; OAAD, ovarian ascorbic acid depletion. Luteinizing hormone subunit nomenclature has varied according to the laboratory involved. We originally designated an ovine S subunit and an A subunit (Ward *et al.*, 1966), shortly thereafter Papkoff and Samy (1967) designated C-I and C-II. In our studies on bovine LH (Reichert *et al.*, 1969) we designated C-1 and C-2. There is growing evidence that the subunits designated S-, C-I, or C-1 all have common properties which appear to relate to other glycopeptide hormones (*e.g.*, Liao and Pierce, 1970); the A subunit, C-II, or C-2 appears to possess a more specific character. In view of this J. G. Pierce and H. Papkoff (personal communication) have proposed a more uniform nomenclature. If uniformly adopted by those working in the field the C-1 subunit of the present study would become murine LH-α, and the C-2, murine LH-β.