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Isolation and Characterization of three Phosphoamido-Neomycins and their Conversion into Neomycin by *Streptomyces fradiae*

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Some amino acids, particularly glycine and serine, favour the accumulation in the fermentation broth of three phosphorylated amino sugar compounds that are intermediates in the pathway of neomycin biosynthesis by *Streptomyces fradiae* 3535. The compounds were separated and purified further by Amberlite IRC-50 (NH_4^+ form). The intermediates were characterized by physicochemical methods as neomycin B pyrophosphate ($C_{23}H_{48}N_6O_{19}P_2,3H_2O$), neomycin C pyrophosphate ($C_{23}H_{48}N_6O_{19}P_2,3H_2O$) and neomycin C dipyrophosphate complex ($C_{24}H_{66}N_8O_{33}P_4$).

We have previously reported (Majumdar & Majumdar, 1969*a*) the isolation of a phosphoamido-amino sugar antibiotic from *Streptomyces fradiae* producing neomycin. During our study on the effect of nitrogen compounds on neomycin production in *S. fradiae*, it was observed that some amino acids favoured the formation of a phosphorylated substance that did not move from the origin in the paper chromatogram by the method of Majumdar & Majumdar (1967*a*). Subsequent work showed that this substance stimulated neomycin biosynthesis and contained three components. The present paper reports the isolation and characterization of these three intermediates and their role in the formation of neomycin.

MATERIALS AND METHODS

Culture and cultural conditions. The neomycin-producing culture of *S. fradiae* 3535 (Waksman & Lechevalier, 1949) was maintained on a potato–yeast extract–mineral-agar slant at 28°C and was subcultured at monthly intervals. A well sporulated slant culture (7 days old) was washed with 2ml of sterile water, and 0.5ml was used to inoculate a 500ml Erlenmeyer flask containing 200ml of medium of the following composition: maltose, 15g; nitrogen source, equivalent to 0.84g of N; K_2HPO_4, 1.0g; $MgSO_4,7H_2O$, 0.5g; $CaCl_2,2H_2O$, 40mg; $FeSO_4,7H_2O$, 5mg; $ZnSO_4,7H_2O$, 0.5mg; water, 1000ml; pH 7.5±0.1 (Majumdar & Majumdar, 1967*b*). The carbon sources and phosphate were sterilized separately and added just before inoculation. The nitrogen source was always included in the basal medium and the medium was adjusted to pH 7.2 and sterilized. After a night stationary, the flasks were placed on a rotary shaker (250 rev./min, eccentricity 1.27cm). Incubation temperature was 28°C. Occasional checking of the flask to resuspend adhering cells into the medium was necessary during the first 48h.

Preparation of washed mycelium. After 60h of shaking, the mycelium from the medium containing $NaNO_3$ as nitrogen source was separated by filtration through a sintered-glass filter and washed with water. It was shaken with 10 vol. of 0.5% KCl solution for 10min and then filtered. This treatment with KCl solution was repeated once again, and the mycelium was thoroughly washed with water and filtered through a sintered-glass filter. Then 1g wet wt. of mycelium was transferred to each 50ml Erlenmeyer flask containing 10ml of the appropriate medium and the flasks were placed on the rotary shaker. Incubation temperature was 28°C.

Separation and determination of phosphorylated compounds from the broth. Phosphorylated compounds accumulating in the fermentation broth were separated by the method of Majumdar & Majumdar (1967*a*). A 20ml portion of broth was passed through a small column (1ml volume; diameter 8mm) of Amberlite IRC-50 (200–400 mesh; NH_4^+ form), washed with 5ml of water and finally eluted with 5ml of 1M-NH_3. The phosphorus content of the eluate was determined by King's (1932) method.

For testing the stimulatory effect of the compounds on neomycin production in a resting-cell experiment, the phosphorylated compounds were separated in the following way. A 400ml portion of broth was passed through a column (1.6cm diam.×6cm) of Amberlite IRC-50 (NH_4^+ form). The column was washed with 50ml of water. Finally it was eluted with 1M-NH_3, and organic phosphate-containing fractions were identified by a spot test (Hanes & Isherwood, 1949) and mixed together. This portion was concentrated under reduced pressure, treated with ethanol and kept overnight at 4°C. The precipitate of crude phosphorylated compounds was washed with ethanol and dried in a vacuum desiccator.

Determination of antibiotic activity and growth. The phosphorylated intermediates were dissolved first in

a minimum quantity of 0.5M-NH_3 and then diluted appropriately with potassium phosphate buffer, pH 8, as described by Grove & Randle (1955). The broth and neomycin B base (used as standard) were diluted with the same buffer. The antibiotic activity was determined by a modified cup-plate method with *Bacillus subtilis* (strain B_3) as the test organism. Results were expressed relative to neomycin B base. Growth was determined as the dry weight of cells (Majumdar & Majumdar, 1967*b*).

Paper chromatography. Unless specified otherwise, paper chromatography was carried out on Whatman no. 1 paper. The solvent systems used for paper chromatography were: I, butan-2-one–2-methylpropan-2-ol–methanol–6.5M-NH_3 (16:3:1:6, by vol.); II, butan-2-one–2-methylpropan-2-ol–methanol–90% (v/v) formic acid–water (16:4:1:2:2, by vol.); III, butan-1-ol–acetic acid–water (2:1:1, by vol.); IV, phenol–water (lower phase); V, butan-1-ol–water–piperidine (42:8:1, by vol.); VI, butan-1-ol–water–pyridine (6:3:4, by vol.); VII, propan-2-ol–water (3:1, v/v)+5g of trichloroacetic acid+0.3ml of 20% NH_3; VIII, butan-1-ol–acetic acid–water (4:1:5, by vol.); IX, propan-1-ol–water–aq. NH_3 (sp.gr. 0.90) (6:1:3, by vol.); X, 2-methylpropan-2-ol–butan-2-one–water (14:3:3, by vol.); XI, 3-methylbutan-1-ol–water–pyridine (5:4:5, by vol.). Compounds containing free amino groups were detected with ninhydrin reagent (composed of 0.25g of ninhydrin, 10ml of methanol, 47ml of butanol, 3ml of water and 50ml of pyridine) and phosphate-containing compounds by ammonium molybdate reagent (Hanes & Isherwood, 1949).

Neamine (Ford *et al.* 1955), neosamine B (Rinehart, Argoudelis, Goss, Sohler & Schaffner, 1960), neosamine C (Rinehart & Woo, 1961) and deoxy-streptamine (Kuehl, Bishop & Folkers, 1951) were prepared from commercial neomycin sulphate. Their *N*-acetyl derivatives were prepared by a method similar to that described by Roseman & Ludowieg (1954).

RESULTS AND DISCUSSION

Effect of nitrogen sources on the accumulation of phosphorylated compounds. Table 1 shows that accumulation of phosphorylated substances in the broth is dependent on supplied nitrogen source but not on the growth of the organism. Thus the medium containing glycine gave the highest yields, but alanine or asparagine, though giving very good growth, did not produce phosphorylated compounds. High neomycin-yielding nitrogen sources like aspartic acid and sodium nitrate did not give phoshorylated compounds. In the subsequent experiments glycine was included for the production of the phosphorylated compounds.

Stimulation test with phosphorylated compounds in resting cell culture. Table 2 shows the stimulatory

Table 1. *Effect of nitrogen sources on the growth of* S. fradiae *neomycin production and accumulation of phosphorylated compounds in a chemically defined medium*

The medium (200ml/500ml Erlenmeyer flask) contained basal mineral salts and 1.5% of maltose and was incubated on a rotary shaker at 250 rev./min at 28°C. Analysis was made on day 6 of fermentation. The phosphorylated compound was isolated from 20ml of broth.

Nitrogen source	Growth (mg/100ml)	Neomycin (μg/ml)	Phosphorus content of phosphorylated compound (μg)
Glycine	372	64	86.4
DL-Serine	385	62	76.2
L-Glutamic acid	352	72	30.6
L-Histidine	365	25	23.0
DL-Alanine	380	113	0
L-Aspartic acid	264	154	0
L-Asparagine	413	58	0
Sodium nitrate	180	252	0

Table 2. *Effect of phosphorylated compounds on neomycin production by washed mycelium of* S. fradiae *in a chemically defined medium*

The washed mycelium was a 60h-old mycelium suspension (6.1mg dry wt./ml). The medium (10ml/50ml Erlenmeyer flask) contained basal salts (K_2HPO_4, 1.0g; $MgSO_4,7H_2O$, 0.5g; $CaCl_2,2H_2O$, 40mg; $FeSO_4,7H_2O$, 5mg; $ZnSO_4,7H_2O$, 0.5mg; in 1000ml of water, pH 7.5±0.1) and was incubated on a rotary shaker at 250 rev./min at 28°C.

Substrate	Time ...	Neomycin (μg/ml) 0h	24h	48h
None		0.5	42	102
Phosphorylated compound (500μg/ml)		72	300	325
Glucosamine (500μg/ml)		0.5	58	118

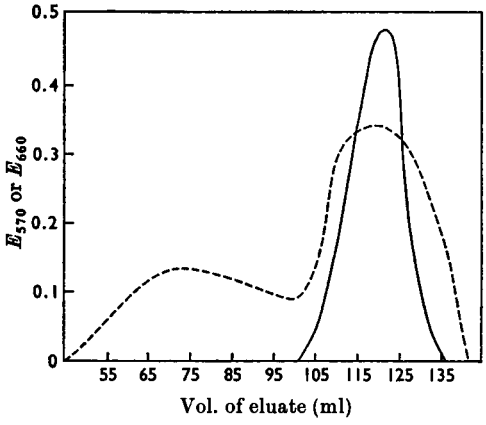

Fig. 1. Isolation of the phosphorylated intermediate by using an Amberlite IRC-50 (NH_4^+ form) column (3.5cm diam.×8cm) with 1M-NH_3 as eluent at a flow rate of 0.2ml/min; 2ml fractions were collected. ----, E_{570} with ninhydrin reagent; ——, E_{660} with ammonium molybdate reagent.

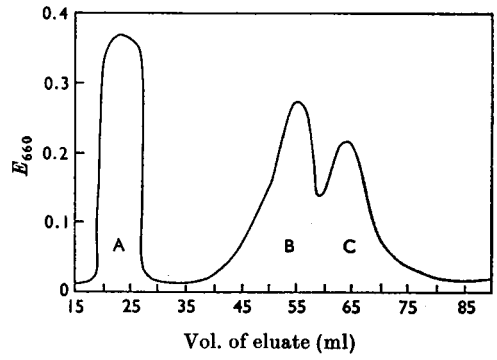

Fig. 2. Fractionation of the phosphorylated intermediate by using an Amberlite IRC-50 (NH_4^+ form) column (1.4cm diam.×65cm) at a flow rate of 0.15ml/min; 1.6ml fractions were collected. ——, E_{660} with ammonium molybdate reagent.

effect of phosphorylated compounds on neomycin production. The compounds have a better precursor effect than glucosamine, which is considered to be an intermediate (Rinehart & Schimbor, 1967) in neomycin biosynthesis. It is noteworthy that *S. fradiae* contains an intracellular repressible alkaline phosphatase, which can hydrolyse both nitrogen–phosphorus and phosphorus–oxygen–phosphorus bonds of pyrophosphoamido-neomycins (M. K. Majumdar & S. K. Majumdar, unpublished work). A study on the alkaline phosphatase activity of cells grown in different media for different periods shows that neomycin formation varies directly with enzyme activity (M. K. Majumdar & S. K. Majumdar, unpublished work). This observation indicates that these phosphorus-containing compounds are intermediates on the main path of neomycin biosynthesis.

Recovery of phosphorylated intermediates from the fermentation broth. On day 6 of fermentation 10 litres of broth were filtered to remove mycelia and then passed through an Amberlite IRC-50 (100–200 mesh; NH_4^+ form) column (3.5cm diam.×8cm) at a flow rate of 700ml/h at 6°C. The column was washed with 300ml of water and eluted with 1M-ammonia. Different eluate fractions were analysed with respect to amino group and organic phosphate by the ninhydrin and ammonium molybdate tests respectively. A typical pattern is shown in Fig. 1. The ninhydrin-positive and ammonium molybdate-positive fractions eluted between 102ml and 136ml were pooled, concentrated under reduced pressure and freeze-dried. This fraction contained neomycins along with phosphorus-containing compounds, and no neomycin was obtained in the fraction after 140ml. About 400mg of this product was dissolved in 3ml of water with a minimum amount of aqueous ammonia and passed at a rate of 0.05ml/min through an Amberlite IRC-50 (200–400 mesh; NH_4^+ form) column (1.4cm diam.× 65.5cm) previously equilibrated with 1500ml of 18mM-ammonia, and the product was eluted with 90mM-ammonia. The effluent was collected in 1.6ml fractions at a flow rate of 0.15ml/min. The results of the ammonium molybdate test of different fractions gave the elution pattern shown in Fig. 2. A relatively sharp peak (fraction A) appearing at 19.2–27.2ml was followed by the two overlapping peaks of fractions B and C. Neomycin, being highly basic, was not eluted from the column by ammonia at the concentration used. To get pure samples, the eluate at 54.4–62.4ml was discarded and the remaining portions of the B and C fractions were further purified by repeating the above chromatographic procedure three times. The freeze-dried fraction A was taken up in water by addition of a minimum amount of aqueous ammonia, and ethanol was then added with shaking to give a very good turbidity. The suspension was kept in a desiccator until a fine white precipitate appeared at the bottom; this was separated off and the supernatant fluid was again treated with fresh ethanol and kept in a desiccator. By repeating the technique, this substance was precipitated completely from the solution. Fractions B and C were concentrated separately and treated with ethanol. On being kept at 4°C overnight, each fraction formed a precipitate and a clear supernatant fluid. The precipitates were centrifuged and washed with ethanol, dried at 4°C over sodium sulphate and evaporated under reduced pressure to give hygroscopic white substances.

General properties. Fractions A, B and C have some common physicochemical properties. The compounds are sparingly soluble in water, soluble in dilute alkali solution but insoluble in all common organic solvents including pyridine. The compounds show no absorption in the u.v. region. They give positive Molisch and carbazole tests for carbohydrate, ammonium molybdate test for organic phosphate (Hanes & Isherwood, 1949), ninhydrin test for amino groups and negative Elson–Morgan test (Levvy & McAllan, 1959). The compounds do not reduce Fehling's or Tollen's reagent. Acid hydrolysates (6M-hydrochloric acid at 100°C for 18h) of the compounds show the absence of amino acids on the paper chromatogram, but give a positive test with the Elson–Morgan reaction. Table 3 shows the difference in properties of the three fractions. The behaviour of fractions A, B and C on chromatography and electrophoresis on paper shows their apparent homogeneity as only one spot was detected for each fraction (Tables 3 and 7). It appears from these studies that these compounds are very closely related and chemically they are phosphorylated amino sugars.

Table 3. *Properties of fractions A, B and C*

The test organism for antibiotic activity was *Bacillus subtilis* (strain B_3).

Fraction	Relative antibiotic activity with respect to neomycin B	$R_{neomycin\ C}$ value in solvent system I on Whatman 3MM paper after 72h run (descending)
A	0.0285	0
B	0.0205	0.095
C	0.0635	0.19

When the compounds were hydrolysed vigorously with 6M-hydrochloric acid they gave furfural, which might arise from degradation of pentose, but mild hydrolysis produced no pentose. Such resistance to acid hydrolysis is characteristic of glycosides of amino sugars (Rinehart, 1964) and is due to electrostatic shielding of the glycosidic bond by ammonium groups in its vicinity. The compounds were *N*-acetylated to neutralize the basic group. *N*-Acetylation of the fractions by the method of Rinehart *et al.* (1960) was unsuccessful, but when the phosphate groups were removed completely by hydrolysis with acid (1M-hydrochloric acid at 100°C for 10min) the compounds could be *N*-acetylated (Roseman & Ludowieg, 1954). The *N*-acetyl derivatives were heated at 95°C for 5h in 3M-hydrochloric acid. After removal of the hydrochloric acid they were chromatographed in solvent system II and spots were developed with ninhydrin and aniline hydrogen phthalate reagent. Hydrolysis of fractions A and B produced three spots whereas that of fraction C gave four spots (Table 4). Different components from fractions A, B and C were separated by chromatography on sheets of Whatman no. 3MM filter paper with the same solvent system. The individual components were cut from the sheet and then eluted with water. Components A_1, B_1 and C_1, with the same R_F value, give colour reactions typical of aldopentose. This was identified as ribose by paper chromatography in solvent systems II, III and IV. Components A_3, B_3, C_3 and C_4 gave a positive reaction with the Elson–Morgan test. All the components were compared by paper chromatography with glucosamine and other amino sugars: neosamine C and neosamine B (positive to ninhydrin and Elson-Morgan tests) and deoxystreptamine (positive to ninhydrin test). Table 5 indicates the identity of

Table 4. *R_F values of the components after acid hydrolysis of fractions A, B and C and their reactions with ninhydrin and aniline hydrogen phthalate reagent*

After dephosphorylation, the fractions were *N*-acetylated and then hydrolysed with 3M-HCl at 95°C for 5h.

Fraction	Components after acid hydrolysis	R_F value in solvent II	Ninhydrin test	Aniline hydrogen phthalate test
A	A_1	0.41	–	+
	A_2	0.041	+	–
	A_3	0.112	+	+
B	B_1	0.41	–	+
	B_2	0.041	+	–
	B_3	0.112	+	+
C	C_1	0.41	–	+
	C_2	0.041	+	–
	C_3	0.112	+	+
	C_4	0.153	+	+

Table 5. *Paper chromatography of some amino sugars and the components of fractions A, B and C and their* N*-acetyl derivatives*

N-Acetylated compounds were detected by the method of Majumdar & Majumdar (1967*a*) and others by the ninhydrin test.

Amino sugars	R_F values of amino sugars		R_F values of *N*-acetyl derivatives		
Solvent system ...	II	III	V	VI	X
Glucosamine	0.198	0.219	0.143	0.371	0.564
Deoxystreptamine	0.041	0.092	0.068	0.218	0.428
Neosamine C	0.114	0.150	0.203	0.425	0.625
Neosamine B	0.154	0.172	0.406	0.515	0.695
Component A_2, B_2 or C_2	0.042	0.094	0.069	0.216	0.426
Component A_3, B_3 or C_3	0.112	0.153	0.201	0.421	0.622
Component C_4	0.153	0.174	0.401	0.517	0.692

components A_3, B_3 and C_3 with neosamine C, component C_4 with neosamine B and components A_2, B_2 and C_2 with deoxystreptamine.

Thus this study indicates the presence of the following moieties in three different fractions: fraction A, ribose, neosamine C and deoxystreptamine; fraction B, ribose, neosamine C and deoxystreptamine; fraction C, ribose, neosamine C, neosamine B and deoxystreptamine.

Acid-stability of phosphate groups of the compounds. Samples of fractions A, B and C (each equivalent to 58 μg of P_i) were dissolved in 2ml of 1M-hydrochloric acid and heated at 100°C for periods up to 60min. The P_i of each hydrolysate was measured (King, 1932), and the hydrolysis rate is expressed as a percentage of the total phosphates released as P_i. As shown in Fig. 3, the P_i concentration increased gradually and reached a final value of 100% in 10min. This acid-lability ruled out the possibility of ester linkage at C-5 of pentose or C-6 of hexose (Roseman & Ludowieg, 1954).

Identification of dephosphorylated fractions. When the fractions were dephosphorylated by heating with acid (1M-hydrochloric acid at 100°C for 10min) for characterization of dephosphorylated fractions, neamine was formed as one of the products of partial hydrolysis. The fractions were dephosphorylated enzymically with calf intestinal alkaline phosphatase in glycine buffer, pH9.0, containing Mg^{2+}, in the presence of toluene for 18h. Dephosphorylated compounds were isolated by using an Amberlite IRC-50 (NH_4^+ form) column (Majumdar & Majumdar, 1967*a*) and chromatographed as free bases and as *N*-acetyl derivatives in different solvent systems (Majumdar & Majumdar, 1967*a*, 1969*b*). Table 6 shows that dephosphorylation of fractions A, B and C yielded neomycin C, neomycin C and neomycin B respectively.

Before chemical analysis, the fractions were dried at room temperature at high vacuum over phosphorus pentoxide. The results of analysis show the strong retention of 3mol prop. of water in both fraction B and fraction C (Found for fraction A: C, 25.7; H, 5.7; N, 10.0; O, 47.5; P, 11.0; $C_{24}H_{66}N_8O_{33}P_4$ requires: C, 25.7; H, 5.9; N, 10.1; O, 47.2; P, 11.1%. Found for fraction B: C, 33.3; H, 6.5; N, 10.1; O, 42.6; P, 7.5; $C_{23}H_{48}N_6O_{19}P_2$, $3H_2O$ requires: C, 33.2; H, 6.5; N, 10.1; O, 42.5; P, 7.5%. Found for fraction C: C, 33.2; H, 6.5; N, 10.1; O, 42.5; P, 7.4; $C_{23}H_{48}N_6O_{19}P_2$,$3H_2O$ requires: C, 33.2; H, 6.5; N, 10.1; O, 42.5; P, 7.5%). Empirical formula for neomycin B and neomycin C: $C_{23}H_{46}N_6O_{13}$.

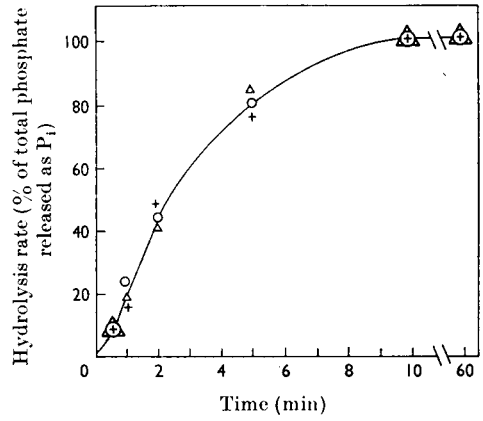

Fig. 3. Hydrolysis rate (expressed as percentage of the total phosphate released as P_i) of fraction A (+), fraction B (○) and fraction C (△) in 1M-HCl at 100°C.

The elemental analysis leaves little doubt about the identity of dephosphorylated products of fractions B and C as neomycins. Moreover, the i.r. spectra of the compounds, shown in Fig. 4, are very similar to those of neomycin B and neomycin C except for the absorption in the regions 1639 and 900–950cm^{-1}, which might be due to bound wate

Table 6. *Relative R_F values of neomycins and dephosphorylated fractions A, B and C*

Compound	$R_{neamine}$ values of free base	$R_{N\text{-acetyl-neamine}}$ values of *N*-acetyl derivatives		
Solvent system	I	V	VI	XI
Neomycin B	0.53	0.70	1.12	1.09
Neomycin C	0.29	0.34	0.74	0.85
Dephosphorylated fraction A	0.30	0.35	0.76	0.85
Dephosphorylated fraction B	0.30	0.35	0.74	0.84
Dephosphorylated fraction C	0.55	0.69	1.1	1.08

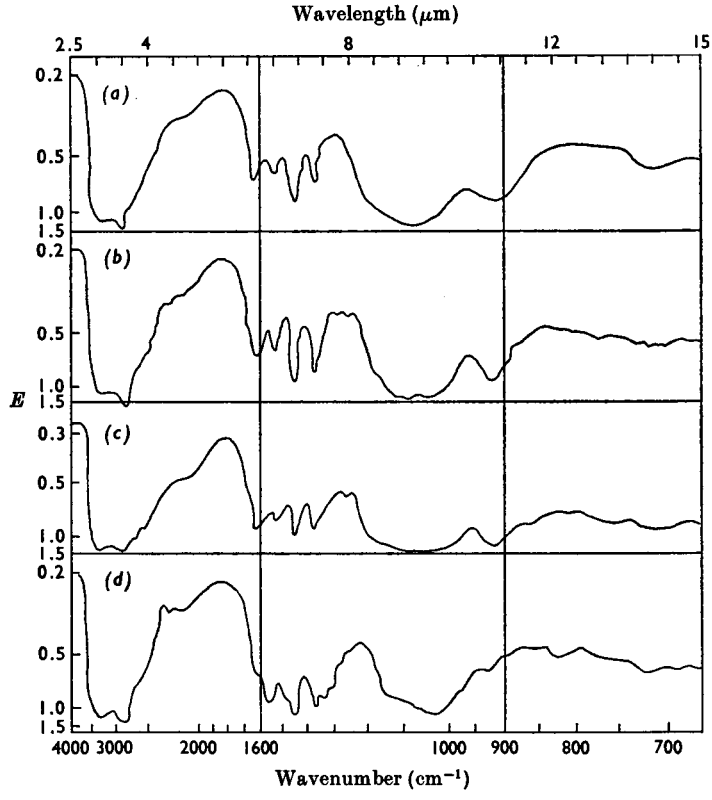

Fig. 4. I.r. spectra of phosphorylated intermediates: [(*a*) fraction A; (*b*) fraction B; (*c*) fraction C] and neomycin B or C (*d*) in Nujol mull.

and phosphate group (Rao, 1963) respectively. However, the absorption of fractions A, B and C at 1520 cm^{-1} compared with the absorption of neomycin at 1570 cm^{-1} is not clear. The results of the elemental analysis of fraction A are discussed below.

Nature of phosphate linkage. As fractions A, B and C are adsorbed by a cation-exchange resin and migrate towards the negative pole during ionophoresis at pH 7 (Table 7), they are basic in nature. This indicates that six positive charges from the six amino groups of neomycin are not neutralized by negative charges from phosphate groups. Table 7 also shows similarity in charges and basicity of fractions B and C, but fraction A is less basic.

Fractions A, B and C are unstable as there is rapid liberation of neomycin in 1 M-hydrochloric acid at room temperature. Fig. 5 shows the rate of neomycin release [measured by a microbiological method, as described by Grove & Randle (1955)] from these compounds at 37°C in 1 M-hydrochloric acid. The liberation of 100% neomycin from each of the compounds in 10 min indicates a similar type linkage. P_i was absent from this hydrolysate, in

Table 7. *Paper electrophoresis of fractions A, B and C and neomycin*

The experiment was carried out on Whatman no. 3MM paper at 140V for 2.5h at 6°C. A solution with ionic strength of 0.2M was used. A negative number signifies migration (cm) towards the negative pole, a positive number migration towards the positive pole.

	Migration (cm)			
pH	Neomycin B or C	Fraction A	Fraction B	Fraction C
2.0 (formic acid)	−14.1	−6.8*	−10.1*	−10.1*
3.5 (formic acid–aq. NH_3)	−9.5	−5.8	−7.6	−7.6
7.0 (formic acid–aq. NH_3)	−5	−1.5	−3.1	−3.1
9.0 (formic acid–aq. NH_3)	−3.3	+0.9	−0.8	−0.8

* Tendency to decompose.

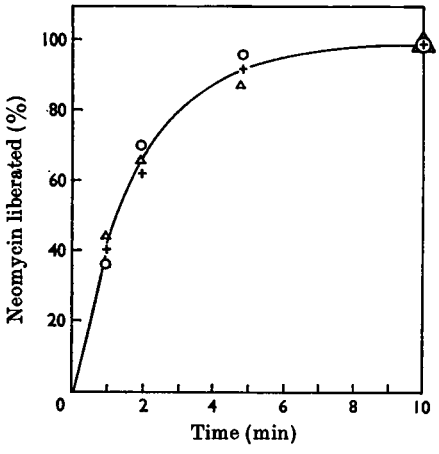

Fig. 5. Neomycin release rate from fraction A (+), fraction B (○) and fraction C (△) in 1M-HCl at 37°C.

contrast with hydrolysis at 100°C (Fig. 3). The compounds were completely dephosphorylated on treatment with 2M-formic acid for 12h and the phosphate component of the hydrolysates was identified as pyrophosphate by paper chromatography (Block, Durrum & Zweig, 1958) in solvent systems VII, VIII and IX.

Fractions A, B and C were *N*-acetylated with 0.035ml of acetic anhydride in the presence of 0.125ml of methanol and 168mg of sodium hydrogen carbonate at 5°C for 12h. Completion of the reaction was tested by lack of a reaction with ninhydrin. The reaction mixture was neutralized with Amberlite IRC-50 (H^+ form) at 4°C. *N*-Acetylated products were dephosphorylated with 2M-formic acid. Mild hydrolysis does not remove *N*-acetyl groups. Table 8 shows the migration of the *N*-acetylated derivatives. *N*-Acetylated fraction C (neomycin B pyrophosphate) after dephosphorylation should show the same migration as *N*-acetylneomycin B if the phosphate group were ester-linked, but migration was found to be slower in the experiment. Similarly, *N*-acetylated fraction B (neomycin C pyrophosphate) after dephosphorylation showed a slower migration than *N*-acetylneomycin C. This slow migration of *N*-acetylated and dephosphorylated products might be due to one or more amino groups becoming free during dephosphorylation. This finding, together with the facts that the compounds are extremely unstable towards acid, but stable in alkali, and that the *N*-acetylated compounds after dephosphorylation are ninhydrin-positive, strongly indicates that these are nitrogen–phosphorus-linked compounds.

Elemental analysis and also simultaneous assay of neomycin C by a microbiological method and phosphate by King's (1932) method of fraction A show that it contains 4g-atoms of phosphorus, i.e. 2mol of pyrophosphate/mol of neomycin C. Table 8 shows that *N*-acetylated fraction A (neomycin C dipyrophosphate) after dephosphorylation migrated even slower than that of the dephosphorylated product of *N*-acetylneomycin C pyrophosphate. This implies that in this compound at least one more position is nitrogen–phosphorus-linked. When two amino groups (minimum possibility) are blocked by two pyrophosphate groups six negative charges from two pyrophosphate groups will not be neutralized by the remaining four positive charges from four amino groups, and the resulting compound will be acidic in nature. However, it has been shown previously that the compound is basic in nature. Elemental analysis shows that this fraction contains in addition to two pyrophosphate groups, two more nitrogen atoms, one carbon atom and more oxygen and hydrogen atoms than neomycin C. These additional atoms somehow form a basic grouping to impart the basicity of the fraction A, which is a dipyrophosphoamido-neomycin C complex (fraction A). The other two fractions are pyrophosphoamido-neomycin C (fraction B) and pyrophosphoamido-neomycin B (fraction C).

Table 8. *Paper chromatography of N-acetyl intermediates after dephosphorylation*

The *N*-acetyl derivatives were dephosphorylated by treatment with 2M-formic acid for 12h at 37°C.

Compound	$R_{\text{neomycin C}}$ values	$R_{\text{N-acetylneomycin C}}$ values
Solvent system	I	V
Neomycin C	1.00*	—
Neomycin B	2.00*	—
N-Acetylneomycin C	—	1.00†
N-Acetylneomycin B	—	2.08†
N-Acetylated fraction A	—	0†
N-Acetylated fraction B	—	0†
N-Acetylated fraction C	—	0†
Dephosphorylated *N*-acetylated fraction A	2.2*	0.086†
Dephosphorylated *N*-acetylated fraction B	6.2*	0.542†
Dephosphorylated *N*-acetylated fraction C	11.5*	1.29†

* Detected with ninhydrin reagent.
† Detected by the methods of Majumdar & Majumdar (1967*a*).

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