

INHIBITION BY NEOMYCIN OF POLYPHOSPHOINOSITIDE TURNOVER IN SUBCELLULAR FRACTIONS OF GUINEA-PIG CEREBRAL CORTEX *IN VITRO*

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Abstract—The addition of 10^{-5} M to 10^{-3} M neomycin to incubations of subcellular fractions of guinea-pig cerebral cortex increased the labelling of phosphatidylinositol phosphate and decreased the labelling of phosphatidylinositol diphosphate by $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The effect was observed in all subcellular fractions tested and depended on the cationic form of the antibiotic. Similar effects on lipid labelling were exerted by related aminoglycosidic antibiotics, by neamine, spermine and poly-L-lysine. Other neomycin fragments, antibiotics, local anesthetics or small polyamines were ineffective. Neomycin also inhibited the enzymatic hydrolysis of ^{32}P -polyphosphoinositides. The addition of the drug to aqueous dispersions of these lipids increased the turbidity and lowered the pH of the suspensions. It is suggested that the effects of neomycin on polyphosphoinositide metabolism result from the formation of an ionic complex between the lipids and the antibiotic.

AMINOGLYCOSIDE antibiotics—neomycin, streptomycin, and related compounds—are potent antibacterial agents, but also exert toxic actions on eukaryotic cells. These actions include block of neuromuscular and ganglionic transmission (PITTINGER & ADAMSON, 1972), nephrotoxicity, and, most prominently, ototoxicity in the cochlea and the vestibular system (HAWKINS, 1970).

We have recently demonstrated (SCHACHT, 1974; ORSULAKOVA *et al.*, 1976) that the incorporation of $^{32}\text{P}_i$ into polyphosphoinositides is altered in inner ear tissues of guinea pigs receiving chronic ototoxic doses of neomycin. In acute experiments with cochlear perfusions we have shown that the change of polyphosphoinositide labelling followed the same dose response as the decrease of cochlear microphonic potential (NUTTALL *et al.*, 1975). Although a physiological role for the polyphosphoinositides (phosphatidylinositol phosphate and phosphatidylinositol diphosphate) has not yet been established, there are strong indications that they are involved in the control of membrane permeability in bioelectric events (MICHELL, 1975). Of all tissues, brain has the highest content of polyphosphoinositides (HAUSER & EICHBURG, 1973) and provides a convenient system to study their metabolism. We therefore investigated the mechanism of neomycin action on the turnover of phosphatidylinositol phosphate and phosphatidylinositol diphosphate in subcellular fractions of guinea-pig cerebral cortex *in vitro*.

Abbreviations used in tables and figures: PhA, phosphatidic acid (1,2-diacyl-sn-glycero-3-phosphate); PhI, PhIP, PhIP₂, phosphatidylinositol, phosphate, diphosphate; Nm, Neomycin B.

MATERIALS AND METHODS

Cerebral cortex from albino guinea-pigs was homogenized in 0.32 M-sucrose in a Teflon-glass homogenizer to yield a 10% homogenate (w/v). This was centrifuged for 10 min at 1000 *g* and washed once. The combined supernatant fractions were centrifuged for 20 min at 13,000 *g* to yield a crude nerve ending-mitochondrial pellet and a supernatant fraction containing cytosol and microsomes. The nerve ending-mitochondrial pellet was suspended in 30 ml of 0.32 M-sucrose, and was layered over three tubes of a discontinuous gradient consisting of 13 ml each of 0.8- and 1.2 M-sucrose, which was centrifuged in an SW 27 rotor for 120 min at 62,000 *g*. Fractions collected at the interfaces were a myelin-rich fraction over 0.8 M-sucrose, a nerve ending-rich fraction over 1.2 M-sucrose and a pellet containing mitochondria. The fractions were pelleted by centrifuging for 30 min at 100,000 *g*. Each pellet was suspended in 0.16 M-sucrose/5 mM-Tris-Cl (pH 7.4) and frozen twice in a dry-ice-acetone bath and thawed.

For lipid labelling, incubations were performed at 37°C in air with shaking. The fractions were incubated for 15 min with 40 mM-Tris-Cl (pH 7.4) and 10 mM-dithioerythritol, in the presence or absence of drugs; all in a volume of 0.25 ml. Then were added (final concentrations) 80 mM-Tris-Cl (pH 7.4), 20 mM-MgCl₂, 0.2 mM-EDTA, and 4 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$; final volume, 0.5 ml. The incubations were terminated by the addition of 1.5 ml of chloroform-methanol (1:2, v/v). After addition of 0.5 ml 2.4 N-HCl and 1.0 ml of chloroform, mixing and centrifugation, the lower phase was collected and the upper phase was reextracted with 1.0 ml of chloroform. Samples of the remaining upper phase were analyzed for $[\text{}^{32}\text{P}]\text{ATP}$ (HUANG, 1970). The combined lower phases were washed with 2.0 ml of methanol-0.5 N-HCl (2:3, v/v) and the upper layer was discarded. Maintenance of high acidity in the upper phase during extraction and washing was necessary for complete recovery of polyphosphoinositides. Portions of the lower phase were removed for the determination of total

lipid- ^{32}P , the rest was dried under N_2 . For TLC the lipids were taken up in a small volume of chloroform-methanol-conc. HCl (6:3:0.1, by vol) and applied to TLC plates 'Silicagel 60' (E. Merck). Routinely, plates were developed in chloroform-methanol-aqueous conc. $\text{NH}_3\text{-H}_2\text{O}$ (45:45:3.5:11, by vol) which resulted in good separation of phosphatidylinositol phosphate, phosphatidylinositol diphosphate and phosphatidic acid. ^{32}P -lipids were located by autoradiography, scraped and counted by liquid scintillation. Lipid standards for identification were commercial (AVANTI, Birmingham, AL) or, in the case of polyphosphoinositides, were prepared according to HENDRICKSON & BALLOU (1964).

Hydrolysis of phospholipids was studied with previously extracted ^{32}P -labelled lipids. The lipid extract was dried under N_2 and suspended in 40 mM-Tris-Cl (pH 7.4) with a Polytron PT 10-35 (Brinkman Instr.). Portions of this suspension were incubated at 37°C with the nerve ending fraction in (final concentrations) 80 mM-Tris-Cl pH 7.4, 1 mM-NaF, and 1 mM-MgCl $_2$, in a total vol of 0.5 ml. Incubations were terminated and lipids extracted as above.

For studies of physicochemical interactions between neomycin and the polyphosphoinositides, the lipids were prepared by chromatography on DEAE-cellulose (HENDRICKSON & BALLOU, 1964) from bovine brain obtained from a local slaughterhouse. The composition of the lipid fraction used in these experiments was estimated after TLC to be 80% phosphatidylinositol diphosphate and 20% phosphatidylinositol phosphate with only traces of other phospholipids. Dispersions of this lipid fraction in 20 mM-Tris-Cl (pH 7.4) were mixed with increasing concentrations of neomycin and turbidity was determined in a spectrophotometer at 520 nm (FEINSTEIN, 1964) after a stable absorbance was reached. Dilutions of the lipid suspension with water served as blanks. Neomycin does not absorb at 520 nm. For pH titrations with neomycin, the lipids were suspended in water and the suspension as well as the drug were adjusted with HCl or NaOH to pH 6.10 \pm 0.03. Changes of pH of the lipid dispersion upon addition of neomycin were monitored with a pH meter.

[γ - ^{32}P]ATP was prepared enzymatically (SCHENDEL & WELLS, 1973). Lipid phosphorus (AMES & DUBIN, 1960) and protein (LOWRY *et al.*, 1951) were determined spectrophotometrically. Neomycin B sulfate and fragments were a gift from The Upjohn Co. (Kalamazoo, MI). Radioactive materials were purchased from New England Nuclear (Boston, MA).

Data presented are generally averages of at least duplicate determinations with variability less than 10%.

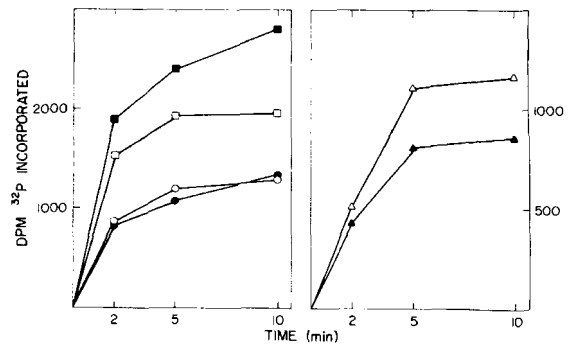


FIG. 1. Time course of ^{32}P -incorporation into phospholipids. A nerve ending fraction (0.8 mg protein) was incubated as described in 'Methods' with 10 μCi ^{32}P -ATP. \circ — \circ phosphatidic acid; \square — \square phosphatidylinositol phosphate; \triangle — \triangle phosphatidylinositol diphosphate. Open symbols: controls; closed symbols: 10^{-4} M-neomycin.

RESULTS

Labelling of subcellular fractions

Fractions obtained by differential and density gradient centrifugation were incubated with [γ - ^{32}P]ATP in the absence or presence of 10^{-4} M-neomycin (Table 1). Phosphatidic acid, phosphatidylinositol phosphate and phosphatidylinositol diphosphate were the only significantly labelled lipids, as confirmed by two dimensional TLC (EICHBERG *et al.*, 1973). The nerve ending fraction showed the highest activity of incorporation per mg protein. The addition of neomycin increased phosphatidylinositol phosphate labelling in all fractions 1.5- to 2-fold, whereas phosphatidylinositol diphosphate labelling was generally decreased. Radioactivity in phosphatidic acid did not seem to be affected with the possible exception of the myelin-rich fraction.

The time course of labelling (Fig. 1) showed linearity of ^{32}P -incorporation for somewhat less than 5 min. The effect of 10^{-4} M-neomycin on polyphosphoinositide labelling was evident at all times, while no influence was observed on phosphatidic acid labelling.

Effect of neomycin

The changes of ^{32}P -incorporation into the polyphosphoinositides depend on the concentration of

TABLE 1. LABELLING OF PHOSPHOLIPIDS IN SUBCELLULAR FRACTIONS

Fraction	mg protein per incubation	PhIP		PhIP $_2$		PhA	
		Control	+Nm	Control	+Nm	Control	+Nm
d.p.m. incorporated							
Mitochondria	0.7	782	1364	186	247	322	385
Nerve endings	0.4	2900	4677	1160	596	695	588
Myelin	0.2	811	1210	322	233	352	192
13,000 g supernatant	1.0	2490	3740	1530	1375	3178	3175

The fractions were incubated with 10 μCi ^{32}P -ATP for 5 min as described in 'Methods'. Neomycin was 10^{-4} M.

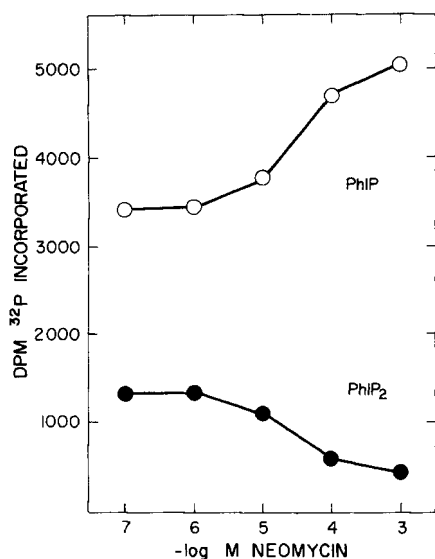


FIG. 2. Effect of neomycin concentration on labelling of polyphosphoinositides. A nerve ending fraction (0.6 mg protein) was incubated as described in 'Methods' with $12 \mu\text{Ci } ^{32}\text{P-ATP}$ for 5 min.

neomycin (Fig. 2). Both the decrease in ^{32}P -phosphatidylinositol diphosphate and the increase in ^{32}P -phosphatidylinositol phosphate are clearly evident at 10^{-4} M-neomycin and in some cases—as in the experiment presented—small effects could be observed at 10^{-5} M drug.

The incubation medium was similar to that of Kai *et al.* (1968) and had been modified to promote the highest rate of ^{32}P -incorporation into the polyphosphoinositides. Variation of its composition did not significantly influence the effect of neomycin except when changes in the magnesium concentration (Table 2) or in pH (Fig. 3) were made. With increasing Mg^{2+} , the basal rate of labelling of phosphatidylinositol phosphate was enhanced about six-fold while the relative effect of neomycin (percentage stimulation) was decreased. Phosphatidylinositol diphosphate also responded to Mg^{2+} , and neomycin which inhibited its labelling at higher Mg^{2+} slightly stimulated it at 1 mM- MgCl_2 .

A rather broad pH optimum is observed for the ^{32}P -incorporation into phosphatidylinositol phos-

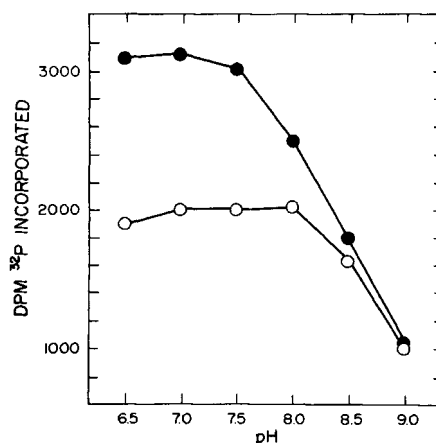


FIG. 3. Effect of pH on neomycin stimulation of phosphatidylinositol phosphate labelling. A nerve ending fraction (2 mg protein) was incubated with $7 \mu\text{Ci } ^{32}\text{P-ATP}$ for 5 min as described in 'Methods' except that MgCl_2 was 5 mM and Tris maleate was substituted for Tris-Cl. Open symbols: controls; closed symbols: 10^{-4} M-neomycin.

phate (and for diphosphate, not shown). At pH 8.5 labelling proceeds at about 80% and at pH 9 at 50% of the maximal rate. In contrast, stimulation by neomycin is essentially abolished at pH 8.5 and 9.0.

Comparison with other drugs

The action of neomycin was compared with that of a number of other drugs (Table 3).

Tobramycin, gentamicin and kanamycin, which are desoxystreptamine antibiotics, as is neomycin, stimulated labelling of phosphatidylinositol phosphate. Streptomycin an aminoglycoside of the streptidine group decreased ^{32}P -phosphatidylinositol diphosphate (Table 3, Exp. A). Of the neomycin fragments (Exp. B) only neamine was found to increase phosphatidylinositol labelling. The magnitude was less than with the intact antibiotic, but a stimulation was consistently observed in a number of experiments. Other inhibitors of bacterial protein synthesis, such as puromycin and clindamycin or local anaesthetics (Exp. C) were ineffective. The polyamine spermine, however, and the polyamino acid poly-L-lysine (Exp. D) altered polyphosphoinositide labelling similarly to neomycin.

In most of these experiments, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was measured at the end of the incubation time. About 30% of the initially added ATP was usually recovered, and 10^{-4} M-neomycin decreased this amount by about 10% (i.e. 25–27% recovered). The increased hydrolysis of ATP in the presence of neomycin may account for some of the drug effect on phosphatidic acid labelling. A consistent decrease of $[\text{}^{32}\text{P}]\text{phosphatidate}$ could only be observed at higher drug concentrations. In contrast to neomycin, poly-L-lysine inhibited the hydrolysis of ATP slightly (about 15%). Other drugs did not show effects that exceeded 10%.

TABLE 2. EFFECT OF MgCl_2 ON NEOMYCIN STIMULATED POLYPHOSPHOINOSITIDE LABELLING

MgCl_2	PhIP		PhIP ₂	
	Control	+Nm	Control	+Nm
	d.p.m. incorporated			
1 mM	736	2250	745	930
5 mM	1787	3895	1932	1655
20 mM	4470	6520	2180	1560

A nerve ending fraction (2 mg protein) was incubated as described in 'Methods' with MgCl_2 as indicated and $18 \mu\text{Ci } ^{32}\text{P-ATP}$ for 5 min. Neomycin was 2×10^{-4} M.

TABLE 3. EFFECT OF VARIOUS DRUGS ON THE LABELLING OF PHOSPHOLIPIDS

Drug (10^{-4} M)		PhIP	PhIP ₂	PhA
		d.p.m. incorporated		
Exp. A	None	5963	1689	3068
	Neomycin	8899	1196	2992
	Tobramycin	8327	1540	3092
	Gentamicin	8098	1670	2911
	Kanamycin	7913	1763	3047
Exp. B	Streptomycin	5683	1374	2932
	None	4407	2433	2346
	Neomycin	6346	1122	1835
	Neamine	5087	2362	2186
Exp. C	Methylneo-biosaminid	4197	2368	2218
	2-Desoxy-streptamine	4144	2341	2307
	None	3534	1896	2077
	Neomycin	6252	1155	1757
Exp. D	Puromycin	3519	1720	2088
	Clindamycin	3573	1714	2262
	Cocaine	3486	1703	1937
	Lidocaine	3668	1993	2127
	Putrescine	3953	1979	2178
	Spermidine	4270	1866	2132
	Spermine	5170	1389	1966
	None	6490	2473	3268
Neomycin	10,290	1068	2368	
Exp. D	L-Lysine	6853	2537	3570
	L-Lysine (1 mM)	6435	2397	3620
	Poly-L-lysine	9644	2217	3548
	Poly-L-lysine (1 mM)	12,416	623	1711

Incubations were performed as described in 'Methods' with 5 mM-MgCl₂.

Exp. A: 1.1 mg protein of nerve ending fraction. 14 μ Ci ³²P-ATP.

Exp. B and C: 1.3 mg protein of nerve ending fraction. 8 μ Ci ³²P-ATP.

Exp. D: 1.3 mg protein of nerve ending fraction. 12 μ Ci ³²P-ATP.

Poly-L-lysine, average MW 3400. Molarity in table is given as molarity with respect to lysine residues for comparison with the monomers. '1 mM' corresponds to 0.04 mM-polymer.

Hydrolysis of polyphosphoinositides

For the study of the enzymatic hydrolysis of polyphosphoinositides, lipids were labelled by [γ -³²P]ATP as described above, extracted and added to assays with the nerve ending fraction. Only little hydrolysis was observed when the conditions of the labelling studies (ATP omitted) were employed. In a medium more suitable for the assay of polyphosphoinositide phosphomonoesterases and phosphodiesterases (SHELTAWY *et al.*, 1972; KEOUGH & THOMPSON, 1972), the rate of hydrolysis was almost linear for 20 min during which about 30% of both lipids were hydrolysed (Fig. 4). There was no non-enzymatic breakdown under these conditions. Neomycin was inhibitory at concentrations of 10^{-4} M or higher (Fig. 5).

In these experiments where a known amount of ³²P-polyphosphoinositides was added to the incubation medium, no influence of neomycin on the recovery could be detected (Fig. 5).

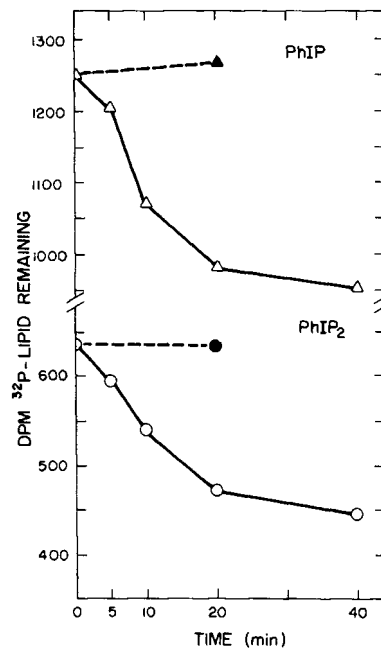


FIG. 4. Time course of hydrolysis of polyphosphoinositides. A nerve ending fraction (1.1 mg protein) was incubated with previously labelled lipids as described in 'Methods'. Closed symbol: incubation with bovine serum albumin substituting for nerve ending fraction.

Drug/lipid interactions

Low concentrations of neomycin increased the turbidity of buffered aqueous dispersions of polyphosphoinositides (Fig. 6). At a lipid concentration of

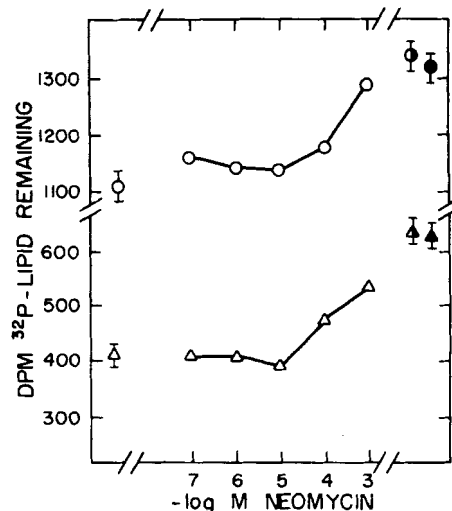
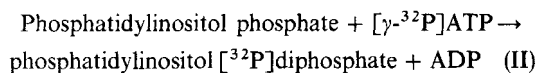
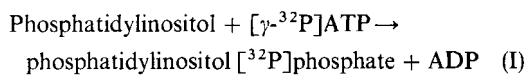


FIG. 5. Effect of neomycin concentration on hydrolysis of polyphosphoinositides. A nerve ending fraction (1.1 mg protein) was incubated with previously labelled lipids for 20 min as described in 'Methods'. \circ — \circ PhIP, \triangle — \triangle PhIP₂; Symbols at the left ordinate: control incubation without drug. Half closed symbols at the right ordinate: ³²P-lipid present at the start of the incubation; fully closed symbols: same, but extraction in the presence of 10^{-3} M-neomycin.

0.4 mM-lipid-phosphate, 40 μ M-neomycin produced maximal turbidity. The same concentration of the drug decreased the pH of an unbuffered aqueous polyphosphoinositide suspension (0.2 mM-lipid-phosphate) from 6.1 to 5.6 indicating an ionic exchange between the hydrogen of the lipid phosphate and neomycin.

DISCUSSION

The rapid labelling of phosphatidylinositol phosphate and phosphatidylinositol diphosphate in brain subcellular fractions *in vitro* by the reactions (COLODZIN & KENNEDY, 1965; KAI *et al.*, 1968):



makes this an easily accessible system for the study of polyphosphoinositide metabolism. In this study we see a pronounced stimulation of ^{32}P -incorporation into phosphatidylinositol phosphate and a decrease of incorporation into phosphatidylinositol diphosphate in the presence of neomycin. Labelling of phosphatidate, the only other highly labelled lipid, remained largely unaffected at low drug concentrations.

The susceptibility to neomycin did not seem to be associated with a particular subcellular fraction. The nerve ending fraction was selected for further studies on the basis of its efficiency of ^{32}P -incorporation into

the lipids. The requirement for high magnesium concentrations for phosphatidylinositol and phosphatidylinositol phosphate kinase reactions is known (KAI *et al.*, 1966, 1968), and was confirmed in our experiments. Low concentrations of Mg^{2+} appeared to favor a higher (percentage) stimulation of phosphatidylinositol phosphate labelling by neomycin, but the stimulatory effect on the diphosphate was inconsistent in a number of experiments. Five mM- Mg^{2+} promoted almost maximal labelling of phosphatidylinositol diphosphate and a pronounced and reproducible effect of the drug on both polyphosphoinositides. Variation of the pH of the incubation medium significantly changed the antibiotic action. With increasing pH, basal labelling of phosphatidylinositol phosphate is only slightly affected while the stimulation by neomycin is reduced. At pH 9, no drug effect is evident. Since the apparent pK of neomycin is approx. 8.2 (KOEPEL & FORD, 1958) this indicates that the cationic form of the antibiotic is the active species in changing the lipid labelling.

It appears unusual that labelling of phosphatidylinositol phosphate and diphosphate are affected in opposite ways. A number of previous studies have indicated that both lipids show a similar response to drugs or experimental conditions that change their turnover (WHITE *et al.*, 1974; SCHACHT & AGRANOFF, 1972a, b; LLOYD *et al.*, 1972). Since phosphatidylinositol kinase and phosphatidylinositol phosphate kinase are distinct enzymes, a differential action by neomycin is not surprising. Inhibition of the latter enzyme might explain all of the observed effects on ^{32}P -incorporation. A stimulation of phosphatidylinositol diphosphate phosphomonoesterase might also be considered to explain the incorporation studies, but this mechanism is contradicted by the inhibition of polyphosphoinositide breakdown evident in experiments in which neomycin was added to previously labelled lipids. Examination of the action of a number of other drugs suggests, however, a third mechanism. The neomycin effect is class-specific. It is shared by structurally-related antibiotics of the aminoglycoside group, but not by other antibacterial agents or local anesthetics. The latter are also known to interact with phospholipids. EICHBERG & HAUSER (1974) reported a stimulation by cocaine and lidocaine of $^{32}\text{P}_i$ incorporation into acidic phospholipids in the intact pineal gland. FEINSTEIN (1964) described the formation of ionic complexes between local anaesthetics and phospholipids. In this study, only polyamines produce changes in lipid labelling like those produced by neomycin. Poly-L-lysine has been reported to form insoluble mixed complexes with $\text{Me}(\text{II})$ ions and phosphatidylinositol diphosphate (FULLINGTON & HENDRICKSON, 1966) and we suggest a similar action for neomycin. The turbidity experiment confirms the formation of a complex between polyphosphoinositides and the drug. The displacement of H^+ from the polyphosphoinositides indicates that the complex is based on ionic interactions, which is supported by the fact

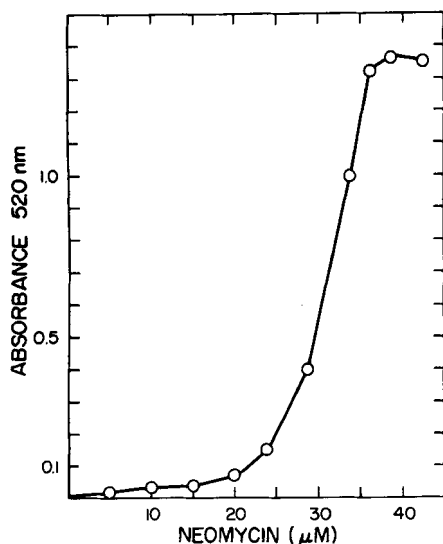


FIG. 6. Effect of neomycin on the turbidity of an aqueous dispersion of polyphosphoinositides. Neomycin (aliquots of a 0.5 mM solution) was added to a dispersion of polyphosphoinositides in 20 mM-Tris Cl pH 7.4, (0.4 mM lipid phosphate) as described in 'Methods'. Turbidity was measured at 520 nm, volume 1 ml.

that the neomycin cation is required for the effect on lipid labelling.

Our study then suggests that the binding of neomycin to the polyphosphoinositides renders these lipids unavailable as substrates for the phosphoesterases and for phosphatidylinositol kinase. This assumption of a complex formation between neomycin and the polyphosphoinositides is sufficient to explain the observed *in vitro* effects as well as the *in vivo* neomycin action on inner ear tissues as we have previously speculated (ORSULAKOVA *et al.*, 1976). Cellular toxicity of polycations including poly-L-lysine is documented for the rat kidney (SEILER, *et al.*, 1975). Ototoxic effects of polycations have, to our knowledge, not been reported. Preliminary studies (Nuttall & Schacht, unpublished) indicate that spermine as well as poly-L-lysine decrease the cochlear microphonic potential when administered by cochlear perfusion. Thus, the ototoxicity of neomycin may well be due to the polyamine character of the drug.

It should be expected that neomycin will also bind to other acidic phospholipids. This question still has to be examined, but this study indicates some preferential action on polyphosphoinositides at low drug concentrations. Moreover, our *in vivo* studies of neomycin toxicity in the inner ear (ORSULAKOVA *et al.*, 1976) and recently in the kidney (SCHIBECI & SCHACHT, 1976) demonstrated a neomycin effect on the polyphosphoinositides but not on other lipids. Further investigations of drug/lipid interactions in artificial membranes (LODHI *et al.*, 1976) should help to elucidate this point. Knowledge of the specificity of the neomycin action and of its physiological effects *in vivo* may contribute to our understanding of the functional significance of the polyphosphoinositides.

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