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EFFECT OF NEOMYCIN ON PHOSPHOINOSITIDE LABELLING AND CALCIUM BINDING IN GUINEA-PIG INNER EAR TISSUES *IN VIVO* AND *IN VITRO*[1]

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Abstract—Phospholipids of guinea-pig inner ear tissues were labelled *in vivo* by perilymphatic perfusion of the cochlea with [^{32}P]orthophosphate or myo-[^{3}H]inositol. After 20–40 min the most highly labelled ^{32}P-lipids were phosphatidylinositol phosphate and diphosphate. Incorporation of [^{3}H]inositol proceeded in the order phosphatidylinositol > phosphatidylinositol phosphate > phosphatidylinositol diphosphate.

After treatment of animals with neomycin for 3 weeks, ^{32}P-incorporation into phosphatidylinositol diphosphate, but not into other lipids, was significantly decreased in the preparations of the organ of Corti and stria vascularis. In homogenates of inner ear tissues, the labelling of the polyphosphoinositides by [γ-^{32}P]ATP was increased and the hydrolysis of these lipids was blocked in the presence of 10^{-4} M-neomycin. Neomycin also competitively inhibited the binding of $^{45}Ca^{2+}$ to homogenates of these tissues.

THEIR potent antibacterial action has made streptomycin, neomycin, and related aminoglycosidic antibiotics indispensable therapeutic agents. The bactericidal mechanism has been linked to the ability of these drugs to inhibit protein synthesis by binding to the 30 S ribosomal subunit of prokaryotic cells (PESTKA, 1971). These aminoglycosides can also exert a number of toxic effects on mammalian tissues. These effects include convulsions after intracisternal injection (MOLITOR & KUNA. 1947), blocking of neuromuscular and ganglionic transmission (PITTINGER & ADAMSON, 1972), nephrotoxicity, and ototoxicity in the cochlea and the vestibular system (HAWKINS, 1970). The biochemical mechanism of these actions on eukaryotic cells remains unclear.

The ototoxicity of the aminoglycosidic antibiotics is the single most important factor that restricts their clinical use. It becomes evident only after chronic administration of the antibiotics, which may be due to the slow accumulation of the drug in the inner ear (STUPP, 1970). That the underlying mechanism may be related to that of the acute actions can be inferred from studies on the lateral line organ. Here, streptomycin rapidly but reversibly suppresses the microphonic output (WERSÄLL & FLOCK, 1964). This and the various neurotoxic effects seem to link the action of the drugs to bioelectrical events at the membrane. The stimulation or blocking of such membrane phenomena by neurotransmitters (HOKIN, 1969), convulsants (SCHACHT & AGRANOFF, 1972), or depressants (MARGOLIS & HELLER, 1966), has been associated with phosphoinositide metabolism. Phosphoinositide metabolism may also be involved in the binding and release of calcium at the membrane (KAI & HAWTHORNE, 1969; BUCKLEY & HAWTHORNE, 1972; REDMAN, 1972). An interference by the aminoglycosides with the calcium equilibrium at the membrane has been proposed for the neuromuscular junction (CORRADO, 1963; VITAL BRAZIL & PRADO-FRANCESCHI, 1969; GOODMAN *et al.*, 1974). We, therefore, investigated the influence of neomycin on the metabolism of phosphoinositides in the inner ear.

METHODS

Albino guinea-pigs (300–500 g) were used in these experiments. The animals were anaesthetized with Nembutal (40–50 mg/kg body wt) and maintained under artificial respiration. The otic capsule was surgically exposed and both the round and oval windows were carefully opened. "Artificial perilymph" (RAUCH & KÖSTLIN, 1964) containing 1 mM P_i and 0·2–0·5 mCi $^{32}P_i$/ml or 1 mCi [^{3}H]myo-inositol/ml was perfused through the perilymphatic spaces once at the start of the experiment and then remained in the cochlea. At the end of the incubation time the cochlea was perfused with 10% neutral formaldehyde (see Results) and the tissues were dissected.

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Abbreviations used: PhA, phosphatidic acid (1,2-diacyl-sn-glycero-3-phosphate); PhC, PhE, PhS, phosphatidyl choline, ethanolamine, serine; PhI, PhIP, $PhIP_2$, phosphatidylinositol, phosphate, diphosphate.

Microdissection of inner ear tissues was performed in 10% neutral formaldehyde under the light microscope. The cochlear bone was cut longitudinally and was removed with spiral ligament and stria vascularis attached. Stria vascularis was lifted from the spiral ligament by pushing a glass rod between the two tissues. This glass rod was prepared by fusing the tip of a microcapillary over a flame and abrading the tip with sandpaper. Spiral ligament was then removed from the bone. The entire organ of Corti including the supporting cells and the osseous spiral lamina was mounted on a glass slide in 12% gelatine. Under high magnification the osseous spiral lamina was dissected with a sharpened piece of a razor blade.

Tissues were collected in chloroform–methanol (1:2, v/v) and a guinea-pig brain homogenate (1 mg protein) was added as a source of carrier lipids. Lipids were extracted with 2·5 ml chloroform–methanol–concentrated HCl (3:2:0·05, by vol)/1·0 ml 1 M-KCl (HAJRA *et al.*, 1968). Aliquots of the resulting upper phase were analyzed for 'HCl soluble ^{32}P'. The lower layer was washed with 2 ml chloroform–methanol–0·04 N-HCl (3:48:47, by vol). Lipids were separated on silica gel plates (Brinkman Silplate 22) in chloroform–methanol–conc aqueous NH_3–H_2O (45:45:3·5:11, by vol). This system gave good and reproducible separations of phosphatidylinositol phosphate and diphosphate from the other lipids but separation of phosphatidylinositol from phosphatidylserine was poor. Lipids were identified by co-chromatography with known standards (Supelco, Bellefonte, PA) in ammoniacal and acetic (SKIPSKI *et al.*, 1964) solvents, or by high voltage electrophoresis of the water soluble products after alkaline methanolysis (HAJRA *et al.*, 1968). Phosphatidylinositol phosphate and diphosphate were a gift of Dr. B. W. Agranoff, University of Michigan. ^{32}P-lipids on TLC plates were located by radioautography, scraped, and counted by liquid scintillation. ^{3}H-lipids were located from the radioautographically established R_f values of ^{32}P-lipids chromatographed on the same plates.

Tissues from more than 50 cochleae were pooled and homogenized in 0·32 M-sucrose for *in vitro* experiments. The homogenates were centrifuged for 10 min at 1000 *g* to remove cellular debris and nuclei. Aliquots of the supernatant fraction were employed in the study of lipid labelling by [γ-^{32}P]ATP, hydrolysis of ^{32}P-lipids, and binding of $^{45}CaCl_2$.

For lipid labelling, incubations were performed at 37°C in air with shaking under the following conditions: a brain lipid extract ("Peak E" of a DEAE cellulose fractionation, HENDRICKSON & BALLOU, 1964) as a source of substrate for polyphosphoinositide labelling was suspended in 0·2 M-Tris–HCl pH 7·4 with a Polytron PT 10–35 (Brinkman Instr.). An aliquot of this suspension (1 μmol lipid-P per assay tube) was incubated with the tissue homogenates for 15 min in the presence or absence of neomycin in 40 mM-Tris–HCl pH 7·4, 10 mM-dithioerythritol; vol 0·25 ml. Then were added (final concn) 80 mM-Tris–HCl pH 7·4, 20 mM-$MgCl_2$, 0·2 mM-EDTA, and 4 mM-[γ-^{32}P]ATP; final vol, 0·5 ml. Thirty min thereafter the incubations were terminated by the addition of 2 ml chloroform–methanol–HCl (1:2:0·03; by vol) and lipids were processed as above.

Hydrolysis of phospholipids was studied with ^{32}P-labelled lipids extracted from guinea-pig kidney after intraperitoneal injection of $^{32}P_i$ or from *in vitro* incubations of guinea-pig cerebral cortex synaptosomes (SCHACHT & AGRANOFF, 1974*b*). The lipid extract was taken to dryness under N_2 and suspended in Tris–HCl pH 7·4 with a Polytron PT 10–35 (Brinkman Instr.). Aliquots of this suspension were incubated at 37°C with the fractions in (final concn) 100 mM-Tris-HCl pH 7·4, 1 mM-$MgCl_2$ and 1mM NaF for 20 min; vol. 0·3 ml. The incubations were terminated and lipids extracted as above.

Calcium binding was measured by incubating the tissue fractions for 10 min in 80 mM-sodium-HEPES buffer pH 7·4 with 1 μCi $^{45}CaCl_2$; vol, 0·25 ml. Bound calcium was assayed by centrifugal sedimentation. The pellet (14,000 *g* for 20 min) was dissolved in 0·5 ml 1 N-NaOH/1 mM-EDTA, an aliquot was neutralized with acetic acid and counted by liquid scintillation spectrometry. [γ-^{32}P]ATP was prepared enzymatically (GLYNN & CHAPPEL, 1964) and protein determined spectrophotometrically (LOWRY *et al.*, 1951). Neomycin B was a gift from The Upjohn Company (Kalamazoo, Mich.). Radioactive materials were purchased from New England Nuclear (Boston, Mass.).

RESULTS

Labelling of inner ear lipids

Of three possible routes of isotope application to the inner ear—intraperitoneal or intracisternal injection, or perilymphatic perfusion—only the last yielded lipids labelled enough to permit analysis of tissues from a single cochlea (SCHACHT, 1974*b*). In view of the high rate of post-mortem hydrolysis of the polyphosphoinositides (HAUSER *et al.*, 1971) various methods were investigated for the quick fixation of inner ear tissues taking the ratio of $PhIP_2$:PhI as a sensitive measure of $PhIP_2$ breakdown (Table 1).

Freezing the cochlea *in situ* with Freon, followed by immersion of the dissected temporal bone into liquid nitrogen and by freeze drying, is an established way to minimize post-mortem events in the inner ear (THALMANN *et al.*, 1972). Fixation with formaldehyde or glutaraldehyde by cochlear perfusion appears equally effective for the preservation of labelled cochlear lipids. Since dissection of tissues is best accomplished after formaldehyde fixation this was the method of choice. Microwave irradiation (1200 W, 30 s) proved unsuitable, longer exposure led to tissue coagulation. It may seem surprising that microwave irradiation which is a rapid way of tissue fixation to prevent post-mortem breakdown of substrates (STAVINOHA *et al.*, 1970), is not effective in the case of the

TABLE 1. COMPARISON OF FIXATION METHODS

Fixation	Dissection	$\frac{\text{d.p.m. } PhIP_2}{\text{d.p.m. PhI + PhS}}$
Freon *in situ* + freeze drying	room temperature	1·6
10% Formaldehyde	cold formaldehyde	1·6
10% Glutaraldehyde	cold glutaraldehyde	1·6
Microwave irradiation	0–4°C	0·4
Microwave irradiation	room temperature	0·1

Guinea-pigs received a perilymphatic perfusion as described in Methods. Incubation time 60 min with 250 μCi $^{32}P_i$/ml. A preparation of spiral ligament and stria vascularis was analyzed. Numbers are average values obtained from 2 animals.

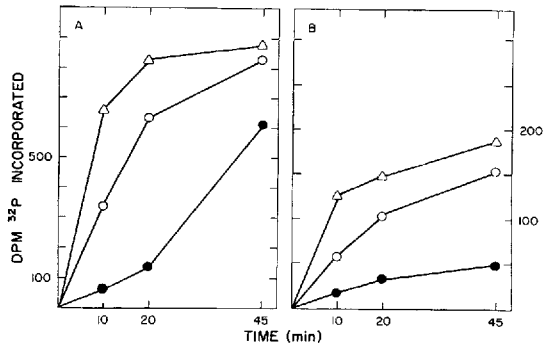

FIG. 1. Time-course of labelling of cochlear phosphoinositides by $^{32}P_i$. Guinea-pigs received a perilymphatic perfusion as described in Methods with 250 μCi $^{32}P_i$/ml. Numbers are mean values from 3 animals. A: Stria vascularis + spiral ligament; B: organ of Corti + osseous spiral lamina. △——△ $PhIP_2$; ○——○ PhIP; ●——● PhI.

polyphosphoinositides. However, $PhIP_2$ phosphodiesterase from ox brain has been reported to be relatively heat stable (KEOUGH & THOMPSON, 1972), and the loss of $PhIP_2$ may be due to enzymatic activity rather than due to secondary effects of irradiation. This is supported by the fact that ^{32}P-$PhIP_2$ is lower when the tissue is handled at room temperature rather than at 0°C.

With these methods, rapid labelling of phosphoinositides in the inner ear can be demonstrated (Fig. 1). $PhIP_2$ and PhIP show the highest initial rate of ^{32}P-incorporation, followed by PhI. Labelling of other lipids proceeds more slowly (Table 2). The one-dimensional TLC routinely used in these experiments does not separate PhI and PhS. However, additional analyses by two-dimensional TLC (PUMPHREY, 1969) showed that there is only minimal ^{32}P-incorporation into PhS at short times. The labelling pattern of the phosphoinositides is reversed with [^{3}H]inositol as the precursor (Table 3). Radioactivity in PhI exceeds that in PhIP. Incorporation into $PhIP_2$ could only be demonstrated in spiral ligament.

Effect of neomycin treatment

Guinea-pigs received subcutaneous injections of neomycin (100 mg/kg body wt) daily for 2 or 3 weeks,

TABLE 2. LABELLING OF COCHLEAR LIPIDS AFTER 180 MIN

Lipid	Stria vascularis + spiral ligament	Organ of corti + osseous spiral lamina
	d.p.m. ^{32}P incorporated	
Phosphatidylinositol diphosphate	6660	2890
Phosphatidylinositol phosphate	4440	2710
Phosphatidylinositol + -serine	3450	2640
Phosphatidyl choline	573	220
Phosphatidyl ethanolamine	282	172
(Lyso-phosphatidyl choline)*	742	220
Unknown	254	129

Guinea-pigs received a perilymphatic perfusion as described in Methods. Perfusion medium (250 μCi $^{32}P_i$/ml) was exchanged every 45 min; total incubation time, 180 min. Numbers are averages from 3 animals.

* Identified by co-chromatography with known standard in one solvent only (see Methods).

TABLE 3. LABELLING OF COCHLEAR PHOSPHOINOSITIDES BY ^{3}H-INOSITOL

Tissue	PhI	PhIP	$PhIP_2$
	d.p.m. ^{3}H incorporated		
Spiral ligament	9235	330	109
Striaa vascularis	1536	82	*
Organ of Corti	891	51	*

Guinea-pigs received a perilymphatic perfusion as described in Methods with 1 mCi[^{3}H]-myo-inositol/ml. Incubation time 2 h. Numbers are mean values obtained from 4 animals.

* Not sufficiently high above background.

and the labelling of their inner ear lipids was compared to that of untreated animals (Table 4). There were 12 animals per experimental condition, and ^{32}P-lipids were analyzed after combining tissues from 2 or 3 animals. The ^{32}P-content of lipids was normalized to the ^{32}P-content in the HCl soluble fraction of the tissue samples to correct for variabilities in dissection and perfusion. Data were subjected to analysis of variance for every possible contrast between pairs. There were no significant changes with drug treatment in the labelling of PhI. ^{32}P-incorporation into PhIP appeared somewhat more variable, and an increase was seen after 3 weeks of neomycin treatment in the organ of Corti ($P = 0{\cdot}02$). $PhIP_2$ labelling showed a consistent trend in response to drug treatment. There was virtually no change in any of the tissues after 2 weeks, but a decrease of labelling was evident after 3 weeks of neomycin injections in the organ of Corti ($P = 0{\cdot}02$), spiral ligament ($P = 0{\cdot}05$) and stria vascularis ($P = 0{\cdot}05$).

To assess further the significance of the decreases in $PhIP_2$ labelling, these results were compared to those obtained in a preliminary experiment. In this case, the drug treatment had been the same, but the perfusion time with $^{32}P_i$ was 30 min. Tissues from four cochleae had been pooled for analysis. Again, we found a decrease in $PhIP_2$ labelling in the organ

TABLE 4. EFFECT OF NEOMYCIN TREATMENT ON LABELLING OF PHOSPHOINOSITIDES *in vivo*

	Drug treatment		
	Control	2 weeks	3 weeks
Spiral ligament	10^3 × d.p.m. lipid-^{32}P/HCl-sol. ^{32}P		
PhI + PhS	9·3 ± 1·9	11·2 ± 2·2	13·4 ± 2·4
PhIP	13·6 + 4·7	23·5 ± 4·9	12·2 ± 2·3
$PhIP_2$	9·5 ± 4·6	14·1 ± 3·9	5·4 ± 1·1*
Stria vascularis			
PhI + PhS	46·4 ± 15·0	47·3 ± 19·7	40·4 ± 13·7
PhIP	54·3 ± 24·3	64·1 ± 22·8	35·7 ± 6·1
$PhIP_2$	24·3 ± 7·8	28·9 ± 10·1	13·2 ± 3·9*
Organ of Corti			
PhI + PhS	12·3 ± 1·9	11·2 ± 3·7	19·0 ± 2·7
PhIP	12·0 ± 2·7	13·4 ± 3·3	19·4 ± 2·4†
$PhIP_2$	5·9 ± 1·1	5·7 ± 1·7	2·7 ± 0·8†

Guinea-pigs received a perilymphatic perfusion as described in Methods, with 250 μCi $^{32}P_i$/ml. Incubation time 60 min. Drug treatment was 100 mg/kg body wt daily. Spiral ligament and stria vascularis: for each of the conditions, four samples were analyzed (tissues from 12 guinea-pigs in pools of 3); spiral ligament, 6 samples (pools of 2 tissues). Numbers are means + S.D.

* $P = 0{\cdot}05$; † $P = 0{\cdot}02$ (One-way ANOVA).

TABLE 5. EFFECT OF NEOMYCIN ON LABELLING OF POLYPHOSPHOINOSITIDES *in vitro*

	PhIP	$PhIP_2$
	d.p.m. ^{32}P incorporated	
Control	6634, 7406	733, 878
10^{-4}M neomycin	8406, 8766	1023, 1253

A homogenate of stria vascularis and spiral ligament (0·24 mg protein) was incubated with 60 μCi [^{32}P]ATP for 30 min as described in Methods. Numbers are values of duplicate incubations.

of Corti (control 4·0, treated animals 2·5 d.p.m./10^3 d.p.m. HCl soluble ^{32}P) and stria vascularis (13·6 vs 7·6), but this time not in spiral ligament (6·9 vs 7·5). The osseous spiral lamina which had also been analyzed in both experiments did not show any significant changes of lipid labelling.

In vitro *experiments*

Labelling of polyphosphoinositides by [γ-^{32}P]ATP was demonstrated in a homogenate of stria vascularis and spiral ligament (Table 5). As with subcellular fractions from brain (SCHACHT & AGRANOFF, 1974*a*), $PhIP_2$ and PhIP are highly labelled. The presence of 10^{-4} M-neomycin appears to increase the labelling of both PhIP and $PhIP_2$.

Hydrolysis of lipids was studied with lipids previously labelled by $^{32}P_i$ in guinea-pig brain synaptosomes (Table 6, expt. I) or kidney (Table 6, expt. II). There is no apparent hydrolysis of lipids other than PhIP or $PhIP_2$. The enzymatic breakdown of the polyphosphoinositides by inner ear tissues is inhibited

TABLE 6. EFFECT OF 5×10^{-4} M NEOMYCIN ON HYDROLYSIS OF PHOSPHOLIPIDS BY INNER EAR TISSUES *in vitro*

Tissue	Protein (μg)	Condition	$PhIP_2$	PhIP	PhI + PhA		
			d.p.m. ^{32}P remaining in lipid				
Expt. I							
None	—	—	1048 ± 14	1201 ± 28		1787 ± 36	
Combined tissues	90	control	768 ± 34*	1112 ± 16†		1772 ± 36	
	90	+ neomycin	956 ± 16	1202 ± 40		1759 ± 44	
			$PhIP_2$	PhIP	PhI + S	PhC	PhE
Expt.II							
None		0 time	1064	687	769	697	1184
	—	60 min	963	630	655	666	1125
Spiral ligament	37	control	913	650	658	673	1264
	37	+ neomycin	1055	648	648	696	1196
Stria vascularis	12	control	767	467	764	727	1128
	12	+ neomycin	1079	581	788	644	1174
Organ of Corti	20	control	731	567	801	678	1146
	20	+ neomycin	1037	541	713	663	1181

Expt. I: A homogenate of combined inner ear tissues was incubated for 60 min with previously labelled and extracted lipids (from guinea-pig synaptosomes, SCHACHT & AGRANOFF, 1974*b*) as described in Methods. Numbers are means ± S.D. of 3 incubations.

* Differs from incubations without enzyme, $P < 0{\cdot}01$; differs from incubations with neomycin, $P < 0{\cdot}01$.

† Differs from incubations without enzyme, $0{\cdot}02 > P > 0{\cdot}01$; differs from incubations with neomycin, $0{\cdot}05 > P > 0{\cdot}02$.

Expt. II: A 1000 *g* supernatant fraction of tissue homogenates was incubated for 60 min with previously labelled and extracted lipids (from guinea-pig kidney) as described in Methods. Numbers are averages of duplicate incubations.

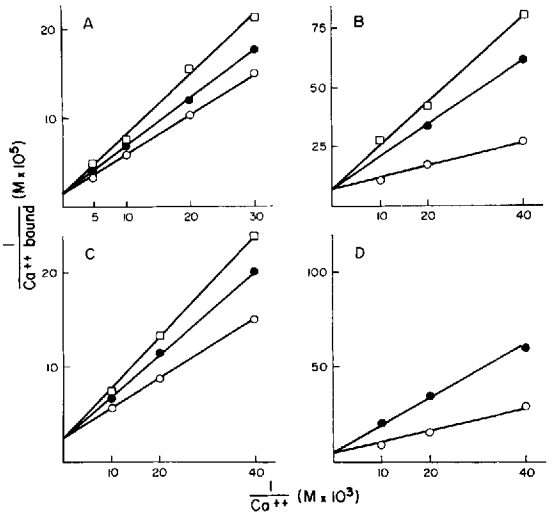

FIG. 2. Inhibition by neomycin of calcium binding. A 1000 *g* supernatant fraction of tissue homogenates was incubated with $^{45}CaCl_2$ (1 μCi) as described in Methods. A: Combined tissues, 18 μg protein; B: Spiral ligament, 35 μg protein; C: Organ of Corti, 13 μg protein; D: Stria vascularis, 28 μg protein. O——O no neomycin; ●——● 5×10^{-5} M; □——□ 5×10^{-4} M neomycin.

significantly by the addition of 5×10^{-4} M-neomycin (expt. I). This effect can also be demonstrated in individual tissues with the possible exception of spiral ligament where only little if any hydrolytic activity is seen (expt. II).

In view of the possible involvement of the polyphosphoinositides in the binding of calcium, the effect of neomycin on this binding was investigated with $^{45}CaCl_2$ (Fig. 2). Neomycin acts as a competitive inhibitor of calcium binding to inner ear tissues. For one of the preparations (Fig. 2A) the binding capacity was extrapolated (SCATCHARD, 1949) and compared to the phospholipid content. There were 280 nmol lipid-*P*/mg protein which could bind 80 nmol calcium. The polyphosphoinositides should not constitute more than 1–3% of the total phospholipids (SHELTAWY & DAWSON, 1969) and thus can only account for a small fraction of the calcium binding sites.

DISCUSSION

A participation of the polyphosphoinositides in the regulation of membrane permeability, possibly via their capacity to bind calcium, has been inferred from various observations. Their concentration is highest in nervous and secretory tissue (DAWSON, 1969), the labelling of their monoesterified phosphate groups is rapid (SANTIAGO-CALVO *et al.*, 1964), and this labelling increases with appropriate stimulation of the tissues (SCHACHT & AGRANOFF, 1972; WHITE *et al.*, 1974). Such a rapid labelling can also be observed in inner ear tissues after introduction of the precursor into the perilymphatic spaces. After short incubation times, $PhIP_2$ and PhIP are by far the most highly labelled lipids with ^{32}P-$PhIP_2$ exceeding PhIP. This

labelling pattern resembles closely that observed in brain (SHELTAWY & DAWSON, 1969), and kidney (TOU *et al.*, 1972). After complete dissection of the ear tissues, the highest rate of ^{32}P-incorporation is seen in stria vascularis. This agrees well with the generally high metabolic activity ascribed to this tissue (THALMANN *et al.*, 1970). The low labelling of PhI at short times suggests that the ^{32}P-incorporation into PhIP and $PhIP_2$ takes place largely into their monoester phosphates via kinase reactions. This is supported by the fact that the incorporation of [^{3}H]inositol which is more a measure of *de novo* synthesis, proceeds in the order PhI > PhIP > $PhIP_2$.

Treatment of animals with neomycin for several weeks produces damage to the auditory system which can be assessed morphologically and electrophysiologically. Such analyses, carried out in parallel with the biochemical experiments indicated that injury to the inner ear by neomycin at this dose level, as judged by decrease in cochlear potentials and histological changes in the organ of Corti, begins to appear in some animals after 2 weeks of treatment and is clearly evident in all of them after 3 weeks (J. E. Hawkins, Jr., personal communication). No apparent changes in phosphoinositide labelling are evident after two weeks of drug treatment. After 3 weeks, however, a decrease of the labelling of $PhIP_2$ can be observed which is significant for the organ of Corti and marginally significant for the other tissues. The labelling of PhIP is somewhat more variable in the drug treated animals and a clear effect on this lipid can only be observed in the organ of Corti. Incorporation of $^{32}P_i$ into PhI remains unaffected. The depression of ^{32}P-$PhIP_2$ after neomycin treatment was also seen in the second experiment in the organ of Corti and stria vascularis but not in spiral ligament. A third experiment (SCHACHT, 1974*b*) had also shown a significant decrease in the labelling of $PhIP_2$ in a preparation of the organ of Corti. Stria vascularis had not been dissected separately, and the combined tissues of stria vascularis and spiral ligament did not show changes in ^{32}P-lipids after neomycin treatment.

Two points seem to be of interest in this context. Firstly, on the basis of morphological evidence, the organ of Corti and stria vascularis are usually considered the primary targets of ototoxic drugs (HAWKINS, 1970). Second, neomycin is also nephrotoxic and kidney tissues and stria vascularis show marked similarities in a variety of modalites (QUICK *et al.*, 1973). In kidney, we were able to demonstrate a decreased labelling of ^{32}P-polyphosphoinositides after neomycin treatment *in vivo* as well as an inhibition of polyphosphoinositide hydrolysis *in vitro* (SCHACHT, in preparation). Thus neomycin seems to alter phosphoinositide metabolism in both these susceptible tissues.

In vitro experiments were conducted to answer two questions. (1) Is the change in $PhIP_2$ labelling a direct action of neomycin or is it a secondary response, and (2) what is the underlying mechanism?

The concentrations of neomycin chosen for the *in vitro* experiments appear to fall well within the physiological range. Aminoglycoside antibiotics are known to accumulate in the inner ear fluids exceeding the serum concentration by an order of magnitude or more. Twelve hours after a single injection of 100 mg neomycin/kg body wt the drug concentration in guinea-pig perilymph is about 10^{-4} M and repeated injections would increase this level (STUPP, 1970). Labelling of PhIP and $PhIP_2$ by [γ-^{32}P]ATP appears to be stimulated in inner ear tissues in the presence of neomycin and the hydrolysis of these lipids is inhibited by the addition of 5×10^{-4} M neomycin. This inhibition may explain the higher labelling of these lipids from [^{32}P]ATP in the presence of the drug. Table 6 also shows that stria vascularis and organ of Corti have a much higher activity of polyphosphoinositide phosphomonoesterases and/or -diesterases than the spiral ligament. It is tempting to speculate that the high rate of polyphosphoinositide metabolism makes these two tissues susceptible to neomycin *in vivo*. However, drug penetration and accumulation may also play an important role in determining the *in vivo* effects.

To explain the decreased $PhIP_2$ labelling *in vivo* by an inhibition of hydrolysis, we may consider, as pointed out above, that the ^{32}P-incorporation *in vivo* probably occurs via phosphatase and kinase reactions:

$$PhIP_2 + H_2O \rightarrow PhIP + P_i$$
$$PhIP + [^{32}P]ATP \rightarrow [^{32}P]PhIP_2 + ADP.$$

An inhibition of $PhIP_2$ phosphomonoesterase can thus lead to a decreased rate of labelling from [^{32}P]ATP if the hydrolysis is the rate-limiting step in the reaction sequence. In the *in vitro* labelling experiment, a phosphoinositide fraction was added to the tissue homogenate so that PhIP (and PhI) availability should no longer be rate limiting for the kinase reactions. Inhibition of hydrolysis would then increase labelled polyphosphoinositides.

A different mechanism of action could be suggested, however, by the calcium binding studies. It is evident that neomycin is a competitive inhibitor of calcium binding to inner ear tissues. An interference of neomycin with calcium at the neuromuscular junction has been discussed (CORRADO, 1968; VITAL BRAZIL & PRADO-FRANCESCHI, 1969), and recently an inhibition of calcium reuptake or rebinding by neomycin has been demonstrated in vascular smooth muscle (GOODMAN *et al.*, 1974). Although the amount of calcium bound by inner ear tissues is greater than can be accounted for by the presence of polyphosphoinositides, the displacement of calcium implies that neomycin can occupy anionic binding sites. Complexes between basic antibiotics and polyanionic compounds are well documented *in vitro* (COHEN, 1947; MORA *et al.*, 1959). Such a complex formation between the acidic polyphosphoinositides and the

polybasic neomycin might render the lipids unavailable as substrates to their degradative enzymes, explaining the observed effects as described above. Moreover, such binding may, in addition, block the PhIP kinase reaction, leading to the decrease in $PhIP_2$ and an accumulation of PhIP as observed *in vivo* in the organ of Corti. A dual mechanism of neomycin action, that is inhibition of $PhIP_2$ phosphomonoesterase and the displacement of calcium from other binding sites, cannot, however, be ruled out from the present studies.

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