Biochemical Pharmacology, Vol. 29, pp. 597–601.

0006-2952/80/0215-0597 $02.00/0

OTOTOXICITY OF AMINOGLYCOSIDES CORRELATED WITH THEIR ACTION ON MONOMOLECULAR FILMS OF POLYPHOSPHOINOSITIDES

SHAHID LODHI, NORMAN D. WEINER, IRIS MECHIGIAN and JOCHEN SCHACHT
College of Pharmacy and Kresge Hearing Research Institute, University of Michigan, Ann Arbor, MI 48109, U.S.A.

(*Received* 27 *June* 1979; *accepted* 21 *August* 1979)

Abstract—The ototoxicities of eight aminoglycoside antibiotics and fragments were measured quantitatively by cochlear perfusion in the guinea pig. Perilymphatic spaces were perfused for 1 hr with 'artificial perilymph' containing 10 mM drug, during which time continuous measurements of cochlear microphonic potentials were made. Kanamycin B and neomycin B caused the most rapid decline of cochlear microphonic potentials, followed by gentamicin C_{1a} ≃ ribostamicin > kanamycin A ≃ G-418. Neamine and methylneobiosamine did not show significant effects. The same drugs were tested for their interaction with monomolecular films of polyphosphoinositides, and relative binding constants were determined. Neomycin B and kanamycin B had the highest affinities to the lipids, followed by the other drugs in the order as seen for toxicity. The correlation between the *in situ* and *in vitro* actions of the drugs was $r = 0.9$. These results support the hypothesis that binding to polyphosphoinositides plays an important role in the decrease of the cochlear microphonic potentials. Furthermore, the good correlation between the drug actions in the two test systems suggests that an *in vitro* assay may be possible for the assessment of aminoglycoside ototoxicity.

The interpretation of drug toxicity is frequently complicated by secondary effects following the initial insult. A necessary prerequisite, however, for understanding the molecular mechanism of a drug action is the determination of a primary site of action.

Aminoglycoside antibiotics have nephrotoxic and ototoxic properties, and a variety of biochemical effects has been reported to result from treatment with such drugs. Recently, effects have been shown on glycolysis [1], nucleic acids [2], mucopolysaccharides [3] and glucose transport [4]. We had proposed, on the basis of *in vivo* and *in vitro* experiments [5–8], that the polyphosphoinositides (phosphatidylinositol phosphate and phosphatidylinositol bisphosphate) serve as *in vivo* receptors for aminoglycosides and render tissues susceptible to these drugs. Subsequently, we isolated these lipids by affinity chromatography on immobilized neomycin [9].

Interactions between drugs and lipids can be measured directly with monomolecular lipid films. Polyphosphoinositides appeared to be unique among various anionic phospholipids with respect to both the type and the magnitude of the interaction when films were challenged with neomycin in the presence of Ca^{2+} [10]. We have proposed that the observed increase in surface pressure is indicative of a very strong preference of the polyphosphoinositide film for neomycin over calcium and other cations.

In this study we wish to present evidence that such an interaction with polyphosphoinositides is a property of ototoxic aminoglycoside antibiotics. We determined quantitatively by perilymphatic perfusion the effect of eight aminoglycosides on cochlear microphonic potentials and compared the *in situ* action to the ability of the drugs to increase the surface pressure of monomolecular films of polyphosphoinositides.

MATERIALS AND METHODS

Materials. Neomycin sulfate, neamine and neobiosamine were obtained from the Upjohn Co., Kalamazoo, MI; kanamycin B and ribostamicin sulfate from Charles Pfizer Inc., New York, NY; gentamicin C_{1a} and antibiotic G-418 from the Schering Corp., Nutley, NJ; and kanamycin A from Bristol Laboratories, Syracuse, NY. Polyphosphoinositides were prepared by chromatography on immobilized neomycin [11]. All water was triple distilled from an all-glass still.

Perilymphatic perfusions. Perilymphatic perfusions were carried out in male albino guinea pigs (200–250 g). An animal with a positive Preyer hearing reflex was anesthetized with 1.0–1.4 ml allobarbital/kg body wt (100 mg allobarbital, 400 mg urethane, 400 mg monoethylurea per ml) given intraperitoneally. The guinea pig was then placed on a heating pad, its body temperature maintained at 38 ± 1°, and artificial respiration provided via tracheal cannulation. Surgical and perfusion techniques were essentially similar to those described by Nuttall *et al.* The head of the animal was secured to the head holder with clamps fastened into slits made over either zygomatic arch. A conventional ventral surgical approach was used to expose the left auditory bulla and cochlea. Two holes were drilled through the bony cochlea at the basal turn, and glass capillaries (0.8 mm O.D.) were inserted through them, one into the scala vestibuli and the other into the

scala tympani. Once the capillaries were implanted, they were sealed with Durelon carboxylate cement (Espe GmbH, Seefeld, Germany).

The inlet capillary to the scala tympani served also to hold the electrode for recording cochlear microphonic potentials. For the electrode, a 5-cm piece of stainless steel annealed wire (AWG 36, Cooner Sales Co., Chatsworth, CA) was inserted into the glass capillary. The excess wire was bent down toward the capillary tip, and the other end of the capillary was inserted into a 35-cm length of polyethylene tubing (Intramedical, PE 60). Duco cement was used to seal the tubing–capillary junction. The outlet capillary was inserted into a 60-cm length of polyethylene tubing. Both capillaries and tubing were filled with 'artificial perilymph' [12] prior to insertion into the cochlea, and flow was started by lowering the outlet tubing.

The perilymphatic spaces were perfused with or without drugs at the rate of approximately 30 μl/min. The perfusate was heated so that the temperature of the solution reaching the cochlea was 32–33°. Cochlear microphonic potentials were measured in response to a sound stimulus of white noise, ranging from 20 to 4000 Hz delivered through an ear phone, and the sound intensity level was adjusted to give a 200–300 μV response. Potentials were read off of a digital Data Precision voltmeter, and recorded on a Beckman 10 in. recorder.

Monolayer studies. A 250-ml Teflon beaker with an inner diameter of 8 cm and a Wilhelmy balance were used to measure surface pressure. The subphase consisted of 100 ml of 50 mM sodium 4-(2-hydropyethyl)-1-piperozine-ethanesulphonic acid (HEPES), pH 7.0, and 1 mM Ca^{2+}. The ionic strength was adjusted to 0.2 with sodium chloride previously heated for 12 hr at 700°. The film was a mixture of phosphatidylinositol phosphate and phosphatidylinositol bisphosphate in an approximate 1:2 molar ratio and was spread as a solution in *n*-hexane–ethanol–chloroform (80:5:15, by vol). The spreading solution was added to the subphase so as to produce a surface pressure of 20 dynes/cm ± 10% which required approximately 20 μg lipid. A stationary 3-ml syringe whose needle remained in the subphase throughout the experiment delivered drugs to the solution beneath the film. A Teflon-coated magnetic bar stirred the solution at slow speed without disturbing the film. Drug concentrations in the subphase were varied from 10^{-8} to 10^{-3} M. After each addition of a drug, the subphase was mixed for 15 min before surface pressure was measured. Two readings each were taken at ten different drug concentrations over the range indicated above. Experiments were acceptable only if duplicate readings agreed within 0.1 dyne/cm; the curves resulting from the average values are shown.

RESULTS

Ototoxicity. Perilymphatic perfusions could usually be carried out for 1–2 hr without significant detrimental effect on cochlear microphonic potentials (Fig. 1, B, and Table 1, controls). There was, however, some variability of the stability of the preparations, and a control perfusion of 30 min preceded each testing of a drug. Effects on cochlear microphonics (Fig. 1, A2) were usually seen within 10 min (range, 3–17 min) after the introduction of the drug into the perfusion fluid (Fig. 1, A1). The decrease of the microphonic potential to the end of the linear period (Fig. 1, A2–A3) was considered the measure of drug toxicity. This linear period usually lasted 15 to 30 min. When no change in slope was apparent (e.g. controls and most perfusions with neamine or methylneobiosamine), the rate of decrease was calculated from approximately 10 to 40 min after addition of the drug (Fig. 1, B2–B3). All rates,

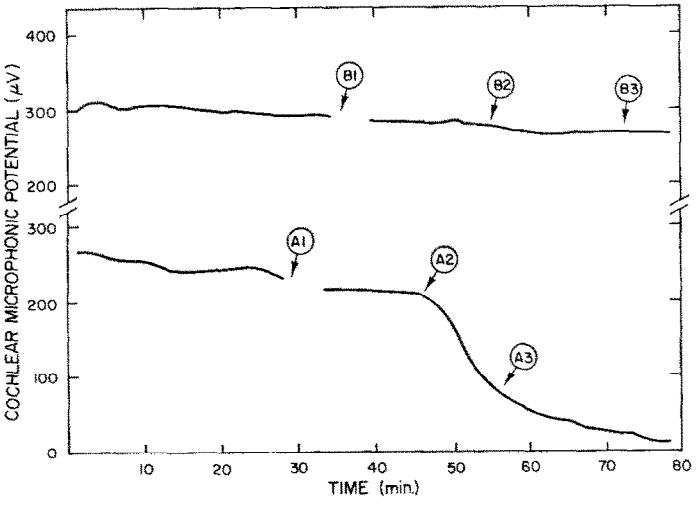

Fig. 1. Recording of cochlear microphonic potentials during perilymphatic perfusions. Perilymphatic spaces were perfused and microphonic potentials recorded as described in Materials and Methods. Perfusions with drugs: A1, addition of drug to artificial perilymph; and A2–A3, initial slope of drug action. Control perfusions without drug: B1, manipulation of perfusion system corresponding to A1; and B2–B3, slope for calculations of loss of microphonic potential (40–70 min).

Table 1.Effects of drugs on cochlear microphonic potentials*

Drug	Loss of cochlear microphonics during perfusion			
	Rate of loss (μV/min)		Significance of drug effect	Percent loss
	without drug	with drug	(P value)	at 30 min
None	0.22 ± 0.15	0.32 ± 0.12	>0.4	7 ± 1
Methylneobiosamine	0.71 ± 0.14	0.91 ± 0.30	>0.5	6 ± 5
Neamine	0.33 ± 0.16	0.68 ± 0.37	>0.3	10 ± 7
G-418	0.29 ± 0.23	1.38 ± 0.19	0.02	24 ± 7
Kanamycin A	0.46 ± 0.18	1.52 ± 0.45	0.025	29 ± 6
Ribostamicin	0.11 ± 0.09	1.83 ± 0.41	<0.01	51 ± 6
Gentamicin C_{1a}	0.30 ± 0.17	2.07 ± 0.34	<0.01	47 ± 4
Neomycin B	0.36 ± 0.20	3.56 ± 0.68	<0.01	79 ± 7
Kanamycin B	0.22 ± 0.15	4.37 ± 0.65	<0.01	76 ± 10

* Perilymphatic spaces were perfused for 30 min with artificial perilymph only (without drug) followed by 60 min with the respective drug. Details are described in Materials and Methods. Values are means ± S.E.M. of five experiments each.

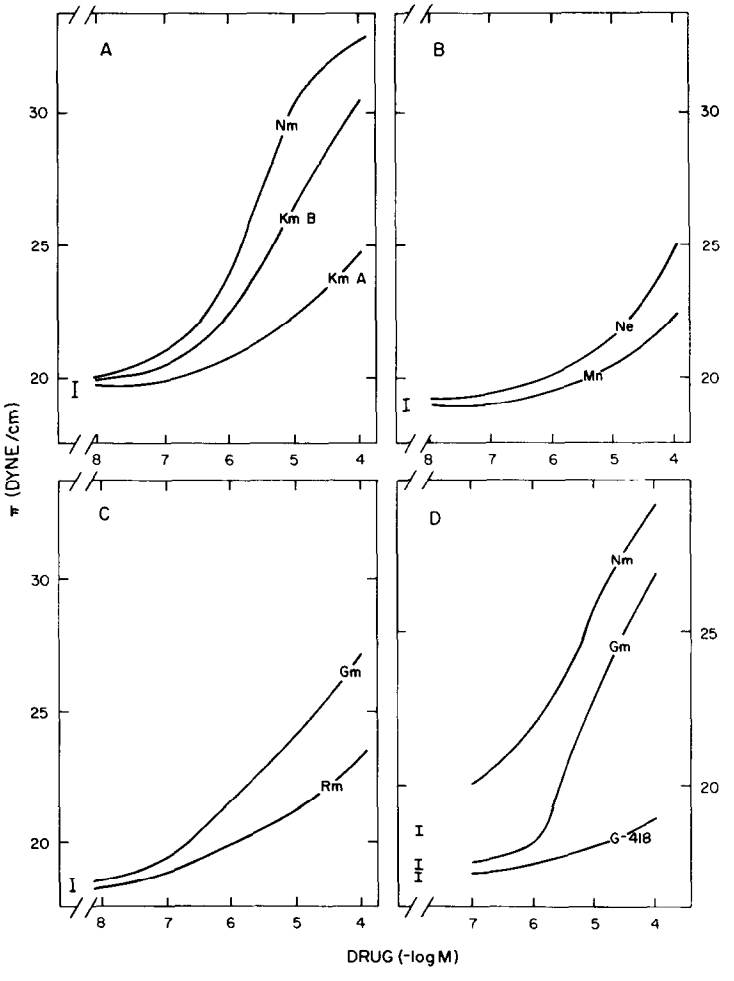

Fig. 2. Effects of aminoglycosides on surface pressure (π) of polyphosphoinositide films. Increasing amounts of a drug were added to the subphase of monomolecular films of polyphosphoinositides as described in Materials and Methods. The ratio of phosphatidylinositol phosphate to phosphatidylinositol bisphosphate was approximately 8:2 in experiment D. Abbreviations: nM, neomycin B; Gm, gentamicin C_{1a}; Km A, kanamycin A; Km B, kanamycin B; Rm, ribostamicin; Ne, neamine; Mn, methylneobiosamine; and G-418, antibiotic G-418.

including those of the preceding control period, were calculated by linear regression analysis of the described slopes based on readings taken 5 min apart (Table 1).

Calculation of the initial slope may overestimate the toxicity of a drug. In a few instances, a steep, but brief (5–10 min), initial slope was observed, followed by a more gradual decline of the microphonic potential. Therefore, all drug effects were also expressed as loss of the cochlear microphonic potential within a 30-min period after the onset of the drug action (Table 1, last column).

Both methods yielded essentially similar results. Four different levels of toxicity could be distinguished. Kanamycin B and neomycin B were clearly the most ototoxic drugs, gentamicin C_{1a} and ribostamicin showed an intermediate degree of toxicity, while kanamycin A and G-418 displayed low but significant effects. Neamine and methylneobiosamine did not decrease cochlear microphonics significantly during the perfusion time.

Monolayer studies. All drugs tested above also interacted with polyphosphoinositides in the presence of 1 mM Ca^{2+}, increasing the surface tension of the lipid films (Fig. 2) The responses, however, were graded, and the largest increases of surface tension were caused by neomycin B and kanamycin B. With these two antibiotics, surface tensions were already increased by drug concentrations three orders of magnitude lower than the concentration of calcium in the experiment. The other drugs had lesser but still significant effects, particularly at higher concentrations. Antibiotic G-418 was tested with a film which contained polyphosphoinositides in a ratio different from the other experiments. It caused only a small increase in surface pressure but neomycin and gentamicin reacted similarly under both experimental conditions.

DISCUSSION

Cochlear perfusion permits a direct and quantitative measure of drug toxicity. The concentration of the drug in the perilymph and the duration of the treatment are under direct experimental control. In contrast, conventional assessments of toxicity by systemic injections are complicated by the rate of absorption, renal handling of the drug, serum levels and levels in the inner ear fluids. Yet, for a rational determination of structure–activity relationships, the knowledge of the potential or intrinsic ototoxicity of a drug is imperative.

The drug concentration in the perfusion studies was 10^{-2} M. Drug levels between 10^{-4} and 10^{-3} M can be reached in inner ear fluids after repeated systemic injection [13, 14]. The tissues susceptible to the drugs—the organ of Corti and the stria vascularis—are surrounded by endolymph, and in perilymphatic perfusions the drugs have to reach these tissues by diffusion. This may cause dilution of the antibiotic and may account for the delay in reaction. The fact that toxic and non-toxic drugs are clearly differentiated at the chosen concentration points to the validity of the approach.

While neither the precise origin nor the physiological role of the cochlear microphonic is known, it seems that such measurements are a reliable method of assessing the toxicity of aminoglycosides. A number of chronic animal studies have demonstrated correlations between effects on cochlear microphonics and other parameters of ototoxicity, e.g. loss of hair cells or whole nerve action potentials [15]. Although our determination of toxicity rests on the effects on the cochlear microphonics alone, the results from these acute perfusions are in good agreement with inferences from chronic studies and findings of clinical ototoxicity. Neomycin is generally considered the most toxic of the antibiotics tested, the toxicity of gentamicin is higher than that of kanamycin A, and neamine has little or no ototoxicity [15, 16]. Kanamycin B was reported in one study to be considerably more ototoxic than kanamycin A [17].

The ototoxicity of at least one drug, antibiotic G-418 [18], does not seem to agree with its systemic toxicity. Antibiotic G-418 was highly toxic in preliminary systemic studies; at 40 mg/kg/day, cats became ataxic or died after 2–3 days (J. Allan Waitz, personal communication). Our perfusions place it at a low level of ototoxicity close to kanamycin A. Although ribostamicin has been studied for its antibacterial properties [19], we are not aware of reports of its ototoxicity. This study showed ototoxicity at the level of gentamicin C_{1a}.

The salient point of this study is the comparison of ototoxicity with drug effects *in vitro*. A first and obvious quantitation of the drug/polyphosphoinositide interaction is the increase in surface pressure at a given drug concentration, e.g. 3.2×10^{-5} M (Table 2). Lower concentrations produce small increases with relatively large variability (±0.1 dyne/cm), while higher concentrations may produce saturation effects (Fig. 2, panel A, neomycin).

The interaction with polyphosphoinositides reflects two properties of an antibiotic: (1) its affinity to the lipid and ability to displace calcium; and (2) the geometry of the molecule which determines the magnitude of increase of surface pressure upon displacement of the small calcium ion. Comparison of surface pressures alone lends considerable weight to the second point, but in order to calculate affinity

Table 2. Effects of drugs on monomolecular films of polyphosphoinositides

Drug	$\Delta\pi$ at 32 μM* (dynes/cm)	Displacement constant^{+}(K_m) (μM)
Neomycin	12.0	3.9
Kanamycin B	9.5	6.1
Ribostamicin	4.0	6.5
Gentamicin	7.0	6.8
Kanamycin A	4.5	8.3
G-418	1.8	8.6
Neamine	4.0	12.0
Methylneobiosamine	3.0	12.9

* $\Delta\pi$ from Fig. 2.

$^{+}$ Displacement constants were calculated by Lineweaver–Burk analysis (7–70 μM drug).

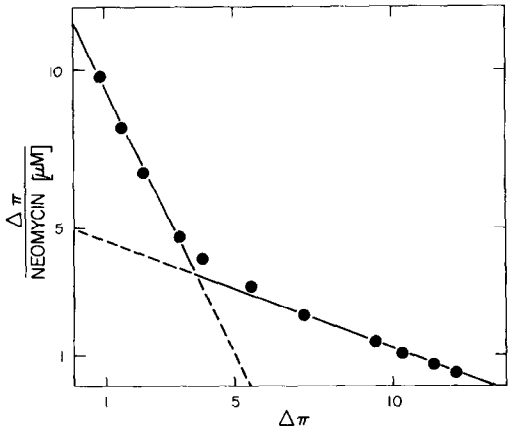

Fig. 3. Scatchard analysis of neomycin interaction with polyphosphoinositide films. As described in the text, $\Delta\pi$ is assumed to be a direct function of bound neomycin. The term at the ordinate is equivalent to $\Delta\pi$/[free neomycin] assuming [total neomycin] >> [bound neomycin].

constants, the amount of bound drug has to be measured. If we assume that the increase in surface pressure is a simple function of the amount of drug bound, we can treat the data by Scatchard or Lineweaver–Burk analysis. Two binding sites of different affinity become evident for neomycin (Fig. 3) and the other drugs. This agrees well with our previous findings of biphasic neomycin actions on synaptosomal membranes and on polyphosphoinositides [10, 20]; a 'calcium-like' action at lower concentrations, also seen with other anionic lipids, and a noncompetitive action at higher levels of drug, only seen with polyphosphoinositides. A K_m (or, better termed 'displacement constant') was calculated for all drugs by Lineweaver–Burk analysis from data in the concentration range, 7–70 μM (Table 2).

The analyses confirm what is apparent from Fig. 2: toxic aminoglycosides show a larger degree of interaction with polyphosphoinositides than the less toxic ones. Indeed, the 'displacement constants' follow the ototoxicity very closely; a linear correlation between these constants and the loss of cochlear microphonics (last column, Table 1) yields a correlation coefficient of $r = 0.93$. While the increase in surface pressure is not in such good agreement with the ototoxicity of some drugs (e.g. G-418 and ribostamicin), it still provides a differentiation between the most toxic and the less toxic compounds. The loss of cochlear microphonic correlates with $\Delta\pi$, $r = 0.86$.

While these comparisons of the *in situ* and *in vitro* effects already provide a reasonably good correlation, modifications of the assay systems remain to be explored. Lower drug concentrations in the perfusions may yield a better differentiation, e.g. of the most toxic drugs, neomycin B and kanamycin B, whose toxicities are not significantly different in this study ($P > 0.4$). Another possible modification is the composition of the monomolecular films. These were a mixture of phosphatidylinositol phosphate and bisphosphate in an approximate molar ratio of 3:7. Variations of this ratio clearly influence the magnitude of the increase in surface pressure (compare neomycin and gentamicin in Fig. 2, panels A + C vs panel D). Moreover, combinations of polyphosphoinositides with other acidic or neutral lipids may more closely approximate physiological conditions and improve the differentiation between the various drugs.

In any case, the present results strongly support our hypothesis that polyphosphoinositides are crucially involved in the ototoxicity [7, 21] and, possibly, nephrotoxicity [6] of aminoglycoside antibiotics. Moreover, they point to the possibility of establishing an *in vitro* system for the determination of aminoglycoside toxicity.

Acknowledgement—This research was supported by Research Grant NS 13792 and Program Project Grant NS 05785 from the National Institutes of Health.

REFERENCES

1. M. Tachibana, O. Mizukoshi and K. Kuriyama, *Biochem. Pharmac.* **25**, 2297 (1976).
2. J. Jarlstedt and D. Bagger-Sjöbĕk, *Acta oto-lar.* **84**, 361 (1977).
3. T. Deguchi, A. Ishii and M. Tanaka, *Antibiotics* **31**, 150 (1978).
4. J. Garcia-Quiroga, C. H. Norris, L. Glade, G. M. Bryant, M. Tachibana and P. S. Guth, *Res. Commun. Chem. Path. Pharmac.* **22**, 535 (1978).
5. A. Orsulakova, E. Stockhorst and J. Schacht, *J. Neurochem.* **26**, 285 (1976).
6. A. Schibeci and J. Schacht, *Biochem. Pharmac.* **26**, 1769 (1977).
7. E. Stockhorst and J. Schacht, *Acta oto-lar.* **83**, 401 (1977).
8. J. Schacht, *J. Neurochem.* **27**, 1119 (1976).
9. J. Schacht, *Arch. oto-rhino-lar.* **224**, 129 (1979).
10. S. Lodhi, N. D. Weiner and J. Schacht, *Biochim. biophys. Acta* **557**, 1 (1979).
11. J. Schacht, *J. Lipid Res.* **19**, 1063 (1978).
12. A. L. Nuttall, D. M. Marques and M. Lawrence, *Acta oto-lar.* **83**, 393 (1977).
13. H-F. Stupp, *Acta oto-lar.* (suppl.), **262**, 8 (1970).
14. L. Voldrich, *Acta oto-lar.* **60**, 243 (1965).
15. J. E. Hawkins, Jr., in *Handbook of Sensory Physiology* (Eds. W. D. Keidel and W. D. Neff), p. 707. Springer, Berlin (1976).
16. J. E. Hawkins, Jr., in *Biochemical Mechanisms in Hearing and Deafness* (Ed. M. M. Paparella), p. 323. Charles C. Thomas, Springfield, IL (1970).
17. J. E. Hawkins, Jr., *Ann. Otol.* **68**, 698 (1959).
18. D. Loebenberg, M. Counelis and J. A. Waitz, *Antimicrob. Agents Chemother.* **7**, 811 (1975).
19. E. Yourassowsky and M. P. Vanderlinden, *Arzneimittel-Forsch.* **26**, 184 (1976).
20. S. Lodhi, N. D. Weiner and J. Schacht, *Biochim. biophys. Acta* **426**, 781 (1976).
21. J. Schacht, N. D. Weiner and S. Lodhi, in *Cyclitols and Phosphoinositides* (Eds. W. W. Wells and F. Eisenberg), p. 153. Academic Press, New York (1978).