Influence of Chemical and Physical Factors on Biological Responses to Neomycins B and C

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The two known components of the neomycin complex—neomycins B and C—have been shown to respond differently under the usual conditions of bioassay. Potassium phosphate, commonly used as a diluent, antagonizes both the activities of the neomycins and the growth of *Staphylococcus aureus*. The antagonism is greater against neomycin C than against neomycin B. The same effect occurs with potassium chloride. Both components adsorb to agar and are released by salts. There is an apparent difference in binding to Bacto agar compared to Noble agar with binding of B and C less on Noble agar. The potency values in neomycin bioassay will vary depending on the B:C ratios of the test and standard preparations and the method used for assay. This is probably the cause of many lab-to-lab differences in potency results. A critical analysis of existing methods and standards and some agreement on the method and the definition of neomycin standards is recommended.

THE TERM "NEOMYCIN" usually refers to mixtures of neomycin B and neomycin C. The "United States Pharmacopeia" (1) makes no direct mention of components or relative amounts of B and C that may be present. Kaiser (2) has shown that production lots from various sources which he examined varied in neomycin C content from 6.2 to 28.5%. The differences in the B:C ratio between neomycin preparations may be a factor in assay problems of day-to-day, lab-to-lab, and method-to-method variations.

The structures of neomycins B and C have been reported by Rinehart, *et al.* (3, 4), as 4-ring compounds; the only difference between the two components, excluding glycosidic linkages which have not all been established, is the optical configuration of an aminomethyl group in a pyranose sugar. As similar as the two structures are, however, the biological activities in broth (5–7) and in agar (7–9) are sometimes markedly different. This paper is a report on some factors which influence the responses to neomycins B and C in liquid and agar media.

METHODS AND RESULTS

Two lots of each component, neomycins B and C, were used in these studies. The neomycin C preparations, 2733-R2 and 2569-MEB, were found to be free (<1%) of neomycin B in a radioisotopic assay (2). The neomycin B preparations, 2732-R2 and 2689-MEB, contained approximately 4 and 11% neomycin C, respectively. Chemical and physical properties of the MEB preparations were described by Ford and co-workers (10).

Effect of Potassium Phosphate on Neomycin Activity.—Salts have been reported to antagonize the antibacterial activity of neomycin (11, 12). Since phosphate buffer is commonly used as a diluent in assay procedures (8, 13–15), the effect of phosphate on the activity of neomycin may be a factor in the assay response.

Minimum inhibitory concentration (MIC) end points were determined for neomycin B and neomycin C in broth, designated SAB, consisting of 0.15% beef extract, 0.3% yeasts extract, and 0.6% peptone adjusted to pH 7.9 before autoclaving. Sterile solutions of potassium chloride and phosphate at pH 7.9 were added to the media after autoclaving for final concentrations of 0.1 *M*. Readings of growth or no growth were made after 24 hours incubation at 32°. Table I shows the greater antagonism of both salts to neomycin C than to B and the greater antagonism to both neomycins with potassium phosphate than with potassium chloride.

Effect of Potassium Phosphate on the Growth of S. aureus.—*Staphylococcus aureus* FDA 209-P is the test organism of choice for neomycin assays in the U. S. Food and Drug Administration laboratories (14). The effect of potassium phosphate on *S. aureus* was examined because any influence on the test organism could be a factor in the antibiotic response in the assay system. The growth with phosphate buffer was compared with the growth with trishydroxymethylamino methane (tris) buffer.

Cell suspensions of *S. aureus* were prepared by diluting a culture grown in SAB 1:1000 with water to approximately the numbers of organisms encountered in an assay plate. Further dilutions and viable counts were made with the usual poured-agar-plate technique. One milliliter of diluted cell suspension was added to one end of the plate, and 1 ml. of water or 10× strength pH 7.9 phosphate or tris buffer was added to the other end. Care was taken so that the buffer did not contact the organisms before the agar was added. Eight milliliters of 10/8 strength Bacto streptomycin assay agar (SAA) or neomycin assay agar (NAA) were added to each plate, and the entire contents was mixed. All plates were incubated for 24 hours at 37°. Three experiments were conducted with results indicating that 0.1 *M* potassium phosphate antagonized the growth of *S. aureus* (Table II), while 0.1 *M* tris buffer did not.

Adsorption of Neomycin on Agar.—Adsorption tests were conducted by adding antibiotic to a 1.5%

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TABLE I.—EFFECT OF SALTS ON THE ACTIVITY OF NEOMYCIN AGAINST *S. aureus* IN pH 7.9 BROTH

Salt	Minimum Inhibitory Concn., mcg./ml.[a] — Neomycin B — Expt. 1	Expt. 2	Expt. 3	Expt. 4	Neomycin C — Expt. 1	Expt. 2
No salt control	0.2 (>0.1)	0.15 (>0.1)	0.1 (>0.05)	0.2 (>0.1)	0.2 (>0.1)	0.5 (>0.2)
0.1 *M* potassium chloride	1.0 (>0.5)	0.75 (>0.5)	0.5 (>0.2)	1.0 (>0.5)	5 (>2)	5 (>2)
0.1 *M* potassium phosphate	10 (>5)	7.5 (>5)	5 (>2)	10 (>5)	50 (>20)	75 (>50)

[a] Numbers in parentheses are the highest concentrations tested in which growth occurred.

TABLE II.—VIABLE COUNTS OF *S. aureus* SUSPENSIONS IN BUFFERED MEDIA

Expt.	Medium	Concn. of Buffer	Viable Count[a]
1	NAA	Water control	6.9×10^5
	NAA	0.05 *M* phosphate	3.7×10^5
	NAA	0.1 *M* phosphate	1.2×10^5
	SAA	Water control	7.0×10^5
	SAA	0.05 *M* phosphate	3.5×10^5
	SAA	0.1 *M* phosphate	1.9×10^5
2	NAA	Water control	4×10^6
	NAA	0.1 *M* phosphate	3×10^5
	SAA	Water control	2.5×10^6
	SAA	0.01 *M* phosphate	2.5×10^6
	SAA	0.05 *M* phosphate	5×10^5
	SAA	0.1 *M* phosphate	3×10^6
3	NAA	Water control	4×10^5
	NAA	0.05 *M* phosphate	2.8×10^5
	NAA	0.1 *M* phosphate	3.2×10^6
	NAA	0.05 *M* tris	4×10^6
	NAA	0.1 *M* tris	4×10^6

[a] On agar containing phosphate, the colonies were appreciably smaller than on agar without buffer. The colonies looked normal on agar with tris buffer.

agar solution at 50°, allowing the agar to gel, spinning the gel in an ultracentrifuge with a force of 50,000 × g for 2 hours, and assaying the supernatant liquid. The radioisotopic assay procedure reported by Kaiser (2) was used for the 1 mg./ml. or higher concentrations. Bioassays were used for the lower concentrations. In the bioassay procedure, standard and test solutions were diluted with supernatant liquid removed from agar gel in order to keep the solutions consistent. Potencies of the supernatant test solutions were estimated from standard curves using neomycin B as standard for B solutions and neomycin C for C solutions. Per cent binding was calculated by subtracting the per cent antibiotic remaining in the supernatant liquid after centrifugation from 100.

Table III shows that neomycin components were less adsorbed to Noble than to Bacto agar. When 3% potassium chloride was added to the system, no adsorption of neomycin B or C was detected.

Effect of the Agar on Neomycin Dose-Responses.—Because of the apparent difference in binding between Bacto and Noble agar, dose responses were obtained with neomycins B and C in media comparing the two agars. Streptomycin assay broth was prepared and adjusted to pH 7.9. Each agar was added to an aliquot of the broth for 1.5% solutions, and both media preparations were autoclaved. Ten-milliliter single layer test plates were made with agar seeded with 1% of a *S. aureus* culture incubated overnight at 32° in SAB. Antibiotic solutions were made in water. Each solution was added to four cylinders, one on each of four plates. The plates were incubated for 18 hours at 32°, and zone diameters were averaged for each solution. (Figure 1 indicates greater sensitivity with medium containing Noble agar than with medium containing Bacto agar.) The responses from neomycin B are almost identical to those from neomycin C.

Effect of Potassium Phosphate on the Response to Neomycin B in an Agar-Diffusion System.—Dose-responses were obtained comparing neomycin B in 0.1 *M* pH 7.9 potassium phosphate buffer to neomycin B in 0.1 *M* pH 7.9 tris buffer. The test plates were made as described in the preceding section, but only with the medium prepared with Noble agar. Figure 2 indicates lesser sensitivity but a slightly steeper slope with tris buffer.

Effect of Salt on the Activity of Neomycin B and Neomycin C.—Neomycin activity is known to be antagonized by salts (11, 12). The activity of neomycin C was reported (7) to be markedly suppressed by potassium chloride, more so than B, in

TABLE III.—ADSORPTION OF NEOMYCIN TO AGAR

Antibiotic	Concn. Antibiotic in Agar Gel, mg./ml.	% Bound[a] — Bacto Agar	Noble Agar
Neomycin B	0.05	...	56, 73
	0.20	...	62
	0.50	...	30, 40
	1.0	58, 67	35
	2.0	...	25
	10.0	...	0
Neomycin C	0.05	...	70, 80
	0.20	...	65
	0.50	...	36, 45
	1.0	43, 46, 53	25, 32
	2.0	...	18
	10.0	...	...

[a] Multiple numbers indicate results of different experiments.

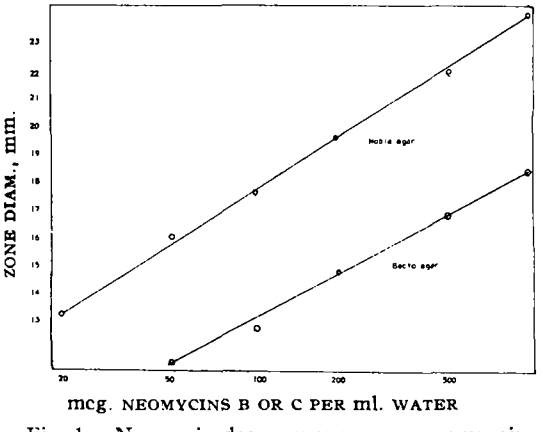

Fig. 1.—Neomycin dose-response curves comparing agars.

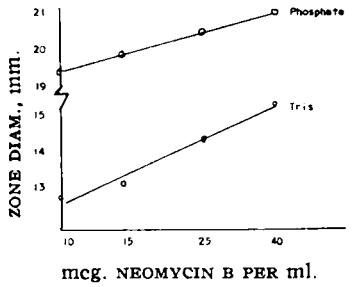

Fig. 2.—Neomycin standard dose-response curves comparing buffers.

an agar-diffusion system. This system was used to assay neomycin B in the presence of neomycin C (8). The difference in response between neomycin B and neomycin C in an agar diffusion system suggested studies on the effect of salt on the activity of each component in broth and agar systems.

Minimum inhibitory concentrations in SAB with and without 3% potassium chloride were determined for neomycin B and neomycin C against *S. aureus*. Each tube was inoculated with 0.1 ml. of a 1:100 dilution of a culture in SAB incubated overnight at 32°. The MIC end points shown in Table IV indicate that the salt antagonized neomycin C activity to a greater extent than it did B.

Since the antagonistic effect of salt may be different in agar and broth systems, a more intensive study was made of antagonism in agar against two species of bacteria reportedly used in neomycin assays, *S. aureus* FDA 209 (1–14) and *Bacillus subtilis* UC564 (8, 13–15). In a series of eight experiments, gradient plates were prepared according to the method described by Szybalski (16) with a 10-ml. SAA lower layer without antibiotic and a 10-ml. upper layer with antibiotic. In the plates with salt, 3% potassium chloride was added to both layers. The concentrations of antibiotic in the upper layer for plates without salt were 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, and 1.0 mcg./ml.; the concentrations for plates with salt were 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 mcg./ml. Each concentration was tested in duplicate plates. Cell suspensions of approximately 10 (7) cells per milliliter were streaked across the gradient with cotton swabs, and the plates were incubated at 32° for 24 hours. The MIC for each plate was determined by multiplying the fractional length of the streak by the antibiotic concentration in the top agar layer. Fractions of one-tenth or less and nine-tenths or more of the streaks were not used in the determinations. The results in Table V indicate a large day-to-day variation in MIC end points, but an analysis of salt antagonism ratios indicates that salt antagonizes neomycin C more than it does B. Also, the extent of antagonism differs with the two test organisms.

DISCUSSION

Many factors contribute to the responses obtained in agar-diffusion assays. To minimize the inescapable day-to-day variations in bioassays, the analyst attempts to standardize those factors which influence the response of the active agent. For some agents, designing an assay is routine; a buffered diluent, a nutrient medium, a susceptible test organism, an optimum temperature for growth, and the usual standardization of steps in the procedure. For many agents, the problems in developing an assay lead to "monumental headaches." The headaches become astronomical when the agent is a complex whose individual components react differently in the commonly accepted assay systems, particularly when the components are difficult to separate and the content ratios vary between test and standard preparations. Such is the case with neomycin.

Phosphate buffer, used as a diluent, is probably a cause of some of the day-to-day variation in neomycin assays. It antagonizes the activity of neomycin, more so than potassium chloride (Table I), but more important, it antagonizes the growth of the commonly used test organism, *S. aureus* (Table II). This is supported by the greater sensitivity to neomycin in phosphate buffer than in tris buffer (Fig. 2). The flatter slope obtained with phosphate buffer may be caused by the antagonism of the phosphate to the growth of the test organism, allowing concentrations of neomycin to be inhibitory which would be subinhibitory without phosphate.

Consider that potassium phosphate and neomycin are diffusing into the agar independently of each other. This is a situation in which two substances influence an assay response and only one is supposedly measured.

Neomycins B and C are both adsorbed to agar. The adsorption appears to be less on Noble agar than on Bacto agar (Table III). This is supported by the increased activity of neomycin in medium with

TABLE IV.—EFFECT OF 3% POTASSIUM CHLORIDE ON THE ACTIVITY OF NEOMYCIN AGAINST *S. aureus* IN BROTH

Concn. Antibiotic, mcg./ml.[a]	Neomycin B[b]				Neomycin C[b]			
	No salt		With KCl		No salt		With KCl	
	35 hrs.	72 hrs.	35 hrs.	72 hrs.	35 hrs.	72 hrs.	35 hrs.	72 hrs.
0	++	++	++	++	++	++	++	++
0.05	+±	+±	...	...	++	++	...	...
0.1	−±	±±	...	...	−−	++	...	...
0.2	−−	−−	++	++	−−	−−	++	++
0.5	−−	...	++	++	−−	−−	++	++
1.0	−−	...	++	++	−−	−−	++	++
2.0	−−	...	++	++	−−	−−	++	++
5.0	...	...	−−	−−	...	...	++	++
10.0	...	...	−−	−−	...	...	+−	++
20.0	...	...	−−	−−	...	...	−−	−−

[a] Each concentration was tested in duplicate. [b] Each sign represents a growth response in one tube. +, Good growth; ±, slight growth; and −, no growth.

TABLE V.—EFFECT OF 3% POTASSIUM CHLORIDE IN AGAR ON THE ACTIVITY OF NEOMYCIN

	Minimum Inhibitory Concn., mcg./ml.							
	B. subtilis				*S. aureus*			
	Neomycin B		Neomycin C		Neomycin B		Neomycin C	
Expt.	No Salt	With Salt	No Salt	With Salt	No Salt	With Salt	No Salt	With Salt
1	0.005	0.09	0.01	1.00	0.02	0.40	0.03	1.80
2	0.01	0.16	0.03	2.70	0.02	0.60	0.05	2.80
3	...	...	...	...	0.02	0.70	0.03	3.20
4	0.01	0.05	0.02	0.34	0.02	0.12	0.03	0.48
5	0.02	0.03	0.03	0.62	0.02	0.16	0.02	0.65
6	0.01	0.08	0.05	1.20	0.02	0.17	0.04	1.00
7	0.01	0.2	0.02	3.20	0.02	0.62	0.02	2.40
8	0.01	0.1	0.03	3.00	0.02	0.52	0.04	2.40
Av.	0.01	0.11	0.03	1.72	0.02	0.41	0.03	1.84
MIC Salt / MIC No salt	11		57		20		61	
C MIC Salt / MIC No salt / B MIC Salt / MIC No salt	5.4 ± 1.4 (95% Conf.)				3 ± 0.5 (95% Conf.)			

Noble agar (Fig. 1). Since salt releases the bound neomycin, a plausible explanation for the change in slope in the presence of salt as reported by Sokolski and Carpenter (8) is that neomycin is not bound in the salt agar and diffuses more freely. Noble agar with the lesser binding of neomycin may be a better agar to use for a lower day-to-day variation in the assay.

Salts appear to antagonize the activity of neomycin C more than B (Tables I and V). It is evident then that the ionic strength of the medium is an important factor for standardizing the assay procedure.

We have attempted to point out some of the factors that affect the responses of neomycins B and C in bioassay systems. Standardization of the neomycin assay necessitates an understanding of these factors.

Since the neomycin C content varies from lot-to-lot, the potency of one particular preparation will depend on the B:C ratio in the test and standard preparations. The variation of assay results between laboratories will depend on these ratios plus the differences in assay systems and how much of the C responds in these systems.

If neomycin C is included in the definition of neomycin, as it apparently is, then the present day assay methods should be made to assay the B and C additively. The Food and Drug Administration assay (14) performed in our laboratories measures only 50% of the C. Since all of the neomycin preparations tested in our laboratories contain some neomycin C, the problem becomes one of definition of potency. If the present concept of neomycin potency is retained, then rigid policies establishing universal standards and assay methods should be adopted. If neomycin C is to be considered as neomycin, then an assay design for the additive response to B and C would be more realistic than the existing methods.

SUMMARY

Salts antagonize the response of neomycin in agar-diffusion assay systems. Potassium phosphate is more antagonistic than is potassium chloride, and both salts antagonize neomycin C more than B.

The effect of 0.1 *M* buffers on *S. aureus*, a commonly used assay organism, indicated that potassium phosphate antagonized growth, while tris did not.

Neomycins B and C are both adsorbed to agar gel and desorbed with potassium chloride. The adsorption phenomenon slows neomycins diffusion in agar. Adsorption is less on Noble agar than on crude agar.

The extent of antagonism in an agar-diffusion system is more pronounced against *S. aureus* than against *B. subtilis*, with neomycin C being more antagonized than B. This emphasizes the importance of choice of test organisms for assays on preparations containing neomycin C.

Agreement on assay methods and standards are recommended for elimination of lab-to-lab differences in neomycin potency estimates.

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