Binding of Gentamicin and Other Aminoglycoside Antibiotics to Mycelium of Various Actinomycetes

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Gentamicin, an aminoglycoside antibiotic, occurs both free in the fermentation filtrate and bound to the mycelium of the producing organism, *Micromonospora purpurea*. The bound gentamicin, which represents the major portion of the total quantity present in the fermentation broth, was released after exposure to acid, alkali, or sonic disruption. Washing the mycelium with distilled water, heat treatment, and the addition of sodium chloride to the fermentation medium were not effective methods for releasing bound gentamicin. Gentamicin and other aminoglycoside antibiotics were effectively adsorbed from neutral aqueous solutions by the acid-extracted mycelium of *M. purpurea*. In addition, the acid-extracted mycelium of actinomycetes other than *M. purpurea* were shown to be able to adsorb gentamicin from solution.

Although the binding of aminoglycoside antibiotics to the mycelium of their respective producing organisms is well known, few studies have been published regarding this phenomenon. In the case of streptomycin, early studies showed that the addition of sodium chloride to fermentation medium before sterilization resulted in an increased yield of the antibiotic in the fermentation broth filtrate (4). A later study (3) showed that the added sodium chloride only effected the release of streptomycin bound to the mycelium rather than causing greater production of streptomycin by the producing culture, and, on the contrary, sodium chloride was found to actually decrease the total streptomycin production of the organism. Treatment of fermentations with acid (3; G. W. Rake, W. L. Koerber, and R. Donovick, U.S. Patent 2461922, 1949), alkali (3), highly dissociatable salts (R. B. McCormack, A. F. Langlykke, and D. Perlman, U.S. Patent 2656300, 1953), and sonic disruption (3) were shown to be efficient methods for releasing bound streptomycin, thereby allowing greater recoverable yields of streptomycin from fermentation broth.

Gentamicin is produced in submerged fermentation as an antibiotic complex consisting of three major components and several minor components. Much of the antibiotic is bound to the mycelium of the producing cul-

ture, *Micromonospora purpurea*, and must be acid extracted for quantitative recovery (6). By means of acid extraction, the bound gentamicin is released from the mycelium and made soluble in the aqueous phase of the fermentation broth. The aqueous solution is separated from the mycelium and other particulate matter by centrifugation or filtration. The three major components are then isolated from the filtrate and purified for use as a chemotherapeutic agent. In this paper, we report the results of our studies on the efficacy of various physical and chemical techniques for releasing gentamicin bound onto *M. purpurea* mycelium. In addition, studies regarding the specificity, or lack of specificity, of the binding of gentamicin and other aminoglycoside antibiotics to mycelium from a variety of *Micromonospora* and *Streptomyces* organisms, including *M. purpurea*, are reported.

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MATERIALS AND METHODS

Organisms. The following species of antibiotic-producing *Micromonospora* were employed in this study: *M. purpurea* NRRL 2953 (gentamicin), *M. megalomicea* NRRL 3274 (megalomicin), *M. carbonacea* NRRL 2972 (everninomicin), *M. halophytica* NRRL 2998 (halomicin) and *M. chalcone* NRRL 3222 (antibiotic 460). Antibiotic-producing

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Streptomyces cultures used in the study consisted of the following: *S. fradiae* ATCC 10745 (neomycin), *S. rimosus* Forma paromomycinus ATCC 14827 (paromomycin), and *S. griseus* ATCC 12475 (streptomycin). Inocula for vegetative stages were maintained at -20°C .

Media. The constituents of the vegetative (6) and fermentation (5) media for the *Micromonospora* cultures were previously reported. The media used for the *Streptomyces* cultures were the same, except for the substitution of dextrose for potato dextrin as the carbohydrate source in the fermentation medium.

Growth conditions. The vegetative stage consisted of inoculating a 250-ml Erlenmeyer flask containing 70 ml of the vegetative medium with 2.5 ml of frozen broth, and incubating it for 48 h at 35°C on a rotary shaker with a 1-inch (2.54 cm) stroke at 300 rpm. The fermentation stage consisted of inoculating a 500-ml Erlenmeyer flask containing 100 ml of fermentation medium with 5 ml of the vegetative stage, and incubating it for 120 h at 28°C on a rotary shaker with a 2-inch (5.08 cm) stroke at 300 rpm.

Antibiotic assay. Supernatants containing gentamicin or sisomicin were assayed by the disk plate method of Oden et al. (2) and reported as the base. Assays of supernatants containing kanamycin, neomycin, and streptomycin were performed as previously described (1).

Preparation of washed cells. One liter of fermentation broth was centrifuged at $1,500 \times g$, and the supernatant was discarded. The packed mycelium was suspended in distilled water, centrifuged, and resuspended in distilled water two additional times. The final suspension was adjusted to the initial volume of the whole fermentation broth.

pH adjustment. Sulfuric acid and sodium hydroxide were used to make pH adjustments of both whole broth and washed cell preparations. Whole broth and washed cell preparations were maintained at the indicated pH values for 1 h prior to centrifugation. Supernatants were neutralized before assays were performed. Volume changes were noted, and assay results were corrected accordingly.

Sonic treatment. A Branson S-125 sonifier was used at full power for the length of time indicated. Samples of 30 ml were sonically disrupted in a 50-ml stainless-steel centrifuge tube cooled in an ice bath. The sonically disrupted whole broth and washed mycelium preparations were centrifuged at $1,500 \times g$ for 15 min, and the resulting supernatants were assayed for antibiotic potency.

Antibiotic adsorption by mycelium. Antibiotics used in these studies were: kanamycin and neomycin sulfates (Sigma Chemical Co., St. Louis, Mo.), streptomycin sulfate (Charles Pfizer and Co., New York, N.Y.), and gentamicin and sisomicin sulfates (prepared by Schering Corp., Bloomfield, N.J.). Antibiotic adsorption experiments were performed as follows: the mycelium was repeatedly acid extracted until antibiotic activity could no longer be detected in the filtrates by disk assay. The mycelium was brought to original volume with distilled water and neutralized with sodium hydroxide. Antibiotics were dissolved in 10-ml portions of the mycelial suspension in test tubes to give $500 \mu\text{g/ml}$ concentrations. The tubes

were then incubated for 2 h at 28°C and centrifuged as previously described, and the supernatants were assayed for antibiotic activity.

Chromatography. Paper chromatography was carried out by the method of Wagman and co-workers (5).

RESULTS

Extraction pH. Figure 1 shows the effect of pH on the quantities of gentamicin in supernatants from whole broth and washed cell preparations of fermentation broth. The supernatants were neutralized prior to assay. The values shown were corrected for the dilution errors incurred during pH manipulations. The supernatants showed highest potencies when the samples were extracted at the pH extremes and were lowest near neutrality. The acidic extracts gave clear supernatants when centrifuged at $1,500 \times g$ for 15 min, but the alkaline extracts were very turbid under these conditions.

To determine whether the pH of extraction had an effect on the ratio of the major components of the gentamicin complex, approximately equal amounts of antibiotic from the pH 1, 3, 5, 7, 9, 11, and 13 supernatants were subjected to chromatography on Whatman no. 1 paper as previously detailed (5). Bioautography of the paper chromatograms showed no gross variations by visual inspection.

For the remaining studies, the pH 2 extract was chosen as the control because it efficiently extracted bound gentamicin and resulted in little dilution error from pH adjustment. A 1-h extraction period was used for convenience since exposure times (at pH 2) of 1 min through 3 h resulted in yields of equal gentamicin potencies.

Addition of sodium chloride to the fermentation medium. In an effort to determine whether the addition of NaCl to the fermentation medium would dramatically increase the potency of gentamicin in supernatants, as has been shown for streptomycin (4), various amounts of NaCl were incorporated into the fermentation medium as indicated in Table 1. In the range of 0.25 to 5.0% (wt/vol) of added NaCl, the data indicate that no significant effects on potency of neutral extracts could be detected. When these same broths were acid extracted prior to centrifugation, again little effect from added salt was seen in the potencies of the supernatants.

Sonic treatment. Sonic disruption was tested as an alternative method for releasing gentamicin bound to mycelium. The effect of sonic treatment on whole broth and washed cell preparations is shown in Table 2. Before sonic treatment, about 40% of the total antibiotic

FIG. 1. Gentamicin potency in supernatants of *Micromonospora purpurea* fermentations after pH adjustment. Closed bars represent activity from whole broth; hatched bars represent activity from washed cells prepared from the whole broth.

TABLE 1. Effect of addition of NaCl to the medium on the potency of gentamicin in supernatants of *Micromonospora purpurea* fermentations^a

% NaCl in medium (wt/vol)	Gentamicin in supernatants (μg/ml)	
	pH 7	pH 2
0	302	671
0.25	306	707
0.5	313	723
1.0	284	716
2.0	290	688
3.0	320	649
4.0	324	659
5.0	327	616

^a The NaCl was added prior to sterilization of the medium.

produced was free in the supernatant from the whole broth and the remainder was bound to the mycelium. Only a short period of sonic treatment was necessary to release another 30% of the total antibiotic from the mycelium. The remaining 30% could be released by acid extraction but not by additional sonic treatment. In washed cell preparations nearly all of the antibiotic is bound to the mycelium and about 60% of this bound antibiotic was released by sonic disruption. In both whole broth and washed cells, about 150 to 170 μg/ml remained bound after sonic treatment but was released by acid extraction.

Heat effects. Although ice baths were employed during sonic disruption, local heating effects could not be prevented. Therefore, the effect of heat on whole broth, washed cell preparations, and aqueous solutions was examined. As shown in Table 3, it was found that the increase of temperature and the lengthening of exposure time resulted in lower potencies of the supernatants of whole broth and washed cell preparations. Heat had no effect on aqueous solutions.

Adjustment of whole broth samples to pH 2 prior to heating prevented most of the loss of

TABLE 2. Gentamicin activity in supernatants after sonic disruption of whole broth and washed cell preparations from *Micromonospora purpurea* fermentations

Sonic disruption (min)	Gentamicin in supernatants (μg/ml)			
	Whole broth		Washed cells	
	pH 7	pH 2	pH 7	pH 2
0	280	743	12	400
1	486	669	194	387
3	518	750	244	406
6	501	704	264	401
10	569	740	262	417

TABLE 3. Effect of heat on gentamicin potencies of whole broth and washed cell preparations of *Micromonospora purpurea* fermentations and on aqueous solutions of gentamicin

Temp (C)	Gentamicin in supernatants (μg/ml)			
	1 h ^a		24 h ^a	
	7°	2	7	2
Whole broth				
30	280	813	256	785
65	253	748	224	672
100	263	658	86	209
Washed mycelium				
30	13	379	15	427
65	9	392	13	358
100	46	394	48	294
Aqueous gentamicin solutions ^c				
30	768	790	803	718
65	721	707	762	733
100	738	749	756	751

^a Exposure time at temperatures shown.

^b Extraction pH.

^c The aqueous solution contained an initial concentration of approximately 750 μg of gentamicin per ml.

potency due to heat. In addition, by adjusting to pH 2 and reheating, most of the activity lost due to heat was recovered.

Specificity of adsorption. To determine whether the binding of gentamicin to the mycelium of *M. purpurea* was specific for gentamicin or if other aminoglycoside antibiotics were adsorbed as well, the mycelium was acid extracted until gentamicin was no longer detected in the pH 2 washes. This extracted mycelium was neutralized and tested for its ability to adsorb gentamicin, sisomicin, kanamycin, neomycin, and streptomycin from solution at pH 7. Concentrations of 500 $\mu\text{g/ml}$ of the above-named antibiotics were dissolved in 10-ml samples of the extracted *M. purpurea* mycelial suspension. After incubation, the supernatants obtained after centrifugation showed less than 10% of the initial potency in all cases. Adjusting to pH 2 before centrifugation allowed full recovery of the added antibiotics (Table 4).

In addition, other species of *Micromonospora* which produce sisomicin, everniomicin, halomycin, and megalomicin, as well as *Streptomyces* species which produce neomycin, paromomycin, and streptomycin, were tested for the ability of their acid-extracted and neutralized mycelium to adsorb gentamicin from solution at pH 7. *M. purpurea*, which produces gentamicin, was included as a control. Quantitatively, the mycelium of these other species of *Micromonospora*, as well as the species of *Streptomyces* adsorbed gentamicin to approximately the same degree as mycelium of *M. purpurea* with the exception of *S. griseus* (Table 5). It is probable that the apparently deficient gentamicin-adsorbing ability of *S. griseus* mycelium was due to decreased cell mass per unit volume resulting from relatively poor growth of the culture in the fermentation medium, rather than a qualitative difference in the adsorption ability of the mycelium.

DISCUSSION

It is apparent from the data presented that the most efficient method for releasing mycelial-bound gentamicin into solution is by pH adjustment of the whole broth to high or low pH values. Sonic disruption was effective for releasing only about one-half of the bound gentamicin. The application of heat to the whole broth and the addition of sodium chloride to the fermentation medium were not efficacious for gentamicin release.

The results of this study also demonstrate the non-specificity of the bond between gentamicin and *M. purpurea* mycelium, and suggest a

TABLE 4. Ability of acid-extracted *Micromonospora purpurea* mycelium to adsorb aminoglycoside antibiotics from solution at pH 7

Aminoglycoside antibiotic ^a	Assays of supernatants ^b ($\mu\text{g/ml}$)	
	pH 7	pH 2
Gentamicin	14	520
Sisomicin	18	483
Kanamycin	44	489
Neomycin	10	470
Streptomycin	35	564

^a The initial concentration of the various antibiotics in their respective solutions was approximately 500 $\mu\text{g/ml}$.

^b Assayed postadsorption.

TABLE 5. Ability of neutralized, acid-extracted mycelium from various species of *Micromonospora* and *Streptomyces* to adsorb gentamicin from solution at pH 7^a

Culture	Gentamicin ^a potencies in supernatants ($\mu\text{g/ml}$) ^b	
	pH 7	pH 2
<i>Micromonospora purpurea</i> (NRRL 2953)	20	524
<i>M. megalomicea</i> (NRRL 3274) ..	54	481
<i>M. carbonacea</i> (NRRL 2972) ...	20	479
<i>M. halophytica</i> (NRRL 2998) ..	49	507
<i>M. chalcea</i> (NRRL 3222)	27	539
<i>Streptomyces fradiae</i> (ATCC 10745)	91	486
<i>S. rimosus</i> Forma <i>paromomycinus</i> (ATCC 14827)	33	500
<i>S. griseus</i> (ATCC 12475)	162	491

^a Initial concentration of gentamicin was approximately 500 $\mu\text{g/ml}$.

^b Assayed postadsorption.

general lack of specificity for aminoglycoside antibiotic binding to mycelium. Acid-extracted and neutralized mycelia from a variety of *Micromonospora* species, producing a number of different classes of antibiotics, were able to bind gentamicin in neutral solutions as effectively as the mycelia from *M. purpurea*, *M. inyoensis*, or the aminoglycoside antibiotic-producing *Streptomyces* species used in the study. In addition, the ability of *M. purpurea* mycelium to bind aminoglycoside antibiotics other than gentamicin indicates a general affinity of aminoglycosides for some common structure found on the cell walls of various *Micromonospora* and *Streptomyces* species, and possibly a variety of other microorganisms. The speed and efficacy of pH adjustment as a means of

liberating bound gentamicin, combined with the non-specificity of the binding phenomenon, suggest some form of ionic bonding as the operating principle.

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