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Effect of Assay Medium on the Antibacterial Activity of Certain Penicillins and Cephalosporins

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It is well known that the composition of the assay medium greatly affects the antimicrobial activity of aminoglycoside antibiotics. A similar response has now been observed with certain penicillins and cephalosporins. In the case of these compounds, this effect is apparently governed by the chemical nature of the penicillin 6- and cephalosporin 7-side chains. In comparison with their activity in Nutrient Broth, the activity of some of the β-lactam antibiotics that have a weakly basic or basic group in their side chain was reduced as much as 40-fold in one or more of the following media: Mueller-Hinton, Trypticase soy, antibiotic assay, and heart infusion broths. In contrast, the assay medium had no effect on the activity of those compounds possessing an acidic or a nonionizable function in their side chain. The extent to which medium influences the antibacterial activity was also dependent upon the assay method and the organism, the effect being more pronounced in broth dilution than in agar dilution tests and occurring more frequently with gram-negative than with gram-positive organisms.

The effect of the composition of assay medium on the antibacterial activity of aminoglycosides has been extensively investigated and is generally attributed to the concentration of ions in the medium (2, 3, 8, 11). Thus, a marked decrease in the activity of gentamicin against *Pseudomonas aeroginosa* was observed in media with high magnesium and calcium concentrations (3, 4). Similarly, the addition of sodium chloride to nutrient broth decreased the activity of gentamicin against strains of *Serratia marcescens* and *Escherichia coli* (6). Kanamycin's inhibitory action against the latter species was reduced in similar fashion when the inoculum was diluted in physiological saline rather than water (5).

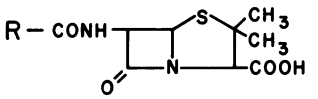
In contrast, very few studies of this nature have been undertaken with penicillins and cephalosporins. Ronald and Turck (9) reported that cephaloglycin was considerably more active when tested in nutrient broth than when tested in Trypticase soy broth because of the greater stability of the compound in the former medium. Wick and Boniece (12) found that significant differences in the activities of cephalothin, cephalexin, and cephaloridine could be obtained in different media if the conditions for assaying were not carefully standardized.

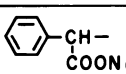
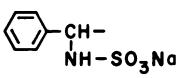
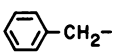
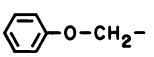
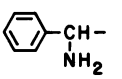
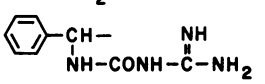
The present report describes the effect of different media on the apparent antibacterial activity of some penicillins and cephalosporins. An attempt has been made to relate the observed medium effects to the chemical structure of 6- or 7-side chains of the β-lactam antibiotics tested.

MATERIALS AND METHODS

Antibiotics. Commercially available compounds used in this study were sodium cephalothin and cephalexin monohydrate (Lilly Laboratories); disodium carbenicillin (Beecham Laboratories); and sodium ampicillin, potassium penicillin G, and sodium penicillin V (Bristol Laboratories). Also evaluated were several new β-lactam antibiotics that had been synthesized at Bristol Laboratories. These were sodium cephapirin; BL-P 1462, disodium 6-[R-α-(sulfoamino)-phenylacetamido]penicillinate; BL-P 1654, sodium-6-[R-α-(guanylureido)phenyl-acetamido penicillinate; BL-S 215, 7-(phenylacetimidoylaminoacetamido)-cephalosporanic acid; BL-S 217, 7-[α-(1-methyl-4-pyridinothio)acetamido]cephalosporanic acid; and BL-S 339, 7-phenylacetimidoylaminoacetamido)-3-(2-methyl-1,3,4-thiadiazol-5-ylthiomethyl)ceph-3-em-4-carboxylic acid. Structures of these compounds are shown in Tables 1 and 2.

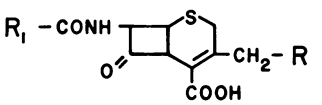
TABLE 1. *Classification and acid dissociation constants of selected penicillins*

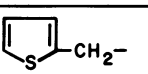
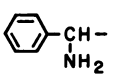
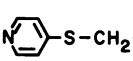
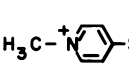
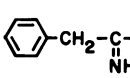
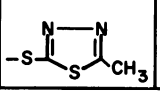
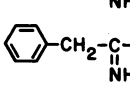


PENICILLIN	R			CLASSIFICATION OF PENICILLIN
	STRUCTURE	(pK_a)	% IONIZED AT pH 7.0	
CARBENICILLIN		3.96 ± 0.08	99.9	ACIDIC
BL-P 1462		<2.0[a]	>99.9	ACIDIC
PENICILLIN G		–	0	NON-IONIZED
PENICILLIN V		–	0	NON-IONIZED
AMPICILLIN		7.24 ± 0.04	50	WEAKLY BASIC
BL-P 1654		7.46 ± 0.05	50	WEAKLY BASIC

[a] VALUE ESTIMATED

TABLE 2. *Classification and acid dissociation constants of selected cephalosporins*



CEPHALOSPORIN	R	R_1			CLASSIFICATION OF CEPHALOSPORIN
	STRUCTURE	STRUCTURE	(pK_a)	% IONIZED AT pH 7.0	
CEPHALOTHIN	$-OCOCH_3$		–	0	NON-IONIZED
CEPHALEXIN	$-H$		7.32 ± 0.03	50	WEAKLY BASIC
CEPHAPIRIN	$-OCOCH_3$		5.35 ± 0.05	1	WEAKLY BASIC
BL-S 217	$-OCOCH_3$		>11.0[a]	>99.9	BASIC
BL-S 215	$-OCOCH_3$		10.5[a]	99.9	BASIC
BL-S 339			10.6 ± 0.1	99.9	BASIC

[a] VALUE ESTIMATED

Organisms. The strains of *Staphylococcus aureus*, *E. coli*, *Klebsiella pneumoniae*, and *P. aeruginosa* used in this study were predominantly of clinical origin. However, organisms were selected so that all members of a particular species responded uniformly to a given β-lactam antibiotic.

Media. The five media utilized in bacterial susceptibility tests were nutrient broth (NB), Mueller-Hinton broth (MHB), and heart infusion broth (HIB) from Difco; and antibiotic assay Broth (AAB) and Trypticase soy broth (TSB) from BBL. These broths were supplemented with 1% Ion-agar No. 2 (Colab Laboratories, Inc.) for agar dilution and disc-agar diffusion tests.

Bacterial susceptibility tests. The two-fold serial broth dilution method was used to study the effect of medium on antibiotic activity. The inocula were prepared by using the appropriate assay broth to make 10^{-4} dilutions of the 18-hr cultures. Tubes of the seeded antibiotic-containing media were incubated overnight at 37 C, after which the minimal inhibitory concentration (MIC) was determined. The MIC is considered to be the lowest concentration of antibiotic that prevents visible growth.

The effect of solid media on antibiotic activity was studied in tests in which agar dilution and disc-agar diffusion techniques were used. In agar dilution tests, the multiple inoculator device of Steers, Foltz, and Graves (10) was used to apply 0.003 ml of 10^{-2} dilutions of overnight broth cultures to the surface of antibiotic-containing agar plates. MIC values were determined after overnight incubation at 37 C. For the disc-agar diffusion technique (1), overnight broth cultures were first adjusted to an optical density of 0.2 at 540 nm in cuvettes (11.5 by 10 mm). The cell suspensions were then diluted 250-fold in water and streaked on the surface of the agar plates. Paper discs containing 20 μg of the appropriate antibiotic were placed on the inoculated agar surface, and the diameter of inhibition zones was measured after overnight incubation of the agar plates at 37 C.

RESULTS

The penicillins and cephalosporins discussed in this report are listed in Tables 1 and 2. They are classified as acidic, basic, or nonionized according to the nature of their 6- and 7-side chain. Cephalosporins with an acidic function in the 7-side chain have not been included in these studies, as all available representatives of this group had little antibiotic activity. Tables 1 and 2 include the structure, the pK_a of the ionizable function in the side chain, and an estimate of the percentage of compound ionized at the *p*H of the assay media (6.8 to 7.2).

The influence of five liquid media on the apparent activity of selected β-lactam antibiotics against representative strains of several bacterial species is considered in Tables 3–8. Three columns of figures are shown: (i) the mean MIC in NB; (ii) the range of MIC values in NB; and (iii) the ratio of the mean MIC obtained in each of the four other media to the mean MIC in NB. NB was chosen as the reference medium because the inhibitory activity of the antibiotics was generally greatest in this medium. For the purposes of this study, a significant medium effect was considered to have occurred when the activity of an antibiotic in a given test medium varied by at least threefold from its activity in the reference medium.

The inhibitory action of the β-lactam antibiotics against eight penicillinase-negative strains of *S. aureus* was not significantly affected by the type of assay medium (Tables 3 and 4). However, for one weakly basic penicillin and two basic cephalosporins (Tables 1 and 2), ratios of their activities in some of the test media to those found in the reference medium approached the cut-off point for significance (3.0). Thus, the penicillin BL-P 1654 was 2.6 to 2.8 times more active in NB than in the other four media (Table 3), and BL-S 215 and BL-S 339 were more active in NB than in TSB by factors of 2.7 and 2.5, respectively (Table 4). The other basic cephalorsporin, BL-S 217, was uniformly active in all test media.

In tests with eight strains of *E. coli* (Tables 5 and 6), the inhibitory action of β-lactam antibiotics with an acidic or nonionized 6- or 7-side chain was not influenced by medium. However, the weakly basic penicillin BL-P 1654 and all of the cephalosporins with a basic side chain were significantly more active in NB than in some of the other media. Differences were particularly striking in the case of BL-P 1654, BL-S 215, and BL-S 339.

The MIC of the weakly basic penicillin BL-P 1654 was almost seven times as high in MHB as in NB. A significant but borderline difference was also found for TSB, and the compound's effectiveness in AAB did not differ from that observed in NB. Potency of BL-S 215 and BL-S 339, which are basic cephalosporins with identical 7-side chains, was significantly lower in all four test media than in NB. The average of the ratios for all media was 8 for BL-S 215 and 40 for BL-S 339. The antibacterial effect of BL-S 217, the remaining basic compound (Table 6), was moderately, but significantly, reduced in MHB (ratio of 3.6) and HIB (ratio of 3.8) as compared to NB. Finally, a value (2.7) approaching significance was obtained for the weakly basic penicillin ampicillin when its MIC in TSB was compared with that found in NB.

The effect of medium on the antipseudomonal activities of β-lactam antibiotics was investigated only with the penicillins carbenicil-

TABLE 3. *Effect of the assay medium on the antibiotic activity of various penicillins against eight Staphylococcus aureus strains*

Penicillin	Nature of 6-side chain	Nutrient broth		Ratio of geometric mean MIC (test broth/nutrient broth)			
		Geometric mean MIC (μg/ml)	Range of MIC	Mueller-Hinton	Trypticase soy	Antibiotic assay	Heart infusion
Carbenicillin .	Acidic	0.28	0.25–0.5	0.82	0.71	0.61	0.71
BL-P 1462 ...	Acidic	2.8	1.25–5.0	0.75	0.71	0.5	0.61
Penicillin G ..	Nonionized	0.02	0.016–0.032	1.8	1.1	0.59	1.3
Penicillin V ..	Nonionized	0.02	0.016–0.032	0.95	1.1	0.55	0.85
Ampicillin ...	Weakly basic	0.1	0.063–0.25	0.48	1.1	0.63	0.58
BL-P 1654 ...	Weakly basic	0.15	0.063–0.25	2.8	2.8	2.8	2.6

TABLE 4. *Effect of the assay medium on the antibiotic activity of various cephalosporins against eight Staphylococcus aureus strains*

Cephalosporin	Nature of 7-side chain	Nutrient broth		Ratio of geometric mean MIC (test broth/nutrient broth)			
		Geometric mean MIC (μg/ml)	Range of MIC	Mueller-Hinton	Trypticase soy	Antibiotic assay	Heart infusion
Cephalothin ..	Nonionized	0.07	0.02–0.16	0.43	2.1	0.43	1.3
Cephalexin ..	Weakly basic	1.1	0.6–1.25	1.6	1.2	1.6	1.8
Cephapirin ..	Weakly basic	0.04	0.02–0.08	2.3	1.8	1.8	0.75
BL-S 217	Basic	0.24	0.16–0.32	0.92	1.1	0.67	0.92
BL-S 215	Basic	0.62	0.16–1.25	1.8	2.7	1.8	1.8
BL-S 339	Basic	0.12	0.04–0.32	2.0	2.5	1.3	2.3

TABLE 5. *Effect of the assay medium on the antibiotic activity of various penicillins against eight Escherichia coli strains*

Penicillin	Nature of 6-side chain	Nutrient broth		Ratio of geometric mean MIC (test broth/nutrient broth)			
		Geometric mean MIC (μg/ml)	Range of MIC	Mueller-Hinton	Trypticase soy	Antibiotic assay	Heart infusion
Carbenicillin .	Acidic	17.7	8–32	0.61	0.55	0.55	0.5
BL-P 1462 ...	Acidic	35.2	16–125	0.91	1.1	0.82	0.91
Penicillin G ..	Nonionized	35.0	16–63	0.61	1.0	0.61	1.1
Penicillin V ..	Nonionized	168.2	125–250	0.89	1.8	1.0	1.3
Ampicillin ...	Weakly basic	1.5	1–4	2.0	2.7	1.1	1.8
BL-P 1654 ...	Weakly basic	0.41	0.25–1	6.6	3.7	1.4	6.7

lin, BL-P 1462, and BL-P 1654, since the other compounds are not active against members of this species (Table 7). Once again, the activity of the two penicillins that possess an acidic side chain was not influenced significantly by medium, whereas the weakly basic compound BL-P 1654 was 3.5- and 4.9-fold more active in the reference medium than in MHB and HIB, respectively.

Medium effects were also determined for the cephalosporins in tests involving eight strains of *K. pneumoniae* (Table 8). Here, significant changes in activity were restricted to BL-S 215 and BL-S 339, although a near-significant difference was found for BL-S 217 when results obtained in HIB and NB were compared. As had been observed with *E. coli* strains, BL-S 339 activity was influenced by medium to a greater extent than was that of BL-S 215.

The preceding studies conducted in liquid

TABLE 6. *Effect of the assay medium on the antibiotic activity of various cephalosporins against eight Escherichia coli strains*

Cephalosporin	Nature of 7-side chain	Nutrient broth		Ratio of geometric mean MIC (test broth/nutrient broth)			
		Geometric mean MIC (μg/ml)	Range of MIC	Mueller-Hinton	Trypticase soy	Antibiotic assay	Heart infusion
Cephalothin ..	Nonionized	9.5	4–16	1.3	0.6	0.55	0.6
Cephalexin ...	Weakly basic	6.2	2–8	0.89	1.5	1.1	1.7
Cephapirin ..	Weakly basic	5.2	2–8	2.6	2.2	1.1	1.2
BL-S 217	Basic	2.1	2–4	3.6	2.3	2.8	3.8
BL-S 215	Basic	5.0	4–8	7.9	8.6	6.4	9.8
BL-S 339	Basic	0.21	0.125–0.5	33.3	53.8	27.1	41.4

TABLE 7. *Effect of the assay medium on the antibiotic activity of various penicillins against eight Pseudomonas aeruginosa strains*

Penicillin	Nature of 6-side chain	Nutrient broth		Ratio of geometric mean MIC (test broth/nutrient broth)			
		Geometric mean MIC (μg/ml)	Range of MIC	Mueller-Hinton	Trypticase soy	Antibiotic assay	Heart infusion
Carbenicillin .	Acidic	177	63–250	0.4	0.42	0.46	0.65
BL-P 1462 ...	Acidic	177	63–250	0.55	0.36	0.36	0.65
BL-P 1654 ...	Weakly basic	1.5	0.25–4	3.5	1.7	0.8	4.9

TABLE 8. *Effect of the assay medium on the antibiotic activity of various cephalosporins against eight Klebsiella strains*

Cephalosporin	Nature of 7-side chain	Nutrient broth		Ratio of geometric mean MIC (test broth/nutrient broth)			
		Geometric mean MIC (μg/ml)	Range of MIC	Mueller-Hinton	Trypticase soy	Antibiotic assay	Heart infusion
Cephalothin ..	Nonionized	4	1–8	1.1	0.78	0.93	1.0
Cephalexin ..	Weakly basic	8	4–16	0.84	0.78	0.78	0.71
Cephapirin ..	Weakly basic	2	1–4	1.7	1.1	1.2	2.4
BL-S 217	Basic	1.1	0.5–2	1.5	2.4	1.2	2.8
BL-S 215	Basic	7.3	4–16	7.3	12.2	4.0	15.8
BL-S 339	Basic	0.6	0.32–1.25	11.8	16.7	9.8	19.8

medium were extended to include an investigation of the possible effects of solid medium on the activity of two medium-sensitive compounds, BL-P 1654 and BL-S 339. Agar dilution and disc-agar diffusion techniques were used to determine the susceptibility of 21 strains of *P. aeruginosa* to BL-P 1654 and the susceptibility of 30 strains of *E. coli* to BL-S 339 (Table 9).

By the agar dilution method, BL-P 1654 was six to seven times more active on nutrient agar (NA) than on Mueller-Hinton agar (MHA) and heart infusion agar (HIA), whereas its activity on antibiotic assay agar and Trypticase soy agar did not differ significantly from its activity in the reference medium. These results are virtually identical with those obtained in liquid medium (Table 7). Moreover, the average diameter of the zones of inhibition produced by 20-μg discs of BL-P 1654 proved to be markedly smaller on MHA and HIA than on the other three media, as would be expected on the basis of results obtained by broth and agar dilution techniques.

TABLE 9. *Influence of the test medium on the activity of BL-P 1654 and BL-S 339 as determined by the agar dilution and disc-agar diffusion tests*

Compound	Organism	Test method	Medium				
			Nutrient	Antibiotic assay	Trypticase soy	Mueller-Hinton	Heart infusion
BL-P 1654	*Pseudomonas aeruginosa* (21 strains)	Agar dilution	1.1[a]	0.6	1.5	6.6	7.0
		Disc-agar diffusion	29.3[b]	28.5	26.0	21.5	21.5
BL-S 339	*Escherichia coli* (30 strains)	Agar dilution	0.85	4.1	5.9	4.3	5.0
		Disc-agar diffusion	31.1	20.1	23.5	23.4	20.4

[a] Geometric mean MIC, in micrograms per milliliter, as determined by the method of Steers et al. (10).
[b] Average zone sizes, in millimeters, obtained with discs containing 20 μg of the indicated antibiotic. Values determined by the method of Bauer et al. (1).

Similarly, MIC values of BL-S 339 were five to seven times lower on NA than on the other solid media (Table 9). Although this result parallels that obtained with liquid media (Table 6), absolute differences were much smaller on agar than in broth. In the disc-agar diffusion method, as was found with broth and agar dilution techniques, BL-S 339 was appreciably more active on NA than on the other media.

DISCUSSION

The preceding studies show that the antibacterial activity of certain β-lactam antibiotics is affected by the assay medium, the extent of this effect varying with the test organism. These results were obtained by use of broth dilution, agar dilution, and disc-agar diffusion techniques.

Medium-induced variations in activity of the β-lactam antibiotics were greatest with gram-negative organisms and were restricted to compounds with a basic function in their 6- or 7-side chain. However, no correlation between the extent of the medium effect and the fraction of positively charged ions in the side chain at *p*H 7 was observed. Two basic compounds, BL-S 215 and 339, whose ionizable function in the 7-side chain has a pK_a value above 10 and is therefore more than 99.9% ionized at *p*H 7 were markedly, but nevertheless variably, affected by the type of assay medium, whereas the third basic compound, BL-S 217, was only minimally affected. The weakly basic compound BL-P 1654, whose ionizable function in the side chain has a pK_a of approximately 7.5 and is thus about 50% ionized at *p*H 7, was significantly affected by medium whereas two other compounds with similar pK_a values, ampicillin and cephalexin, showed little or no response to the test medium. The fourth weakly basic compound, cephapirin, whose side chain function is about 1% ionized at *p*H 7, was also virtually unaffected by medium. Thus, the mere presence of a basic function in the side chain gives no assurance that a compound will vary in its effectiveness in different media.

The antibiotic activity of aminoglycosides possessing strongly basic groups is known to be affected by the concentration of cations in the assay medium. Preliminary data have now been obtained which suggest that the cation content of the assay medium may also be responsible for the medium effects observed among basic penicillins and cephalosporins. Additional studies are planned which may clarify the precise role that cations play in modifying the activity of β-lactam antibiotics.

One practical consideration resulting from this study is that the variables affecting susceptibility tests must be noted early in the investigation of β-lactam antibiotics so that their true therapeutic value can be recognized. This also raises the question as to whether any standardized method of testing can be applied to all drugs. For example, data obtained in experimental infections of the mouse showed that the degree of therapeutic effectiveness of BL-P 1654 is more accurately predicted by susceptibility test results obtained on Mueller-Hinton medium than on NA (K. E. Price, *unpublished data*), whereas Misiek et al. (7) found that the reverse situation applies in the case of BL-S 339. Appropriate clinical laboratory studies should ultimately establish the medium and test conditions that most accurately predict the degree of therapeutic efficacy of these compounds in man.

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