SIDE CHAIN STEREOISOMERISM AND ANTISTAPHYLOCOCCAL POTENCY OF PENICILLINS *

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Penicillins may be regarded as acylation products of 6-aminopenicillanic acid (Figure 1). This bicyclic dipeptide results from cyclization of L-cysteinyl-L-valine (1) and is loosed into the culture medium in good yield when a strain of *Penicillium chrysogenum* (W.51.20) is cultured in the absence of side chain precursors suitable for acylation at the 6-amino grouping (2). With the attainment of commercially feasible fermentive production of 6-aminopenicillanic acid, the organic chemist can now carry out industrially practical terminal synthesis of known or new penicillins.

Alpha-phenoxyethylpenicillin [penicillin B, 6-(α-phenoxypropioamido)-penicillanate, or phenethicillin—see Figure 1], the first marketed product of such industrial terminal penicillin synthesis, has not been identified as a naturally occurring penicillin. Special biological properties have been ascribed to this compound which also distinguish it from other penicillins (3–5). These include: *1*) resistance to acid (gastric) degradation; *2*) excellent absorption from the gastrointestinal tract; *3*) greater antibacterial potency. Slow penicillinase inactivation quite reasonably would be reflected in apparent *in vitro* superiority in antibacterial effectiveness against penicillinase-producing bacteria. However, enhanced antibacterial effectiveness against nonpenicillinase-elaborating bacteria would require other explanation.

In part, the augmented antibacterial potency claimed for α-phenoxyethylpenicillin has been ascribed to the stereoisomerism inherent in the α-phenoxyethyl side chain characteristic of this penicillin. The L-isomer was reported to be generally more active than the D-isomer, and the racemate [1] more effective than either enantimorph (3). In addition, both L- and DL- forms were alleged to be superior to phenoxymethylpenicillin (V) in antistaphylococcal effectiveness (3).

Aminocarboxybutylpenicillin (penicillin N, synnematin B, cephalosporin N), a fermentation product of certain *Cephalosporium* species, is a penicillin of natural occurrence (7). In the side chain characteristic of this compound there is, also, an asymmetric carbon atom (see Figure 1); the structural configuration of this side chain α-aminoadipic acid moiety is D- (8).

The influence of side chain optical activity upon the antibacterial potency of penicillins can be assessed thoroughly with α-phenoxyethylpenicillin, since L-, D- and DL- mixtures are all available. Useful comparison of potency data regarding α-phenoxyethylpenicillins with other penicillins requires simultaneous assay of the antibacterial potencies of all of the penicillins under consideration, using identical test conditions. Accordingly, the antistaphylococcal effectiveness of L-, D- and racemic α-phenoxyethylpenicillin was tested at the same time as benzypenicillin (penicillin G), phenoxymethylpenicillin and aminocarboxybutylpenicillin, using a broth tube-dilution method.

Tellurite-positive (9) staphylococci, isolated from clinical specimens, were used for testing. Staphylococci are notorious for many reasons and were chosen for evaluation of the potency of penicillins not only because of the clinical challenge presented by staphylococcal infections, but also because a spectrum of intensity of exposure to penicillinase would be assured. Any large group of staphylococci of recent clinical isolation is cer-

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[1] A product of an organic chemical synthesis, α-phenoxyethylpenicillin should be 50 per cent levo- and 50 per cent dextrorotary, with regard to side chain optical activity. However, federal law requires commercial α-phenoxyethylpenicillin to have at least 55 per cent L-isomer to a maximum of 75 per cent L-isomer (6). *Racemate, raceme,* and *racemic,* when applied to α-phenoxyethylpenicillin in this article, will refer to DL-α-phenoxyethylpenicillin of legal constitution.

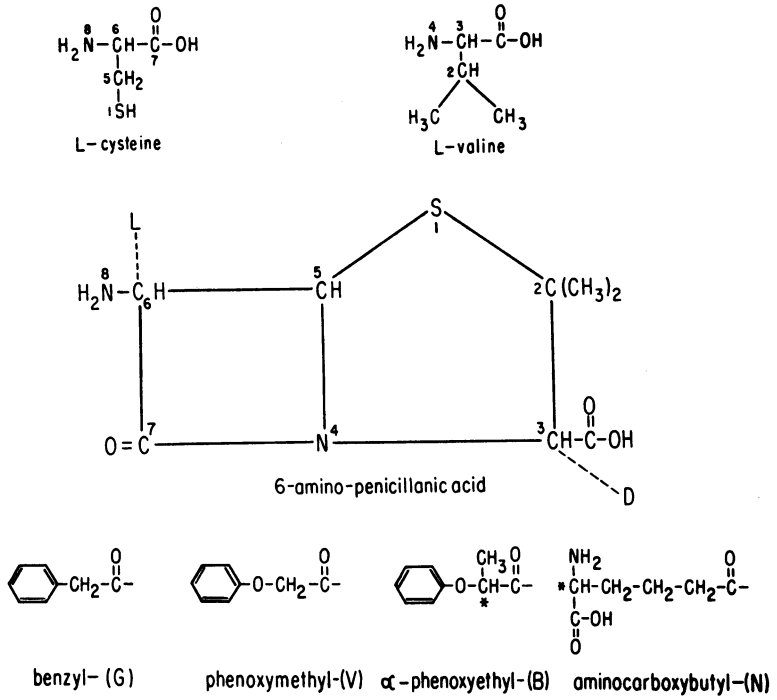

FIG. 1. 6-AMINOPENICILLANIC ACID (6-APA), AVAILABLE VIA BIOSYNTHESIS, RESULTS FROM CYCLIZATION OF THE DIPEPTIDE, L-CYSTEINYL-L-VALINE (1). 6-APA is an optically active compound, with configuration at carbon atoms 3 and 6 as indicated, which has but a low order of antibacterial potency. Both structural and optical configuration must remain intact for any derived penicillin to have a high order of antibacterial activity. Penicillins can be derived from 6-aminopenicillanic acid by acylation of the L-cysteine moiety ($^{8}_{N}$). The acyl radicals indicated are precursor to the side chains which characterize the penicillins studied. Both aminocarboxybutyl- and α-phenoxyethyl- have optically active carbon atoms, indicated by asterisks. The former, in fermentation-produced penicillin N, is levorotary; the latter, in penicillin B, a product of terminal organic synthesis, is available as a quasi-racemate.[1]

tain to include strains capable of penicillinase elaboration—these are the penicillin-resistant staphylococci of clinical significance (10). Penicillinase production appears to vary quantitatively among staphylococcal strains which are competent in this regard. There are penicillin-resistant staphylococci which have not been shown to elaborate penicillinase and, of course, those staphylococci which are markedly susceptible to penicillins do not make penicillinase. Techniques for detection of penicillinase activity are simple and sensitive; assay for this means of penicillin resistance was carried out with all of the staphylococci under study, using benzylpenicillin, phenoxymethylpenicillin and racemic α-phenoxyethylpenicillin.

MATERIALS AND METHODS

Staphylococci. Human associated staphylococci of community-wide origin (Salt Lake City) were used in testing. The total of 200 tellurite-positive (9) isolates were obtained by culture of: *1*) lesions acquired by patients in the course of hospitalization, 50 isolates; *2*) anterior nares of normal hospital personnel, 50 isolates; *3*) lesions acquired outside the hospital, cultures taken prior to any antibacterial therapy, 50 isolates; *4*) anterior nares of normal high school seniors, 50 isolates. Details regarding collection and storage of these isolates are the same as those given in an earlier communication (11).

TABLE I

Some properties of the penicillins studied are listed along with the microgram and, where appropriate, bioassay unit equivalents of the test concentrations

Side chain			Salt tested		μmole, μg and bioassay unit equiv. of test concentrations per ml		
Kind		Optical activity	Cation	Mol wt	0.001 μmole	0.010 μmole	0.100 μmole
Benzyl-	G	None	K^+	372.47	0.37 μg 0.59 U	3.73 μg 5.88 U	37.24 μg 58.82 U
Phenoxymethyl-	V	None	K^+	388.47	0.39 μg	3.89 μg	38.85 μg
α-Phenoxyethyl-	B	Levo- or dextro-rotary	K^+	402.49	0.40 μg	4.03 μg	40.25 μg
Aminocarboxybutyl-	N	Levorotary	di-Na^+	403.37	0.40 μg 0.27 U	4.03 μg 2.66 U	40.34 μg 26.62 U

Penicillins. D-,[2] L-,[2] and racemic [3] α-phenoxyethylpenicillins, as well as phenoxymethylpenicillin,[4] were sterilized by exposure of weighed portions of the respective potassium salts to ethylene oxide gas (12) for 18 hours at room temperature. Potassium benzylpenicillin [3] and di-sodium aminocarboxybutylpenicillin [5] were obtained as preweighed sterile, dry powders in ampules. All of these penicillins were dissolved and diluted for testing in tryptic digest of casein-papaic digest of soybean broth.

Stock solutions were prepared which were 0.00100 M, 0.00010 M and 0.00001 M for each of the penicillins studied. Preparation for testing involved transfer of 0.1 ml portions of stock solutions to appropriately labeled series of 13 × 100 mm screw-capped culture tubes before storage at − 22° C. In use, addition of 0.9 ml tryptic digest of casein-papaic digest of soybean broth, containing the inoculum of test staphylococci, resulted in final test concentrations for each penicillin of 0.001, 0.010, and 0.100 μmole per ml. Some characteristics of the penicillins studied and the relationships of micromoles, mass and bioassay units are presented in Table I.

Testing. Test staphylocccci from frozen storage were grown out overnight in tryptic digest of casein-papaic digest of soybean broth. According to a direct count in the Petroff-Hausser chamber, a dilution yielding 10,000 staphylococci per 0.9 ml was prepared in sufficient quantity (tryptic digest of casein-papaic digest of soybean broth) to permit inoculation of the requisite 19 tubes from a common supply (3 test concentrations per penicillin; 6 penicillins; 1 control tube containing 0.1 ml sterile broth).

After incubation for 24 hours at 37° C the tubes were inspected. Gross turbidity was accepted as evidence of resistance; absence of visible growth was taken to indicate inhibition. All tubes in which there was inhibition of growth were mixed by swirling, before a 3 mm loopful was removed to inoculate an eighth sector of a tryptic

[2] D-α-phenoxyethylpenicillin, potassium salt, lot 3168–11B4 and L-α-phenoxyethylpenicillin potassium salt, lot L9810, supplied by E. R. Squibb & Sons (Dr. John T. Groel), N. Y.

[3] Racemic α-phenoxyethylpenicillin, (batch 02295) with composition given as 35 per cent D-isomer and 65 per cent L-isomer, supplied by Charles Pfizer & Co. (Mr. Andrew J. Schmitz, Jr.), Brooklyn, N. Y. Potassium benzylpenicillin was supplied through the kindness of Dr. Fredrick L. Fink, also of Charles Pfizer & Co.

[4] Potassium phenoxymethylpenicillin, lot 755838, supplied by Eli Lilly & Co. (Dr. G. E. Maha), Indianapolis, Ind.

[5] Di-sodium aminocarboxybutylpenicillin, supplied as Salmotin test powder, lot 2085-CP, by Abbott Labs. (Dr. J. T. Sylvester), North Chicago, Ill.

TABLE II

Two tellurite-positive staphylococcal isolates were subjected to fivefold repetition of the testing procedure according to which the susceptibility of 200 recent clinical isolates of the tellurite-positive staphylococci to six penicillins was judged *

Isolate no.	μmole ml	Benzylpenicillin					Phenoxymethylpenicillin					α-Phenoxyethylpenicillin L-isomer					α-Phenoxyethylpenicillin D-isomer					α-Phenoxyethylpenicillin DL-mixture					Aminocarboxybutylpenicillin				
163	0.001	R	R	R	S	R	C	C	C	C	C	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
	0.010	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	R	R	R	R	R
	0.100	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	R	S	S	R	R
263	0.001	C	C	S	S	S	C	C	C	C	C	S	S	S	S	S	C	C	S	C	S	C	C	C	C	C	R	R	R	R	R
	0.010	C	C	C	C	S	C	C	C	C	C	S	S	S	S	S	C	C	C	C	C	C	C	C	C	C	R	R	R	R	R
	0.100	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	S

* There was concordance of susceptibility within replication units 15 times (of 18 trials) with Isolate 163 and 13 times with Isolate 263. By this assessment, a single determination of susceptibility, by the method described, will give the same result as 5 times repeated testing 88% of the time. R: resistant; S: bacteriostatic; C: bactericidal.

digest of casein-papaic digest of soybean agar plate. Following 24 hours at 37° C in a moist air-candle jar, these agar subcultures were examined. Absence of growth was interpreted as indicating a bactericidal effect, growth as evidence of a bacteriostatic effect in the broth test culture from whence the agar subculture was inoculated. Data supporting such loop subculture differentiation of the nature of the observed inhibitory effect have been presented elsewhere (11).

Assessment of the reproducibility of the results of susceptibility testing by the method described was provided by 5 repetitions of testing with 2 staphylococcal isolates. Each test proceeded from an overnight tryptic digest of casein-papaic digest of soybean broth culture inoculated from the frozen storage culture with counting, dilution, inoculation of penicillin tubes, incubation and interpretation as described. Considering 5 tests at a given concentration of a particular penicillin with one strain of staphylococcus to be a unit of testing, there were 18 units per isolate (Table II). There was agreement in 15 units with Isolate 163; 13 units with Isolate 263. Thus, if testing according to the method described be repeated 5 times, there will be accord in results about 88 per cent of the time; i.e., a single test would suffice 88 per cent of the time. The pattern of disagreements was that of stepwise gradation in effectiveness—variation in effect was between resistant and bacteriostatic, or, bacteriostatic and bactericidal; not resistant and bactericidal.

Penicillinase assay. Gots plates (13) were prepared, using a strain of *Sarcina lutea*[6] which was inhibited in agar pour plates (*Sarcina* inoculum, 1:100 final dilution of a 24 hour broth culture) by 5×10^{-6} μmoles benzylpenicillin per ml. Each staphylococcal isolate was inoculated from an overnight broth culture in the pattern of a small V described on a sixth sector of each of 3 kinds of plates—containing 10^{-4} μmoles per ml of either benzyl-, phenoxymethyl- or DL-α-phenoxyethylpenicillin; all plates having been seeded with *S. lutea* (a 24 hour broth culture in 1:100 final dilution. The plates were incubated at 37° C in moist air-candle jars and inspected at 24 hour intervals for 5 days for appearance of colonies of *S. lutea* juxtaposed to staphylococcal growth.

RESULTS

Since antistaphylococcal effectiveness was observed at three concentrations for each of the six

[6] Kindly supplied by Mr. R. E. Rhodes, Microbiology Section, Smith, Kline & French Labs., Philadelphia, Pa.

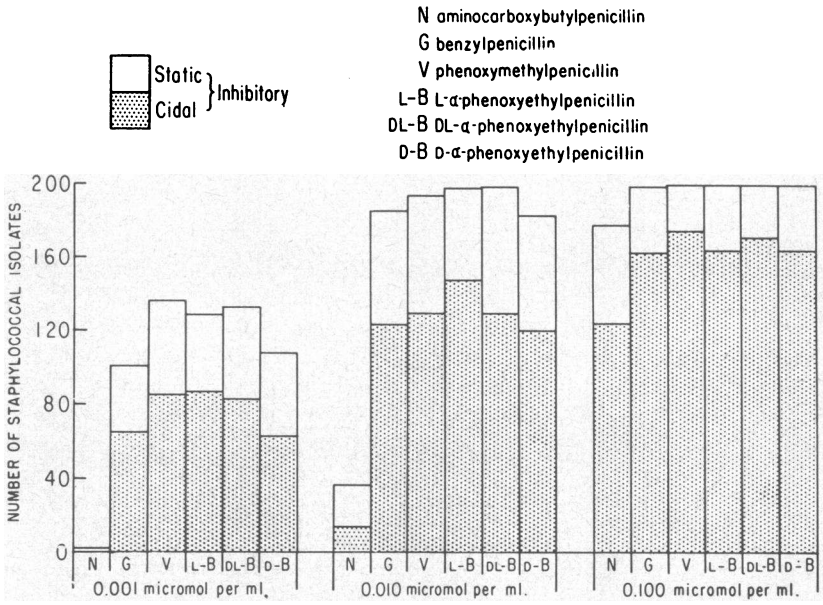

FIG. 2. THE TOTAL HEIGHT OF THE COLUMNS INDICATES THE EXTENT OF INHIBITION OF GROWTH (BACTERIOSTATIC PLUS BACTERICIDAL EFFECT) OF 200 CLINICAL ISOLATES OF TELLURITE-POSITIVE STAPHYLOCOCCI BY 6 PENICILLINS APPLIED AT 3 TEST CONCENTRATIONS. Aminocarboxybutylpenicillin was least potent. Benzylpenicillin and D-α-phenoxyethylpenicillin were about equally effective; both were superior to aminocarboxybutylpenicillin but were less potent than phenoxymethylpenicillin, L-α-phenoxyethylpenicillin or DL-α-phenoxyethylpenicillin; the latter 3 penicillins were nearly identical in potency. Bactericidal potency, the stippled area of each column, varied in the same manner as did inhibition of growth. All of the penicillins tested were more effective when present in high concentrations.

penicillins studied, the results of susceptibility testing are presented (Figure 2) as three groups of data corresponding to the three concentrations employed. It is apparent that with the decimally-stepped increments in penicillin concentration, differences in effectiveness tended to decrease, if not disappear. Thus, aminocarboxybutylpenicillin was virtually impotent at 0.001 μmole per ml, effecting just bacteriostasis with only 2 of 200 isolates. But this same penicillin, when present in a concentration of 0.100 μmole per ml, inhibited the growth of 177 of these same 200 staphylococcal isolates. The other penicillins studied were much more effective than aminocarboxybutylpenicillin, even at 0.001 μmole per ml, but all displayed increased effectiveness at greater concentrations.

Since all six penicillins were effective against most of the test staphylococci at some concentration, differences in effectiveness were quantitative; i.e., potency varied. In order to determine whether or not potency could be related to side chain stereoisomerism, chi square comparison of inhibitory effectiveness (bacteriostatic plus bactericidal effect) and of bactericidal effectiveness was carried out. These results (Figure 3) are best considered one test concentration group at a time.

0.001 μmole per ml. Using phenoxymethylpenicillin, a clinically and pharmacologically well known penicillin as basis for comparison, benzyl-, D-α-phenoxyethyl- and aminocarboxybutylpenicillins were all significantly ($p \leq 0.050$) less effective inhibitors for 200 tellurite-positive staphylococci of clinical origin. Racemic and L-α-phenoxyethylpenicillins were also significantly more active than the D-isomer of penicillin B.

No difference in over-all inhibitory potency distinguished phenoxymethylpenicillin, DL-α-phenoxyethylpenicillin and L-α-phenoxyethylpenicillin. However, detailed comparison revealed that, while 51 of the 200 isolates were resistant, 117 were susceptible to these three penicillins. The remaining 32 isolates were resistant to one or two of the three penicillins, with 4 resistant to phenoxymethylpenicillin alone, 5 resistant only to DL-α-phenoxyethylpenicillin and 6 resistant just to L-α-phenoxyethylpenicillin (Figure 4).

In terms of bactericidal action, the same potency relationships prevailed as were described for inhibition of growth.

0.010 μmole per ml. Both aminocarboxybutylpenicillin and D-α-phenoxyethylpenicillin were again significantly less effective inhibitors of staphylococci than was phenoxymethylpenicillin. At this test concentration, however, benzylpenicillin was not markedly inferior to phenoxymethylpenicillin. Again, DL- and L-α-phenoxyethylpenicillin were significantly more effective than the D-isomer of this penicillin.

Over-all, DL-α-phenoxyethylpenicillin, L-α-phenoxyethylpenicillin and benzylpenicillin were not significantly different from phenoxymethylpenicillin in effectiveness as inhibitors of staphylococci. From isolate-by-isolate comparison, there were 182 of the 200 isolates tested which were susceptible, and 1 which was resistant, to all of these four penicillins. Seventeen were resistant to one or more, but not to all four penicillins. One isolate was resistant to phenoxymethylpenicillin alone, 1 was resistant only to L-α-phenoxyethylpenicillin and 10 were resistant just to benzylpenicillin (Figure 4).

In terms of bactericidal action, phenoxymethylpenicillin was significantly superior to aminocarboxylbutyl penicillin, but was not more active than the other penicillins tested. The pure diastereoisomers and the DL-mixture of α-phenoxyethylpenicillin were not significantly variable in potential for lethal effect.

0.100 μmole per ml. Aminocarboxybutylpenicillin was significantly less effective in securing either inhibition or death of staphylococci than was any of the five other penicillins—all of which were equally effective. Only one isolate was resistant to all six penicillins; another isolate, resistant to benzylpenicillin as well as to aminocarboxybutylpenicillin, was susceptible to the remaining four penicillins tested (Figure 4).

These data suggest the following sequence of antistaphylococcal potency for the penicillins tested:

phenoxymethylpenicillin
L-α-phenoxyethylpenicillin
DL-α-phenoxyethylpenicillin
} > benzyl- > D-α-phenoxyethyl- > aminocarboxybutylpenicillin

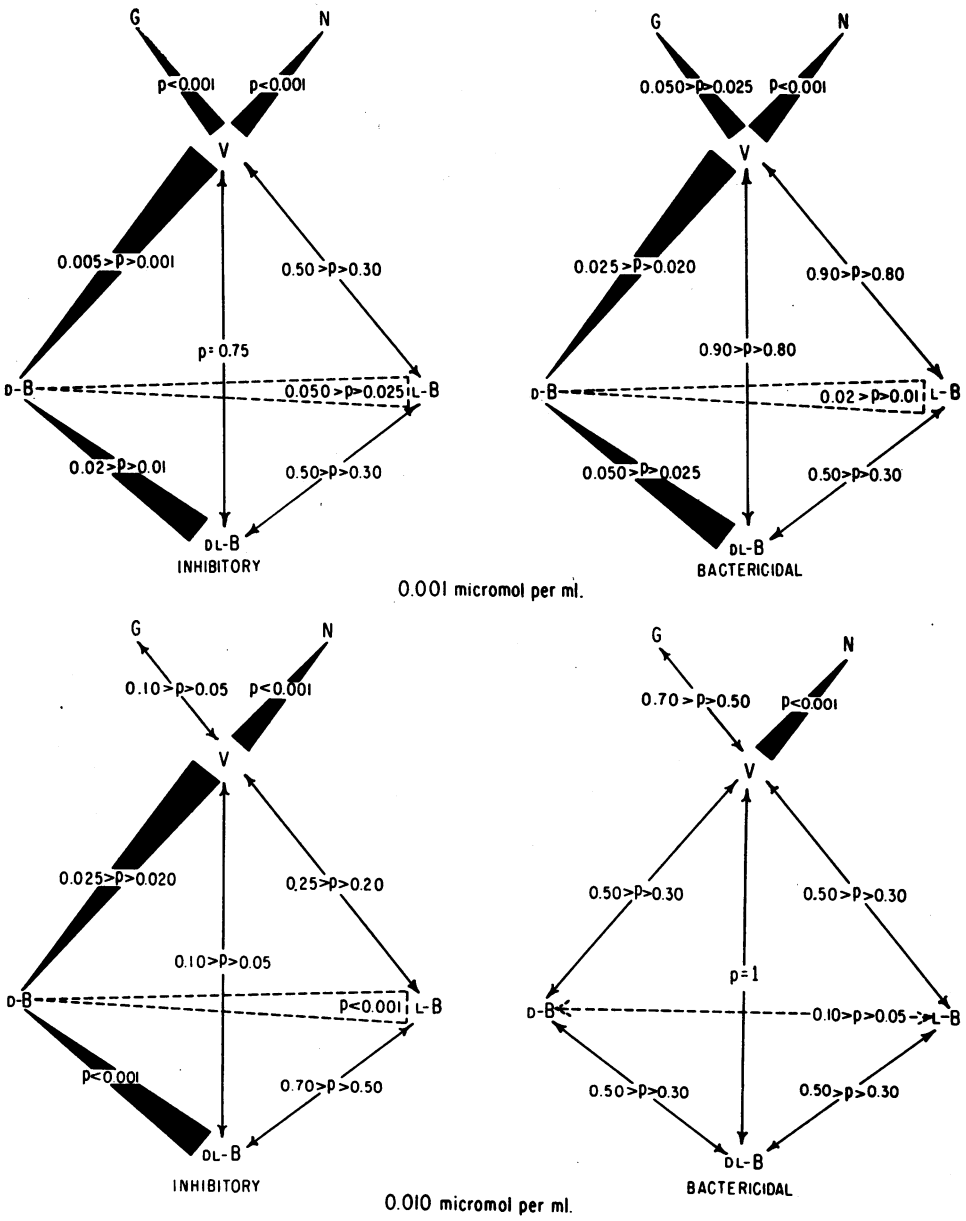

FIG. 3. THE ANTISTAPHYLOCOCCAL POTENCY OF AMINOCARBOXYBUTYLPENICILLIN (N), BENZYLPENICILLIN (G), D-α-PHENOXYETHYLPENICILLIN (D-B), DL-α-PHENOXYETHYLPENICILLIN (DL-B) AND L-α-PHENOXYETHYLPENICILLIN (L-B) WAS EVALUATED BY COMPARISON WITH PHENOXYMETHYLPENICILLIN (V). Two hundred recent clinical isolates of tellurite-positive staphylococci were exposed to all 6 penicillins at the same time, under identical test conditions.

TOP. (0.001 μmole penicillin per ml) These were the relationships whether over-all inhibition of growth or bactericidal effect was considered: penicillin V was significantly ($p \leq 0.050$) more effective than penicillins N, G and D-B; penicillins L-B and DL-B were more effective than D-B; penicillins V, L-B and DL-B were not significantly different.

MIDDLE. (0.010 μmole penicillin per ml) Inhibitory effect: penicillin V was significantly more effective than penicillins N and D-B; penicillins L-B and DL-B were significantly more effective than D-B; penicillins V, L-B and DL-B were not significantly different. Bactericidal effect: penicillin V was significantly more potent than was penicillin N, but was not significantly more often associated with lethal antistaphylococcal effect than were penicillins G, D-B, DL-B or D-B.

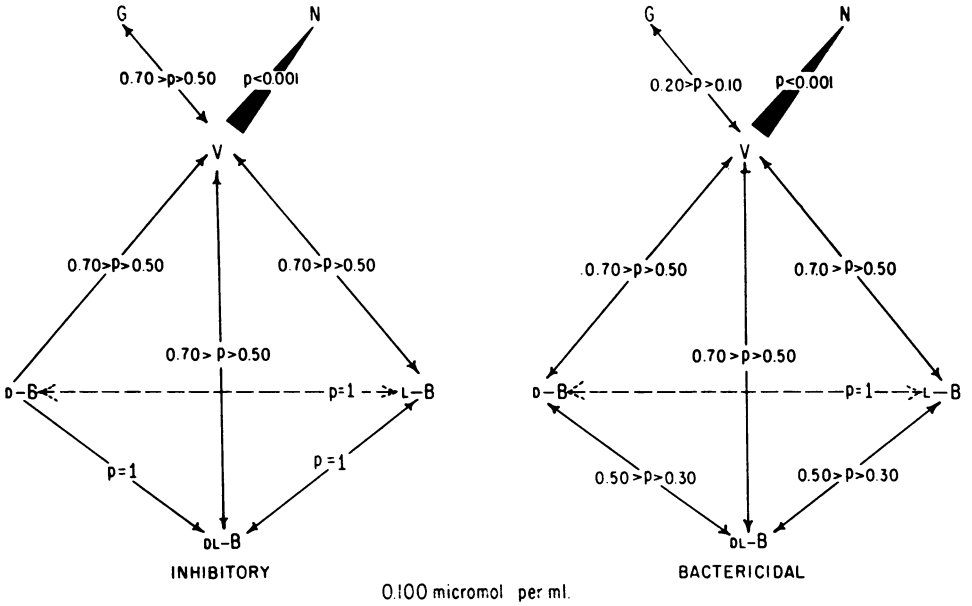

FIG. 3—*Continued*

BOTTOM. (0.100 μmole penicillin per ml) In terms of both inhibitory and bactericidal effect, penicillin N was significantly less effective than penicillins V, G, D-B, DL-B and L-B.

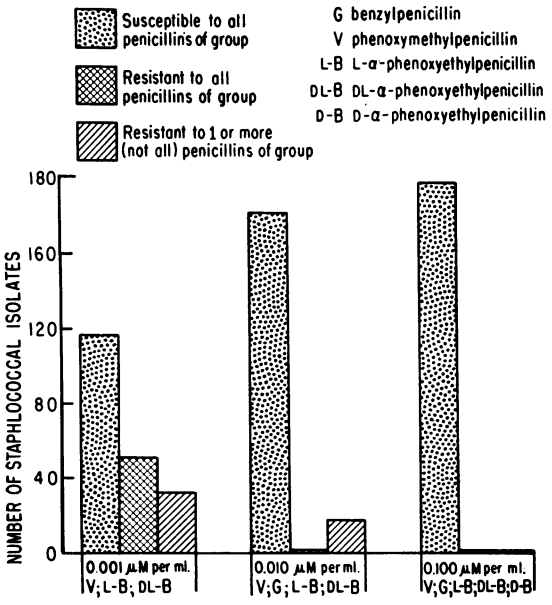

FIG. 4. THE PLOTTED DATA REFER TO ISOLATE-BY-ISOLATE COMPARISON OF THE ANTISTAPHYLOCOCCAL EFFECTIVENESS OF PENICILLIN GROUPS. These groups were delineated at each test concentration by lack of significant variation ($p \leq 0.050$) in over-all inhibitory effect on 200 staphylococcal isolates comparing benzyl-, aminocarboxybutyl-, L-, DL- and D-α-phenoxyethylpenicillins with phenoxymethylpenicillin. As the test concentration increased, the number of penicillins in each group increased and the number of staphylococcal isolates inhibited by all penicillins of a group increased also. Although aminocarboxybutylpenicillin never achieved statistical parity in effectiveness with the other penicillins tested, the major difference between these penicillins in antistaphylococcal effectiveness appears to be quantitative.

Penicillinase activity was first perceptible after 48 hours' incubation of the Gots plates. The *Sarcina* colonies were tiny at this time and were little pigmented. While the total number of positive reactions was greater at 72 than at 48 hours' incubation, there was little increase thereafter. However, observation at 120 hours was considered most reliable, since the deep golden color of the *Sarcina* colonies was then fully developed. Under the test conditions described, penicillinase activity was evident with: 122 isolates against benzylpenicillin; 131 isolates against phenoxymethylpenicillin; 134 isolates against DL-α-phenoxyethylpenicillin. The differences between these three values are not significant ($0.50 > p > 0.30$).

DISCUSSION

Molar description of penicillin concentrations used for susceptibility testing may seem an undue refinement; yet, penicillins are compounds of

known chemical constitution which become irreversibly fixed to bacteria to an extent directly proportional to susceptibility to lethal injury (14). Bound penicillins appear to work their effects by bringing about serious enzymatic dislocations which are only partially understood. Penicillins are antimetabolites and as such are effective as whole molecules. It follows that rational assessment of potency must proceed from description of concentration in molar terms.

In 1957 Sheehan and Henery-Logan (15) reported the laboratory synthesis of phenoxymethylpenicillin. Of little significance in terms of commercial manufacture of penicillins, this chemical milestone was a feat of great significance in permitting demonstration of the essential nature of certain structural features of the bicyclic ring nucleus common to all penicillins. It was known that lysis of the C_7—N_4 bond of the β-lactam ring (see Figure 1) to produce a penicilloic acid, as accomplished by bacterial penicillinases (16), resulted in inactivation of penicillins. From comparison of synthetic with natural penicillins, it became clear that an intact 6-aminopenicillanic acid nucleus was also required in terms of the 5-membered ring and its substituents. More pertinent to the present study was the finding that, for antibacterial activity, all of the optically active centers of 6-aminopenicillanic acid must have the configuration uniformly present in natural penicillins. These observations with 6-aminopenicillanic acid, together with the demonstrated essentiality of particular optical configuration with chloramphenicol (17) and cycloserine (18), are precedent for the affirmation of significant variation in antistaphylococcal effectiveness of penicillins, differing only in optical configuration in the side chain.

Widely publicized in advertisements, the report of Gourevitch, Hunt and Lein (3) indicated that the L-isomer of α-phenoxyethylpenicillin displayed *in vitro* greater antibacterial activity than did the D-isomer against three of five strains of *Staphylococcus aureus* designated: 52-34, 52-75, WR 188, BRL J, BRL O. The superiority of L- over D-α-phenoxyethylpenicillin was attested by respective minimal inhibitory concentrations in micrograms per milliliter of: 1.6 vs 3.1, 3.1 vs 6.2, 3.1 vs 3.1, 0.8 vs 0.8, 0.8 vs 1.6. In the same report, a DL- mixture of α-phenoxyethylpenicillin was: *1*) also more active than D-α-phenoxyethylpenicillin with four of the five test strains of staphylococcus—respective minimal inhibitory concentrations in micrograms per milliliter: 0.8 vs 3.1, 3.1 vs 6.2, 1.6 vs 3.1, 0.8 vs 0.8, 0.8 vs 1.6; *2*) more active than L-α-phenoxyethylpenicillin with strains 52-34 and WR 188—respective minimal inhibitory concentrations in micrograms per milliliter: 0.8 vs 1.6, 1.6 vs 3.1; *3*) equally as active as L-α-phenoxyethylpenicillin with strains 52-75, BRL J and BRL O (3.1, 0.8 and 0.8 μg per ml, respectively).

In addition to indicating that L- and DL-α-phenoxyethylpenicillin were superior in antistaphylococcal effectiveness to D-α-phenoxyethylpenicillin, these meager data were represented as indicating that D- and L-stereoisomers worked in complementary fashion with each other so that a DL-mixture was superior to either single enantiomorph in antistaphylococcal effect. While mouse protection, following on Smith strain staphylococcus infection, was reported to be more successful with

TABLE III

Isolate-by-isolate comparison of the inhibitory effect of the pure diastereoisomers and a mixture (35% D- + 65% L-) of α-phenoxyethylpenicillins on 200 clinical isolates of tellurite-positive staphylococci in a uniform, broth tube-dilution test system *

α-Phenoxyethylpenicillins, μmole/ml	Number of isolates inhibited by					
	D-		L-		DL-	
	Alone	And/or L-, DL-	Alone	And/or D-, DL-	Alone	And/or D-, L-
0.001	1	108	5	129	6	133
0.010	0	182	1	197	1	198
0.100	0	199	0	199	0	199

* Both L- and DL- mixtures were more often solely effective than was the D-isomer. However, since the DL-mixture was not significantly more often alone effective than was the L- form, there is no indication from these data that mixing stereoisomers augments antistaphylococcal potency.

racemic than with either pure diastereoisomer of α-phenoxyethylpenicillin, the authors noted that the differences were not statistically significant.

Determination of the susceptibility of a significant number of clinical isolates of tellurite-positive staphylococci, as described in the present study, indicates that both L- and DL-α-phenoxyethylpenicillins are superior to D-α-phenoxyethylpenicillin.

There was no support from these data for the claim of augmented antistaphylococcal potency on mixing stereoisomers. Over-all, DL-α-phenoxyethylpenicillin was not significantly different in potency from L-α-phenoxyethylpenicillin at any of the concentrations tested (Figure 2). In addition, when isolate-by-isolate comparison of the effectiveness of these three α-phenoxyethylpenicillins was carried out to determine just how often each was the sole inhibitor of a staphylococcal isolate (Table III), no differences were found that were not apparent from the over-all susceptibility data (Figure 2). If complementary effectiveness were gained from mixing D- and L-isomers, the DL- mixture should have been most often the sole effective form of α-phenoxyethylpenicillin. This was not so—these data do not support the notion of augmented potency ascribed to DL- mixture of α-phenoxyethylpenicillin. Yet, in affirming that the L-form was more potent than the D-form, the critical importance of optical configuration is asserted even in the side chain of penicillin. This importance is likely not critical in terms of clinical usage of α-phenoxyethylpenicillin since, generally, overtreatment is clinical practice. However, because chemical variation in acyl radical appears to be the only feasible approach to synthesis of new penicillins, side chain stereoisomerism should be considered in designing new penicillins for synthesis.

Garrod (19) compared *in vitro* the antistaphylococcal activity of benzylpenicillin, phenoxymethylpenicillin and α-phenoxyethylpenicillin (since isomeric designation was not made, it is assumed that a DL- mixture of α-phenoxymethylpenicillin was tested). With 36 "penicillin-sensitive" strains, there were no significant differences in effectiveness of the three penicillins by either plate or tube-dilution testing.

Thirty-eight penicillin-resistant (penicillinase-forming) strains were also tested as part of the same study. When a small inoculum (an 0.02 ml drop of a 1:500 dilution in saline of a broth culture) was applied to an agar culture medium containing penicillin in plate-dilution testing, the three kinds of penicillin were again effective to the same extent. However, by tube-dilution tests in which a large inoculum (an 0.02 ml drop of an undiluted broth culture) was used, both phenoxymethylpenicillin and α-phenoxyethylpenicillin were superior to benzylpenicillin. Moreover, α-phenoxyethylpenicillin was generally more effective than was phenoxymethylpenicillin. As noted by the author, tube-dilution testing with a large inoculum reflects primarily upon the effect of the preformed penicillinase added to the test system as part of the inoculum.

Preparation of inocula in the present study proceeded from broth cultures which by direct count usually had around 10^9 staphylococci per ml; accordingly, dilution to achieve the inoculum, 10,000 staphylococci per 0.9 ml, was at least 100,000-fold. If Garrod's broth cultures for inoculation had attained bacterial populations of density similar to ours—say 5×10^9 per ml—his small inoculum would have delivered about 200,000 and his large inoculum about 250 million staphylococci (as added to 2.5 ml broth, there would have been about 10 million staphylococci per ml). From these considerations, our test situation was more nearly comparable (staphylococci versus penicillins and penicillins versus preformed penicillinase) to Garrod's small inoculum plate-dilution trials than to his large inoculum tube-dilution system. Even so, there remain differences—inoculum size, relative accessibility of penicillins to staphylococci, agar versus broth culture—of such significance that disagreement in results, if not inevitable, is at least not surprising.

Comparison of aminocarboxybutylpenicillin with benzylpenicillin by *in vitro* assay of antistaphylococcal effectiveness, using 30 isolates (20) and 7 isolates (21), indicated that penicillin N was markedly less inhibitory for staphylococci than was penicillin G. The data of the present study, relating to 200 staphylococcal isolates of recent, community-representative origin, support these findings—benzylpenicillin had greater antistaphylococcal potency than had aminocarboxybutylpenicillin. In addition, data were presented which assert that phenoxymethyl- and the

α-phenoxyethylpenicillins are also, individually, more potent antistaphylococcal penicillins than is aminocarboxybutylpenicillin. However, penicillin N is remarkably more active than penicillin G against many species of gram-negative bacilli. Moreover, in addition to being an unusual penicillin in origin from *Cephalosporium* species—a non-*Penicillium* genus of the class *Fungi imperfecti*—the side chain of penicillin N is a hydrophilic, aminoacyl chain which is optically active (8). Unfortunately, we can only speculate on what would be the antistaphylococcal activity of penicillins enantiomorphic and racemic to the tested D-aminocarboxybutylpenicillin.

Application of dense inocula of staphylococci to agar media containing low concentrations of penicillins (about five times the minimal concentration inhibitory for the indicator strain of *S. lutea* which was heavily seeded in the agar media) provided settings designed for demonstration of even meager penicillinase production. On the other hand, the broth susceptibility testing method used exposed small inocula of staphylococci, virtually without preformed penicillinase, to 10-, 100- and 1,000-fold greater concentrations of penicillins than were present in the tests for penicillinase activity. Thus, susceptibility testing and penicillinase assay conditions were sufficiently disparate in design (as in aim) to make reasonable the finding that fewer of the 200 staphylococcal isolates tested were inhibited by even 0.001 μmole of the penicillins per ml than were found to be capable of inactivating these same penicillins. However, assay for penicillinase activity against benzyl-, phenoxymethyl- and DL-α-phenoxyethylpenicillin was carried out to determine whether or not there were differences among these penicillins in terms of susceptibility to staphylococcal penicillinase. Under the conditions of testing employed, there were no significant qualitative differences—results which are in agreement with those reported by McCarthy, Hirsch and Finland (5).

SUMMARY

On the basis of broth tube-dilution susceptibility testing with 200 recent clinical isolates of tellurite-positive staphylococci, the potency of D-, L- and DL-α-phenoxyethylpenicillins was compared with simultaneously and identically tested benzyl-, phenoxymethyl- and aminocarboxybutylpenicillins.

Differences in antistaphylococcal potency were noted which were quantitative: all of the penicillins were more effective when applied in higher concentrations. A potency sequence of the penicillins tested was apparent:

phenoxymethyl-
L-α-phenoxyethyl-
DL-α-phenoxyethyl- } > benzyl-> D-α-phenoxyethyl-> aminocarboxybutylpenicillin

While both L- and DL-α-phenoxyethylpenicillin were superior to D-α-phenoxyethylpenicillin, there was no indication of augmented potency on the part of the DL-mixture in comparison with pure L-α-phenoxyethylpenicillin.

Although side chain optical configuration of α-phenoxyethylpenicillin had significant reflection in antistaphylococcal potency *in vitro,* the major therapeutic implication of this observation lies in the designing of new penicillins for terminal synthesis.

Alpha-phenoxyethylpenicillin, in any of its optical forms, was not a more potent inhibitor of staphylococci than was phenoxymethylpenicillin.

Benzylpenicillin, phenoxymethylpenicillin and DL-α-phenoxyethylpenicillin did not differ qualitatively in susceptibility to inactivation by staphylococcal penicillinase.

Despite the presence of an asymmetric carbon atom in the aminoacyl side chain of aminocarboxybutylpenicillin, the single enantiomorph studied was least effective of the penicillins tested as an inhibitor of staphylococci.

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