MICROBIOLOGICAL ASPECTS OF SYNTHETIC PENICILLIN RESEARCH

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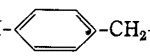
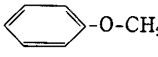
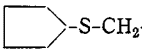
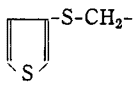
It is indeed a pleasure to be asked to talk to this Society on a topic whose early history is so closely associated with scientists from this Country. The chemical 6-aminopenicillanic acid (6-APA) is the necessary precursor of synthetic penicillins. The first report of an experimental attempt to prepare 6-APA was an article published by SAKAGUCHI and MURAO[1] in 1950 which presented the structure of 6-APA under the name penicin. However, this compound has had a long and difficult road to travel before it took the very prominent place it now has in pharmaceutical research. It is my intention today to discuss the events that led to the synthetic penicillins, the techniques used to evaluate these new penicillins, and the latest addition to the synthetic penicillin family, namely oxacillin.

Before discussing the synthetic penicillins, it will be necessary to go back to the early days of penicillin research when it was found that penicillin was not a single substance but a mixture of substances. As structural studies proceeded, it became apparent that the penicillins differed only in their side chains. A notable advance was made when it was found that different penicillins could be formed by adding different precursors to the media. Some of these new penicillins are shown in Table 1 which lists also the activity and the differential assyl ratio which is the ratio of activity against *B. subtilis* to *S. aureus*. Many new penicillins were synthesized and only a few are indicated in the table. It will be seen that different side chains impart different degrees of activity to the penicillin. Thus *n*-butylthiomethyl penicillin has an activity of 3,400 u/mg while methylthiomethyl penicillin has only 550 u/mg. Also, it should be noted that the relative activity against different organisms varies with different side chains. In the list will be seen phenoxymethyl penicillin or penicillin V. This penicillin was found to be more stable to acid and consequently better absorbed when given orally than penicillin G.

While a great variety of new penicillins could be made in this manner, there was a definite restriction as to the types of penicillin that could be made by fermentation. All such compounds had to have a free methylene group adjacent to the carbonyl group. This limited greatly the type of penicillin that could be made. The differences in activities and the differential antimicrobial effects seen with different penicillins indicated that if other types of side chains could be added to the penicillin nucleus, perhaps important therapeutic advantages could be obtained.

In 1950 SAKAGUCHI and MURAO published their paper indicating that the mycelium of the mold *Penicillium* could split penicillin G into phenylacetic acid and 6-aminopenicillanic acid[1]. This report stimulated similar work in many laboratories since it was recognized that 6-aminopenicillanic acid might be the key to preparing penicillins that could not be prepared by fermentation. Unfortunately until recently, such work was unsuccessful. However, ERICKSON and BENNETT reported in 1961[2] that they were able to form 6-aminopenicillanic acid in small yields from penicillin G using *Penicillium chrysogenum* mycelium. A few years after the first paper was published, MURAO published two additional articles on the enzyme[3]. In 1953, KATO[4] published two papers indicating that the penicillin nucleus was present in fermentation broth which had no precursor added to it. The data he presented were

Table 1 Activity and differential assay value of a series of biosynthetic penicillins

Penicillin	R	Activity μ/mg	Differential assay value
p-Iodobenzyl-		2,425	0.67
Phenoxymethyl-		1,670	0.87
Metylthiomethyl-	CH_3-S-CH_2-	550	1.50
n-Butylthiomethyl-	CH_3-CH_2-CH_2-CH_2-S-CH_2-	3,400	0.53
Cyclopentylthiomethyl-		3,400	0.43
3-Thiophenethiomethyl-		2,000	0.76

based on differential biological and chemical assays and were quite convincing. During this time, a very stubborn organic chemist, JOHN C. SHEEHAN continued to work on the total chemical synthesis of penicillin designing new coupling reactions to overcome the lability of the strained β-lactam ring of the molecule. SHEEHAN[5] was able to announce the chemical synthesis of 6-APA during a special symposium in 1958. While his synthesis led to only small quantities of material, he was able to show that through this compound many new penicillins could be made.

A very notable advance was made in 1959 when a team of scientists from the Beecham Research Laboratory[6] announced the isolation from fermentation broths of the penicillin nucleus and named it 6-aminopenicillanic acid based on SHEEHAN's nomenclature. They isolated the compound in crystalline form and showed that it could be acylated by simple chemical methods to form active penicillins. Many new penicillins were synthesized in their laboratories. Bristol Laboratories, at this time, joined with the Beecham Research Laboratories for a limited period to develop this area. In a short period of time, over 1,500 new penicillins were made and evaluated by these groups. Also, Bristol Laboratories, extensive fermentation and isolation experience were utilized to produce 6-APA on a commercial scale.

At the same time, work continued in a number of laboratories attempting to obtain 6-APA through the use of enzymes. In 1960, Bristol Laboratories[7], Beecham Research Laboratories[8], Pfizer Laboratories[9], and Bayer[10] independently announced that penicillin G could be split by an enzyme present in various bacteria to form 6-APA in good yield. Thus, the penicillin nucleus became available through both a fermentation and an enzyme process. Scientists were no longer restricted in the types of side chains that could be used and chemotherapy was entering a fascinating period.

As work started in this area, goals were set with regard to the desirable types of penicillins. Some of these goals represented small gains in therapy and some important gains if they could be realized. Since many staphylococci strains, especially in hospital environments, were resistant to penicillin G by virtue of the penicillinase they produced, we

Table 2 The *in vivo* and *in vitro* properties of α-aryloxyalkyl penicillins

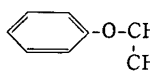
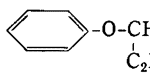
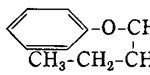
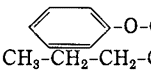
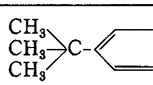
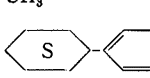
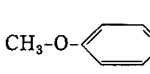
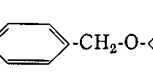
	Compound No.	Side chain	CD_{50}	Minimum inhibitory concentration with *Staph. aureus* in heart infusion broth		
				Smith strain	Smith strain plus 50% serum	BLK strain
Altering the length of the side chain	152		0.5	0.08	0.16	6.25
	284		1.1	0.08	0.32	3.12
	299		3.7	0.16	1.25	3.12
	291		4.0	0.08	0.625	25
Substitution in the phenyl group	216		9.0	0.08	0.625	05
	329		9.0	0.08	2.5	25
	309		0.8	0.32	0.16	25
	383		2.5	0.03	0.625	12.5
	303		20.0	1.25	5.0	6.25

Table 3 Comparison of CD_{50} values of different penicillins using *Staphylococcus aureus*

Penicillins	CD_{50} mg/kg							
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	Geometric mean
α-Phenoxyethyl penicillin	0.46	0.62	0.18	0.45	0.45	0.4	0.4	0.41
Penicillin V	0.62	1.90	0.6	0.26	0.7	1.2	0.85	0.75
Penicillin G	1.0	1.9	0.64	1.1	0.9	1.2	0.9	1.04

were particularly interested in obtaining penicillins resistant to the action of staphylococcal penicillinase. Also, in view of the fact that the natural penicillins had some activity against Gram-negative organisms but not sufficient to be generally useful, it was hoped that synthetic penicillins with increased activity against Gram-negative pathogens could also be found. We were also interested in penicillins that had properties somewhat similar to penicillin G or penicillin V but which had the additional attribute of being better absorbed when taken orally. We considered the possibility of producing a nonallergenic penicillin, but thought the likelihood of this very slight. While penicillins of decreased allergenicity could probably be found, clinical proof in quantitative terms would be very difficult. Workers in various laboratories set out to synthesize numerous penicillins hoping to find some with these various properties. Different degrees of success have been attained in each of these areas after only a few years of intensive laboratory and clinical investigation.

Literally thousands of new penicillins have been screened. Organic chemists in many laboratories have been very attaching various types of side chains to 6-APA and the resulting penicillins have been evaluated with respect to their properties. In order to give you an appreciation of the different types of structures that have been evaluated, I shall take data from one of our publications[11] which lists some of the compounds made in a particular series and gives certain microbiological information as well. In Table 2 is listed a series of α-aryloxyalkyl penicillins. The structure of the side chain and the values for the CD_{50}'s and the minimum inhibitory concentrations (MIC) against certain strains are given. The CD_{50}, which is the median curative dose obtained in an experimental mouse infection, was run against the Smith strain of *Staphylococcus aureus*. This is a virulent strain which is sensitive to penicillin G. The minimum inhibitory concentrations were done using the Smith strain and the Smith strain in the presence of 50% human serum. In addition, a penicillinase-producing strain designated as BLK was also used.

Examination of the data reveals that the type of chemical substitution on the side chain has a marked effect on the microbiological properties. Some penicillins are very active while others have relatively poor activity. In some cases, the activity is decreased very markedly in the presence of 50% human serum. It may also be noticed that in this series the different penicillins show different activities against the penicillinase-producing strain. We rely to a great extent on such tests in trying to determine how interesting is a particular penicillin. We feel the *in vivo* tests are particularly important and use two different types of tests. One, the CD_{50} as explained before is a determination of the median curative dose. In this case, an overwhelming infection is used. Generally, the infection is very acute and the end point is death or survival of the animal. By taking great precautions as to the strain, inoculum, the condition of the mice, and other factors, suitable quantitative data can be obtained which permits comparison of different penicillins. An example of this is shown in Table 3 which lists the CD_{50}'s obtained in a series of experiments comparing phenethicillin with penicillin G and penicillin V, using the intramuscular route of administration and the Smith strain of *Staphylococcus aureus* as the infecting organism.

Another type of infection that we have used is a subacute subcutaneous test that was first described by SELBIE and O'GRADY[12] and made use of by

Fig. 1 The *in vivo* measurement of penicillin action using the leg swelling test.

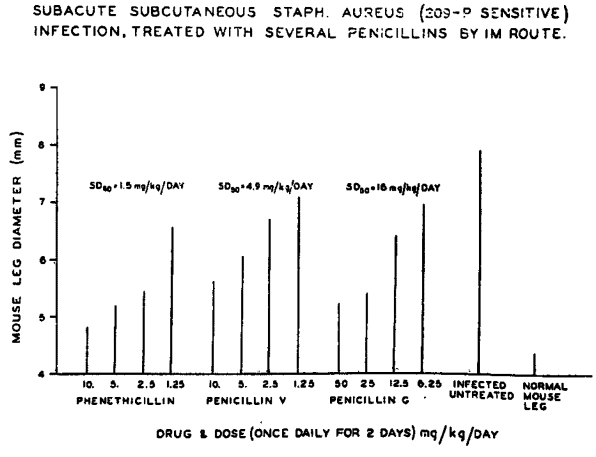


Table 4 Effect of inoculum size of a Penicillinase-producing *S. aureus* strain on minimum inhibitory concentration of α-phenoxyalkyl penicillins

Penicillin	MIC in μg/ml Inoculum dilution			
	10^2	10^3	10^4	10^5
Phenoxymethyl	>100	>100	6.2	0.4
α-Phenoxyethyl	>100	100	1.6	0.8
α-Phenoxypropyl	>100	25	0.8	0.8
α-Phenoxyisopropyl	>100	12.5	1.6	0.8
α-Phenoxybutyl	>100	100	6.2	3.12
α-Phenoxyisobutyl	12.5	1.6	0.8	0.8
Controls :				
Benzylpenicillin	>100	>100	12.5	0.8
Dimethoxyphenyl penicillin	3.2	3.2	1.6	1.6

BROWN and ACRED[13] in evaluating penicillins. In this case, the infecting organism is injected into the thigh of the mouse and the resultant swelling noted. The ability of the penicillin to decrease the swelling is used as a measure of the effectiveness of the penicillin. This is shown in Figure 1 which compares the same three penicillins by this test using *S. aureus* 209 P as the infecting organism. You will note that phenethicillin in this type of experiment also is the most effective of the penicillins tried.

Fig. 2 The rate of destruction of benzylpenicillin, phenethicillin, and methicillin by staphylococcal penicillinase.

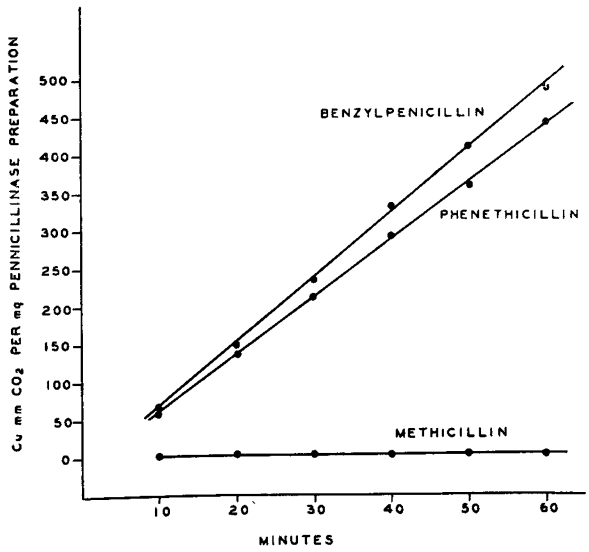


Table 5 Synthetic penicillins sold commercially

Penicillin G spectrum

1. Phenethicillin
2. Propicillin
3. α-Phenoxybenzyl Penicillin

Increased Gram-negative activity

1. Ampicillin

Penicillinase resistant

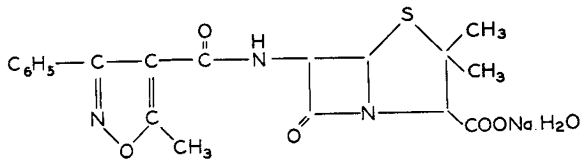
1. Methicillin
2. Oxacillin

When we evaluate penicillins which are active against penicillin G resistant *S. aureus* organisms, we use other tests to judge their effectiveness. These tests are designed to determine how resistant the penicillin is to the enzyme penicillinase. A simple way of obtaining information is to determine the minimum inhibitory concentration in presence of increasing amounts of inoculum. The results of such an experiment comparing a variety of penicillins are shown in Table 4. It will be seen that the MIC's of those penicillins which are not very resistant to penicillinase increase markedly at higher inocula levels while with a penicillin such as methicillin, the MIC's are scarcely affected. Alternatively, the ability of staphylococcal penicillinase to destroy the penicillin can also be determined directly. Figure 2 shows the results obtained using the manometric technique of HENRY and HOUSEWRIGHT[14] which actually measures the rate of destruction of penicillin by staphylococcal penicillinase. You will note that this rate is very slow for methicillin in contrast to penicillin G and phenethicillin. This type of data gives good quantitative estimations of stability to penicillinase but it

should be mentioned that the actual values obtained will depend on the substrate concentration used since in these experiments the enzyme is not totally saturated by the penicillin. In any case, using such experiments one can get a reliable estimate of resistance to penicillinase and consequently make judgments as to usefulness in penicillin G resistant *Staphylococcus* infections.

Using such types of evaluative procedures, a great many penicillins have been studied. In Table 5 are listed those penicillins which have been thought to offer a sufficient advantage to warrant their being sold commercially. The penicillins are divided into three sections, those used against the usual types of infections treated with penicillin G, the one penicillin showing increased activity against Gram-negative organisms, and those penicillins active against penicillin G resistant staphylococci by virtue of their resistance to destruction by staphylococcal penicillinase. Since there is not sufficient time to discuss all of these penicillins, I will concentrate the remaining portion of my lecture on the latest synthetic penicillin to be introduced namely 5-methyl -3-phenyl-4-isoxazolyl penicillin, known generically as oxacillin and sold commercially by Bristol Laboratories in the United States under the trade name Prostaphlin. It was evaluated microbiologically[15] and clinically under the designation of penicillin P-12 and is also known by this name. The penicillin was first synthesized by DOYLE, *et al*[16], and its structure is shown in Figure 3. Since the penicillin is active in the same general area as methicillin, comparison will be made with this antibiotic. Table 6 shows the spectrum of oxacillin compared with methicillin and penicillin G. It will be seen from the data that oxacillin is active against a variety of Gram-positive cultures. Like methicillin, oxacillin is less active than penicillin G. However, in comparing the activities of oxacillin with those of methicillin, it will be seen that oxacillin is considerably more active against *S. aureus* strains. It is also more active than methicillin against *D. pneumoniae* and *Streptococcus pyogenes* strains.

Fig. 3 Structure of oxacillin (sodium salt, monohydrate).



The most interesting property of oxacillin is its activity against penicillin G resistant staphylococci.

Table 6 Comparison of the minimum inhibitory concentration of penicillin P-12, methicillin, and penicillin G using a variety of organisms

Organism	Medium	Minimum inhibitory concentration, μg/ml		
		Penicillin P-12	Methicillin	Penicillin G
*A. aerogenes**	†	>200	>200	200
*B. anthracis**	†	0.3	0.08	0.016
B. cereus PCI 213	†	25	1.6	2.5
B. circulans ATCC 9961	†	25	100	1.6
*C. xerosis**	†	0.3	0.16	0.016
*D. peneumoniae***	§	0.13	0.5	0.016
E. coli ATCC 8739	†	>100	>100	50
*Gaffkya tetragena**	†	0.16	0.62	0.03
M. flavus ATCC 10240	†	0.62	0.62	0.031
N. catarrhalis ATCC 8176	§	0.16	0.08	0.008
P. vulgaris ATCC 9920	†	>200	200	200
*Ps. aeruginosa**	†	>200	>200	>200
*Sal. typhosa**	†	>100	>100	12.5
S. lutea ATCC 10054	†	0.03	0.03	<0.002
Staph. aureus 209 P	†	0.3	1.25	0.016
Staph. aureus Smith	†	0.16	1.25	0.016
Str. faecalis ATCC 8022	†	50	>100	6.25
Str. pyogenes Digonnet 7	§	0.06	0.25	0.008
*Vibrio comma**	†	25	2.5	3.12

* Cultures from Yale collection brought to Bristol Laboratories by George Valley.
† Heart infusion broth, Difco.
** Type II, St. Louis City Hospital, D-11008. Brought to Bristol Laboratories by G. A. HUNT.
§ Heart infusion broth containing 10 per cent pooled human serum.

Table 7 Comparison of the minimum inhibitory concentration of penicillin P-12, methicillin, and penicillin G using resistant *Staph. aureus* obtained from clinical sources

Staph. aureus strain		Minimum inhibitory concentration, μg/ml		
		Penicillin P-12	Methicillin	Penicillin G
Test	2964	0.8	3.12	50
	2614	0.4	3.12	50
	2992	0.4	3.12	50
	3008	0.4	3.12	50
	2462	<0.2	1.6	12.5
	2960	0.4	1.6	50
	2949	0.4	3.12	100
	2972	0.4	3.12	100
	3013	0.4	3.12	100
	3010	0.4	3.12	>100
Control	209 P	0.3	1.25	0.016

Table 8 Effect of inoculum size and serum concentration on the minimum inhibitory concentration of penicillin P-12, methicillin, and penicillin G

Staph. aureus strain	Per cent serum	Minimum inhibitory concentration, μg/ml					
		Penicillin P-12		Inoculum dilution methicillin		Penicillin G	
		10^2	10^5	10^2	10^5	10^2	10^5
Smith	0	0.16	0.08	1.25	1.25	0.016	0.016
Smith	50	2.5	0.625	2.5	1.25	0.12	0.031
1633-2	0	0.625	0.31	2.5	2.5	>100	0.8
1633-2	50	5.0	1.25	5.0	2.5	>100	3.1
52-75	0	1.25	0.31	5.0	2.5	>100	1.6
52-75	50	5.0	1.25	5.0	2.5	>100	3.1

In Table 7, data are compiled on the susceptibility of clinically isolated *S. aureus* strains to oxacillin, methicillin, and penicillin G. It will be seen that oxacillin has a lower minimum inhibitory concentration than methicillin against all such strains tested were uniformly sensitive to oxacillin. The effect of inoculum size and serum is shown in Table 8. The data show that inoculum size has little effect on the minimum inhibitory concentration of either oxacillin or methicillin, indicating that they are both very resistant to the penicillinase produced by the resistant strains 1633-2 and 52-75. The minimum inhibitory concentrations of penicillin G are greatly affected, however, by the inoculum size because it is not penicillinase resistant. The data also show that oxacillin is bound to serum to a greater extent than is methicillin. This experiment indicates in qualitative terms the resistance of oxacillin to penicillinase.

In Table 9 is shown a more quantitative determination of the degree of resistance to staphylococcal penicillinase using the method of HENRY and HOUSEWRIGHT as described above. The rates of destruction were calculated in terms of the rate of penicillin G arbitrarily set at 100. It will be seen that the resistance of oxacillin is of the same order of magnitude as that observed with methicillin.

Figure 4 compares the acid stability of oxacillin with that of penicillin G, and penicillin V using 0.004 M citrate buffer at pH 2.0. It will be seen that oxacillin is considerably more acid stable than penicillin G. This raises the possibility of oxacillin being used orally.

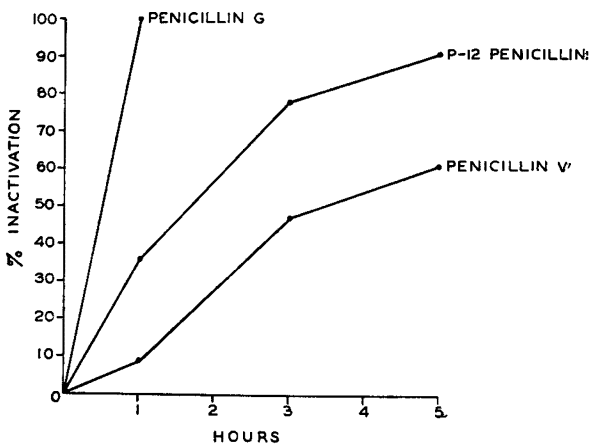
In vivo experiments done with mice confirmed the activity of oxacillin found in the *in vitro* experiments. In Table 10, the median curative doses or CD_{50}'s of oxacillin against penicillin susceptible and penicillin resistant strains of *Staphylococcus aureus* using intramuscular and oral administration of the drug

Table 9 Relative rates of destruction of penicillin P-12, methicillin, penicillin V, and penicillin G by Staphylococcal and *B. cereus* Penicillinases

Compound	Staphylococcal Penicillinase	*B. cereus* Penicillinase
Penicillin G	100	100
Penicillin V	110	126
Methicillin	0.4	2.3
Penicillin P-12	0.6	1.2

are shown. Oxacillin is not as active as penicillin G against the susceptible organism, but gives better protection by both routes than methicillin against the resistant organism. Results of the treatment of subacute subcutaneous infection described above are shown in Figure 5 using the penicillin G resistant *S. aureus* Russell strain. No effect is seen following treatment with penicillin G at doses as high as 300 mg/kg. Both oxacillin and methicillin were effective

Fig. 4 Acid stability of penicillin G, oxacillin, and penicillin V.



at doses of 100 and 300 mg/kg, with oxacillin being more effective. In this case, the drugs were given by the intramuscular route.

Summarizing the microbiological evaluation, oxacillin was found to be extremely resistant to staphylococcal penicillinase. It was more active than methicillin against penicillin G susceptible and resistant *Staphylococcus aureus* strains, *Diplococcus pneumoniae*, and *Streptococcus pyogenes*. Oxacillin was more acid stable than methicillin and penicillin G and also was bound by serum to a greater extent. Such results indicated the feasibility of using this penicillin orally against penicillin G resistant staphylococcal infections. The penicillin was clinically evaluated and found to be highly effective against resistant *Staphylococcus* infections by the oral route in doses of 500 mg given four times a day. It has indeed been a gratifying experience to see how well the clinical findings conformed to the microbiological evaluation.

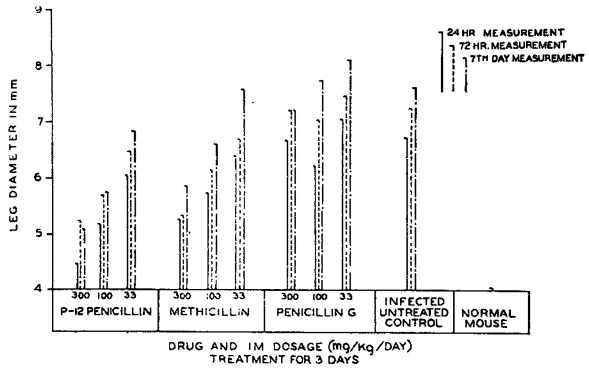
I have attempted to present to you a survey of this fascinating new area of chemotherapy. It should be borne in mind that the synthetic penicillins have been with us only a few years and we can certainly expect further advances. However, new synthetic penicillins probably will not be found as rapidly as the old ones because essentially all available acids have already been used by the organic chemists to make penicillins. Now, they must synthesize new acids for the side chain and hope to impart to the penicillin molecule properties superior to those penicillins in current use. This means that there must be a considerably larger investment in time and effort to make new synthetic penicillins than the old ones. Nevertheless, it is expected that penicillins with greater activity against resistant and nonresistant strains including Gram-negative organisms will be found. Also, new penicillins which are absorbed better and give more prolonged blood levels will probably be made. Perhaps as our evaluative procedures improve, we may be able to detect penicillins with decreased ability to sensitize people. I do not believe we will reach the point where all these attributes will be combined in a single penicillin. I think rather it is more likely that a series of penicillins will be made available each with its own specific advantage. With knowledge as to what these penicillins can and cannot do, the clinician will have at his disposal very powerful antimicrobial agents with which to combat bacterial infections.

Table 10 Acute CD_{50}'s of penicillin P-12 in experimental mouse infection produced by penicillin G-susceptible and resistant strains of *Staph. aureus*

Staph. aureus strain	CD_{50}*, mg/kg			
	Intramuscular		Oral	
	Penicillin P-12	Control †	Penicillin P-12	Control
Smith (penicillin G susceptible)	13.8	1.8	21	1.1
1633-2 (penicillin G resistant)	17	37	72	197

* Geometric mean of at least five independent determinations.
† For susceptible organism, penicillin G served as control. With resistant organism, methicillin was used.

Fig. 5 Activity of oxacillin, methicillin, and penicillin G in leg swelling test.



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